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RESEARCH PAPER

Heat stress increases the use of cytosolic pyruvate for isoprene biosynthesis

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Abstract

The increasing occurrence of heatwaves has intensified temperature stress on terrestrial vegetation. Here, we investigate how two contrasting isoprene-emitting tropical species, *Ficus benjamina* and *Pachira aquatica*, cope with heat stress and assess the role of internal plant carbon sources for isoprene biosynthesis in relation to thermotolerance. To our knowledge, this is the first study to report isoprene emissions from *P. aquatica*. We exposed plants to two levels of heat stress and determined the temperature response curves for isoprene and photosynthesis. To assess the use of internal C sources in isoprene biosynthesis, plants were fed with ^{13}C position-labelled pyruvate. *F. benjamina* was more heat tolerant with higher constitutive isoprene emissions and stronger acclimation to higher temperatures than *P. aquatica*, which showed higher induced isoprene emissions at elevated temperatures. Under heat stress, both isoprene emissions and the proportion of cytosolic pyruvate allocated into isoprene synthesis increased. This represents a mechanism that *P. aquatica*, and to a lesser extent *F. benjamina*, has adopted as an immediate response to sudden increase in heat stress. However, in the long run under prolonged heat, the species with constitutive emissions (*F. benjamina*) was better adapted, indicating that plants that invest more carbon into protective emissions of biogenic volatile organic compounds tend to suffer less from heat stress.

Keywords: *Ficus benjamina*, heat stress, isoprene biosynthesis, *Pachira aquatica*, photosynthesis, pyruvate.

Introduction

Climate models predict an increase in global ambient air temperatures of 1–5 °C by the end of this century (IPCC, 2013). Heatwaves, defined as prolonged periods of excessive heat (consecutive days that are excessively hotter than average; Perkins *et al.*, 2013), are already increasing in frequency and models predict further increases in the future (Kleist *et al.*, 2012; Perkins *et al.*, 2012; IPCC, 2013; Deryng *et al.*, 2014; Perkins-Kirkpatrick and Gibson, 2017). Tropical regions in particular are expected to experience extremely large increases in heatwaves (Herold *et al.*, 2017), with their cumulative durations predicted to last up to 120 extra days per season under the scenario of global warming of 5 °C (Perkins-Kirkpatrick and Gibson, 2017).

As sessile organisms, plants have evolved a wide variety of strategies to thrive in their natural habitats, such as adaptations to heat and drought (Rennenberg *et al.*, 2006; Arab *et al.*, 2016);

however, when environmental factors shift beyond a certain threshold, plants are subject to stress (Lichtenthaler, 1998; Wahid *et al.*, 2007). In particular, when stresses persist, metabolic homeostasis can be disrupted, while an adjustment of the metabolic pathways may result in acclimation (Lichtenthaler, 1998; Shulaev *et al.*, 2008). However, excessive heat stress, i.e. a rise in temperature beyond optimal functioning, can cause irreversible damage to plant growth and development (Wahid *et al.*, 2007). The severity of heat stress depends on the intensity and duration as well as species-specific adaptations to tolerate elevated temperatures. In order to cope with heat, plants have developed numerous mechanisms, such as scavenging of the reactive oxygen species (ROS) that are induced by excess heat through the biosynthesis of antioxidants (Wahid *et al.*, 2007; Dietz *et al.*, 2016). Another important mechanism to alleviate the consequences of heat stress is the maintenance of thylakoid membrane stability, which helps protect the photosynthetic apparatus and allows photosynthetic CO_2 fixation to continue at elevated temperatures (Rennenberg *et al.*, 2006; Zhang and Sharkey, 2009; Niinemets, 2018). Volatile isoprenoids such as isoprene and monoterpenes are believed to constitute an important component in the plant's arsenal against oxidative stress (Holopainen and Gershenzon, 2010). These volatiles reduce the levels of damaging ROS within the leaf and thus increase thermotolerance (Loreto and Velikova, 2001; Affek and Yakir, 2002; Loreto and Fares, 2007; Sharkey *et al.*, 2008), although this comes at a very high carbon and energetic cost (Harvey *et al.*, 2015). In addition, volatiles such as isoprene may contribute to the stabilization of cell membranes and integrated membrane proteins (Singsaas *et al.*, 1997; Sharkey and Yeh, 2001; Peñuelas and Munné-Bosch, 2005; Behnke *et al.*, 2007; Velikova *et al.*, 2011), although it has been shown more recently that the isoprene content in thylakoid membranes may be too low to affect the membrane dynamics (Harvey *et al.*, 2015). A fascinating role of isoprene as signalling compound has been proposed by (Harvey and Sharkey (2016) as this it seems to modulate plant gene expression (Zuo *et al.*, 2019).

Isoprene is produced in chloroplasts via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Schwender *et al.*, 1996; Lichtenthaler *et al.*, 1997; Lichtenthaler, 1999; Dudareva *et al.*, 2006), which forms the precursor of isoprenoids, dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP). The MEP pathway involves the formation of 1-deoxy-d-xylulose 5-phosphate (DXP), which is derived from glyceraldehyde-3-phosphate (GA-3P) and the decarboxylation of pyruvate (Schwender *et al.*, 1997; Sharkey *et al.*, 2008). Because these compounds are products of the Calvin–Benson cycle, isoprenoid biosynthesis in plastids is directly coupled to photosynthesis. Due to this close connection, environmental factors that influence photosynthesis also exert control over isoprenoid biosynthesis. The main drivers for isoprene emission are therefore solar radiation and temperature (Monson *et al.*, 1992; Harley *et al.*, 1996). Consistent with this view, $^{13}CO_2$ labelling experiments have demonstrated that under normal, non-stress conditions, ~80% of the carbon emitted as isoprene is directly derived from recently fixed CO_2, while the remaining ~20% may be provided from 'alternative' C sources internal to the leaf, such as starch or sugars transported in the xylem (Kreuzwieser *et al.*, 2002; Schnitzler *et al.*, 2004). The mechanism by which such alternative C enters isoprenoid biosynthesis is still not fully understood; the observation that the extent of isoprene labelling reflects the labelling of intermediates of the Calvin–Benson cycle may support the view that all carbon in isoprene comes directly out of the cycle (Delwiche and Sharkey, 1993; Sharkey and Monson, 2014). However, alternative carbon sources for isoprene biosynthesis might be cytosolic phospho*enol*pyruvate (PEP) or pyruvate entering the MEP pathway (Rosenstiel *et al.*, 2004) or other compounds that are directly channeled into the Calvin–Benson cycle after uptake into the chloroplasts, for example as triose-phosphates via P_i-transporters (Anoman *et al.*, 2016; de Souza *et al.*, 2018). Under stress conditions that cause reduction of photosynthesis, the portion of leaf internal carbon sources can indeed increase (Kreuzwieser *et al.*, 2002; Funk *et al.*, 2004; Trowbridge *et al.*, 2012).

In the present study, we selected two species, *Pachira aquatica* (Malabar chestnut) from the Malvaceae family and *Ficus benjamina* (weeping fig) from the Moraceae family, that originate from contrasting habitats. *P. aquatica* is a woody evergreen wetland species native to a region stretching from southern Mexico to north-eastern Brazil (Oliveira *et al.*, 2000; Lorençon *et al.*, 2016; Cheng *et al.*, 2017) and is abundant in mangrove areas (Infante-Mata *et al.*, 2014). In contrast, *F. benjamina* is native to mixed tropical and sub-tropical deciduous forests in Australia and South-East Asia (Veneklaas *et al.*, 2002), and is also abundant in disturbed areas (Pakkad *et al.*, 2001). According to their natural habitats, *P. aquatica* is highly tolerant to flooding, drought, and salt stress (Infante-Mata *et al.*, 2014) whilst *F. benjamina* shows high tolerance to drought and heat stress (Cokuysal *et al.*, 2006; Qados, 2015). Both species possess a very active secondary metabolism, and store large amounts of chemical defense compounds in different tissues (Lansky *et al.*, 2008). For example, *P. aquatica* accumulates high contents of the fungitoxin isohemigossypolone in the outer bark of the below-ground part of the swollen trunk (Shibatani *et al.*, 1999) and it has been found to be a weak isoprene emitter (Taylor *et al.*, 2018). *Ficus* species contain numerous secondary plant metabolites such as alkaloids (Baumgartner *et al.*, 1990; Khan *et al.*, 1993; Novelli *et al.*, 2014) and in contrast to *P. aquatica* they have been found to be strong isoprene emitters (Wyche *et al.*, 2014). Many bioactive constituents such as cinnamic acids, lactose, naringenin, quercetin, caffeic acid, and stigmasterol as well as 28 different alkaloids have been identified in *F. benjamina* (Novelli *et al.*, 2014).

The aim of the study was to investigate how these two contrasting species would cope with a prolonged 36-d heatwave: with global warming of 3 °C, heatwaves in the tropics are predicted to be as long as 40–50 d, Perkins-Kirkpatrick and Gibson, 2017). We tested the hypotheses that the strong isoprene emitter *F. benjamina* is better adapted to heat than the weaker isoprene emitter *P. aquatica*, and that prolonged exposure to heat stimulates the use of internal C sources for isoprene biosynthesis due to reduced rates of photosynthetic CO_2 fixation. We further hypothesized that both plants would induce higher isoprene emissions under heat stress as long as carbon sources were available to maintain biosynthesis.

Material and methods

Plant material and growth conditions

Individual plants of *Ficus benjamina* L. and *Pachra aquatica* Aubl. were purchased from a garden center and were grown in 3-l pots filled with 2/3 soil multiplication substrate (Floragard, Oldenburg, Germany) and 1/3 sand. Since the plants were purchased from a single source, a high level of genetic homogeneity is likely. All plants were supplied with 50 ml Wuxal fertilizer solution (AGLUKON Spezialdünger, Duesseldorf, Germany) once per week. The plants were watered according to their demand between 100–250 ml three times a week. They were grown in a climate-controlled chamber (ThermoTec Weilburg, Germany) under conditions of 25 °C day/night air temperature at 60% relative humidity, with a 12-h photoperiod at a photon flux density of ~600 µmol m^{-2} s^{-1}. The number of replicates used for the treatments in each experiment is shown in Supplementary Fig. S1 at *JXB* online.

Experimental set-up

The cuvette system used for the measurement of volatiles consisted of 600-ml cuvettes made of either borosilicate glass (Kummer, Freiburg, Germany) for *F. benjamina*, or of FEP foil (PFTE Spezialvertrieb, Germany) for *P. aquatica*. Two different types of cuvettes were necessary due to the very different shapes of twigs and leaves of the two species. In the *P. aquatica* cuvettes, a small ventilator (Conrad Electronic, Germany) was used to improve air mixing. Branches of both species were inserted into the bottomless cuvettes which were then closed tightly at the stem with FEP foil, taking care not to damage the stem surface. Hydrocarbon-free, humidified, and CO_2-controlled (400 ppm) air was generated using a specially constructed 'zero air' generator (Fasbender *et al.*, 2018). The air flow at the cuvette inlets was regulated by mass-flow controllers (Omega Engineering, Stanford, USA) and kept constant at 500 ml min^{-1}, which resulted in a calculated residence time of 60 s in the cuvettes. The outlet air flow was split between the different analytical devices (see below). The response-time of air leaving the cuvettes and reaching the analytical devices was less than 10 s. In operational mode, the analytical instruments were supplied with an air flow of ~400 ml min^{-1} resulting in a slight overpressure in the cuvettes. To ensure chemical inertness, all the parts of the cuvette system were made of glass or FEP, and the outlet lines were isolated and continuously heated to 60 °C to prevent adsorption of biogenic volatile organic compounds (BVOCs) or water condensation. The cuvette air temperature increased by 2 °C (from 25±0.5 °C to 27±0.5 °C) compared to the growth chamber during the light period, which is an inherent property of cuvette systems and was taken into account in the data analysis. Due to the lack of temperature sensors, the actual leaf temperatures were unknown. In all experiments one cuvette type for each plant species was kept empty as a reference and these were always measured for 10 min every hour for the heat-stress and temperature-dependence experiment, and for 10 min at the beginning and the end of labelling experiments.

Experimental design

Heat stress

Six plants of each species were kept under normal growth conditions as detailed above at 25 °C day/night for 90 d to determine control rates of net CO_2 assimilation and isoprene emission. Thereafter, all plants were exposed to a temperature of 33 °C for 15 d and then to 38 °C for a further 21 d. Throughout the experiment, the plants were watered according to their demand to avoid the occurrence of drought stress, particularly during the period of the heat treatment. During the experiment, *F. benjamina* grew by 5–10 cm while *P. aquatica* did not show any signs of growth. Moreover, *F. benjamina* developed many new leaves during the 33 °C heat treatment, which became significantly larger than those grown at 25 °C. In contrast, *P. aquatica* exhibited clear signs of leaf senescence at 38 °C. Consequently, the heat intensity was limited to a maximum of 33 °C in subsequent labelling experiments because we were more interested in following carbon allocation during acclimation rather than after irreversible damage.

Temperature dependence of isoprene emission

To characterize the temperature dependence of isoprene emission of control and heat-stressed plants, six plants of *F. benjamina* and four plants of *P. aquatica* were used. Plants of each species were kept under control (25 °C day/night) and heat stress (33 °C day/night) for 5 d. Following this, branches were placed in the cuvettes the night before the experimental treatments to ensure stabilization of conditions. At 2 h before the beginning of the photoperiod the chamber temperature was set to 20 °C. From the start of the photoperiod, the chamber temperature was increased in 5 °C steps every 2 h. The temperature steps were set to 20, 25, 30, 35, and 40 °C, after which the temperature was abruptly set back to 20 °C. As it took ~15 minutes for each new temperature level to be attained in the climate chamber, only the last data points for each temperature step were used for analysis.

Labelling method

Plants of *P. aquatica* and *F. benjamina* were subjected to either 25 °C or 33 °C for 5 d and measurements were taken at these temperatures. To ensure stabilized conditions, branches were placed into cuvettes the evening before the experimental treatments. To characterize the rates of isoprene emission of the intact plants, each branch was measured for 5 min. Following this, the branches were carefully cut without changing their position in the cuvette. To avoid xylem embolism, the branches were immediately recut under water and the cut end was placed in deionized water. To feed ^{13}C-labelled pyruvate to the leaves via the transpiration stream, after 10 min the deionized water was replaced by a solution of 10 mM sodium pyruvate (pH 6.5) with the pyruvate 99% labelled with ^{13}C at the [1-C]- or [2,3-C]- position (Cambridge Isotope Laboratories, MA, USA). The concentration was selected so as not to induce any changes in the metabolism (Priault *et al.*, 2009), as the pyruvate pools is small but with a high turnover rate. It should be noted that this labelling approach is sufficient for tracing the carbon flow through different metabolic pathways but it is not expected to fully label the internal pyruvate pool. Emissions of the three isoprene isotopologues (m/z 69.07, 70.07, and 71.07 for 0, 1, and 2 ^{13}C-atoms, respectively) were followed for 60 min after applying the ^{13}C-label. The amount of pyruvate uptake did not differ significantly between the two species and the two heat regimes (*F. benjamina* took up 0.25±0.08 g h^{-1} and 0.27±0.08 g h^{-1} for control and heat conditions, respectively; and *P. aquatica* took up 0.50±0.17 g h^{-1} and 0.43±0.25 g h^{-1}, respectively).

Determination of leaf area

The leaf area of the branches in each cuvette was determined after each experiment using a CanoScan LiDE 110 scanner (Canon) and the GSA Image Analyser software v.4.09. Emission rates are presented on the basis of m^{-2} leaf area.

Analytical methods

Online isoprene measurements were conducted using a 4000 ultra proton-transfer-reaction–time-of-flight–mass spectrometer (PTR-TOF-MS) (Ionicon Analytic, Innsbruck, Austria). The PTR-TOF-MS was operated at 2.7 mbar drift pressure, 600 V drift voltage, at an E/N of 120 Td, and drift tube heated to 80 °C. More detailed information can be found in Fasbender *et al.* (2018). We measured ^{12}C-labelled isoprene (m/z 69.07) and its isotopologues containing one ^{13}C-atom (m/z 70.07, hereafter referred to as the single-labelled isotopologue) and two ^{13}C-atoms (m/z 71.07, hereafter referred to as the double-labelled isotopologue). Humidity-dependent calibration of isoprene was done with a multicomponent calibration gas standard (1000 ppb ±5%, Ionicon Analytic) using a Liquid Calibration Unit (LCU, Ionicon Analytic).
Isoprene emissions were calculated as:

$$e = (u_i/s) \times (c_o c_i) \quad (1)$$

Where u_i is the molar flux into the cuvette (mol s^{-1}), s is the leaf area in the cuvette in m^2, c_o is the mixing ratio at the outlet of the cuvette, and c_i is the mixing ratio at the cuvette inlet (both mol mol^{-1}).

Photosynthetic gas exchange was quantified using a differential infrared gas analyser (LI-7000 CO_2/H_2O Analyzer; LI-COR) as the differences of CO_2 and H_2O concentrations between empty and plant-containing cuvettes. Transpiration, assimilation rate, and stomatal conductance to water vapor were calculated according to von Caemmerer and Farquhar, (1981).

$^{13}CO_2$ flux (nmol m^{-2} s^{-1}) was quantified by accounting for the difference in CO_2 isotopic composition and CO_2 concentration between empty and plant-containing cuvettes as analysed by a Delta Ray Isotope Ratio Infrared Spectrometer (IRIS, ThermoFisher Scientific). The analyser operated at a sample flow rate of 80 ml min^{-1} with a temporal resolution of 1 s and an analytical precision of $\delta^{13}CO_2$ of 0.15‰. More information about operation and calculation of net fluxes of $^{13}CO_2$ can be found in Fasbender *et al.* (2018).

Cross-validation of identification of isoprene

The PTR-TOF-MS method is a mass-selective technique, and hence isomers could interfere with the measured masses: isoprene measurements could be affected by another compound with the same mass, i.e. prenol. Therefore, thermodesorption–GC-MS (TD-GC-MS) was used to verify the results of PTR-TOF-MS, as this technique is able to distinguish between compounds with the same mass. Samples were collected using adsorbent tubes filled with two different sorbents: 180 mg Tenax TA 60/80 (35 m^2 g^{-1}) followed by 130 mg of Carbograph 1 (90 m^2 g^{-1}). More information can be found in Kesselmeier *et al.* (2002). The adsorbent tubes were analysed using an Agilent 7890B Gas-Chromatograph (GC) using an INSIGHT® flow modulator (SepSolve Analtical, UK). The GC was coupled with a time-of-flight-mass-spectrometer (BenchTOF Select, MARKES International, UK) as a detector. Samples were desorbed into the GC system using a two-stage automated thermal desorber (TD100-xr, MARKES International). The carrier gas used was helium. The sample was then removed from the adsorbent tube at a temperature of 250 °C and a flow of 50 ml min^{-1} for 10 min, and was pre-concentrated onto a cold trap (general purpose carbon, MARKES International) at 30 °C. The cold trap was then purged with helium for 1 min with a flow of 50 ml min^{-1} before being rapidly heated to 300 °C. The sample was removed from the cold trap with a flow of 3.5 ml min^{-1} and injected into the column.

The separation of the sampled compounds was achieved in the first dimension using a 25-m MEGA-DEX DMT-Beta column with 0.1 mm internal diameter and a film thickness of 0.15 µm. The separation in the second dimension was achieved using a 4-m mid-polarity BPX50 column with an internal diameter of 0.25 mm. A 22.5-cm sample loop with an internal diameter of 0.53 mm was used to temporarily store the sample components that had already been separated in the first dimension before introduction into the second column. A 4-m deactivated bleed line with an internal diameter of 0.1 mm was also connected to the modulator plate. A modulation time of 3 s was used. The temperature program used was as follows: 40 °C for 5 min, then 40 °C to 150 °C at 3 °C min^{-1} and 150 °C to 200 °C at 30 °C min^{-1}. Following the separation of the compounds, identification was achieved using the BenchTOF operating in tandem ionization mode at 70 eV and 14 eV so that two chromatograms were obtained per sample for each ionization energy. The common ion fragment m/z 67 of isoprene and prenol was first extracted from the total ion count (TIC) of the 70-eV chromatogram to uncover the peaks of isoprene and prenol. Their mass spectra were then compared with the NIST 70 eV electron ionization library. For further confirmation, the headspace of a liquid standard of isoprene was collected onto an adsorbent tube to confirm its retention time. The signal peaks belonging to isoprene and prenol were found in each of the samples. The integrated area of isoprene was found to be a factor of ~100 greater than the integrated area of prenol. Therefore, it was concluded that since the area of the peak of prenol remained relatively low compared to that of isoprene, the prenol emission by the two species could be considered negligible.

Statistics

The number of replicates varied among the different experiments. For heat-stress duration, six replicates for each species were used. For the temperature-dependency curve under control conditions, six replicates were used for *F. benjamina* and four for *P. aquatica*. Under heat conditions, four replicates for each species were used. For the labelling experiment, a minimum of three replicates for each species were used for each of the different treatments. For statistical analysis, Student's *t*-tests were performed using the Sigma plot 2017 software (Systat, USA).

Results

Effect of heat duration on gas exchange and isoprene emission

Exposure to heat (15 d at 33 °C and followed by 21 d at 38 °C) produced strong differences between the two species. Net CO_2 assimilation in *F. benjamina* was relatively constant at 3.6±0.42 µmol m^{-2} s^{-1} at 33 °C and only decreased slightly to 2.74±0.36 µmol m^{-2} s^{-1} by the end of the period of severe stress, although this was not significant (Fig. 1A). Furthermore, *F. benjamina* seemed to acclimate to higher temperatures, which was reflected in initial, transient decreases in assimilation when temperature was increased from 25 °C to 33 °C and from 33 °C to 38 °C followed by a clear recovery within 2 d. In contrast, *P. aquatica* suffered more from heat stress. It was able to maintain CO_2 assimilation at a relatively constant level at 33 °C, but declined rapidly at 38 °C to only 0.3±0.2 µmol m^{-2} s^{-1}; however, this indicated that net photosynthesis was still counterbalancing respiratory losses during the day. Chlorosis of leaves was apparent by day 12, during the 33 °C period.

In both species there was a significant (P<0.001) transient increase in isoprene emission when the temperature increased from 25 °C to 33 °C, and this was more pronounced in *P. aquatica* than in *F. benjamina* (Fig. 1B). From day 6 there was a continuous decline in emissions in *P. aquatica* that became

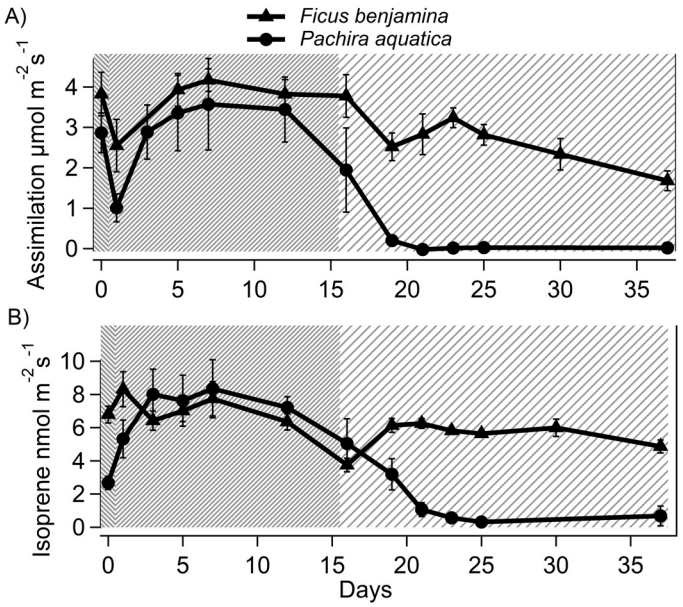

Fig. 1. Effects of prolonged exposure to increasing levels of heat in *Ficus benjamina* and *Pachira aquatica*. Plants were grown at 25 °C for 90 d (indicated by dark shading), and then the temperature was increased to 33 °C (medium shading) and then to 38 °C (light shading). (A) Effects on net CO_2 assimilation rate and (B) on isoprene emission rate. Data are mean midday values (±SE). AQ1

more pronounced when the temperature was further increased to 38 °C. Interestingly, however, the decrease in isoprene emission was not as rapid as that observed for net CO_2 assimilation. In contrast, *F. benjamina* seemed to acclimate to the conditions, and an initial drop in isoprene emission at 38 °C was followed by recovery to levels that were not significantly lower than at 33 °C.

Heat stress modifies the temperature dependence of isoprene emission

Net CO_2 assimilation of *F. benjamina* subjected to control conditions (25 °C) showed a temperature optimum at ~27 °C (T_{opt}) of 5.5±0.3 μmol m^{-2} s^{-1} (Fig. 2A, left). Exposure to 33 °C for 5 d altered the shape of the temperature response curve (Fig. 2A, right); although the maximum CO_2 assimilation rate remained unaffected, net assimilation was greater at higher temperatures compared to control plants. For example, at 42 °C assimilation of *F. benjamina* plants that had been exposed to heat was 3.8±0.7 μmol m^{-2} s^{-1}, which was ~2.5-fold higher than in control plants measured at this temperature. Isoprene emission of *F. benjamina* showed a T_{opt} at 37 °C for plants grown under control conditions, with a rate of 6.4±0.9 nmol m^{-2} s^{-1} (Fig. 2B, left). Exposure to heat shifted the maximum to at least 42 °C and increased the maximum emission rate to at least 10.2±3.2 nmol m^{-2} s^{-1} (Fig. 2B, right).

Exposure to heat exerted similar effects on *P. aquatica* plants, although assimilation and isoprene emission rates were lower than for *F. benjamina*. The T_{opt} for net CO_2 assimilation was 27 °C with a rate of 3.6±0.4 μmol m^{-2} s^{-1}. Following exposure to heat conditions, both T_{opt} and the maximum assimilation rate remained in a comparable range. Similarly, there was no pronounced heat effect on the T_{opt} of isoprene emission; however, the emission rate increased significantly from 2.1±0.5 nmol m^{-2} s^{-1} in control plants to 7.1±0.8 nmol m^{-2} s^{-1} in plants that had been exposed to heat.

Incorporation of ^{13}C into isoprene depends on the labelled position of the pyruvate applied

In order to evaluate the use of alternative carbon sources for isoprene biosynthesis, branches of control and heat-stressed *F. benjamina* and *P. aquatica* plants were fed with position-specific [1-^{13}C]- or [2,3-^{13}C]-labelled pyruvate. For the plants used in this experiment, heat exposure increased isoprene emission in *F. benjamina* from 0.49±0.3 nmol m^{-2} s^{-1} to 2.8±0.4 nmol m^{-2} s^{-1} in control and heat-stressed plants, respectively, whilst in *P. aquatica* fluxes remained relatively constant at 2.9±0.8 nmol m^{-2} s^{-1} to 2.4±0.2 nmol m^{-2} s^{-1}, respectively.. Net CO_2 assimilation remained relatively constant during the course of the experiment (Fig. 3) suggesting that the branches were not appreciably influenced by cutting or application of pyruvate at least for *P. aquatica*.

When the carboxyl group (C1, first carbon in the pyruvate molecule) was decarboxylated from pyruvate at the metabolic branching point, a considerable increase in $^{13}CO_2$ emission was detected when [1-^{13}C]-pyruvate was applied (Fig. 3). Remarkably, we observed a 2-fold (*F. benjamina*) to 3-fold (*P. aquatica*) increase in release of $^{13}CO_2$ under heat conditions in both species: i.e. day-respiration was stimulated by heat, resulting in higher $^{13}CO_2$ release from the plant. Release of $^{13}CO_2$ occurred at higher rates in *F. benjamina* than in *P. aquatica* despite the latter taking up more labelled pyruvate during the course of the experiment (*F. benjamina* control 0.07±0.02 mg s^{-1}, heat 0.08±0.02 mg s^{-1}; *P. aquatica* control 0.14±0.05 mg s^{-1}, heat 0.12±0.07 mg s^{-1}). In contrast, release of $^{13}CO_2$ was considerably lower for both control and heat-treated plants if they were labelled with [2,3-^{13}C]-pyruvate.

Incorporation of ^{13}C into isoprene strongly depended on the form of labelled pyruvate applied in both species (Figs 4, 5). Under [1-^{13}C]-labelling, the isotopologue containing one ^{13}C atom (m/z 70) always increased more than the double-labelled ^{13}C isotopologue (m/z 71.07). In *P. aquatica*, the emission of

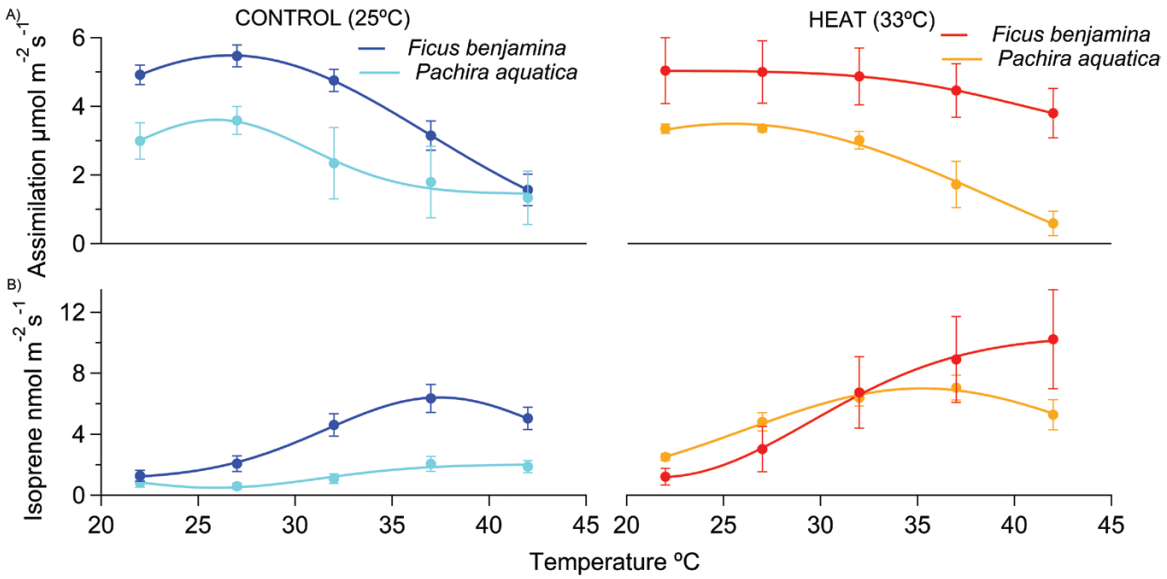

Fig. 2. Temperature dependence of (A) net CO_2 assimilation rate and (B) isoprene emission rate in *Ficus benjamina* and *Pachira aquatica* plants exposed to either control conditions (25 °C) or to heat stress of 33 °C for 5 d. Data are means (±SE) of six and four replicates for controls of *F. benjamina* and *P. aquatica*, respectively, and of four replicates for each species under heat conditions.

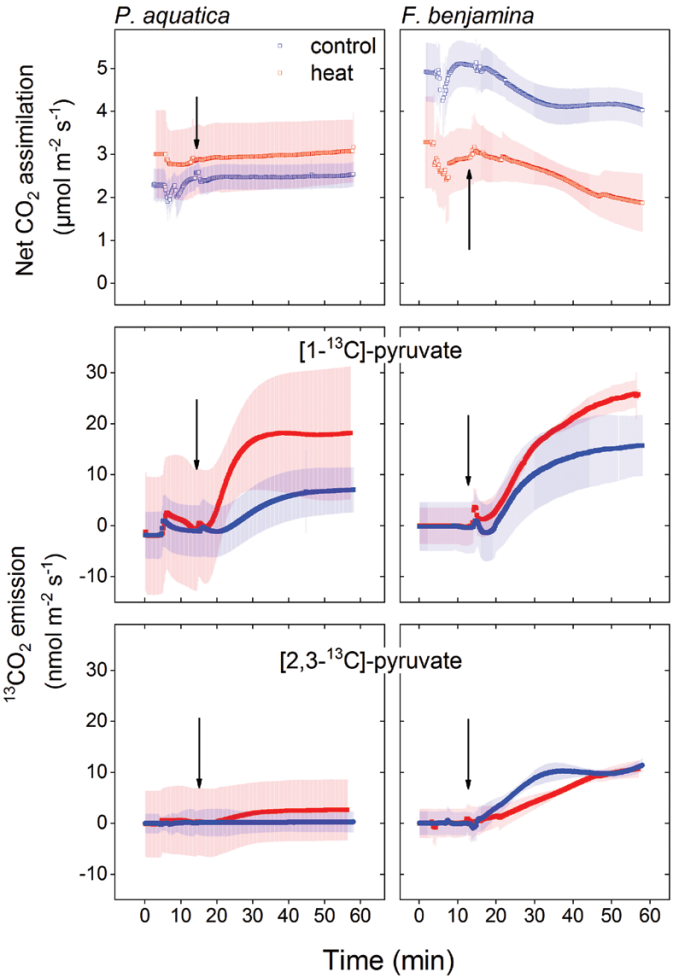

Fig. 3. Effect of heat stress on net CO_2 assimilation rates and $^{13}CO_2$ release in plants of *Ficus benjamina* and *Pachira aquatica*. Plants were exposed to either control conditions (25 °C) or to heat stress at 33 °C for 5 d. Measurements were taken after feeding excised twigs with position-specific labelled pyruvate, namely [1-^{13}C]-pyruvate and [2,3-^{13}C]-pyruvate (time of feeding indicated by arrows). Data are means (±SE) of 4–5 replicates.

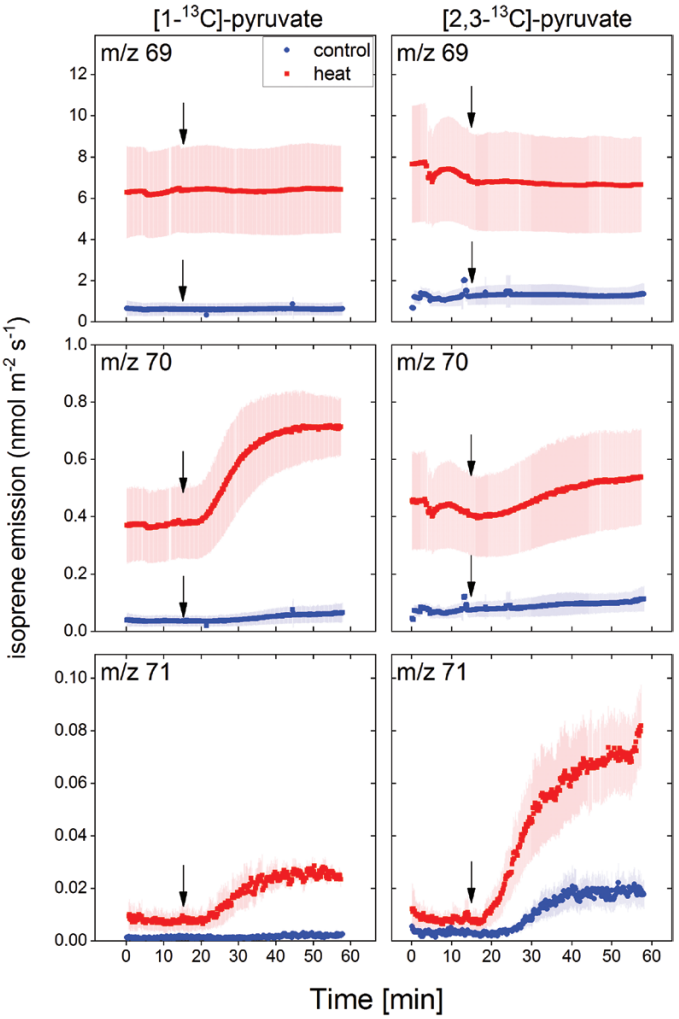

Fig. 4. Temporal dynamics of fluxes of unlabelled and ^{13}C-labelled isoprene isotopologues in plants of *Pachira aquatica* exposed to heat stress. Plants were exposed to either control conditions (25 °C) or to heat stress at 33 °C for 5 d. Measurements of isoprene isotopologues containing no ^{13}C atoms (*m/z* 69), one ^{13}C atom (*m/z* 70), and two ^{13}C atoms (*m/z* 71) were taken after feeding excised twigs with position-specific labelled pyruvate, namely [1-^{13}C]-pyruvate and [2,3-^{13}C]-pyruvate (time of feeding indicated by arrows). Data are means (±SE) of 4–5 replicates.

the single-labelled isotopologue increased by ~2-fold from 0.3±0.1 nmol m^{-2} s^{-1} (pre-label) to 0.7±0.1 nmol m^{-2} s^{-1} (post-label) under heat conditions, whereas it remained constant at 0.06±0.03 nmol m^{-2} s^{-1} in control plants (Fig. 4). The double-labelled isotopologue (*m/z* 71) was only detectable in trace amounts if [1-^{13}C]-pyruvate was applied in control plants, but was ~10-fold higher in heat stressed plants (0.02±0.002 nmol m^{-2} s^{-1}). In contrast, the effects of [1-^{13}C]-pyruvate were less pronounced in *F. benjamina*, although the levels of emission of the single-labelled isotopologue (*m/z* 70) were still ~2-fold higher in heat-stressed plants compared to controls (Fig. 5). There were no really clear differences in the emissions for the double-labelled isotopologue (*m/z* 71).

Feeding [2,3-^{13}C]-pyruvate in *P. aquatica* resulted in relatively small increases in emissions of the single-labelled isotopologue (*m/z* 70) whereas emissions of the double-labelled isotopologue (*m/z* 71) were increased more considerably, particularly in heat-stressed plants (Fig. 4). Feeding [2,3-^{13}C]-pyruvate in *F. benjamina* (Fig. 5) produced broadly similar results to *P. aquatica* in that emissions of the single-labelled isotopologue (*m/z* 70) increased only slightly and emissions of the double-labelled isotopologue (*m/z* 71) were greater. However, there were no clear differences between control and heat-stressed plants between the rates of increase between the single- and double-labelling. Notably, total isoprene emissions (sum of all isotopologues including *m/z* 69.07) were significantly increased in response to heat in both species.

Heat affects carbon partitioning into isoprene in a species-dependent manner

Heat affected the fraction of assimilated carbon released as isoprene in a species-dependent manner (Fig. 6). The fraction was not affected by heat in *F. benjamina*, where it amounted to ~4.2% in plants grown under both treatments (25 °C and 33 °C). In contrast, it significantly increased in response to heat in *P. aquatica*, from ~2.5% to ~5.5%. The fraction of assimilated carbon emitted as isoprene in *P. aquatica* exceeded that of *F. benjamina* at 33 °C, although the levels of emission were

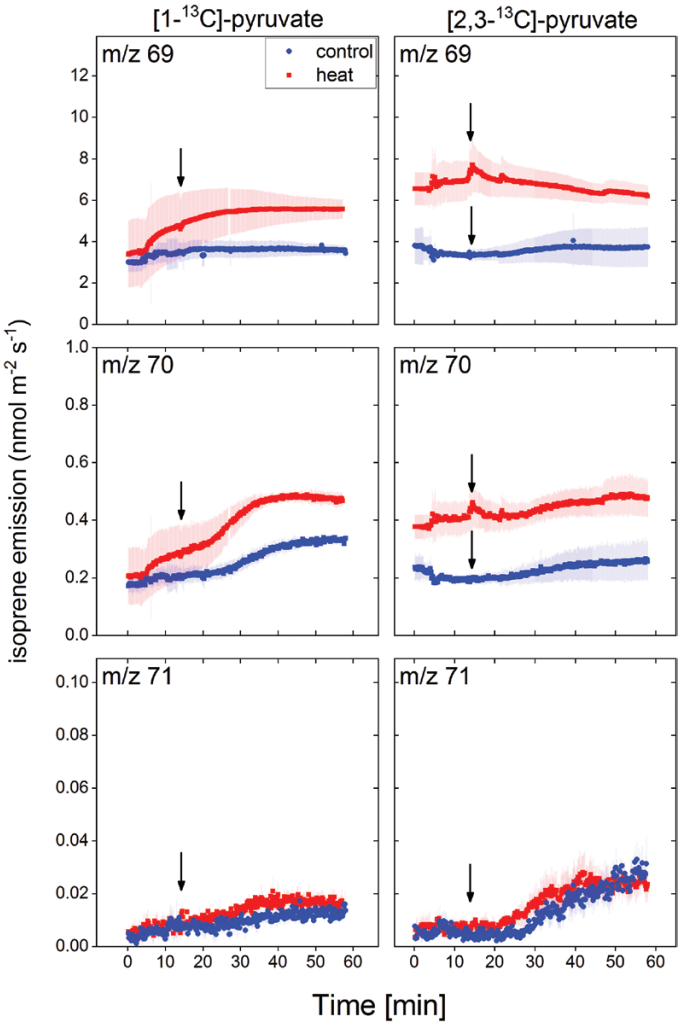

Fig. 5. Temporal dynamics of fluxes of unlabelled and ^{13}C-labelled isoprene isotopologues in plants of *Ficus benjamina* exposed to heat stress. Plants were exposed to either control conditions (25 °C) or to heat stress at 33 °C for 5 d. Measurements of isoprene isotopologues containing no ^{13}C atoms (*m/z* 69), one ^{13}C atom (*m/z* 70), and two ^{13}C atoms (*m/z* 71) were taken after feeding excised twigs with position-specific labelled pyruvate, namely [1-^{13}C]-pyruvate and [2,3-^{13}C]-pyruvate (time of feeding indicated by arrows). Data are means (±SE) of 4–5 replicates.

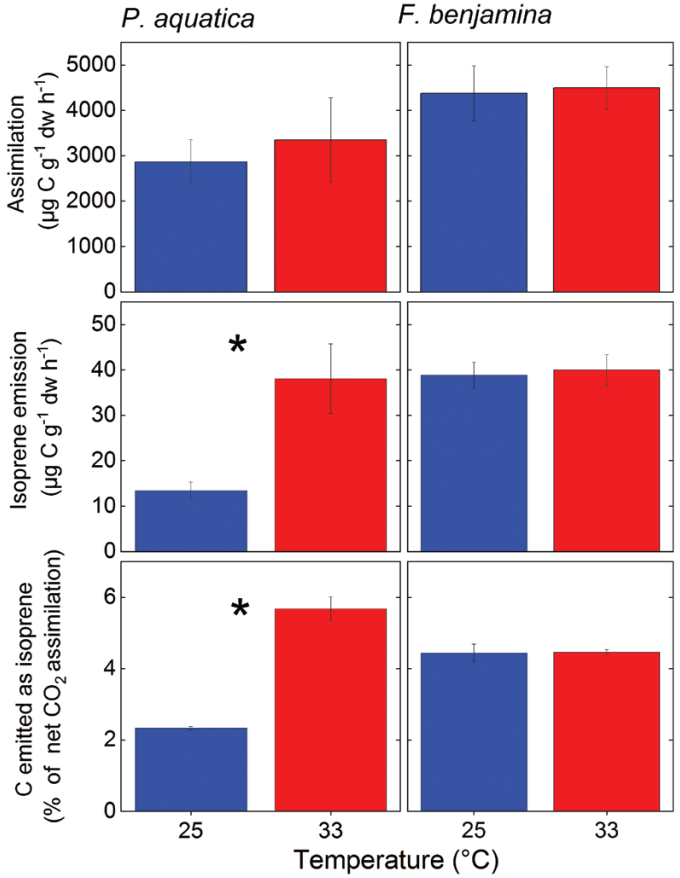

Fig. 6. Effects of heat stress on net CO_2 assimilation, isoprene emissions, and the carbon released as isoprene as a portion of the CO_2 fixed in photosynthesis for plants of *Pachira aquatica* and *Ficus benjamina* exposed to heat stress. Plants were exposed to either control conditions (25 °C) or to heat stress at 33 °C for 5 d. Data are means (±SE) of six replicates. Significant differences between means were determined using Student's *t*-test: *P<0.001.

the same in both species at this temperature. In contrast, both the fraction and the level emitted at 25 °C were significantly lower in *P. aquatica*.

Discussion

Using two tropical species, we tested the hypothesis that plants with high isoprene emission are better adapted to prolonged heatwaves than plants with lower emission, and we also investigated the role of internal carbon sources for isoprene biosynthesis with regards to thermotolerance. Under control conditions (25 °C), *F. benjamina* emitted isoprene at a rate three times higher rates that of *P. aquatica* (6.8 nmol m^{-2} s^{-1} versus 2.7 nmol m^{-2} s^{-1}; Fig. 1), which is in agreement with previous reports for *F. benjamina* (0.03–25 µg C g^{-1} h^{-1}; Sun and Leu, 2004; Carvalho *et al.*, 2005; Geron *et al.*, 2006). There have been no previous reports for isoprene emission rates in *P. aquatica*. Under control conditions, the optimum temperature (T_{opt}) for isoprene emission was ~37 °C for *F. benjamina* and ~39–40 °C for *P. aquatica* (Fig. 2), which for both species exceeded the T_{opt} of net CO_2 assimilation by 12–15 °C, similar to responses reported previously for *Quercus robur* (Schnitzler *et al.*, 2004) and *Phoenix dactylifera* (Arab *et al.*, 2016). This pattern seems to reflect acclimation of a plant to the prevailing environmental conditions. Higher T_{opt} of isoprene emission than of net CO_2 assimilation enables the plant to respond quickly to short-term increases in leaf temperature, for example during periods of high irradiation. It has been demonstrated that isoprene quickly mediates thermotolerance for the photosynthetic apparatus by modulation of membrane protein dynamics (Harvey *et al.*, 2015) and/or ROS quenching (Loreto and Velikova, 2001; Affek and Yakir, 2002; Sharkey *et al.*, 2008), thereby allowing net CO_2 assimilation to be maintained even at temperatures clearly exceeding T_{opt} of photosynthesis.

After 5 d of acclimation to 33 °C, the temperature response of isoprene emission of both species changed (Fig. 2). The T_{opt} of emission in *F. benjamina* increased from 37 °C to at least 42 °C whilst the emission capacity also increased, as indicated by higher emission rates. An increase in emission rates was also observed for *P. aquatica*, but in this case the T_{opt} of emission did

not change. These results were in good agreement with previous data for date palm where both the capacity and T_{opt} of isoprene emission have been shown to increase when plants are cultivated at elevated air temperature (Arab *et al.*, 2016). Thus, our results demonstrated that the higher isoprene emissions induced by elevated temperature in both species was most likely a response to acclimate the leaves to heat. However, since the constitutive isoprene emission was already high in *F. benjamina* under control conditions, the portion of induced emission was lower than in *P. aquatica*. The latter showed very low constitutive emission under control conditions, but highly induced emission in response to heat stress. This is consistent with previous findings that have demonstrated constitutive BVOC emissions from healthy, non-stressed plants (Holopainen, 2004) and additional, stress-induced emissions due to stimulated *de novo* biosynthesis of volatile compounds (Paré and Tumlinson, 1997; Paré *et al.*, 2005). The high constitutive isoprene emission capacity of *F. benjamina* might underlie its high tolerance to short-term increases in temperature.

Interestingly, we found that net CO_2 assimilation of *F. benjamina* exposed to 33 °C was significantly higher than in control plants grown at 25 °C (Fig. 3), which clearly highlights its considerable acclimation potential to higher temperatures. This finding supports a thermoprotectant function of isoprene for the photosynthetic apparatus, as high isoprene-emitting species generally display higher rates of photosynthesis under heat stress, and also the role of isoprene in fast recovery after heat stress (Velikova and Loreto, 2005). In contrast, no such effect was observed for *P. aquatica*, which seemed to be more sensitive to temperature increases and probably reached its upper temperature limit at 33 °C: further exposure to higher temperature (38 °C) resulted in detrimental stress effects. This was further reflected under prolonged exposure to elevated temperatures (Fig. 1). *Pachira aquatica* was not able to maintain isoprene biosynthesis over extended periods even at 33 °C, as evidenced by some individuals that had a midday assimilation rate of 1.4 μmol m^{-2} s^{-1} at the beginning of the 33 °C treatment, dropping to 0.04 μmol m^{-2} s^{-1} after 19 d of exposure (data not shown); this occurred simultaneously with a drop in isoprene emissions.

Under such stress conditions, isoprene emission seemed to become decoupled from net CO_2 assimilation, resulting in an impaired leaf C budget, i.e. reduced photosynthetic CO_2 fixation but increased C loss via isoprene emission. Previous studies have demonstrated that C losses via isoprene can account for up to 25% of the C fixed in photosynthesis if plants are stressed (Sharkey *et al.*, 1996; Brüggemann and Schnitzler, 2002a, 2002b), which greatly exceeds the loss in leaves under non-stressed conditions (0.2–2%; Kesselmeier *et al.*, 2002). *Ficus benjamina* maintained high levels of photosynthetic CO_2 fixation under prolonged periods of exposure to 33 °C. A further increase in temperature to 38 °C only had a relatively small effect on net CO_2 assimilation in *F. benjamina* (Fig. 1), and isoprene emissions under these conditions were maintained at a high level, thus, contributing to the acclimation of the plant to heat stress.

Maintenance of high levels of isoprene emission under conditions of reduced net CO_2 assimilation implies that the portion of leaf internal C sources used for isoprene biosynthesis is increased (Kreuzwieser *et al.*, 2002; Funk *et al.*, 2004). It is generally assumed that under non-stress conditions up to 80% of C used for isoprene biosynthesis is provided from instantaneously fixed CO_2. The remaining ~20% C might be provided by alternative C sources (Delwiche and Sharkey, 1993; Karl *et al.*, 2002b; Affek and Yakir, 2003; Schnitzler, 2004) such as xylem sap sugars (Kreuzwieser *et al.*, 2002), starch (Monson *et al.*, 1994; Schnitzler, 2004), or cytosolic sources (de Souza *et al.*, 2018). This alternative carbon might be imported into the chloroplast as pyruvate, PEP, or as glyceraldehyde-3-phosphate (GA-3P) by specific transporters such as triose-phosphate transporters (Bamberger *et al.*, 1975; Hoefnagel *et al.*, 1998) pyruvate transporters (Furumoto *et al.*, 2011), and PEP transporters (Fischer *et al.*, 1997; Flügge, 1999). The observation from ^{13}C-labelling experiments that intermediates of the Calvin–Benson cycle are labelled similarly to isoprene (Delwiche and Sharkey, 1993) may support the view of a contribution of GA-3P uptake into plastids. Our position-specific pyruvate ^{13}C-labelling experiments were able to shed new light on the question of alternative C sources and potential changes in contributions to isoprene biosynthesis under heat stress, as they enabled us to follow the fate of the ^{13}C atoms of pyruvate into isoprene.

Feeding cytosolic [1-^{13}C]- and [2,3-^{13}C]-pyruvate caused production and emission of ^{13}C-labelled isoprene in both plant species (Figs 4, 5). As expected, the resulting isoprene isotopologues differed depending on the form of pyruvate applied. If [1-^{13}C]-pyruvate was fed to plants under control conditions (25 °C), we observed considerable emissions of single ^{13}C-labelled isoprene (m/z 70) and only traces of the double ^{13}C labelled isoprene (m/z 71). The occurrence of single ^{13}C-labelled isoprene most likely resulted from re-fixation of $^{13}CO_2$ within the leaf (Kreuzwieser *et al.*, 2002; Tcherkez *et al.*, 2017; Fasbender *et al.*, 2018) from the decarboxylation of the C1 carboxylic group of pyruvate. Thus, the substantial emissions of $^{13}CO_2$ during [1-^{13}C]-pyruvate labelling (Fig. 3) may have been used in the Calvin–Benson cycle, hence contributing to the formation of intermediates that are used as precursors of the MEP pathway. On the other hand, if pyruvate is converted to GA-3P and channeled into the chloroplasts via the triose-phosphate transporter (via reversible conversion to dihydroxyacetone phosphate), it might enter the MEP pathway directly. In contrast, if [2,3-^{13}C]-pyruvate was applied, double ^{13}C-labelled isoprene was the main volatile product; although single ^{13}C-labelled isoprene appeared as well. Emission of double ^{13}C-labelled isoprene can be explained by transport of pyruvate into the chloroplasts where it acts as the precursor of the MEP pathway, thus leading to formation of double ^{13}C-labelled isoprene (Figs 4–6). Transport of cytosolic pyruvate into plastids can either be direct, as mediated by a sodium-pyruvate transporter (Furumoto *et al.*, 2011), or indirect, after conversion of pyruvate to PEP, by a PEP transporter located in the chloroplast membrane (Fischer *et al.*, 1997; Flügge, 1999). Alternatively, abundant pyruvate in the cytosol could be converted into GA-3P or other triosephosphates and be channeled into the Calvin–Benson cycle after uptake into the chloroplast, which can be mediated by bi-directional phosphate translocators that have been described previously (Bamberger *et al.*, 1975; Hoefnagel

et al., 1998). A diagram of the different responses to heat of the processes involved in isoprene biosynthesis is shown in (Fig. 7).

It should be noted that the amount of ^{13}C incorporated into isoprene upon ^{13}C-pyruvate feeding was relatively low, and therefore it did not reflect the ~20% of C expected to be derived from alternative (other than CO_2) sources. This can be explained by the low amount of pyruvate fed to the leaves and the assumed dilution of labelled pyruvate within the cytosolic and plastidic pyruvate pools. The pyruvate pool has a fast turnover and ^{13}C-lablled pyruvate is quickly (within minutes) incorporated into several chloroplastic volatile organic compounds (VOCs) such as monoterpenes or benzenoids (Fasbender *et al.*, 2018). Consequently, the ^{13}C-signal in VOCs quickly vanishes when the labelled solution is exchanged with water (Jardine *et al.*, 2014). Thus, whilst our results are efficient in tracing the metabolic fluxes within mesophyll cells, they are not sufficient to provide quantification of the fluxes. Moreover, C from other alternative sources (e.g. sugars) may well have contributed to isoprene biosynthesis, hence further diluting the ^{13}C-pyruvate signal.

When both species were exposed to heat for 5 d, the relative (%) increases in emissions of ^{13}C-labelled isoprene did not change compared to controls. However, because in *P. aquatica* total isoprene emissions increased due to the heat increase from 25 °C to 33 °C (Fig. 1) and the portion of ^{13}C-labelled isoprene remained the same as in controls (data not shown), we conclude that the rate of pyruvate transport from the cytosol into chloroplasts and the use of pyruvate for isoprene biosynthesis were increased in this species to the same extent as isoprene formation increased (Fig. 6). In conclusion, uptake of pyruvate into chloroplasts seems to be controlled by pyruvate consumption in the organelles.

In both species, feeding [1-^{13}C]-pyruvate caused strong release of $^{13}CO_2$ from the leaves in the light (Fig. 3). This process was considerably enhanced when the plants were exposed to heat stress. Importantly, although it is well known that heat increases mitochondrial respiration in plants, we deduce a more significant contribution to enhanced $^{13}CO_2$ release from other metabolic processes. We conclude this from the fact that the plants showed only slightly enhanced dark respiration (*F. benjamina*: control –0.4±0.01 μmol m^{-2} s^{-1}, heat –1±0.02 μmol m^{-2} s^{-1}; *P. aquatica*: control –0.4±0.01 μmol m^{-2} s^{-1}, heat –0.6±0.05 μmol m^{-2} s^{-1}) and, more importantly, from the lower amount of $^{13}CO_2$ emitted from each species after feeding with [2,3-^{13}C]-pyruvate as compared to feeding with [1-^{13}C]-pyruvate . With high respiration rates, turnover in the tricarboxylic acid (TCA) cycle would be high, resulting in a greater release of $^{13}CO_2$ from the C2–C3 moiety of [2,3-^{13}C]-pyruvate. Our results instead suggest that the TCA cycle was inhibited under light conditions, suggesting a light-dependent inhibition of the TCA cycle, which is similar to previous findings in other species (Priault *et al.*, 2009; Sweetlove *et al.*, 2010; Araújo *et al.*, 2012; Cheung *et al.*, 2014; Tcherkez *et al.*, 2017). Consequently, other processes must have contributed to $^{13}CO_2$ release after [1-^{13}C]-pyruvate feeding. Numerous reactions are known to cause pyruvate decarboxylation in plant metabolism, such as the those catalysed by pyruvate dehydrogenase (PDH; Sharkey *et al.*, 1991) and pyruvate decarboxylase (PDC; Kreuzwieser *et al.*, 1999; Karl *et al.*, 2002a, 2002b), and other enzymatic reactions that use pyruvate as a substrate for decarboxylation (e.g. see KEGG, https://www.genome.jp/kegg). The relative contributions of each of these processes to overall emissions of $^{13}CO_2$ cannot be determined from our present data. To identify these

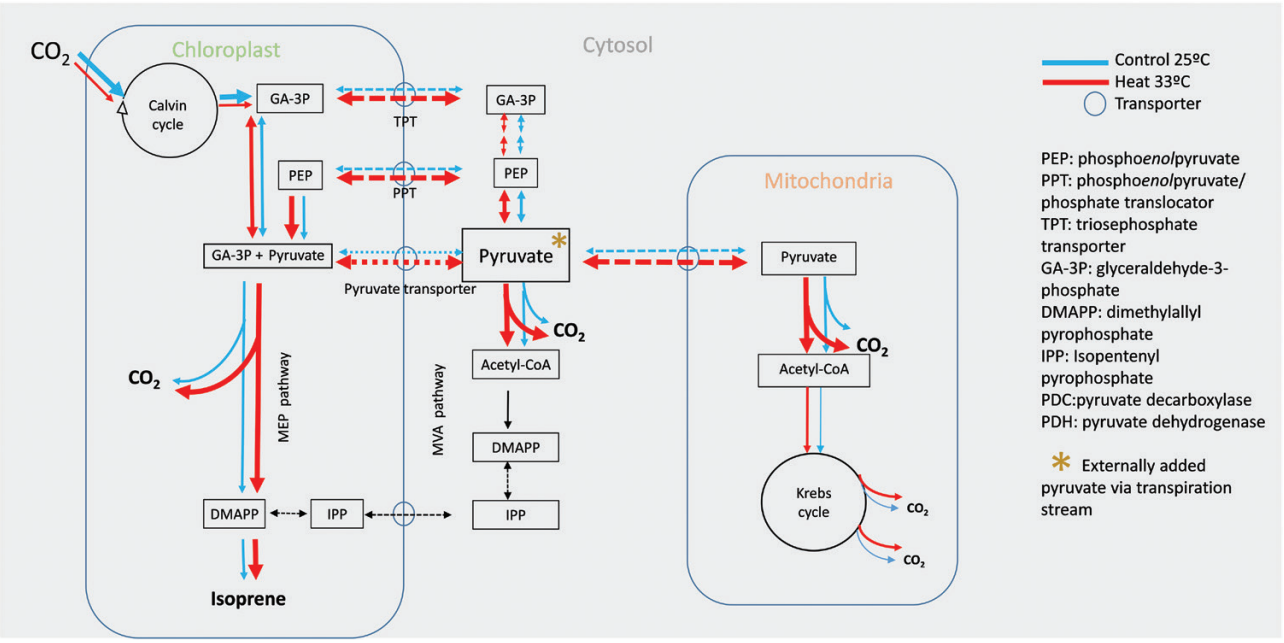

Fig. 7. Diagram illustrating the differential effects of heat stress on the processes involved in isoprene biosynthesis. Control conditions are represented in blue and heat-stress conditions in red. The thickness of the lines indicates the magnitude of the flux, and dashed lines indicate the action of transporters. Black arrows represent processes that have not been elucidated with the data from this study.

enzymatic decarboxylation processes more detailed analysis of ^{13}C-labelled metabolites such as fatty acids, amino acids, and others would be required.

Our comparison of the two tropical plants *F. benjamina* and *P. aquatica* demonstrated that they have very different responses to heat stress. These differences might be a result of adaptation to their different natural habitats, namely well-watered tropical forests with relatively constant temperature regimes for *P. aquatica* and sites that are probably drier and hotter sites for *F. benjamina* (Imran *et al.*, 2014; Infante-Mata *et al.*, 2014). The more heat-tolerant *F. benjamina* seems to constitutively invest more C for isoprene biosynthesis than *P. aquatica*. Consequently, the photosynthetic apparatus of *F. benjamina* is better thermoprotected and net CO_2 assimilation can be maintained at higher levels during heat stress, similar to other isoprene-emitting tropical species (Taylor *et al.*, 2019). In contrast, *P. aquatica* seems to follow a different strategy by investing less C into isoprene as a photo-protective mechanism during non-stress conditions; however, under short-term heat stress it greatly activates its capacity for isoprene biosynthesis to protect its leaves. This strategy might be advantageous as C is only invested when it is actually needed (Spinelli *et al.*, 2011) and is otherwise used for other processes such as biomass production (i.e. growth processes, Schippers *et al.*, 2015). However, under prolonged heat stress, *P. aquatica* was not able to maintain isoprene biosynthesis and thus subsequently net CO_2 assimilation declined, resulting in shedding of leaves. The plants clearly exceeded their heat-stress threshold in our experiments, and it caused irreversible damage (Kask *et al.*, 2016). Irreversible effects of heat can result in decreased plant performance, including denaturation of enzymes involved in isoprene biosynthesis or reduced isoprene formation due to lower substrate supply (Kleist *et al.*, 2012). Thus, the severe effects of heat stress and the induced senescence in *P. aquatica* can be regarded as a coordinated response to remobilize nutrients and to secure reproductive success, thus providing phenotypic plasticity to help to adapt to adverse environmental conditions (Schippers *et al.*, 2015). Moreover, the stems of *P. aquatica* contain large amounts of carbohydrates (Duarte and Golambiuk, 2014), which enables the plant to immediately produce new shoots and leaves when stress is released; thus, stress recovery at the whole-plant level should also be examined in order to obtain a more holistic view of this species thermoprotective mechanisms.

Conclusions

We investigated the thermotolerance of two contrasting tropical plant species, *Ficus benjamina* and *Pachira aquatica*, in their responses to abrupt and prolonged heat stress. Although *F. benjamina* emitted more isoprene than *P. aquatica* under control conditions, both species appeared to emit isoprene as means for thermoprotection. *Ficus benjamina* showed higher heat tolerance than *P. aquatica*, which showed higher induced isoprene emissions upon increases in temperature. The greater heat sensitivity of *P. aquatica* was further reflected in a different carbon allocation strategy. Under control conditions, a portion of cytosolic pyruvate was incorporated and metabolized within the plants for isoprene biosynthesis. Under heat stress, both isoprene emissions and the proportion of cytosolic pyruvate allocated into isoprene synthesis increased. This represents a mechanism that *P. aquatica*, and to a lesser extent *F. benjamina*, has evolved as an immediate response to sudden increases in heat stress. However, in the long run under prolonged heat, the species with higher constitutive emissions (*F. benjamina*) was better adapted, indicating that plants that invest more carbon into protective BVOC emissions tend to suffer less from heat stress. These different strategies in carbon allocation need to be considered in environmental models if they are to have a better predictive capability of the effects of increasing heatwaves in the future.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Diagrams showing the designs of the experiments.

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