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Reassimilation of Leaf Internal CO$_2$ Contributes to Isoprene Emission in the Neotropical Species *Inga edulis* Mart.

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Abstract: Isoprene (C_5H_8) is a hydrocarbon gas emitted by many tree species and has been shown to protect photosynthesis under abiotic stress. Under optimal conditions for photosynthesis, ~70%–90% of carbon used for isoprene biosynthesis is produced from recently assimilated atmospheric CO_2. While the contribution of alternative carbon sources that increase with leaf temperature and other stresses have been demonstrated, uncertainties remain regarding the biochemical source(s) of isoprene carbon. In this study, we investigated leaf isoprene emissions (*Is*) from neotropical species *Inga edulis* Mart. as a function of light and temperature under ambient (450 µmol m^{-2} s^{-1}) and CO_2-free (0 µmol m^{-2} s^{-1}) atmosphere. *Is* under CO_2-free atmosphere showed light-dependent emission patterns similar to those observed under ambient CO_2, but with lower light saturation point. Leaves treated with the photosynthesis inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) failed to produce detectable *Is* in normal light under a CO_2-free atmosphere. While strong temperature-dependent *Is* were observed under CO_2-free atmosphere in the light, dark conditions failed to produce detectable *Is* even at the highest temperatures studied (40 °C). Treatment of leaves with ^{13}C-labeled sodium bicarbonate under CO_2-free atmosphere resulted in *Is* with over 50% containing at least one ^{13}C atom. *Is* under CO_2-free atmosphere and standard conditions of light and leaf temperature represented 19% ± 7% of emissions under ambient CO_2. The results show that the reassimilation of leaf internal CO_2 contributes to *Is* in the neotropical species *I. edulis*. Through the consumption of excess photosynthetic energy, our results support a role of isoprene biosynthesis, together with photorespiration, as a key tolerance mechanism against high temperature and high light in the tropics.

Keywords: abiotic stress; alternative carbon sources; CO_2-free air; decarboxylation process; photosynthesis

1. Introduction

Isoprene (2-methyl-1,3-butadiene, C_5H_8) is a reactive hydrocarbon gas emitted in large amounts to the atmosphere by many plants [1,2]. Approximately 500 Tg C year^{-1} is released as isoprene by vegetation [3]. Tropical rainforests are an important source of isoprene to the atmosphere and estimates suggest that they are responsible for 80% of global isoprene emissions (*Is*) [4]. Isoprene has an

important role in atmospheric chemistry involving air quality and climate [5]. Due to its high chemical reactivity with respect to photooxidation, isoprene impacts the atmospheric concentrations of ozone, methane, and secondary organic aerosols, resulting in strong effects on the radiation balance of the Earth [6–8]. Despite great advances in our knowledge on the roles of plant *Is* on atmospheric chemistry, much less is known about the physiological roles that isoprene plays in plants [9–11]. The emerging view is that isoprene production can protect photosynthesis during abiotic stress through mechanisms including excess photosynthetic energy consumption, physical stability of biological membranes, and direct roles as an antioxidant through reactions with reactive oxygen species that accumulate under stress conditions [12,13]. However, recent work has noted that protection of photosynthesis through isoprene via the physical stabilization of membranes may not be possible and that the mechanisms of protection from oxidative stress are still unclear [14].

Isoprene is synthesized in the chloroplast by the 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway [15–18]. The synthesis is initiated by the condensation of two primary precursors; pyruvate and glyceraldehyde 3-phosphate (G3P), a direct product of the Calvin–Benson cycle [15,19,20]. The fact that isoprene cannot be stored in the leaves results in *Is* from production, with the majority of carbon derived from recent photosynthesis [21]. Under steady-state conditions of light and temperature (1000 μmol m^{-2} s^{-1} and 30 °C) ~70%–90% of the carbon used for isoprene synthesis is produced from recently assimilated atmospheric CO_2 [21–23]. It was observed that plants exposed to $^{13}CO_2$ rapidly incorporate ^{13}C into isoprene molecules [21,23–25]. Despite this rapid incorporation of ^{13}C, ~10%–30% of the carbon atoms in the isoprene molecules emitted have been reported to remain unlabeled, even when the leaves are continuously exposed to $^{13}CO_2$ [26]. This indicates a contribution of carbon sources other than the recently assimilated CO_2. Those alternative carbon sources are important under stress conditions [27] and increase under high temperatures, while net photosynthesis (*Pn*) is reduced at the expense of photorespiration [25]. Therefore, under stressful situations that reduce the uptake of atmospheric CO_2 due to partial stomatal closure while stimulating internal sources of CO_2 (e.g., respiration and photorespiration), *Is* can be increased even if *Pn* is substantially reduced [28–30].

Several studies have identified that potential alternative carbon sources for *Is* can be greater when environmental conditions are limiting *Pn*, including xylem-transported carbohydrates [26,31], starch degradation [26,32], pyruvate [33], stored carbon pools [17], and extrachloroplastic intermediates [34]. In addition, the incorporation of CO_2 released by intercellular decarboxylations, e.g., during mitochondrial respiration, photorespiration or decarboxylation of pyruvate during formation of MEP was suggested [25–27]. For example, photorespiratory CO_2 release is strongly stimulated under high temperatures and stomatal closure due to the decrease of atmospheric CO_2 uptake and the relative increase of O_2 in relation to CO_2. Therefore, under stressful conditions that promote stomatal closure, such as drought and high temperature, an increase in the release of photorespiratory CO_2 inside the leaf could provide alternative carbon sources for isoprene production [35]. However, the identity and quantitative importance of alternative carbon sources for isoprene synthesis in tropical species under abiotic stress is still unclear.

In this study, we investigated the incorporation of CO_2 from decarboxylation process into isoprene molecules by quantifying *Is* under CO_2-free reference air in temperature- and light response curves from a neotropical specie *Inga edulis* Mart. Taking off the CO_2 from the leaf chamber, we stimulated the photorespiration process and the release of internal CO_2 in the leaf. We then compared leaf *Is* in CO_2-free reference air with *Is* in ambient CO_2 under standard conditions of light and temperature. We further investigated the potential for the reassimilation of CO_2 release by internal decarboxylation processes using an inhibitor of photosynthesis under CO_2-free reference air. Finally, we directly evaluated the potential of internal leaf CO_2 to be incorporated into *Is* by providing ^{13}C-sodium bicarbonate (NaH$^{13}CO_3$) solutions to detached leaves under CO_2-free reference air. We suggest that CO_2 released by internal decarboxylation processes is a quantitatively important source of alternative carbon for isoprene formation. Together with photorespiration, isoprene production may be a key

tolerance mechanism under plant stress that leads to a decrease in stomatal conductance and CO_2 uptake. Under these conditions, isoprene could still be synthesized using the CO_2 released by photorespiration, offering a protective mechanism by consuming excess photosynthetic energy and reducing equivalents. These results deepen our understanding about *Is* by tropical species under different environmental conditions commonly experienced in the tropics, such as high irradiance and temperature, and contributes to the modeling of *Is* from terrestrial ecosystems in the tropics under climate change.

2. Materials and Methods

2.1. Plant Material and Experimental Design

Six *I. edulis* trees with a height ranging from 5 to 10 m were used in this study. The experimental trees occur naturally near the Laboratory of Plant Physiology and Biochemistry at the National Institute for Amazonian Research (INPA) campus III in Manaus, Brazil. This tropical species was selected because of its high reported *Is* and for its ability to maintain high transpiration rates for many hours following branch detachment from the tree [25]. From October 2014 to February 2016 *Pn* and *Is* rates were quantified from healthy mature leaves, located in the upper third of the canopy. For each day of the study, one branch near the top of one of the plants was cut and placed in tap water before being recut and transported to the laboratory. The leaf gas exchange measurements occurred between 9:00 AM and 5:00 PM, with the branch exposed to ambient light and temperature conditions.

2.2. Isoprene Emissions and Net Photosynthesis

Pn and *Is* rates were quantified from *I. edulis* leaves using a portable open gas exchange system (IRGA) (LI-6400XT, LI-COR Inc., Lincoln, NE, USA) with an artificial light source 6400-02B Red Blue. The flow rate entering the LI-6400XT leaf chamber was set to 537 mL min^{-1}. A fraction of air exiting the leaf chamber was used to determine *Is* using two methods: quantified in real-time using a high sensitivity quadrupole proton transfer reaction-mass spectrometry (PTR-MS, Ionicon Analytik, Innsbruck, Austria) or thermal desorption (TD) gas chromatography–mass spectrometry (GC-MS, 5975C series, Agilent Technologies, Santa Clara, CA, USA). Using four-way junction fitting, air exiting the leaf chamber was delivered or to the PTR-MS (40 mL min^{-1}) or to the TD tube (100 mL min^{-1} when collecting), with the remainder of the flow diverted to the vent/match valve within the LI6400XT. The excess flow entering the vent/match valve was maintained to at least 200 mL min^{-1} [25]. Background measurements were performed with an empty leaf chamber, at the beginning and at the end of each experiment.

2.3. Light and Temperature Response of Isoprene

For each *I. edulis* leaf, either a light or temperature response curve was generated. Leaves were evaluated for their response of *Pn* and *Is* to changes in PPFD (Photosynthetic Photon Flux Density) at 0, 25, 50, 75, 100, 250, 500, 750, 1000, 1500, and 2000 μmol m^{-2} s^{-1} under constant leaf temperature (30 °C) in both a CO_2-free atmosphere (0 μmol mol^{-1}, 4 leaves) and ambient CO_2 (450 μmol mol^{-1}, 7 leaves). To achieve a CO_2-free atmosphere inside the LI-6400XT leaf chamber, the CO_2 mixer was turned off and the soda lime was put to fully scrub. Leaf temperature response curves (25→ 30→ 35→ 40 °C) were measured under constant PPFD (1000 μmol m^{-2} s^{-1}), again in both CO_2-free atmosphere ($n = 3$) and ambient CO_2 ($n = 4$). Before each series of measurements, the leaves were acclimated for ~15 min in the chamber, until the stomatal conductance and photosynthesis were stable.

To quantify the ratio of *Is* between CO_2-free atmosphere (0 μmol mol^{-1} of CO_2, 8 leaves) and ambient CO_2 concentrations (450 μmol mol^{-1} of CO_2, 8 leaves), gas exchange was measured with leaves of *I. edulis* under standard conditions of light and leaf temperature (1000 μmol m^{-2} s^{-1} PPFD, 30 °C leaf temperature).

2.4. Isoprene Emission under Limiting Conditions for Net Photosynthesis

To evaluate *Is* under limiting conditions for *Pn* we measured gas exchange under two limiting situations: (a) using a specific photosynthesis inhibitor, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) or (b) in the dark. DCMU blocks photosynthetic electron flow from photosystem II to the plastoquinone pool [36], inhibiting photosynthesis.

For the inhibitor experiment, a 180 μM DCMU solution, was prepared and adjusted to pH 7.5. A leaflet was cut from a leaf and placed in the DCMU solution for two hours, until the inhibitor took effect. This inhibition time was determined during previous experiments to be sufficient to decrease *Pn* to near 0 μmol m^{-2} s^{-1} in leaflets under standard conditions (i.e., with CO_2, PPFD and leaf temperature at 450 μmol mol^{-1}, 1000 μmol m^{-2} s^{-1}, and 30 °C, respectively). Following uptake of the DMCU solution by the transpiration stream, a light response curve was measured under CO_2-free atmosphere and constant leaf temperature (30 °C).

For the dark experiment, a temperature response curve was established in the absence of light. During the measurements the leaves were kept under CO_2-free atmosphere and zero PPFD. The samples were collected in TD tubes, using the LI-6400XT/GC-MS methodology [25].

2.5. ^{13}C-labeling of Leaf Isoprene Emissions Using Sodium Bicarbonate [13]C Delivered through the Transpiration Stream

In order to observe possible reassimilation of internal CO_2 by photosynthesis through isoprene labeling molecules, measurements of *Is* under CO_2-free atmosphere were carried out on detached fully expanded *I. edulis* leaflets in a solution of ^{13}C labeled sodium bicarbonate ($NaH^{13}CO_3$) (*n* = 5). Fresh solutions of sodium bicarbonate [13]C (20, 60, 120 mM) were prepared with the pH adjusted to 7.0. For each concentration five leaflets were cut and recut in the sodium bicarbonate ^{13}C solution, and leaf gas exchange measurements were initiated. In sequence, the measurements were made for two hours, under CO_2-free atmosphere at constant PPFD (1000 μmol m^{-2} s^{-1}) and temperature (30 °C) using the LI-6400XT/GC-MS methodology. Isoprene ^{13}C-labeling is reported as the ^{13}C/^{12}C isotope ratio of $[^{13}C_1]$isoprene/$[^{12}C_5]$isoprene, $[^{13}C_2]$isoprene/$[^{12}C_5]$isoprene, $[^{13}C_3]$isoprene/$[^{12}C_5]$isoprene, $[^{13}C_4]$isoprene/$[^{12}C_5]$isoprene, and $[^{13}C_5]$isoprene/$[^{12}C_5]$isoprene by calculating the peak area ratios (7.1 min retention time) of m/z 69/68, 70/68, 71/68, 72/68, and 73/68, respectively.

3. Results

3.1. Isoprene Emissions under CO_2-Free Atmosphere are Stimulated by Light but are Blocked When Photosynthesis is Inhibited

Under ambient CO_2 and leaf temperature of 30 °C *Is* and *Pn* were stimulated together as PPFD increased (Figure 1a). However, while *Pn* saturated at roughly 1000 μmol m^{-2} s^{-1} PPFD, *Is* showed no sign of saturation up to the highest fluxes of PPFD applied (2000 μmol m^{-2} s^{-1}) (Figure 1a). When *Is* was expressed in μmol C m^{-2} s^{-1} (*IsC*) and plotted against *Pn* (Figure 1b) the results show that during high PPFD intensities, *Is* keeps increasing, representing a higher fraction of *Pn* relative to lower PPFD intensities.
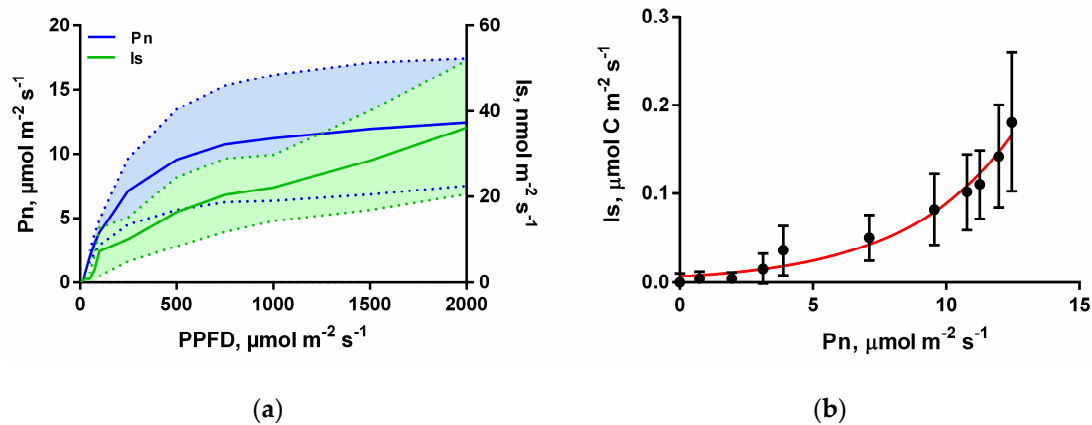
Figure 1. (**a**) Leaf isoprene emissions (*Is*, nmol m^{-2} s^{-1}) and net photosynthesis rates (*Pn*, μmol m^{-2} s^{-1}) from *I. edulis* leaves as a function of photosynthetic photon flux density (PPFD) intensity under constant leaf temperature (30 °C) and ambient CO_2 (450 μmol mol^{-1}); the solid lines are the averages and the hatching area the standard deviation for *n* = 7. (**b**) Isoprene emissions expressed in μmol C m^{-2} s^{-1} (*IsC*) against *Pn* (μmol m^{-2} s^{-1}) as PPFD intensity increases in the same conditions; means, and standard deviation for *n* = 7.

The light response curve in a CO_2-free atmosphere shows that *Pn* remained negative, while light stimulated *Is* in a similar pattern to that observed under ambient conditions of CO_2 (Figure 2a). However, under CO_2-free atmosphere, average *Is* at maximum PPFD reached 59% of the observed under ambient CO_2 conditions (21.3 nmol m^{-2}s^{-1} at CO_2-free atmosphere vs. 36 nmol m^{-2}s^{-1} at ambient CO_2, respectively). The fact that *Pn* remained negative while the leaf was in the chamber demonstrated that the enclosure CO_2 concentrations were below that of the CO_2 compensation point, and that the leaf was a net source of CO_2 to the air, likely due in-part to the stimulation of photorespiration. When real-time *Is* data was collected, *Is* saturated at lower PPFD intensity (1500 μmol m^{-2} s^{-1} PPFD, using average values) under CO_2-free atmosphere relative to ambient CO_2 conditions (Figure 2a vs. Figure 1a), indicating a limitation of carbon for isoprene production.
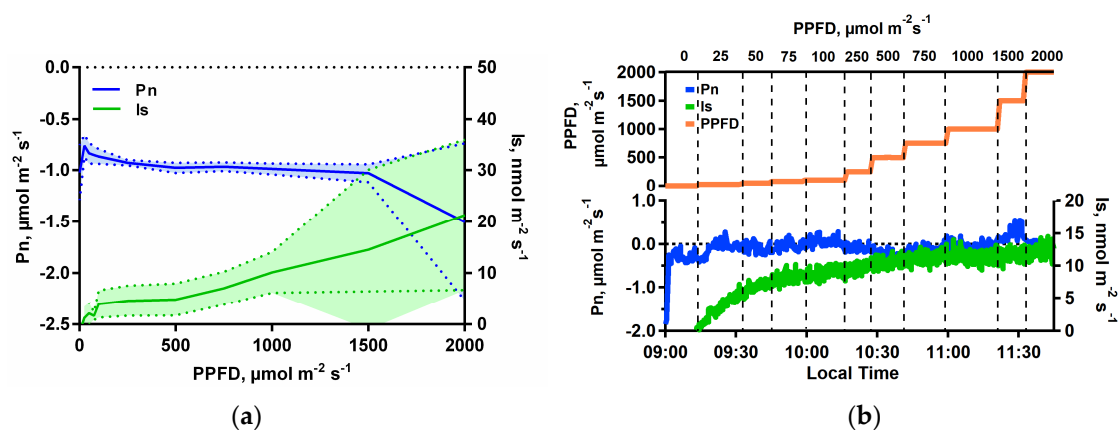

Figure 2. (**a**) Leaf isoprene emissions (*Is*, nmol m^{-2} s^{-1}, measured by gas chromatography-mass spectrometry (GC–MS)) and net photosynthesis rates (*Pn*, μmol m^{-2} s^{-1}) from *I. edulis* leaves as a function of PPFD intensity under constant leaf temperature (30 °C) and CO_2-free atmosphere (0 μmol mol^{-1}). The solid lines are the averages and the hatching area the standard deviation (*n* = 4); (**b**) Example of leaf isoprene emissions (*Is*, nmol m^{-2} s^{-1}) measured by high sensitivity quadrupole proton transfer reaction-mass spectrometry (PTR-MS) and net photosynthesis rates (*Pn*, μmol m^{-2} s^{-1}) from an *I. edulis* leaf under constants leaf temperature (30 °C) and CO_2-free atmosphere, as a function of time with PPFD increases at the marked intervals.

To extend the study of light responses of *Is* and *Pn*, a specific inhibitor of photosynthesis (DCMU—3-(3,4-dichlorophenyl)-1,1-dimethylurea) was provided to a *I. edulis* leaflet under CO_2-free air. Although light-stimulated *Is* could be observed from the detached leaf placed in water as a control (up to 15 nmol m^{-2} s^{-1}), *Is* were small to negligible and did not increase as a function of PPFD when DCMU was provided to the detached leaflet (Figure 3). As stomatal conductance (*gs*) in the DCMU fed leaflet was similar or higher than *gs* in the water control leaf, this effect could not be attributed to a potential stomatal closure induced by DCMU application.
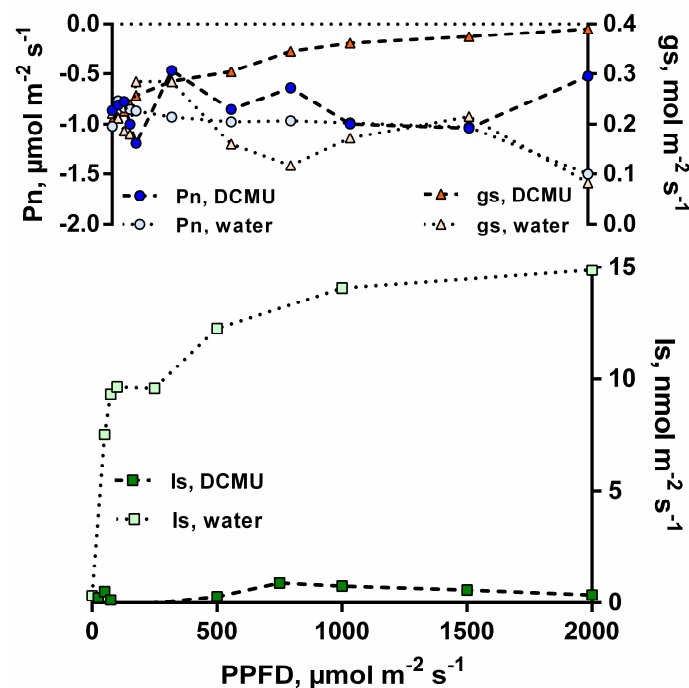

Figure 3. Leaf isoprene emissions (*Is*, nmol m^{-2} s^{-1}), net photosynthesis rates (*Pn*, μmol m^{-2} s^{-1}), and stomatal conductance (*gs*, mol m^{-2} s^{-1}) from an *I. edulis* leaflet under constant leaf temperature (30 °C) as a function of PPFD intensity under CO_2-free atmosphere with and without the addition of the photosynthesis inhibitor DCMU in the transpiration stream (*n* = 1).

3.2. Isoprene Emissions under CO_2-Free Atmosphere are Strongly Stimulate by Leaf Temperature Increase but are Eliminated in the Dark

Under temperature response curves with constant light and CO_2 conditions (1000 μmol m^{-2} s^{-1} and 450 μmol mol^{-1}) *Is* was strongly stimulated as leaf temperature increased and did not show a clear observable saturation point while reaching an average of 76 nmol m^{-2} s^{-1} at the highest leaf temperature (40 °C, Figure 4a). When *IsC* was plotted against *Pn* there was a negative relationship between these two parameters (Figure 4b), which represents the uncoupling between the *Pn* and *Is* as leaf temperature increases. This is driven by both the decrease in *Pn* and the increase in *Is* with increasing leaf temperature.
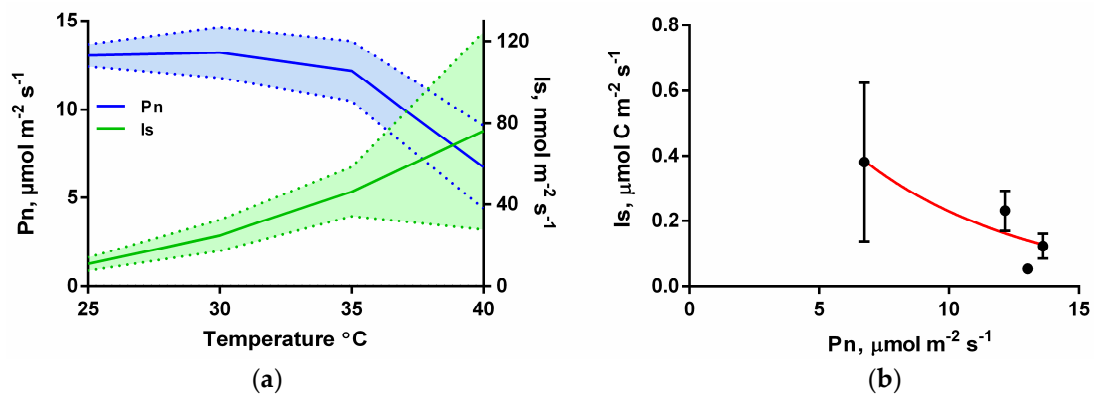
Figure 4. (**a**) Leaf isoprene emissions (*Is*, nmol m^{-2} s^{-1}) and net photosynthesis rates (*Pn*, μmol m^{-2} s^{-1}) from *I. edulis* leaves as a function of temperature increases under constant PPFD intensity (1000 μmol m^{-2} s^{-1}) and ambient CO_2 (450 μmol mol^{-1}); the solid lines are the averages and the hatching area the standard deviation (*n* = 4). (**b**) Isoprene emissions expressed in μmol C m^{-2} s^{-1} (*IsC*) against *Pn* (μmol m^{-2} s^{-1}) as temperature intensity increases in the same conditions; means and standard deviation with *n* = 4.

When the same measurements were made under a CO_2-free atmosphere, *Is* continued to increase with leaf temperature up to 14–20 nmol m^{-2} s^{-1} at 40 °C while *Pn* remained near-zero or slightly negative (e.g., −1.0 μmol m^{-2} s^{-1}) (Figure 5).
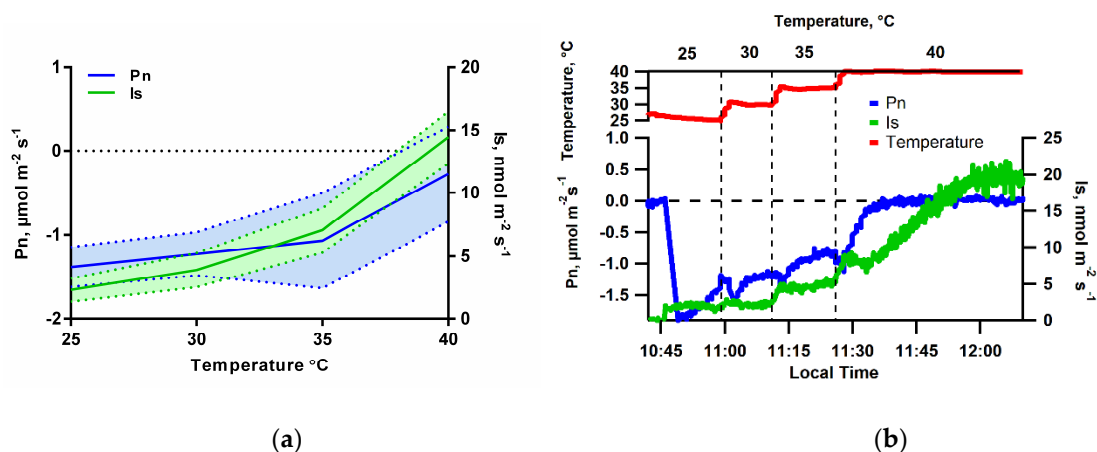
Figure 5. (**a**) Leaf isoprene emissions (*Is*, nmol m^{-2} s^{-1}) and net photosynthesis rates (*Pn*, μmol m^{-2} s^{-1}) from *I. edulis* leaves as a function of temperature under constant PPFD (1000 μmol m^{-2} s^{-1}) and CO_2-free atmosphere (0 μmol mol^{-1}); the solid lines are the averages and the hatching area the standard deviation (*n* = 3). (**b**) Example of leaf isoprene emissions (*Is*, nmol m^{-2} s^{-1}) measured by PTR-MS and net photosynthesis rates (*Pn*, μmol m^{-2} s^{-1}) from an *I. edulis* leaf under constants PPFD intensity (1000 μmol m^{-2} s^{-1}) and CO_2-free atmosphere, as a function of time with temperature increases at the marked intervals.

In order to further evaluate the dependence on *Is* and *Pn*, a measurement of these variables was made as a function of leaf temperature, but in the dark to eliminate photosynthesis. When a temperature response curve was performed in the dark under a CO_2-free atmosphere, *Is* was not detectable, even at the highest temperatures studied (40 °C) (Figure 6). In contrast, in the light, increases in leaf temperature stimulated *Is*, which continued to increase up to the highest leaf temperature studied (40 °C).

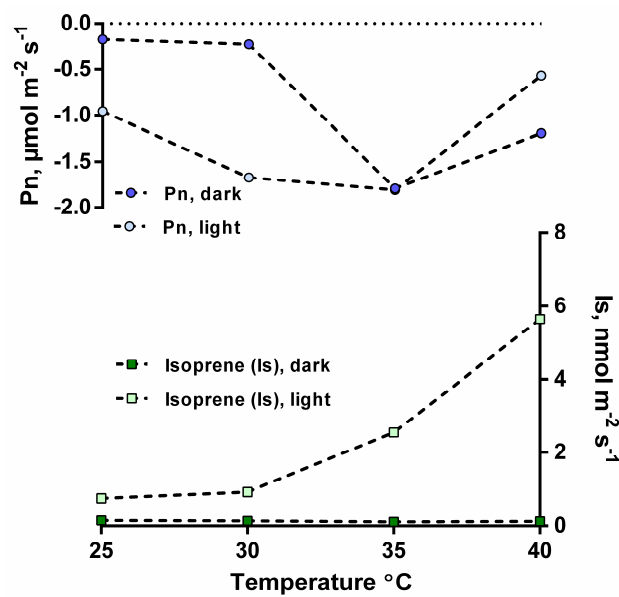**Figure 6.** Leaf isoprene emissions (*Is*, nmol m^{-2} s^{-1}) and net photosynthesis rates (*Pn*, μmol m^{-2} s^{-1}) from an *I. edulis* leaf as a function of temperature under CO_2-free atmosphere, in the presence and absence of light (PPFD intensity 1000 μmol m^{-2} s^{-1}) (*n* = 1).

As it was determined that *Is* are stimulated by increases in light and leaf temperature under CO_2-free air, we calculated *Is* under a CO_2-free atmosphere relative to ambient CO_2 under standard conditions of light and temperature (1000 μmol m^{-2} s^{-1} PPFD, 30 °C, *n* = 8). When *Is* emissions under a CO_2-free atmosphere were quantified from eight *I. edulis* leaves, it was determined that those emissions amounted to 19% ± 7% of emissions under ambient CO_2.

3.3. Sodium Bicarbonate ^{13}C Leaf Feeding

In order to verify the contribution of CO_2 reassimilation from the decarboxylation process in isoprene molecules, sodium ^{13}C-bicarbonate solutions were provided to *I. edulis* leaflets under CO_2-free atmosphere with PPFD and temperature constant (1000 μmol m^{-2} s^{-1}, 30 °C). Within minutes of placing the leaflet in the solution, emissions of ^{13}C-labeled isoprene could be observed (Figure 7).
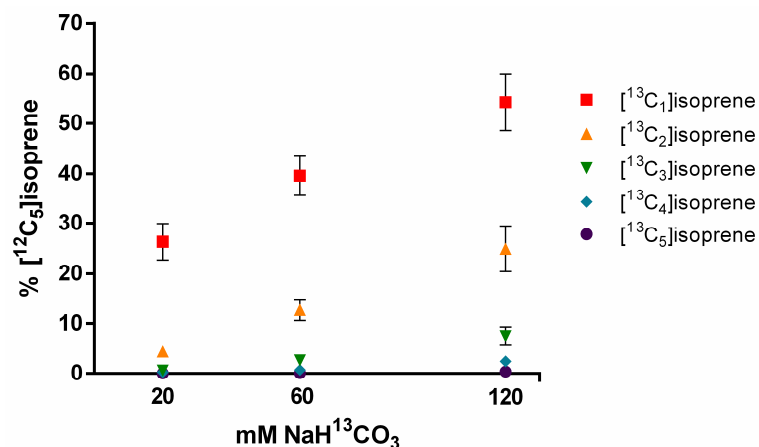

Figure 7. Percentage of ^{13}C-labeled isoprene emissions ($^{13}C_{1-5}$) relative to unlabeled isoprene emissions ($^{12}C_5$) from detached *I. edulis* leaves fed with three concentrations of NaH$^{13}CO_3$ solutions (20, 60, and 120 mM) through the transpiration stream; means and standard deviation for *n* = 5.

When leaflets were placed in the 20 mM solution of sodium bicarbonate ^{13}C they emitted *Is* with one labeled carbon, representing 26% of the emissions of unlabeled isoprene molecules. Emissions of completely labeled molecules [^{13}C$_5$]isoprene were nearly undetectable. With the increase of the molarity (20–120 mM), the percentage of *Is* with ^{13}C-labeling also increased relative to the unlabeled *Is*, as did the number of carbon atoms ^{13}C-labeled per molecule. In the solution with 120 mM of sodium bicarbonate ^{13}C, the percentage of molecules with one ^{13}C atom reached 54%, with two labeled carbons 25%, with three labeled carbons 8%, with four labeled carbons 2%, and with all labeled carbons reaching up 0.35% (relative to unlabeled *Is*).

4. Discussion

4.1. Under CO$_2$-Free Air, Isoprene Emissions Display a Similar Light and Temperature Response Pattern to That under Ambient Conditions, But Is Eliminated when Photosynthesis Is Inhibited

The high correlation between *Is* and *Pn* as a function of light (Figure 1) is widely discussed in literature, since isoprene production via the MEP pathway utilizes, as the first precursor, a product of the Calvin–Benson cycle, glyceraldehydes-3-phosphate (G3P), which is responsible for three of the five carbons in the isoprene skeleton and requires 14 mol of NADPH and 20 mol of ATP for each mole of isoprene synthesized [37–42] (Figure 8). Thus, it is expected that the relationship between *Pn* and *Is* be close under ambient CO$_2$ conditions (Figure 1b).

The production of isoprene from the tropical species *I. edulis* under a CO$_2$-free atmosphere in this study is consistent with previous research on several temperate plant species including myrtle (*Myrtus communis*), buckthorn (*Rhamnus alaternus*), velvet bean (*Mucuna pruriens*), gray poplar (*Populus x canescens*), aspen (*Populus tremuloides*), and tasmanian bluegum (*Eucalyptus globulus*) [16,27,32,43]. We demonstrate for the first time that *Is* from *I. edulis* under CO$_2$-free air are stimulated by light and temperature in a similar fashion to emissions under ambient CO$_2$, albeit with important differences (Figures 2b and 5b). Relative to emissions under ambient CO$_2$, *Is* under a CO$_2$-free atmosphere were reduced and saturated at low light levels (e.g., 100 µmol m^{-2} s^{-1} PPFD) versus no clear light saturation under ambient CO$_2$ up to 2000 µmol m^{-2} s^{-1} PPFD. Other studies that observed *Is* under limited or zero CO$_2$ have suggested that alternative carbon sources, that do not come directly from photosynthesis, such as stored starch and carbohydrates transported in the xylem stream help to maintain the carbon supply for isoprene when a reduced atmospheric CO$_2$ source is limiting G3P formation, while NADPH and ATP are still producing by the photochemical phase of photosynthesis [26]. In this study, we provide quantitative evidence that most carbon alternatives for isoprene production are derived from the reassimilation of internal CO$_2$ sources.

Previous studies have sought to understand how these alternative carbon sources can maintain high rates of *Is* when *Pn* is reduced under stress [25,34]. However, the decrease in *Pn* does not necessarily mean that gross photosynthesis rates are decreasing, but rather that decarboxylation processes are being stimulated (especially photorespiration), increasing the CO$_2$ released directly into the cell. At increasing temperatures the oxygenating reaction of RuBisCO has a relative advantage over the carboxylation reactions because (1) stomatal closure reduces the uptake of atmospheric CO$_2$, (2) the solubility of CO$_2$ declines stronger than the solubility of O$_2$, and (3) the kinetic properties of RuBisCO decrease the affinity for CO$_2$ more strongly than that for O$_2$, causing a proportional increase in photorespiration [44].

Indeed, the *Is* is stimulated by elevated temperatures (Figure 4), and this dependence is a consequence of high temperature optimum for isoprene synthase and the use of excess energy not used in the Calvin–Benson cycle by the enzymatic reactions of the MEP pathway [45–47].

However, when leaves were fed with the photosynthesis inhibitor DCMU under a light response curve it was not possible to observe *Is* anymore (Figure 3). The same result occurred when the light was turned off during a temperature response curve (Figure 6). This confirms that *Is* is dependent on the photochemical products of photosynthesis (NADPH and ATP). Once these energetic cofactors are no longer produced, it is not possible to maintain the isoprene synthesis by the MEP pathway

demonstrating that the *Is* is dependent on these energetic cofactors products but can be maintain without the external carbon supply during several hours.

4.2. CO_2 Reassimilation from Decarboxylation Process as an Alternative Carbon Source to Isoprene Synthesis

With respect to the molecular composition, 60% of carbon that comprises the isoprene molecule is assigned to G3P, as one of initial precursors of the MEP pathway [15,48]. When the external CO_2 source was removed from the reference air stream, 19% ± 7% of *Is* continued relative to *Is* observed under ambient CO_2. This percentage is in the range observed by studies that analyzed the contribution of alternative carbon sources to isoprene synthesis by providing atmospheres of $^{13}CO_2$ to leaves. In these studies, it was observed that ~10%–30% of the carbon atoms were not from atmospheric CO_2, but instead originated from other sources [23,26,27]. Although the possibility of reassimilated CO_2 as a source of carbon for isoprene has been discussed in the literature, our results support a possibility that it represents the main alternative carbon source for isoprene and is largely responsible for maintaining high *Is* rates under abiotic stress conditions like high temperatures, which decrease *Pn*.

A study carried out in leaves of *Populus* sp. under 0 μmol mol^{-1} CO_2 observed that *Is* rates were little affected relative to the measures carried out under ambient CO_2 [32]. However, the authors observed that, when photorespiration was inhibited by the absence of O_2, it was no longer possible to observe *Is*. In our study, we observed that the *Is* rates are high under photorespiratory conditions by stimulating the photorespiratory process by removing the CO_2 from the reference air.

In agreement with these results, a previous study provided a [2−^{13}C]glycine (a photorespiratory intermediate) solution to *I. edulis* leaves under high temperature and observed *Is* with labeled carbon atoms [25]. The authors suggested that, in situations where photorespiration is stimulated, reassimilation of photorespiratory CO_2 by photosynthesis acts as a protective mechanism to avoid oxidative stress by consuming excess ATP and NADHP produced by the light reactions. Likewise, our results suggest that increasing the carbon flux through the MEP pathway, which also consumes excess photosynthetic ATP and NADPH, further increases plant tolerance to abiotic stress including high temperatures and drought. Thus, the coupled activity of enhanced photorespiration and the MEP pathway together with reassimilation of internal CO_2 sources may offer plants protection against abiotic stress by helping balance the sources and sinks of photosynthetic ATP and NADPH [44] (Figure 8).
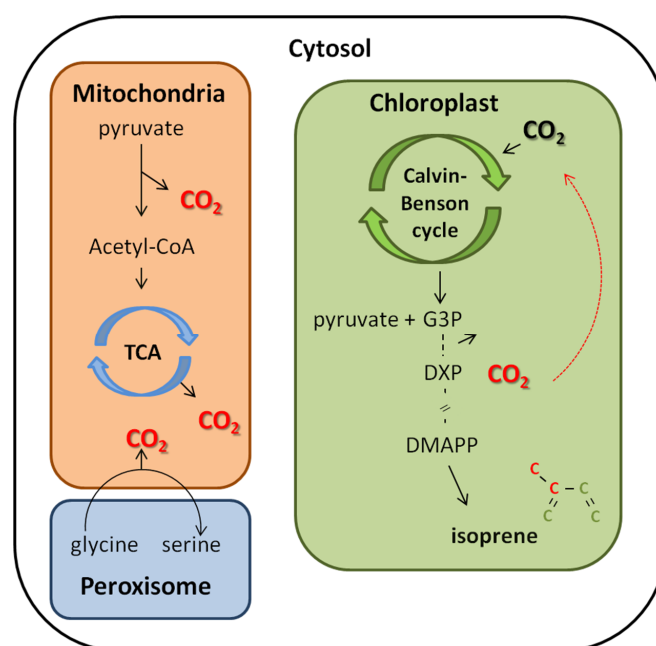

Figure 8. Simplified scheme of the main decarboxylation processes that may contribute as alternative sources for isoprene synthesis: respiration, photorespiration, and the first reaction of the DOXP/MEP pathway.

When sodium bicarbonate ($NaHCO_3$) was provided to *I. edulis* leaves under CO_2-free air, the bicarbonate could be decarboxylated by carbonic anhydrase enzyme. The CO_2 released by the decarboxylation could be fixed by the Calvin–Benson cycle, constituting a G3P molecule, being able to enter into the DOXP/MEP pathway and compose isoprene molecules as observed (Figure 7).

Under conditions of limiting CO_2, a potential alternative source to isoprene synthesis is the CO_2 released into the cell by the decarboxylation process. A previous study estimated that the refixation rate of mitochondrial respiration was related to the incomplete carbon labeling of *Is*, suggesting that respiratory CO_2 can contribute to isoprene formation [24]. Another study in *Populus deltoides* Barr. leaves measured *Is* under N_2 and $^{13}CO_2$ atmosphere and observed that the inhibition of photorespiration did not remove the ^{12}C in isoprene released from leaves under a $^{13}CO_2$ atmosphere and suggested that the unlabeled carbon in isoprene molecules is not derived from photorespiratory carbon [23]. On the other hand, in *I. edulis* under $[2–^{13}C]$glycine, feeding a strong labeling of isoprene molecules was observed, suggesting that the CO_2 released from photorespiration can be incorporated into isoprene molecules [25]. In addition to (photo)respiration, other decarboxylation processes could be acting as alternative carbon sources to isoprene synthesis, including biosynthetic pathways such as the DOXP/MEP, fatty acid, mevalonate, fermentation, and the oxidative pentose phosphate pathway [48–50].

The incorporation of labeled CO_2 released by bicarbonate decarboxylation into isoprene molecules was observed within minutes after placing the leaf in a ^{13}C sodium bicarbonate solution (Figure 7). This confirms the contribution of CO_2 reassimilation to isoprene synthesis and suggests that other decarboxylation processes can contribute to isoprene synthesis as a primary carbon source under reduced atmospheric CO_2 availability. These results have relevance for the next decades of climate change predictions [51], since higher temperatures increase respiration and photorespiration and consequently the amount of CO_2 released in to the leaf. This CO_2 can contribute to *Is* in stress situations that could decrease the stomatal conductance and the carbon uptake.

4.3. The Reassimilation of CO_2 in the Cell Interior Linked to Isoprene Emission: Its Importance for Plant Physiological Functioning under Changing Environmental Factors

In our experiment, high temperatures and light intensities under a CO_2-free atmosphere caused a remarkable depression of *Pn* and substantial increase in *Is*, suggesting that other sources than recently assimilated carbon are used for isoprene formation. Subsequently, when labeled sodium bicarbonate was provided, the emissions of labeled isoprene indicate that the reassimilation of CO_2 released in the chloroplast can contribute as a carbon source to isoprene synthesis. This is in line with evidence from Loreto et al. [24] who showed refixation of unlabeled CO_2 derived from respiration is an alternative source of carbon for isoprene in nonstressed leaves.

Under normal conditions, the reassimilation of CO_2 in the chloroplast can reach up to 40% [52] and increase to more than 80% under low CO_2 conditions and high temperature [53]. The amount of photorespiratory or respiratory CO_2 emitted or recycled by leaves have been associated with an improvement of the photosynthetic carbon gain [52,53]. Therefore, the reassimilation of CO_2 in the chloroplast can contribute significantly for photosynthetic carboxylation efficiency by minimizing the loss of photosynthetic carbon caused by stimulus of photorespiration and respiration.

Previous report have pointed out that the stimulus of internal recycling of photorespired and respired CO_2 in plants may become more important at conditions with high temperatures, low CO_2, water stress, or high irradiance [54–57]. However, in some cases, the respiratory rate is dramatically inhibited by water stress or extreme temperature conditions, while the photorespiration rate increases significantly [58]. Efficient internal recycling of photorespired CO_2 linked to isoprene synthesis might improve tolerance against high irradiance and temperature, commonly experienced in the tropics, besides increasing the overall carbon budget of plants. Nevertheless, the contribution of each of these decarboxylation processes to the CO_2 balance is highly variable, depending on the species, stage of growth, biotic and abiotic factors [48,50–52]. In addition, there is little evidence for widespread uses of

an effective mechanism to trap and reassimilate photorespired CO_2 in tropical species, and particularly with relation with *Is*. Thus, subsequent research is needed to clarify the overall significance of trapping of CO_2 in the cell interior and its flux to isoprene production, particularly in tropical forests, where the bulk of the world's *Is* occur, and both temperature and drought risk are expected to increase according to most climate scenarios [59–61].

5. Conclusions

Previous studies have shown that plants can use nonatmospheric carbon sources to produce isoprene. Most studies have focused on stored carbon sources including carbohydrates (starch and glucose) and their metabolites (pyruvate and phosphoenolpyruvate) as potentially important alternative carbon sources and few studies are made with tropical species. In this study, we show that the CO_2 released by decarboxylation processes is an important carbon source for *I. edulis*—a widespread tropical specie in Amazon forest. Once decarboxylation processes release CO_2 within the leaf mesophyll, our observations suggest that this process acts as a quantitatively important carbon source for isoprene syntheses mainly during stress situations when the stomata are closed and the amount of internal CO_2 decreases. This study enhances our knowledge of isoprene emission by tropical species under conditions that plants often experience in the tropics, where the amount of irradiance and temperature, the two main environmental drivers of isoprene fluxes, are high throughout the year. The reassimilation of released CO_2 within the leaf and its use for isoprene synthesis may be a key tolerance mechanism against a specific type of stress, for example high irradiance and high temperature in tropical environments.

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