Phytogenic biosynthesis and emission of methyl acetate

KOLBY JARDINE1, FREDERIK WEGENER2,3, LEIF ABRELL1, JOOST VAN HAREN1 & CHRISTIANE WERNER2

1Climate Science Department, Earth Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA, 2AgroEcosystem Research, University of Bayreuth, Universitätsstr. 30, 95447 Bayreuth, Germany, 3Experimental and Systems Ecology, University of Bielefeld, Universitätsstrasse 25, D-33615 Bielefeld, Germany, 4Department of Chemistry & Biochemistry, Department of Soil, Water and Environmental Science, and 5Biosphere 2, University of Arizona, Tucson, AZ 85721, USA

ABSTRACT

Acetylation of plant metabolites fundamentally changes their volatility, solubility and activity as semiochemicals. Here we present a new technique termed dynamic 13C-pulse chasing to track the fate of C1–3 carbon atoms of pyruvate into the biosynthesis and emission of methyl acetate (MA) and CO2. 13C-labelling of MA and CO2 branch emissions responds within minutes to changes in 13C-positionally labelled pyruvate solutions fed through the transpiration stream. Strong 13C-labelling of MA emissions occurred only under pyruvate-2-13C and pyruvate-2,3-13C feeding, but not pyruvate-1-13C feeding. In contrast, strong 13CO2 emissions were only observed under pyruvate-1-13C feeding. These results demonstrate that MA (and other volatile and non-volatile metabolites) derive from the C2,3 atoms of pyruvate while the C1 atom undergoes decarboxylation. The latter is a non-mitochondrial source of CO2 in the light generally not considered in studies of CO2 sources and sinks. Within a tropical rainforest mesocosm, we also observed atmospheric concentrations of MA up to 0.6 ppbv that tracked light and temperature conditions. Moreover, signals partially attributed to MA were observed in ambient air within and above a tropical rainforest in the Amazon. Our study highlights the potential importance of acetyl coenzyme A (CoA) biosynthesis as a source of acetate esters and CO2 to the atmosphere.

Key-words: acetyl fragment; dynamic pulse chase; methyl acetate; pyruvate positional labelling; secondary metabolism; stable carbon isotopes; volatile organic compound.

INTRODUCTION

Emissions from terrestrial vegetation are the largest source of volatile organic compounds (VOCs) to the atmosphere. These compounds affect a number of important atmospheric processes crucial to air quality and climate, including the production of ground level ozone and secondary organic aerosols (Went 1960; Rasmussen 1972). Although their identities, quantities and biological functions remain uncertain, model estimates suggest that oxygenated VOCs contribute up to 22% of the 1015 g emitted into the atmosphere annually as biogenic VOCs (Guenther et al. 1995, 2012). One group of oxygenated compounds emitted by plants that play important ecological roles, including plant–plant and plant–insect interactions, are volatile acetate esters (Engelberth et al. 2004; Chehab et al. 2008, 2010). Volatile acetate esters have particularly pleasant odours and are key aroma compounds in flowers (Shalit et al. 2003; Knudsen et al. 2004), fruits (Hansen et al. 1992; Ueda et al. 1992), spices (Herderich et al. 2010; Szewczyk, Wolfe & Mencer 2011) and beverages (Ramey & Ough 1980; Wieland et al. 2012). An enormous array of volatile acetate esters can be produced and emitted from plants, including short chain acetate esters (e.g. methyl acetate, ethyl acetate) (Suchet et al. 2011), medium chain acetate esters (e.g. propyl acetate, butyl acetate, pentyl acetate) (Takeoka, Buttery & Flath 1992), monoterpenes (e.g. S-3-hexen-1-yl acetate, heptyl acetate) (Dudareva et al. 1998; Guterman et al. 2006) and fatty acid-derived acetate esters (e.g. Z-3-hexen-1-yl acetate, heptyl acetate) (Fall et al. 1999; Jardine et al. 2012; Pellegrini et al. 2012).

In plants, acetate esters of metabolites and biopolymers normally derive from the transfer of an acetyl moiety from acetyl coenzyme A (acetyl CoA) to an alcoholic substrate catalyzed by an alcohol acetyltransferase enzyme (Shalit et al. 2003). Acetyl CoA is a central plant metabolite involved in a number of fundamental plant processes, including anaerobic and catabolic metabolism as well as signalling and regulatory mechanisms. It is a product of photosynthesis and carbohydrate, amino and fatty acid catabolism while serving as a substrate in mitochondrial respiration. It is also a key substrate used in the biosynthesis of a very large array of both primary and secondary organic compounds. In addition to many non-volatile organic compounds (fatty acids, some amino acids, flavonoids, phenolics, alkaloids, stilbinoids, etc.) (Oliver, Nikolau & Wurtele 2009), acetyl CoA and its precursors pyruvate and phosphoenolpyruvate, provide substrate for a large array of VOCs, including volatile isoprenoids (isoprene, monoterpenes, sesquiterpenes), oxygenated VOCs (acetaldehyde, ethanol, acetic acid, acetone) and fatty acid oxidation products (C6 green leaf volatiles) (Fig. 1).

Acetylation is a widespread mechanism employed in a large array of processes including the regulation of energy balance, metabolism, and gene expression via protein acetylation (Xing & Poirier 2012), regulating the structural and chemical properties of cell walls through O-acetylation (Pauly & Scheller 2000), and controlling the volatility, solubility, reactivity and biological activity of low molecular
weight metabolites (Oliver et al. 2009, Xing & Poirier 2012). Recently, we demonstrated that photoassimilation of 13CO2 by mesquite branches (Prosopis velutina) resulted in strong and rapid 13C-labelled oxygenated VOCs (acetaldehyde, ethanol and acetic acid) and the acetyl group of (Z)-3-hexen-1-yl acetate following light-dark transitions (Jardine et al. 2012). These observations support the presence of a pyruvate dehydrogenase bypass pathway (PDH bypass) in plants (Wei et al. 2009), which may contribute to acetyl-CoA pools used in a variety of biosynthetic processes and the acetylation of metabolites. Supporting this view, we have utilized pyruvate positional 13C-labelling as an innovative tool to trace plant metabolic pathways associated with acetyl CoA metabolism in individual leaves. We showed that like acetyl CoA, the biosynthesis of volatile isoprenoids (isoprene, monoterpenes and sesquiterpenes) and oxygenated VOCs (acetaldehyde, ethanol, acetic acid and acetone) is associated with pyruvate C1 decarboxylation reactions (Jardine et al. 2010c). Pyruvate-2,13C feeding of mango leaves (Mangifera indica) resulted in a significant incorporation of the 13C-label into volatile isoprenoids and oxygenated VOCs. However, when pyruvate-1,13C was used, less 13C-label was observed in the VOCs produced. In contrast, during a separate study focusing on CO2 emissions of the Mediterranean shrub Halimium halimifolium (Priault, Wegener & Werner 2009; Wegener, Böyschlag & Werner 2010), when pyruvate-1,13C was supplied, δ13CO2 continuously increased during the light period. However, when pyruvate-2,13C was supplied, the evolved CO2 was more depleted in 13C.

Here we combined techniques for isotopic analysis of VOCs [proton transfer reaction-mass spectrometry (PTR-MS) and gas chromatography-mass spectrometry (GC-MS)], CO2 [cavity ringdown spectroscopy (CRDS)] and positional labelling which enables real-time tracing of the fate of the C1,3 atoms of pyruvate into biosynthetic processes or decarboxylation to CO2. We detected diurnal emissions of methyl acetate (MA), the simplest acylated metabolite, from vegetative tissue as a tracer of acetyl CoA metabolism in leaf tissues. Currently, sources of MA in the atmosphere are mainly considered anthropogenic and include fossil fuel burning via atmospheric oxidation of ethers and other industrial processes. However, MA is generally considered a common fermentation product in fruits and flowers (Matich et al. 2003), but emissions to the atmosphere are rarely reported from leaf tissues. Previously, MA emissions were observed from creosotebush ( Larrea tridentata ) in the Sonoran Desert (Jardine et al. 2010a) and mechanically wounded leaves of legumes (Davison et al. 2008), suggesting potentially important biogenic sources to the atmosphere. In this study, we present a novel technique termed dynamic pulse chasing with pyruvate positional 13C-labelling, which reveals, in real time, the differential fate of C1 versus C2,3 atoms of pyruvate to MA versus CO2 production from intact leaves and branches. Further, we investigate the extent to which the biosphere might represent a significant source of MA to the atmosphere. We evaluate the dependence of MA emissions from tropical plants on light and air temperature within a large diverse tropical rainforest mesocosm, and quantify vertical atmospheric MA concentration gradients within and above a primary tropical rainforest in the central Amazon during the 2010 dry and 2011 wet seasons.

MATERIALS AND METHODS

In this study, we utilized 20 potted H. halimifolium L. plants grown under controlled light, temperature, and humidity conditions in a walk-in growth chamber at the University of Bielefeld, Germany. Attached or detached branches of H. halimifolium were enclosed in one of two glass branch chambers (500 mL volume) and flushed continuously (800 mL min⁻¹) with hydrocarbon-free air, generated by passing room air through a series of three high-purity hydrocarbon traps (Restek Inc., Bellefonte, PA, USA). A third empty glass chamber was used simultaneously as a control. VOC identities, enclosure concentrations and isotopic composition were determined using an online PTR-MS (Ionicon Analytik, Innsbruck, Austria) and a GC-MS (5975C series, Agilent Technologies, Bellefonte, PA, USA) with sample collection/injection using thermal desorption (Unity 2, Markes International, Llantrisant, UK) as described below. In addition, enclosure concentrations of CO2 and H2O as well as the stable carbon isotopic composition of CO2 were determined using a cavity ringdown spectrometer (CRDS, G2101-i, Picarro Inc., Santa Clara, CA, USA). CO2 and H2O concentrations entering the enclosures were those of the ambient air and a large (~300 L) buffer volume was utilized to dampen out high frequency variations. Gas samples from control and plant enclosures were connected to a Teflon solenoid valve system (PTFE, Cole-Parmer, Vernon Hills, IL, © 2013 John Wiley & Sons Ltd, Plant, Cell and Environment

Figure 1. Simplified schematic diagram of CO2 and VOC metabolism in photosynthetic cells including one putative mechanism for MA biosynthesis from the acetylation of methanol with acetyl CoA. Shown in red are the volatile products of the pyruvate dehydrogenase bypass, mevalonic acid, 2-C-methyl-D-erythritol 4-phosphate (MEP) and fatty acid pathways. CO2 shown in green represents carbon originating from the C1 of pyruvate whereas CO2 shown in red represents carbon originating from C2,3 of pyruvate. CoA, coenzyme A; MA, methyl acetate; VOC, volatile organic compound.

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13C-labeling experiments

We followed online VOC and CO2 emissions and their stable carbon isotope composition using our new dynamic pulse chasing technique, based on positional-specific 13C-pyruvate branch feeding. Fresh 20 mM solutions of pyruvate, pyruvate-1-13C, pyruvate-2-13C and pyruvate-2,3-13C were prepared 1 day prior to each pulse chase experiment by dissolving the appropriate amount of each component in 10 mL of tap water. Following the introduction of an attached branch in the enclosure, the stem was cut, placed in tap water and immediately re-cut under water. Thereafter, the stem was quickly transferred into a second pyruvate solution in a different Eppendorf tube. The solutions were changed in this manner up to 14 times for a single branch. For the first branch in the light, pyruvate solutions were fed during a pulse chase experiment in the following sequence: unlabelled pyruvate, pyruvate-2,13C, pyruvate-1,13C, pyruvate-2,3,13C, unlabelled pyruvate, pyruvate-2,13C and pyruvate-1,13C. For the second branch, pulse chasing of pyruvate-1,13C with pyruvate-2,3,13C in the light was repeated seven times, with water (H2O) delivered at the beginning and end of the experiment as well as after the first pulse chase. Four additional branches were pulse chased with pyruvate-1,13C and pyruvate-2,3,13C three times in the light, but with darkening of the branch enclosure during the last ~15 min in each solution (Supporting Information Figs S1 & S2). Throughout the pulse-chase experiments, continuous online measurements of the isotopic composition of VOCs (PTR-MS) and CO2 (CRDS) were made. In a separate set of experiments, three thermal desorption (TD) tube samples were collected in the light and analysed by GC-MS from each of four treatments: (1) a detached branch during continuous pyruvate-1,13C feeding; (2) a detached branch during continuous pyruvate-2,13C feeding; (3) an attached branch as a control; and (4) an empty enclosure to determine the system background.

Thermal desorption GC-MS

Enclosure air samples were collected by drawing 200 mL min−1 of enclosure air through a TD tube for 30 min (6.0 L) by connecting a mass flow controller and a pump downstream of the tube. TD tubes were purchased commercially, filled with Tenax TA, graphitized carbon and Carboxen 1000 adsorbents (Markes International). The tube samples were analysed utilizing a Unity 2 thermal desorption system (Markes International) interfaced with a 5975C series gas chromatograph/electron impact mass spectrometer with a triple-axis detector (Agilent Technologies). After loading a tube in the Unity 2 thermal desorption system, the collected samples were dried for 3 min with 30 mL min−1 of ultra-high purity helium (all flow vented out of the split vent) before being transferred to the Unity 2 cold trap (water management cold trap) held at 30 °C by heating to 300 °C for 5 min with 50 mL min−1 of helium. During injection, the trap was heated to 300 °C for 3 min while back-flushing with carrier gas at a flow of 6.5 mL min−1; 5 mL min−1 vented through the split and 1.5 mL min−1 directed to the column (Agilent DB624 60 m × 0.32 mm × 1.8 µm), temperature programmed with an initial hold of 3 min at 40 °C followed by an increase to 220 °C at 6 °C min−1 followed by a hold at 220 °C for 1 min. The mass spectrometer was configured for trace analysis with a 15 times detector gain factor and operated in scan mode (m/z 40–300). Identification of MA in TD tube samples was confirmed by comparison of mass spectra with the NIST mass spectral library and with a MA permeation tube standard (Kin-Tek Labs, La Marque, TX, USA) and by comparison of retention times. Identification of other volatiles such as monoterpenes (MTs) and sesquiterpenes (SQTs) was based solely on mass spectra comparison with the NIST mass spectral library.

PTR-MS

The target volatiles in ambient air and enclosure samples were analysed using a commercial high sensitivity PTR-MS (Ionicon Analytik, Innsbruck, Austria) that was operated in standard conditions with a drift tube voltage of 600 V, temperature of 40 °C and pressure of 2.0 mb. The following mass to charge ratios (m/z) were monitored during each PTR-MS measurement cycle: 21 (H316O+), 32 (O2+), 37 (H2O-H2O+) with a dwell time of 20 ms each. The following m/z values were also sequentially measured with a 2 s dwell time and correspond to the protonated molecular weights (parent ions) of the following compounds: m/z 33 (methanol), m/z 43 (acetyl fragment), m/z 45 (acetaldheyde), m/z 47 (formic acid + ethanol), m/z 59 (acetone), m/z 61 (acetic acid), m/z 69 (isoprene), m/z 75 (methyl acetate), m/z 137 (monoterpenes) and m/z 205 (sesquiterpenes). During pyruvate 13C-labelling studies, PTR-MS signals with a 2 s dwell time were monitored including m/z 75 (methyl acetate-1,2,13C), m/z 76 (methyl...
acetate-$^{13}$C), m/z 77, (methyl acetate-$^{1,2,3}$C), m/z 43, (acetyl fragment-$^{13}$C), m/z 44 (acetyl fragment-$^{1,2}$C), m/z 45 (acetyl fragment-$^{1,2,3}$C + acetaldehyde). Under the oxidizing conditions of the Amazon atmosphere, m/z 75 is assumed to be the combination of MA and hydroxyacetone (HA), a second-generation oxidation product of isoprene (Karl et al. 2007; Yokelson et al. 2009; Warneke et al. 2011). To quantify MA in branch and mesocosm air samples, the PTR-MS was calibrated to MA using the dynamic solution injection technique as previously described (Jardine et al. 2010b). Given that we used the calibration factor based only on MA to estimate the combined atmospheric concentrations of MA + HA within the Amazon canopy, these measurements should be considered rough estimates.

**CRDS**

The CO$_2$ concentration and isotopic composition of the sample gas was measured with a CRDS (G2101-i, Picarro Inc.), which enables real-time analysis of the isotopologues $^{13}$CO$_2$ and $^{12}$CO$_2$ as well as the $\delta^{13}$CO$_2$ ratio (‰) of the sample gas relative to the factory-calibrated reference standard Vienna Pee Dee Belemnite (VPDB). Isotope ratios are presented in $\delta$-notation as:

$$\delta^{13}C = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \% $$

where $R_{\text{sample}}$ is the measured $^{13}$C/$^{12}$C isotope ratio of the sample and $R_{\text{standard}}$ is the $^{13}$C/$^{12}$C isotope ratio of the standard (VPDB). One-minute averages were calculated from the $\delta^{13}$C values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS. The CRDS was regularly calibrated with a laboratory reference gas and cross-calibrated to values provided by the CRDS.

**Amazon field study**

Ambient measurements of MA + HA at six heights within and above a primary rainforest canopy were carried out at the TT34 tower (02°35.657° S, 060°12.557° W) in the Reserva Biologica do Cueiras in central Amazonia, 60 km NNW of the city of Manaus, Brazil. The site is run by INPA (Instituto Nacional de Pesquisas da Amazonia) under the Large Scale Biosphere-Atmosphere Experiment in Amazonia (LBA) programme (Martin et al. 2010). The vegetation in this area is considered to be undisturbed, mature, terra firme rainforest, with a leaf area index of 5–6 and an average canopy height of 30 m (Jardine et al. 2011). PTR-MS measurements of ambient MA + HA occurred during the 2010 dry season (2 September–9 November 2010) and 2011 wet season (6 December 2010–27 January 2011). The gradient measurement scheme employed ambient air inlets at six different tower heights (2, 11, 17, 24, 30 and 40 m) sequentially analysed for MA + HA at m/z 75 with a 5 s dwell time (10 min at each inlet, one complete canopy profile per hour). Ambient air (~0.1 m from the tower) was drawn through 1/4 in O.D. heated Teflon tubing using an oil-free diaphragm pump with a sample point to detector delay time of <15 s.

**Biosphere 2 mesocosm study**

Atmospheric measurements of MA within the Biosphere 2 tropical rainforest (44 × 44 m with a total volume of 26 700 m$^3$) were obtained by drawing air through heated Teflon tubing to a nearby (~25 m) laboratory. The rainforest mesocosm currently comprises 91 species of tropical plants from 41 families, including 73 trees under a flat-topped pyramidal glass enclosure operated as a semi-closed system (Allen, Nelson & Alling 2003). The sampling port was located ~20 m off the ground within 3–5 m of a tower equipped with PAR (SO120 Quantum sensors, Apogee Instruments, Logan, UT, USA) and vented and shielded temperature and humidity sensors (HMP45c, Vaisala Instruments, Tucson, AZ, USA). Within the laboratory, the PTR-MS analysed the Biosphere 2 tropical rainforest air continuously for MA concentrations between 22 January and 10 March 2010. Every hour, the MA background was determined from hydrocarbon-free air measurements.

**RESULTS**

**Controlled laboratory studies**

Attached branches of *H. halimifolium*, a mediterranean shrub of the Cistaceae family previously suspected to be a high VOC emitter (Praulet et al. 2009), were found to be a significant emitter of MTs and SQTs (Fig. 2). For the three attached branches studied, mean midday emission rates (1200–1300 h) were 0.10 ± 0.06 nmol m$^{-2}$ s$^{-1}$ for total MTs and 0.26 ± 0.21 nmol m$^{-2}$ s$^{-1}$ for total SQTs (mean ± one standard deviation for three branches). In addition, emissions of the oxygenated VOCs acetaldehyde, methanol, ethanol, acetic acid, acetone, and MA were observed (Fig. 2 and Supporting Information Fig. S3). Surprisingly, mean midday emission rates of MA were high (0.39 ± 0.22 nmol m$^{-2}$ s$^{-1}$) and thus in a comparable range to those of MTs and SQTs. However, while daytime emissions for MTs and SQTs were much higher than at night, significant night-time emissions were observed for MA, particularly early in the morning before the light turned on. Continuous volatile emission rate measurements at high-time resolution revealed enhanced MA and methanol diurnal dynamics. In the dark at night, a slow increase in MA and methanol emissions occurred, followed by a large morning burst within 5 min of switching on the light source (Fig. 2b).

As MA emissions from vegetative tissues are rarely reported, we used GC-MS to verify its presence in the volatile emission blend from attached *H. halimifolium* branches (Fig. 3a). A strong peak was observed in the GC-MS chromatogram at 8.12 min in the enclosure samples with a branch while the empty enclosure samples did not produce a detectable peak. The identification of the GC-MS peak at 8.12 min was verified as MA by a strong match (>95% confidence) with the NIST 2008 mass spectral library and by the identical retention time and mass spectra of an MA gas phase permeation tube standard (Fig. 3a). Under the conditions studied, MA produced the acetyl fragment (CH$_3$CO, m/z 43) in both the PTR-MS and GC-MS. While MA under proton
transfer ionization in the PTR-MS produced a strong signal at m/z 75 corresponding to the protonated parent ion, electron ionization in the GC-MS produced the parent ion at m/z 74. Using the dynamic solution injection technique for calibration (Jardine et al. 2010b), the PTR-MS response to MA at m/z 75 was found to be highly sensitive (175 cps/ppbv) and linear \( R^2 = 0.999 \) across gas-phase concentrations in the range of 0–28.2 ppbv. These observations are consistent with a previous study on PTR-MS fragmentation patterns of MA which found the majority of signal at the protonated parent ion (m/z 75) with 9% occurring as the acetyl fragment (m/z 43) (Buhr, van Ruth & Delahunty 2002).

In order to further explore the metabolic origin of MA emissions from \textit{H. halimifolium} branches, we performed positional-specific \(^{13}\text{C}\)-pyruvate branch feeding experiments to evaluate the hypothesis that pyruvate is used as a substrate in the biosynthesis of MA. The results of GC-MS analysis of air samples collected from detached branches placed in the pyruvate-\(^{13}\text{C}\) solution versus the pyruvate-\(^{2}\text{\textsuperscript{13}\text{C}}\) solution is shown in Fig. 3b. Upon electron impact ionization, the mass spectrum of MA is dominated by the acetyl fragment (m/z 43) and the parent ion (m/z 74). Ions at m/z 44 and m/z 75 represent the singly \(^{13}\text{C}\)-labelled versions of the acetyl fragment and parent ions, respectively. Relative GC-MS mass spectra of MA peaks from attached \textit{H. halimifolium} branches and detached \textit{H. halimifolium} branches under pyruvate-\(^{1}\text{\textsuperscript{13}\text{C}}\) solution displayed similar relative mass spectra, with relative ion ratios consistent with natural \(^{13}\text{C}\)-abundance samples. For example, expected relative ion intensities m/z 43/(43 + 44) for the acetyl fragment for natural abundance samples is 2.2% and measured values were 3.6 ± 0.4% and 4.6 ± 0.1% for samples collected from attached branches and branches fed with pyruvate-\(^{1}\text{\textsuperscript{13}\text{C}}\), respectively. Likewise, expected relative ion intensities m/z 74/(74 + 75) for the MA parent ion is 3.3% and measured values were 3.9 ± 0.6% and 6.0 ± 0.2% for samples collected.

Figure 2. Representative time series plot showing diurnal dynamics of VOC emissions from attached \textit{H. halimifolium} branches with shaded areas indicating dark (grey) and light (yellow) periods. (a) Mean diurnal emission rates of MA, total MTs, and total SQTs from three attached \textit{H. halimifolium} branches. Shaded regions represent mean emission rates ± one standard deviation. (b) High temporal resolution measurements of MA and methanol emission rates together with transpiration rates showing a large emission burst shortly after switching on the light source. MA, methyl acetate; MT, monoterpenes; SQT, sesquiterpenes; VOC, volatile organic compound.

Figure 3. GC-MS analysis of MA emissions from \textit{H. halimifolium} under natural conditions and during positional-specific \(^{13}\text{C}\)-labelled pyruvate branch feeding. (a) Example GC-MS total ion count chromatograms showing the presence of MA from a permeation tube standard containing MA and the volatile emissions of an attached \textit{H. halimifolium} branch. (b) GC-MS relative ion abundances of methyl acetate peaks (mean ± 1 standard deviation, \( n = 3 \)) over the ranges m/z 43–44 (\(^{12}\text{C}\) and \(^{13}\text{C}\)-acetyl fragment ions) and m/z 74–75 (\(^{12}\text{C}\) and \(^{13}\text{C}\)-methyl acetate ions) in air samples including: (1) emissions from an attached \textit{H. halimifolium} branch; (2) emissions from a detached \textit{H. halimifolium} branch in 20 mM pyruvate-\(^{1}\text{\textsuperscript{13}\text{C}}\); and (3) emissions from a detached \textit{H. halimifolium} branch in 20 mM pyruvate-\(^{2}\text{\textsuperscript{13}\text{C}}\) (red, blue and green bars respectively). GC-MS, gas chromatography-mass spectrometry; MA, methyl acetate.

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from attached branches and branches fed with pyruvate-1-13C, respectively. Thus, no notable effect occurred from detaching the branches, nor was a large labelling of MA recorded after feeding pyruvate-1-13C. However, the slight enhancement in 13C-MA under pyruvate-1-13C may be due to the refixation of the C1 carbon atom of pyruvate during photosynthesis, followed by incorporation into MA as was previously observed for isoprene and monoterpenes (Jardine et al. 2010c). In contrast, a strong 13C-enrichment in both the acetyl fragment ion and the MA parent ion was observed in samples collected from branches fed with pyruvate-2-13C. Relative ion intensities for the acetyl fragment ion and the MA parent ion from these samples was 41.1 ± 0.6% and 43.0 ± 0.5%, respectively.

In order to investigate the real-time dynamics of MA biosynthesis and to further elucidate the different fates of the C1, C2 and C3 carbon atoms of pyruvate, we developed a technique termed ‘Dynamic 13C-pulse chase’ which couples rapid changes in positional-specific 13C-pyruvate plant feeding solutions with real-time MA and δ13CO2 detection using PTR-MS and CRDS. In the first experiment, 20 mm solutions of pyruvate, pyruvate-1-13C, pyruvate-2-13C and pyruvate-2,3-13C were fed sequentially through the transpiration stream of a detached H. halimifolium branch for 20–70 min each, followed by an additional cycle through 20 mm pyruvate, pyruvate-2-13C and pyruvate-1-13C solutions.

Figure 4 shows real-time measurements of MA and its acetyl fragment together with δ13CO2 in response to the feeding solutions. We found that the emission dynamics of MA and its acetyl fragment isotopologues with 0–2 13C atoms can be monitored in real time using PTR-MS. Upon placing the branch in the unlabelled pyruvate solution (at 1110 h local time), emissions of 12C-MA and its 13C-acetyl fragment dominated emissions of all other MA isotopologues (green lines Fig. 4). During this time, δ13CO2 ranged slightly above the background values (−12‰ ± 1‰ throughout the experiment) due to photosynthetic discrimination against 13C. Within 10 min after placing the branch in the pyruvate-2,3-13C solution (at 1125 h), emissions of MA-13C and its acetyl-13C fragment began to decline at the same time that strong emissions of MA-13C and its acetyl-13C fragment emerged. Concurrent with the increase in singly 13C-labelled MA was an increase in δ13CO2. At 1225 h, the solution was switched to pyruvate-1,3-13C and within 10 min, emissions of MA-13C and its acetyl-13C fragment began to increase again while emissions of MA-12C and its acetyl-13C fragment began to decline. At the same time, δ13CO2 values increased strongly. At 1325 h, the solution was switched to pyruvate-2,3-13C and within 10 min declining emissions of MA-13C and acetyl-13C fragment was detected with an increase in MA-1,2-13C and acetyl-1,2-13C fragment. At the same time, δ13CO2 markedly decreased. At 1445 h, the branch was returned to the unlabelled pyruvate solution and within 10 min emissions of MA-12C and its acetyl-13C fragment began to increase while emissions of MA-1,2-13C and its acetyl-1,2-13C fragment began to decline. However, the PTR-MS signal for the acetyl-1,2-13C fragment (m/z 45) began to increase again and is likely due to an interference by acetaldehyde (also appearing at m/z 45). At 1545 and 1650 h, the branch was placed back in the pyruvate-2-13C solution and the pyruvate-1-13C solution, respectively, with similar MA, acetyl fragment and CO2 13C-labeling patterns as the first pulse chase in these solutions.

A separate detached H. halimifolium branch was used to demonstrate the possibility of rapidly changing the positional specific 13C-pyruvate solutions in order to produce a continuous non-steady state time series highlighting the best response in 13C-labelled MA and CO2 emissions, and to repeatedly demonstrate the different fates of the pyruvate C1 atoms versus the C3,3 atoms into MA and CO2 emissions by chasing pyruvate-2,3-13C with pyruvate-1-13C multiple times on the same branch (Fig. 5). In this experiment, the branch was first placed in tap water before chasing pyruvate-2,3-13C with pyruvate-1-13C (in each solution for approximately 20 min), followed by incubation in tap water. Thereafter, pyruvate-2,3-13C was again chased with pyruvate-1-13C six additional times before finishing back in tap water. The results show the same pattern as described in the experiment shown in Fig. 4. Under pyruvate-2,3-13C feeding, emissions of the doubly labelled MA-2,3-13C and acetyl-2,3-13C fragment are induced at the expense of MA-13C and acetyl-13C fragment emissions. In contrast, under pyruvate-1-13C feeding, emissions of the MA-13C and acetyl-13C fragment increase again at the expense of the doubly labelled MA-1,2-13C and acetyl-1,2-13C fragment. While pyruvate-2,3-13C feeding lead to a 13C-enrichment in CO2 emissions relative to tap water feeding, pyruvate-1-13C feeding resulted in a much higher 13C-enrichment in CO2 emissions. Throughout the pulse-chase experiment, CO2 concentrations within the enclosure remained relatively constant with a range of 307–331 ppmv with an incoming CO2 concentration in the range of 400–410 ppmv. This indicates that 20 mm pyruvate feeding does not significantly inhibit photosynthesis or alter net CO2 assimilation.
Tropical rainforest mesocosm and field studies

Mean ambient air concentrations of MA together with air temperature and PAR at 20 m height within the Biosphere 2 mesocosm is shown in Fig. 6 (917 h during 22 January–10 March 2010). Whole mesocosm ambient air concentrations of MA displayed a strong diurnal cycle with maxima occurring in early afternoon (∼1400 h) with values up to 0.6 ppbv. Maximum air temperature also peaked in early afternoon (∼14:00 h) whereas PAR peaked earlier at midday (∼1230 h). Significant ambient concentrations of MA up to 0.2 ppbv occurred at night, supporting the possibility of night-time emissions as observed in the laboratory study (Fig. 2). It is apparent that ambient MA concentrations inside the rainforest mesocosm reported here and those of the oxygenated VOCs (acetaldehyde, ethanol, acetic acid and acetone), and volatile isoprenoids (isoprene, monoterpenes and sesquiterpenes) previously reported (Pegoraro et al. 2005; Jardine et al. 2010c) all share a similar diurnal pattern. These observations are consistent with the positional-specific 13C-pyruvate labelling experiments that demonstrate pyruvate as a common precursor. While these results suggest that tropical ecosystems can emit MA into the atmosphere, field studies are needed to further explore this possibility.

In order to investigate the potential role of a primary tropical rainforest in the central Amazon in the biosphere–atmosphere exchange of MA + HA during the 2010 dry and 2011 wet seasons, we collected continuous in situ vertical concentration profiles within and above the ∼30 m canopy, utilizing a high-sensitivity PTR-MS (Fig. 7a–d). Similar to the tropical mesocosm studies, ambient air concentrations at all heights followed strong diurnal patterns with maxima in the early afternoon (1200–1300 h). MA + HA concentrations in the dry season were generally higher than those in the wet season with maximum mean values similar to those in the Biosphere 2 rainforest mesocosm (dry: 0.3 ppbv, wet: 0.1 ppbv, Biosphere 2: 0.4 ppbv). During the 2010 dry season, the mean vertical gradient of ambient MA + HA in the early afternoon (1300–1400 h) indicated net ecosystem deposition at the Amazon forest tower site, with the concentrations highest above the canopy and declining with depth through the canopy (Fig. 7b). In contrast, during the 2011 wet season, the highest ambient concentrations were observed within the canopy (17 m) rather than above the canopy (40 m) (Fig. 7d). This implies that the ecosystem was a net emission source during the wet season. In both the wet and the dry seasons, the lowest ambient concentrations observed were near the ground (2 m). This may be due to low MA production rates by soils and understory vegetation under reduced light and temperature conditions, together with dry/wet deposition and active consumption of MA.

DISCUSSION

Although MA is generally considered a common fermentation product in plants as it is often found in fruits (de Avila et al. 2012), vegetables (Forney, Mattheis & Austin 1991) and flowers (Matich et al. 2003), MA emissions are rarely reported from leaf tissues. Currently, sources of MA in the atmosphere are mainly considered anthropogenic and include fossil fuel burning via atmospheric oxidation of ethers used in automotive fuel additives (Christensen, Ball &
to follow the differential allocation of C\textsubscript{1} versus C\textsubscript{2} and C\textsubscript{3} carbon atoms of pyruvate into biosynthetic and carbon decarboxylation metabolism in plants in real time and may potentially be applied at a number of spatial scales from individual leaves to whole trees. Using this technique at the branch scale, we found that the C\textsubscript{1} atom of pyruvate is decarboxylated during MA biosynthesis while the C\textsubscript{2} and C\textsubscript{3} atoms are directly incorporated into MA. Unlabelled pyruvate and pyruvate-1\textsuperscript{-13}C branch feeding did not result in strong \textsuperscript{13}C-labelling of MA and its acetyl fragment. In contrast, feeding with pyruvate-2\textsuperscript{-13}C and pyruvate-2,3\textsuperscript{-13}C resulted in the biosynthesis and emission of singly and doubly \textsuperscript{13}C-labelled MA and its acetyl fragment, respectively, at the expense of their corresponding \textsuperscript{12}C isotopologues. Relative to feeding with unlabelled pyruvate, branch feeding with pyruvate-1\textsuperscript{-13}C resulted in a strong \textsuperscript{13}C-enrichment in emitted CO\textsubscript{2}. \textsuperscript{13}C-enrichment in emitted CO\textsubscript{2} was also observed to be significant during pyruvate-2\textsuperscript{-13}C and to a greater extent pyruvate-2,3\textsuperscript{-13}C branch feeding, albeit to a lower extent than pyruvate-1\textsuperscript{-13}C. These results indicate that the C\textsubscript{2,3} atoms of pyruvate are incorporated into MA during de novo biosynthesis but that a certain proportion gets also decarboxylated in the light. The tricarboxylic acid cycle (TCA) has been found to be markedly inhibited in the light (Tcherkez et al. 2005; Werner et al. 2009) as it may undergo a major re-organization during illumination (Sweetlove et al. 2010). Some CO\textsubscript{2} may have also evolved from the heterotrophic tissue (stems) of the enclosed branches. Nevertheless, decarboxylation from the C\textsubscript{1} atom of pyruvate was much enhanced pointing towards marked decarboxylation during MA biosynthesis and other processes in the light.

While stronger \textsuperscript{13}C-labelling of MA (Figs 4 & 5, Supporting Information S1 & S2) and MTs (Supporting Information Figs S4 & S5) were observed under pyruvate-2\textsuperscript{-13}C and pyruvate-2,3\textsuperscript{-13}C than pyruvate-1\textsuperscript{-13}C, their temporal \textsuperscript{13}C-labelling responses to changes in \textsuperscript{12}C-pyruvate solutions showed important differences. Whereas MA and CO\textsubscript{2} responded within minutes (Figs 4 & 5) to changes in \textsuperscript{12}C-pyruvate solutions, a large delay (20–30 min) was observed for MTs (Figs S4 & S5). If allowed to reach steady state under a given \textsuperscript{13}C-pyruvate solution, the dynamics following an immediate change to a different \textsuperscript{13}C-pyruvate solution could be exploited to gain new understanding of biosynthesis and degradation rates and pool sizes. Our observations suggest that pyruvate and acetyl CoA are rapidly turned over in plants and hypothesize that MA may derive from the acetylation of methanol by acetyl CoA catalyzed by an alcohol acetyl-transferase enzyme. In contrast, the kinetics of \textsuperscript{13}C incorporation into MT emissions is slower, possibly due in part to a larger pool size and higher number of C\textsubscript{2,3} pyruvate equivalents needed for MT biosynthesis.

The lower incorporation of the C\textsubscript{1} atom of pyruvate into MA relative to the C\textsubscript{2} atom was also observed for the oxygenated VOCs acetaldehyde, ethanol and acetic acid (Supporting Information Fig. S6). This is consistent with our previous findings from mango leaves where the incorporation of the C\textsubscript{1} atom of pyruvate into VOCs including volatile isoprenoids (isoprene, MTs, SQTs) and oxygenated VOCs...
(acetaldehyde, ethanol, acetic acid and acetone) was lower relative to the C₃ atom (Jardine et al. 2010c). These observations highlight the fact that the biosynthesis of a large array of both primary and secondary organic compounds is associated with pyruvate C₁ decarboxylations, which represent a non-mitochondrial source of CO₂ that must contribute to net CO₂ fluxes. Still, little is known on the quantitative contribution from different CO₂ sources originating from different metabolic pathways to the overall CO₂ flux (Werner et al. 2011). For example, the biosynthesis of many non-volatile organic compounds is well known to derive from acetyl CoA (fatty acids, some amino acids, flavonoids, phenolics, alkaloids, stilbenoids, etc.) (Oliver et al. 2009), and therefore must also be associated with pyruvate C₁ decarboxylations. Thus, our study uses MA emissions as a tracer of CO₂ production not directly associated with TCA decarboxylations and its associated ATP and reductant production during oxidative phosphorylation reactions in mitochondria. As mitochondrial respiration is known to be partially inhibited in the light (Atkin, Evans & Siebke 1998) while biosynthetic processes are active, future studies should aim to quantitatively evaluate the role of pyruvate C₁ versus C₂,₃ decarboxylations during photosynthesis.

We also presented preliminary evidence that a whole tropical forest mesocosm and a primary rainforest ecosystem in the central Amazon can emit MA at rates sufficiently high to be detected in the atmosphere using PTR-MS. However, previous atmospheric studies using PTR-MS focusing on m/z 75 have suggested that this signal in complex forest atmospheres arises not only from MA but also from hydroxyacetone (HA), a second-generation oxidation product of isoprene (Karl et al. 2007; Yokelson et al. 2009), and therefore discussions from Pawel Misztal, Nick Hewitt and Peter Harley.

**CONFLICT OF INTEREST**

The authors have a patent submitted for the Dynamic ¹²C-pulse chase technique.

**REFERENCES**


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**Figure S2.** Emissions (nmol m$^{-2}$ s$^{-1}$) of MA and $^{13}$C-MA together with $\delta^{13}$CO$_2$ during a dynamic pulse-chase experiment with two detached *H. halimifolium* branches B3 and B4 showing real-time $^{13}$C-labelling dynamics.

**Figure S3.** Representative time series plots showing diurnal dynamics of oxygenated VOC emissions (nmol m$^{-2}$ s$^{-1}$) from an attached *H. halimifolium* branch.

**Figure S4.** Representative emissions (nmol m$^{-2}$ s$^{-1}$) of monoterpenes together with $\delta^{13}$CO$_2$ during a dynamic pulse-chase experiment with a single detached *H. halimifolium* branch showing real-time $^{13}$C-labelling dynamics at low frequency.

**Figure S5.** Representative emissions (nmol m$^{-2}$ s$^{-1}$) of monoterpenes and the dominant monoterpane fragment together with $\delta^{13}$CO$_2$ during a dynamic pulse-chase experiment with a single detached *H. halimifolium* branch showing real-time $^{13}$C-labelling dynamics at high frequency.

**Figure S6.** Representative $^{13}$C-labelling dynamics of oxygenated VOCs and CO2 emissions during dynamic pulse-chase experiments with pyruvate-1-$^{13}$C and pyruvate-2-$^{13}$C on a single detached *H. halimifolium* branch.