Temperature tolerance threshold and mechanism of oxidative damage in the leaf of Coffea arabica 'Typica' under heat stress

Koji Yamane, Moena Nishikawa, Yoshihiro Hirooka, Yusaku Narita, Tsukasa Kobayashi, Misako Kakiuchi, Kazuya Iwai and Morio Iijima

ABSTRACT
Coffea arabica, an economically important crop, accounts for most of the coffee consumed globally. Increasing temperature due to climate change can cause a decrease in productivity in many crops, including coffee plants. The maximum temperature at which damage is induced has been reported for many crops, but it remains unclear in coffee plants. Here, we investigated the effect of different temperatures and the physiological damage induced by heat stress using both leaf disks and intact plants of Coffea arabica 'Typica'. In the experiment using intact plants, we observed leaf damage by a decrease in soil plant analysis development value, and an increase in electrolyte leakage after exposure to 45°C for 96 h, whereas no leaf damage was observed for 72 h. The leaf surface temperatures after exposure to 45°C for 72 and 96 h were 44.0 and 46.3°C, respectively. Thus, a tolerance threshold in leaves of C. arabica 'Typica' under heat stress are likely between 44.0 and 46.3°C. The activities of catalase (CAT) and superoxide dismutase (SOD) decreased at 45°C in both leaf disks and intact plants. The decrease in the activities of SOD and CAT under heat stress may be responsible for the increased levels of reactive oxygen species, such as O₂⁻ and H₂O₂, and the resulting cellular damage. Our findings provide valuable insights into the physiological responses of Coffea arabica 'Typica' to heat stress, which may contribute to the breeding and screening of tolerant cultivars in the future.

CONTACT Morio Iijima iijimamorio@nara.kindai.ac.jp
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Introduction

Coffee is cultivated in the inter-tropical zone, from 20–25°N to 24°S (DaMatta & Ramalho, 2006). Among the 90 species of the genus *Coffeea*, *C. arabica* L. (Arabica coffee) and *C. canephora* Pierre ex A. Froehner (robusta coffee) are the most economically important species worldwide, with arabica coffee accounting for approximately 60% of the coffee consumed globally (DaMatta & Ramalho, 2006). Global warming caused by an increase in the average global temperature has recently become a cause of concern worldwide, as it is speculated that climate alteration will induce a decrease in crop production (Kang et al., 2009). Craparo et al. (2015) reported that coffee yield in Tanzanian highlands was reduced by increased average night temperatures. Nearly 80% of the land in hot, dry regions, such as northern Minas Gerais State in Brazil, parts of India, and Nicaragua, are the areas that currently give some of the highest yields of Arabica coffee, but they are in danger of becoming unsuitable for coffee production by 2050 (Bunn et al., 2015). Thus, studies aiming at breeding coffee strains with heat stress tolerance properties are needed.

Coffee was originally grown in the understory of African tropical forests. The optimal temperature range for the growth of Arabica coffee is from 18 to 23°C (DaMatta et al., 2018), and early studies indicated that photosynthesis in coffee plants is affected by temperatures above 20–25°C (DaMatta & Ramalho, 2006). However, the temperature tolerance threshold at which damage is induced could be higher than that at which the inhibition of photosynthesis occurs. Martins et al. (2016) reported that *C. arabica* is tolerant to heat stress at 37°C, but the temperature tolerance threshold is reached at 42°C. This suggests that heat stress above 40°C may induce damage in *C. arabica*. Temperature tolerance threshold has been defined in many crops (Luo, 2011); however, its value remains unclear in coffee plants.

Heat stress induces the production of reactive oxygen species (ROS), such as superoxide radical \( \text{O}_2^- \), hydrogen peroxide \( \text{H}_2\text{O}_2 \), singlet oxygen \( \text{O}_2^+ \), and hydroxyl radical \( \text{OH}^- \), causing damage to plant tissue. Plants react to heat stress by activating the ROS scavenging systems, including antioxidant enzymes and low molecular antioxidants, to prevent cellular damage (Suzuki & Mittler, 2006). Under high temperatures, \( \text{H}_2\text{O}_2 \) is one of the key inducers of oxidative damage in cells, and its excessive production in leaves occurs due to an imbalance in antioxidant enzymes. Heat stress (35°C) increases the activity of superoxide dismutase (SOD) in tomato plants, while it decreases the activities of \( \text{H}_2\text{O}_2 \) scavenging enzymes such as ascorbate peroxidase (APX) and catalase (CAT), leading to a significant increase in \( \text{H}_2\text{O}_2 \) (Rivero et al., 2004). Similar trends were observed in *C. arabica* at 42°C (Martins et al., 2016). However, Gill and Tuteja (2010) reported that the mechanism of oxidative damage induced by the imbalance of antioxidant enzymes is different in each cultivar under various stresses. Thus, the mechanism of oxidative damage under heat stress must be studied in each target cultivar, which may help to develop protocols that will alleviate oxidative damage.

Here, we investigated the maximum temperature at which damage is induced and the effect of heat stress on physiological aspects of leaves of *C. arabica* ‘Typica’, an economically important cultivar in Hawaii. As increased average temperature is an eminent consequence of global warming, it is crucial to identify the temperature that causes damage in coffee plants. In addition, studying oxidative damage under heat stress will contribute to future initiatives in breeding of heat-tolerant coffee plants.

Materials and methods

Plant material

Coffee seeds (*C. arabica* ‘Typica’) were sown in plastic cell trays (50 x 50 x 60 mm) with an 8-mm-diameter hole at the bottom and filled with commercial soil (Green Plaza Yamacho original culture soil; Nara, Japan). The soil was composed of peat moss and ripe bark compost with coarse sand materials. The soil pH (\( \text{H}_2\text{O} \)) and EC were 5.39 and 0.85 mS m\(^{-1} \), respectively. Total C and N in the soil were 177 and 4.1 g kg\(^{-1} \), respectively. After sowing, the seeds were kept in a growth chamber at 27/23°C (day/night) until germination. After four months, the cotyledons of the seedlings were fully expanded, and the seedlings were transplanted into 500 mL pots filled with the same soil. The seedlings were grown in the greenhouse of Kindai University in Nara Prefecture (34°40′N, 135°43′E). The temperature in the greenhouse was monitored hourly, and the average temperature during the growing period was 25.7°C. The day length was adjusted to 14 h with artificial lighting from a metal halide lamp. The average photosynthetically active radiation on the top of the canopy was approximately 320 µmol m\(^{-2} \text{s}^{-1} \). In addition, 10–14-month-old plants were used in the experiments.

Heat stress treatment

We investigated the effect of oxidative stress on coffee leaves under heat stress using leaf disks and intact plants. Leaf disks (12 mm) were cut from fully expanded uppermost leaves and exposed to heat stress by
incubation in 5 mM MES buffer (pH 6.0) at 25 (control), 30, 35, 40, 45, and 50°C under light conditions for 24 h (50 µmol m⁻² s⁻¹) in a growth chamber (LH-30-8CT; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). After exposure to heat stress treatment, the leaf disks were used for further analyses. Analyses were performed with three to nine replicates containing four leaf disks each.

To assess the effect of heat stress on coffee plants, 10–14-month-old plants were exposed to heat stress in a growth chamber (LPH-2205; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) by incubation at 40/30°C (day/night) for 10 days or 45/35°C for 4 days. Control plants were incubated at 25/20°C during the treatments. The photoperiod was 12 h light, and the radiation on the top of the canopy was approximately 350 µmol m⁻² s⁻¹. The plants were irrigated regularly during the experiments to prevent drought stress. The fully expanded uppermost leaves were used for further analyses. Analyses were performed with four replicates containing one plant each.

**Chlorophyll and chlorophyll fluorescence measurements**

The damage induced by heat stress to the photosynthetic apparatus of coffee plants was estimated by chlorophyll parameters of leaf disks and intact plants. Leaf disks exposed to different heat stress treatments were homogenized with 100% ethanol, and the homogenate was kept in the dark for three days. The absorbance of the obtained chlorophyll extract was measured spectrophotometrically at 665 nm and 649 nm, and chlorophyll content was calculated according to the equation of Knudson et al. (1977). Chlorophyll of intact plants was measured non-destructively using a soil plant analysis development (SPAD) meter (SPAD 502; Konica Minolta, Inc., Tokyo, Japan). Measurements were performed on three points on the leaf, and the average SPAD value was calculated.

Chlorophyll fluorescence was measured with a handheld chlorophyll fluorometer (Flour Pen FP110/S; Photon Systems Instruments, Drásov, Czech Republic). Leaf disks and intact plants were placed in the dark for 30 min. The maximum quantum yield of Fv/Fm of photosystem II (PSII) in the leaf samples was calculated using the parameters of the minimal (F₀) and maximum (Fₘ) fluorescence yields.

**Electrolyte leakage**

We determined electrolyte leakage, which is often used as an indicator of cell membrane damage by oxidative stress, according to the protocol described by Dionisio-Sese and Tobita (1998). Leaf disks were washed with deionized water and then incubated in 40 mL deionized water at 25, 30, 35, 40, 45, and 50°C for 24 h under light conditions (50 µmol m⁻² s⁻¹). After the incubation, the initial conductance (Cᵢ) of the solution was measured using a conductance meter (CT-27112B; DKK-TOA Corp, Tokyo, Japan). To measure electrolyte leakage of intact plants, four leaf disks were punched from fully expanded uppermost leaves of plants exposed to aforementioned heat stress treatments. Leaf disks were incubated in 40 mL deionized water at 25°C for 24 h and then the Cl solution of the solution was measured. The solutions from both experiments were autoclaved at 120°C for 20 min for electrolyte extraction. The conductance (Cₘₐₓ) of the autoclaved solution was determined after cooling to room temperature (approximately 25°C). Relative electrolyte leakage was calculated as (Cᵢ/Cₘₐₓ) × 100.

**O₂⁻ and H₂O₂**

The production of O₂⁻ and H₂O₂ was quantitatively determined by the methods described by Kuźniak et al. (1999) and Orendi et al. (2001), respectively, with slight modifications. For the determination of O₂⁻, leaf disks were incubated in 10 mM potassium phosphate buffer containing 10 mM NaN₃ and 0.05% nitro blue tetrazolium. The production of O₂⁻ was assessed by the ability of leaf disks to reduce nitro blue tetrazolium in the medium. For the determination of H₂O₂ generation, leaf disks were incubated in 50 mM potassium phosphate buffer composed of 0.05% of guaiacol and 2.5 Unit of peroxidase from horseradish. In both experiments, leaf disks were incubated in the reaction mixture at 25, 35, 40, and 45°C for 6 h under continuous light condition, and then the absorbance at 580 nm and 470 nm was measured spectrophotometrically to estimate O₂⁻ and H₂O₂ contents, respectively. The velocity of O₂⁻ and H₂O₂ production was calculated after 6 h of exposure of leaf disks to heat stress.

**Enzyme activity**

All assays were performed at 0–4°C. For the determination of SOD activity, 0.1 g of leaf disks and the leaves from intact plants were homogenized with 50 mM HEPES buffer (pH 7.6) containing 0.1 mM Na₂EDTA. The homogenate was centrifuged at 15,000 × g for 20 min at 4°C to obtain a crude extract. The crude extract was dialyzed using centrifugal filters (Amicon Ultra-10 K; Merck Millipore Ltd., Cork, Ireland) to remove low-molecular-weight compounds interfering with SOD activity. SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium according to Yamane et al. (2004).
For the determination of APX and CAT activities, 0.1 g of leaf disks and leaves from intact plants were homogenized in 50 mM potassium phosphate buffer (pH 7.8) with 1 mM EDTA, 7 mM mercaptoethanol, 0.1% (w/v) Triton X-100, 5 mM sodium ascorbate, and 1.0% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 × g for 10 min and the supernatant was used for further assays. The activities of APX and CAT were measured according to Nakano and Asada (1981) and Aebi (1974), respectively.

Protein in the supernatant was estimated by the Coomassie brilliant blue dye binding method using bovine serum albumin as a standard according to Bradford (1976).

**Figure 1.** Chlorophyll content and electrolyte leakage of coffee leaf disks incubated at different temperatures under light conditions. Data are means of six replicates. Vertical bars indicate standard errors. The symbols * and ** indicate significant differences from the control 25°C treatment at p < 0.05 and p < 0.01, respectively, according to Dunnett's multiple comparison test.

For the determination of APX and CAT activities, 0.1 g of leaf disks and leaves from intact plants were homogenized in 50 mM potassium phosphate buffer (pH 7.8) with 1 mM EDTA, 7 mM mercaptoethanol, 0.1% (w/v) Triton X-100, 5 mM sodium ascorbate, and 1.0% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 × g for 10 min and the supernatant was used for further assays. The activities of APX and CAT were measured according to Nakano and Asada (1981) and Aebi (1974), respectively.

Protein in the supernatant was estimated by the Coomassie brilliant blue dye binding method using bovine serum albumin as a standard according to Bradford (1976).

**Measurement of leaf temperature**

Leaf temperature of intact plants was continuously monitored using infrared thermography (G100; Nippon Avionics Co., Ltd, Yokohama, Japan). The average temperature was calculated from three points on the leaf.

**Statistical analysis**

Means were compared using Student's t-test in Excel with an add-in software (Esumi, Co., Ltd, Tokyo, Japan) when the sample size was two; statistical significance was set at p < 0.05. The mean values were compared at p < 0.05. When the sample size was more than three, one-way
analysis of variance (ANOVA) was applied for statistical analysis using the same software. If the result of the ANOVA was significant, post hoc analysis was conducted using Dunnett’s multiple comparison test or Tukey’s multiple comparison test, with the levels of statistical significance set at $p < 0.05$ and $0.01$.

**Results**

**Leaf disk analyses**

In comparison with the control (25°C), chlorophyll content of leaf disks under light conditions increased at 35°C but significantly decreased at higher temperatures (Figure 1). The electrolyte leakage in leaf disks significantly increased at 45 and 50°C compared with that at 25°C under light conditions (Figure 1).

PSII photoinhibition was measured as a decrease in $F_v/F_m$ (Figure 2). A significant decrease in $F_v/F_m$ was observed at temperatures above 40°C compared with that at 25°C, and this decrease was due to the reduction in $F_m$.

The content of O$_2^-$ and H$_2$O$_2$ tended to increase with increasing temperatures compared with that at 25°C. However, a significant increase was observed only at 45°C when compared with those at 25°C.

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**Figure 2.** Chlorophyll fluorescence in coffee leaf disks incubated at different temperatures under light conditions. $F_v/F_m$, maximal efficiency of photosystem II (PSII) photochemistry in the dark-adopted state. $F_v = F_m - F_0$, where $F_0$, minimal fluorescence yield; $F_m$, maximum fluorescence yield. Data are means of six replicates. Vertical bars indicate standard errors. The symbols * and ** indicate significant differences from the control 25°C treatment at $p < 0.05$ and $p < 0.01$, respectively, according to Dunnett’s multiple comparison test. The term of ND means not detected.

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**PSII photoinhibition**

$F_v/F_m$, $F_0$, $F_m$, $F_0$, $F_m$.
Figure 3. Content of $O_2^-$ and $H_2O_2$ in coffee leaf disks incubated at different temperatures under light conditions. Data are means of nine replicates. Vertical bars indicate standard errors. The symbols * and ** indicate significant differences from the control 25°C treatment at $p < 0.05$ and $p < 0.01$, respectively, according to Dunnett's multiple comparison test.

Figure 4. Antioxidant enzyme activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) in coffee leaf disks incubated at different temperatures under light conditions. Data of APX and CAT are means of four replicates. Data of SOD are means of three replicates. Vertical bars indicate standard errors. The symbols * and ** indicate significant differences from the control 25°C treatment at $p < 0.05$ and $p < 0.01$, respectively, according to Dunnett's multiple comparison test.
Figure 5. Photographs and thermographic images of coffee leaves under control and heat stress during the 10-day experiment. Leaf photographs were taken before the treatments. Red and green lines indicate leaf contours under control and heat stress treatments, respectively. Temperature was measured at three points (A, B, and C) on the leaf, and their means plotted on the graph were calculated from three replicate plants. Vertical bars (red) indicate standard errors. The symbol ++ indicates significant difference among the treatments at \( p < 0.01 \) according to ANOVA.
The activities of the antioxidant enzymes under heat treatments were measured (Figure 4). SOD activity decreased with increasing temperature, and a significant decrease was observed at 45°C compared with that at 25°C. While APX activity significantly increased at 40°C, the activities at the other temperatures were comparable to that at 25°C. Moreover, CAT activity decreased with increasing temperature, and significant decreases were observed at 40 and 45°C compared with that at 25°C.

**Intact plant analyses**

The leaf temperatures of control plants incubated at 25°C ranged from 26 to 28°C during the experiment. However, the leaf temperature of plants incubated at 40°C was approximately 39°C during the experiment (Figure 5). The temperatures of leaves under control and heat stress were constant throughout the 10-day incubation period (Figure 5). In contrast, the surface leaf temperature of intact plants exposed to 45°C increased

![Figure 6](image_url). Photographs and thermographic images of coffee leaves under control and heat stress conditions during the four-day experiment. Photographs were taken before the treatments. Red and green lines indicate leaf contours under control and heat stress treatments, respectively. Temperature was measured at three points (A, B, and C) on the leaf, and the means plotted on the graph were calculated from three replicate plants. Vertical bars (red) indicate standard errors. The symbol ++ indicates significant difference among the treatments at $p < 0.01$ according to ANOVA. Significant results of the two-way ANOVA were analyzed post hoc using Tukey's multiple comparison test. The same letters indicate no significant differences between different hours in each temperature at $p < 0.01$ according to Tukey's multiple comparison test.
during the four-day experiment to 38.9, 41.6, 44.0, and 46.3°C at 24, 48, 72, and 96 h of the experiment, respectively (Figure 6).

Both SPAD and electrolyte leakage were affected only by 45°C after 96 h; the SPAD values were significantly decreased and electrolyte leakage was significantly increased in comparison with those of the control (Figure 7).

The $F_{v}/F_m$ ratio and $F_0$ of the leaves were not affected by exposure to 40°C for 10 days; the slight decrease of $F_{v}/F_m$ was due to the significant decrease in the value of $F_m$ (Figure 8). The $F_{v}/F_m$ of the leaves of intact plants incubated at 45°C after 72 h significantly decreased compared with that at 25°C, and the decrease was due to a significant decrease in $F_m$ (Figure 8). The $F_{v}/F_m$ at 45°C drastically decreased after 96 h, with a significant increase and decrease in $F_0$ and $F_m$, respectively (Figure 8).

The effect of heat treatments on the activities of SOD, APX, and CAT was investigated (Figure 9). The temperature of 45°C significantly decreased the activities of SOD, APX and CAT in comparison with the temperature at 25°C.

Discussion

Temperature resistance threshold of coffee plants

A few reports have suggested that the temperature at which damage is induced in coffee species is relatively high compared to that of other plants (Martins et al., 2016; Rodrigues et al., 2016). In the experiment using intact plants, the SPAD value and electrolyte leakage in leaves exposed to 45°C for 72 h were similar to that of the control (Figure 7). However, the decrease in $F_{v}/F_m$ was observed (Figure 8). The decrease in $F_m$ was observed (Figure 8, left and middle), which may indicate an increase in non-photochemical quenching (Bolhar-Nordenkampf et al., 1989). In contrast, the increase in $F_0$, which may correlate with the damage of thylakoid membranes (Yamane et al., 2008), was not observed. These results suggest that excess energy in chloroplasts could be dissipated by heat, resulting in the suppression of leaf damage after exposure to 45°C for 72 h.

The leaf surface temperature in an intact coffee plant exposed to 45°C for 72 h was 44.0°C (Figure 6). In leaves exposed to 40°C for 10 days, the SPAD value and electrolyte leakage were similar to those in the control (Figure 7),

![Figure 7. Soil plant analysis development (SPAD) value and electrolyte leakage of coffee leaves exposed to 40°C for 10 days (left), 45°C for 72 h (middle), and 96 h (right). Control plants were incubated at 25°C. Data are means of four replicate plants. Vertical bars indicate standard errors. The symbol ** indicates significant differences from the control 25°C treatment at p < 0.01 according to Student’s t-test.](image-url)
there was no decrease in $F_v/F_m$ (Figure 8). The leaf surface temperature was 39.1°C (Figure 5). These results suggest that *C. arabica* 'Typica' might be able to suppress leaf damage if its surface temperature remains below 44°C.

Using intact coffee plants, we observed a decrease in SPAD value, an increase in electrolyte leakage (Figure 7), and a decrease in $F_v/F_m$ with the increase in $F_0$ (Figure 8) in leaves exposed to 45°C for 96 h. The decrease in $F_v/F_m$ owing to the increase in $F_0$ correlates with the damage of thylakoid membranes (Yamane et al., 2008). The surface temperature of the leaf in an intact coffee plant exposed to 45°C for 96 h was 46.3°C (Figure 6). The results indicated that a tolerance threshold in leaves of *C. arabica* 'Typica' under heat stress might be between 44.0 and 46.3°C. In field conditions, however, other stresses such as high light and drought are simultaneously generated with heat stress.
(Choudhury et al., 2017). Thus, the temperature that may induce damage in coffee plants under field conditions may be lower than that observed in the present study.

In leaf disks, chlorophyll content and \( F_{v}/F_{m} \), both serving as the indicators of chloroplast damage, significantly decreased at 40°C compared with those at 25°C (Figures 1 and 2). However, those decreases were not observed in intact coffee plants even after their exposure to 40°C for 10 days (Figures 7 and 8). Although the detailed mechanisms of this decrease in leaf disks are still unknown, we considered that chloroplasts become sensitive to heat stress due to the separation from intact plants. Another possibility is that coffee plants exposed to 40°C for 10 days were able to acclimate during the stress treatment, resulting in the maintenance of \( F_{v}/F_{m} \) (Figure 8).

The trend of \( F_{0} \) was also different between intact and cut leaves (Figures 2 and 8). Considering that the leaf damage, indicated by the decrease in chlorophyll content and the increase in electrolyte leakage, was observed in leaf disks at 45°C, it was speculated that \( F_{0} \) in leaf disks would increase. However, the \( F_{0} \) value at 45°C was similar to that in the control (25°C). This could be the artifact of leaf disk method. Thus, it may be challenging to use \( F_{0} \) as an indicator of thylakoid damage in leaf disks under heat stress. The indicator of \( F_{0} \) may be effective in the estimation of severe thylakoid damage for longer stress treatment using intact plants.

**The mechanism of oxidative damage under heat stress**

We observed that higher temperatures decreased the activities of SOD and CAT in both experiments and increased the production of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in the leaf disk experiment. These results suggest that the maintenance of SOD and CAT activities in a cell under heat stress is important for an effective scavenging of ROS. In the experiment using leaf disks, the temperatures above 40°C significantly reduced the activity of CAT compared with that at 25°C (Figure 4). However, the activity of APX significantly increased at 40°C compared with that at 25°C, and the activity at 45°C was comparable to that of the control (Figure 4). These results suggest that as APX localizes in both cytosol and chloroplasts (Shigeoka et al., 2002), \( \text{H}_2\text{O}_2 \) generated in the region may be scavenged by the APX, and APX may control \( \text{H}_2\text{O}_2 \) concentrations in leaf disks. However, as this phenomenon was observed only in leaf disks, the \( \text{H}_2\text{O}_2 \) control by APX under heat stress in *C. arabica 'Typica'* is controversial. In this variety, SOD and CAT activities in both leaf disks (Figure 3) and intact plants (Figure 8) significantly decreased at 45°C, and \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) content in leaf disks significantly increased (Figure 3). Thus, the decrease in the activity of SOD and CAT under heat stress may be responsible for the increase in \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \), resulting in cellular damage.

**Conclusion and future prospects**

For the future of coffee breeding, efforts should focus on screening heat tolerant varieties. As growing coffee plants takes several months, destructive sampling to assess their stress tolerance is ineffective. In addition, homogeneous growth is needed for screening. These features may hinder selection and breeding of tolerant coffee plants. Here, we used leaf disks and intact plants to determine temperature tolerance threshold and the mechanism of oxidative damage under heat stress. The temperature tolerance
threshold, especially observed in electrolyte leakage parameter, was similar between leaf disks and the leaves of intact plants. Thus, the leaf disk method might be useful for the future screening of heat-tolerant coffee cultivars. However, a few contradictions were observed between leaf disks and intact plants. Leaf greenness in leaf disks significantly decreased at 40°C, although the SPAD value in intact plants was similar to that of the control. A similar trend was observed for $F_v/F_m$. Taken together, these results suggest that electrolyte leakage is a good parameter to estimate heat tolerance.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

ORCID

Koji Yamane http://orcid.org/0000-0002-0332-8672
Yoshihiro Hirooka http://orcid.org/0000-0001-9457-6382
Morio Iijima http://orcid.org/0000-0002-2006-0700

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