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RESEARCH PAPER

Emissions of putative isoprene oxidation products from mango branches under abiotic stress

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Abstract

Although several per cent of net carbon assimilation can be re-released as isoprene emissions to the atmosphere by many tropical plants, much uncertainty remains regarding its biological significance. In a previous study, we detected emissions of isoprene and its oxidation products methyl vinyl ketone (MVK) and methacrolein (MACR) from tropical plants under high temperature/light stress, suggesting that isoprene is oxidized not only in the atmosphere but also within plants. However, a comprehensive analysis of the suite of isoprene oxidation products in plants has not been performed and production relationships with environmental stress have not been described. In this study, putative isoprene oxidation products from mango (Mangifera indica) branches under abiotic stress were first identified. High temperature/light and freeze–thaw treatments verified direct emissions of the isoprene oxidation products MVK and MACR together with the first observations of 3-methyl furan (3-MF) and 2-methyl-3-buten-2-ol (MBO) as putative novel isoprene oxidation products. Mechanical wounding also stimulated emissions of MVK and MACR. Photosynthesis under 13CO2 resulted in rapid (<30 min) labelling of up to five carbon atoms of isoprene, with a similar labelling pattern observed in the putative oxidation products. These observations highlight the need to investigate further the mechanisms of isoprene oxidation within plants under stress and its biological and atmospheric significance.

Keywords: 2-Methyl-3-buten-2-ol, 3-methyl furan, methacrolein, methyl vinyl ketone, reactive oxygen species, volatile organic compounds.

Introduction

Isoprene (C5H8) is emitted in large quantities by many terrestrial plants directly into the atmosphere, where it acts to fuel atmospheric chemistry leading to the formation of a number of species with high relevance to air quality and climate change.

Abbreviations: 3-MF, 3-methyl furan; 3-MT, 3-methyl thiophene; GC-MS, gas chromatography/mass spectrometry; GLV, green leaf volatiles; MACR, methacrolein; MBO, 2-methyl-3-buten-2-ol; MVK, methyl vinyl ketone; NIST, the National Institute of Standards and Technology; PAR, photosynthetically active radiation; PTR-MS, proton transfer reaction/mass spectrometry; ROS, reactive oxygen species; sccm, standard cm3 min–1; slpm, standard l min–1; TD, thermal desorption.

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including organic nitrates (Lockwood et al., 2010), ground level ozone (Pierce et al., 1998), and secondary organic aerosols (Kroll et al., 2006) that may act as effective cloud condensation nuclei (Engelhart et al., 2011). Due to its high annual global emission rate of 500–750 Tg year$^{-1}$ (Guenther et al., 1995) and high reactivity to atmospheric oxidants such as the OH radical (Atkinson and Arey, 2003), isoprene emissions from terrestrial vegetation exert a controlling effect on the oxidative power of the lower atmosphere. Once emitted, isoprene is rapidly oxidized in the atmosphere (1–2 h lifetime with respect to oxidation by OH radicals) with first-order oxidation products dominated by formaldehyde (50–70%), methyl vinyl ketone (MVK, 25–39%), methacrolein (MACR, 17–27%), and 3-methyl furan (3-MF, 4.2–5.4%) (Atkinson and Arey, 2003). However, efforts to quantify the magnitude of isoprene chemistry–climate feedbacks have been hindered by major uncertainties as to why plants dedicate a significant fraction (a few per cent under normal physiological conditions) of assimilated carbon to isoprene emissions in the first place (Pacifico et al., 2009). In contrast to its role in atmospheric chemistry, very little is known about the behaviour and effects of isoprene in planta, including potential redox reactions and products.

Reactive oxygen species (ROS) including singlet oxygen ($O_2^*$), superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical (OH) are continuously generated in plants due to the incomplete reduction of oxygen. While initially considered toxic byproducts of aerobic metabolism, ROS are now widely recognized to play a multitude of signalling roles during the regulation of biological processes including growth and development (Foreman et al., 2003), cell cycle (Feher et al., 2008), programmed cell death (Overmyer et al., 2003), hormone signalling and stomatal regulation (Kwak et al., 2003; Pei et al., 2000), and biotic and abiotic stress responses (Mittler, 2002; Mullineaux and Karpinski, 2002). While ROS concentrations within plants are generally kept low by cellular antioxidant defence systems, ROS accumulation during stress can overwhelm these systems, leading to the initiation of defence signalling mechanisms (Miller et al., 2010). A growing body of evidence suggests that isoprene protects plants under stress by functioning as an effective antioxidant (Vickers et al., 2009a) and/or by stabilizing membranes (Velikova et al., 2011). Thus, isoprene may protect photosynthesis during stress by stabilizing thylakoid membranes and directly scavenging ROS (Velikova et al., 2012). For example, isoprene increases the plant’s ability to conduct photosynthesis at elevated temperatures (Singhsaas et al., 1997) and, in the presence of ozone, exogenously supplied isoprene reduces the accumulation of ROS (Loreto et al., 2001; Loreto and Velikova, 2001; Velikova et al., 2004; Vickers et al., 2009b).

The isoprene oxidation products MVK and MACR are known to be highly active defence signalling compounds, and can initiate the expression of a wide array of defence genes (Almeras et al., 2003). In line with this, fumigation of plants with MVK resulted in the upregulation of genes involved in defence signalling and ROS metabolism (Kari et al., 2010). This enhanced biological activity derives from the fact that MVK and MACR act as reactive electrophiles due to the presence of $\alpha,\beta$-unsaturated carbonyl groups, and therefore show high reactivity with nucleophiles (Uchida et al., 1998). Reactive electrophiles may potentially affect gene expression at all levels by chemically reacting with nucleic acids, proteins, and small molecules as well as by indirectly lowering pools of cellular reductants (Farmer and Davoine, 2007).

Recently, we obtained evidence that isoprene is oxidized not only in the atmosphere but also within plants. Using gas chromatography/mass spectrometry (GC-MS) and proton transfer reaction/mass spectrometry (PTR-MS), we detected gas-phase emissions of isoprene and its oxidation products MVK and MACR from high temperature/light-stressed desert (Jardine et al., 2010a) and tropical (Jardine et al., 2012) plants. We demonstrated at the leaf, branch, and enclosed mesocosm scales that higher emissions of MVK+MACR relative to isoprene occurred during high temperature/light stress. In addition, leaf feeding of pyruvate-$^{13}$C (a precursor of isoprene) through the transpiration stream was used to track $^{13}$C into de novo isoprene biosynthesis and its oxidation products MVK and MACR, suggesting that these compounds are produced directly from isoprene reactions.

In this study, using a coupled PTR-MS, GC-MS, and photosynthesis system, we first investigated branch-level emissions of isoprene and putative isoprene oxidation products MVK and MACR from a high isoprene-emitting species, Mangifera indica (mango), and a non-isoprene-emitting species Annona muricata (soursop) under abiotic stress including high temperature/light, freeze– thaw/dehydration, and mechanical wounding. Next, we quantified emissions of the putative novel isoprene oxidation products 3-MF, 3-methyl thiophene (3-MT), and 2-methyl-3-buten-2-ol (MBO) from mango branches in response to abiotic stress. Finally, we explored the possibility that these compounds derive from isoprene oxidation within plants (Fig. 1) by tracking isoprene biosynthesis and volatile emissions during photosynthesis under $^{13}$CO$_2$.

**Materials and methods**

**Experimental setup**

A recent synthesis paper on isoprene emissions from terrestrial ecosystems found that only three out of 20 publications reviewed reported isoprene emissions from tropical regions, even though the tropics were estimated to contribute $\sim$80% of global annual isoprene emissions (Guenther et al., 2006). Previous studies (Harley et al., 2004; Jardine et al., 2010b, 2012) found high isoprene emissions from leaves of tropical mango trees (Mangifera indica). We therefore used mango as a representative tropical species from which to study isoprene biosynthesis, oxidation, and emission. For this purpose, a single mango tree ($\sim$7 m tall) and soursop tree (Annona muricata, a non-isoprene emitter, $\sim$5 m tall) located inside the large environmentally controlled mesocosm at Biosphere 2 (Allen et al., 2003) were used for the branch enclosure experiments. In addition, three potted ($\sim$1 m tall) mango trees (Top Tropicals), grown under controlled growth conditions at Lawrence Berkeley National Laboratory, USA were also used to evaluate the relationship between leaf temperature, net photosynthesis, stomatal conductance to water vapour, and isoprene emissions using a LI-6400XT photosynthesis system (LI-COR Biosciences).

Measurements of leaf isoprene emissions (one leaf on each of the three potted plants) in relation to plant physiological variables was made by modifying the LI-6400XT such that 100 standard cm$^3$ min$^{-1}$
Following this, background samples (enclosure blanks) for each of the two TD tubes were collected from an empty enclosure in the light in parallel with PTR-MS and LI-7000 measurements prior to introduction of the branch. This procedure resulted in clean GC-MS backgrounds with negligible concentrations of the volatiles of interest observed in the empty enclosure (<0.02 ppbv). To prevent room air from being measured while the branch was being installed, the three-way valve was switched to the position that diverted a portion of the air entering the enclosure to the PTR-MS and LI-7000. After 5–10 min following the installation of the branch in the enclosure, the three-way valve was switched such that air inside the enclosure was analysed. PTR-MS and LI-7000 trace gas measurements continued in real-time while samples were collected on the TD tubes manually for 30 min and immediately run on the GC-MS. The second TD tube was attached for sample collection immediately after the first TD tube was detached for analysis so that consecutive samples were taken.

### 13C-labelling experiments

Carbon used for isoprene biosynthesis is strongly linked with photosynthesis under unstressed conditions (Karl et al., 2002). We therefore measured the incorporation of 13C into volatile emissions under 13CO2. These experiments were designed to help determine whether the putative isoprene oxidation products were indeed associated with the rapid de novo biosynthesis of isoprene without artificially altering precursor substrate pools (e.g. pyruvate). During 13C-labelling experiments, 99% 13CO2 (Cambridge Isotope Laboratories) was introduced at a flow rate of 1.3–3.0 sccm into 3.0 slpm hydrocarbon-free airflow entering the enclosure to generate 13CO2 concentrations between 429 and 989 ppmv. Following introduction of the mango branch into the enclosure, four to six TD samples were collected and analysed by GC-MS in parallel with online PTR-MS and LI-7000 measurements. Although the LI-7000 has reduced sensitivity to 13CO2 relative to 12CO2 (~10%), net assimilation of 13CO2 during photosynthesis could still be monitored. Real-time labelling patterns of isoprene were monitored using PTR-MS signals at m/z 69–74, which represent 13C-labelling of between zero and five carbon atoms, respectively (Karl et al., 2002). Significant PTR-MS interferences between 13C-labelled isoprene and its putative oxidation products occurred (e.g. the 13C-labelled ion were also determined. As the parent ion of MBO is not present in significant amounts due to extensive fragmentation during electron impact ionization in the GC-MS, we used a major four-carbon fragment at m/z 71 for 13C-labelling analysis of MBO. Thus, relative mass spectra were determined for isoprene (m/z 68–73), MBO (m/z 71–75), MVK and MACR (m/z 70–74), 3-MF (m/z 82–87), and 3-MT (m/z 98–103). The experiment was repeated on three different mango branches.

### Abiotic stress treatments

Abiotic stress experiments in the laboratory were performed on detached branches and included high light/temperature, freeze-thaw/desiccation, and mechanical wounding stress. High temperature/light stress experiments (five mango branches and three soursop branches) were performed under air (3.0 min−1 dry hydrocarbon-free air flowing into the enclosure). After the branch was allowed to stabilize inside the enclosure for ~1 h (two TD tube samples collected), the lamp used to illuminate the chamber was lowered until PAR intensities at branch height increased from 275–640 to 2000–2500 μmol m−2 s−1 and enclosure air temperatures increased from 19–25 to 35–45°C. Following the lowering of the grow lamp, four TD tube samples were collected and analysed by GC-MS in parallel with
Enclosure air samples (6.0 l) were collected by drawing enclosure air through one of two TD tubes for 30 min at a flow rate of 200 scm. Sample airflow rate was regulated by a mass flow controller and a pump downstream of the TD tube. Each of the two TD tubes was filled with Tenax TA, graphitized carbon, and Carboxen 1000 adsorbents. The adsorbents were selected in order to minimize water vapour collection and to quantitatively collect ppv levels of C₅-C₁₀ volatiles on the graphitized carbon and Carboxen 1000 adsorbents. The Tenax TA adsorbent reversibly bound higher-molecular-weight compounds (e.g., C₅-C₁₀) which prevented their irreversible binding to the stronger graphitized carbon and Carboxen 1000 adsorbents. Immediately following sample collection, the TD tube sample was analysed utilizing a UNITY 2 thermal desorption system (Markes International), which was directly connected to a UNITY 2 capillary GC equipped with a cross-linked 100% silicone capillary column (60 m×0.32 mm×1.8 μm, D-B624; Agilent). Identification of putative isoprene oxidation product emissions from mango branches was performed with the intent of studying branch emissions with the intent of studying branch emissions in the laboratory. The following steps were performed: (1) the branch was cut with a pair of scissors three times (~5 cm per cut with a typical leaf length of 25 cm); (2) after removing the branch, the TD tube was closed and the enclosure air was collected and analysed by GC-MS in parallel with online PTR-MS and LI-7000 measurements.

Identification of putative isoprene oxidation product emissions from mango branches

Detached branches of mango were studied with the intent of identifying primary emissions of putative isoprene oxidation products. In order to increase the enclosure concentrations to aid in compound identification of volatile species in the initial experiments, a low flow rate of hydrocarbon-free air (1.0 ml min⁻¹) was introduced into the ~4 l branch enclosure. Fig. 2 shows an example GC-MS chromatogram from headspace samples with isoprene [retention time (RT): 7.00 min; 3-MF; m/z 70, 3-MT; m/z 71, 3-MT; m/z 97] by the calibration slope.

The target volatiles were analysed from the enclosure and ambient air samples using a high-sensitivity PTR-MS (IONICON, Austria). The PTR-MS, which is based on quadrupole mass spectrometry (Lindner and Hansel, 1997), was operated under standard conditions with a drift tube voltage of 600 V and drift tube pressure of 2.0 mb. The following m/z values were monitored during each PTR-MS measurement cycle: 21 (H₁³O²⁺), 32 (O₂⁺), and 37 (H₂O–H₃O⁺), with a dwell time of 20 ms each. The following m/z values were also measured sequentially and corresponded to the protonated molecular weights (parent ions), with a 2 s dwell time: m/z 69 (isoprene), m/z 71 (MVK+MACR), m/z 83 [3-MF+C₅ green leaf volatiles (GLVs)], m/z 87 (MBO+C₅ green leaf volatiles), and m/z 99 (3-MF+C₆ GLVs). The PTR-MS was calibrated using the same dynamic liquid injection method as described previously for GC-MS but with the use of cyclohexane as the solvent. A cyclohexane solution was prepared by dissolving 20 μl each of isoprene, MVK, MACR, MBO, and 3-MT liquid standards (Sigma-Aldrich) in 10 ml of cyclohexane. 3-MF was not added to the solution due to formation of precipitates upon addition to the solvent. Therefore, the calibration slope of 3-MF was assumed identical to that of 3-MT.

Although good quantitative agreement was obtained between GC-MS and PTR-MS concentration measurements of MVK and MACR in mango enclosures (total MVK+MACR concentrations measured by PTR-MS), those for MBO, 3-MF, and 3-MT were overestimated by PTR-MS relative to GC-MS. GC-MS chromatograms revealed that this was probably due to the emissions of C₅ and C₆ GLVs, which are known to be released from plants under stress and probably interfere with PTR-MS signals at m/z 87 (MBO+C₅ GLVs), m/z 83 (3-MF+C₆ GLVs), and m/z 99 (3-MF+C₆ GLVs) (Fall et al., 1999, 2001). For example, abiotic stress treatments induced the emissions of C₅ GLVs (e.g., 3-pentanone, 3-penten-2-ol, 2-penten-1-ol, 2-pentene, 2-penten-3-ol, 1-pentanol, 4-penten-1-ol) and C₆ GLVs (e.g., 3-hexenal, 3-hexen-1-ol, 3-hexen-1-yl acetate) identified only from mass spectra comparison with the NIST mass spectral library (and not quantified). Therefore, we used GC-MS to quantify enclosure concentrations of MBO, 3-MF, and 3-MT.

Results

Identification of putative isoprene oxidation product emissions from mango branches

Detached branches of mango were studied with the intent of identifying primary emissions of putative isoprene oxidation products. In order to increase the enclosure concentrations to aid in compound identification of volatile species in the initial experiments, a low flow rate of hydrocarbon-free air (1.0 ml min⁻¹) was introduced into the ~4 l branch enclosure. Fig. 2 shows an example GC-MS chromatogram from headspace samples with isoprene [retention time (RT): 7.00 min; 301.6 ppbv] and five putative oxidation products including MACR (RT: 9.58 min; 5.9 ppbv), MVK (RT: 10.45 min; 6.1 ppbv), 3-MF (RT: 10.59 min; 6.2 ppbv), MBO (RT: 11.39 min; 2.5 ppbv), and 3-MT (RT: 16.93 min; 6.7 ppbv). When the experiment was repeated with a different mango branch, high concentrations of all putative isoprene oxidation products except...
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3-MT were observed (MACR, 13.6 ppbv; MVK, 15.5 ppbv; 3-MF, 4.1 ppbv; MBO, 84.5; 3-MT, 0.1 ppbv). An analysis of enclosure blanks under identical conditions but without a mango branch revealed low background concentrations of these compounds (<0.2 ppbv). The identification of these putative isoprene oxidation products emitted from mango branches was verified by GC-MS with authentic standards by comparison of retention times and mass spectra. Although the possibility exists that the oxidized volatiles observed arose from isoprene oxidation in the enclosure following isoprene emissions rather than from a reaction in planta, it is very unlikely. While gas-phase oxidant concentrations (e.g. ozone) were not measured in the branch enclosure, their role in gas-phase isoprene oxidation was considered negligible due to the use of a high-purity hydrocarbon-free air generator with an ozone output rated less than 1 ppbv (737 Pure Air Generator; AADCO Instruments). In addition, ozone production within the enclosure itself was prevented through the use of a light source without UV light output (600 W LED grow light output wavelengths: red, 630–660 nm; blue, 460 nm; orange, 612 nm; AIBC International). Finally, the residence time in the enclosure was short, in the order of a few minutes.

13CO2-labelling experiments

In order to examine further the possible role of isoprene oxidation within mango leaves as a within-leaf source of MVK, MACR, MBO, 3-MF, and 3-MT emissions, 13CO2 labelling was performed on detached mango branches inside the laboratory under controlled lighting conditions. During these experiments, continuous measurements of 13CO2 and volatile emissions using online LI-7000 and PTR-MS and near real-time GC-MS were made. While the LI-7000 has greatly reduced sensitivity to 13CO2 relative to 12CO2, net assimilation of 13CO2 during photosynthesis could still be monitored by comparing the measured signal from air with known 13CO2 entering the branch enclosure with that of air inside the enclosure. For example, upon placing the branch in the enclosure with ~500 ppmv 13CO2 flowing in, the 13CO2 enclosure concentrations decreased reaching a minimum after ~30 min (Fig. 3, top panel) and then increased over the remainder of the experiment to the levels of the incoming 13CO2 concentrations. Online PTR-MS observations of branch enclosure air showed that photosynthesis under 13CO2 led to the rapid 13C-labelling of isoprene emissions (Fig. 3, top panel), as observed previously (Karl et al., 2002; Vickers et al., 2011). However, while the total isoprene concentration increased until peaking after ~30 min, it then declined over the remainder of the experiment. A decline in overall isoprene emission levels over time after the original peak was a common feature of all detached mango branches studied; we presumed that this was due to declines in photosynthesis, as 13CO2 concentrations in the enclosure (a proxy for photoassimilation) were inversely correlated with total isoprene emissions. Loss of photosynthetic capacity and isoprene emissions may be a stress response to severing the branch and placing it inside the enclosure.

Upon initially placing the mango branch shown in Fig. 3 in the enclosure (10:27 a.m.), emissions of fully unlabelled isoprene (m/z 69) dominated. However, within 3 min of replacing 13CO2 with 13CO2 (10:30 a.m.), emissions of all 13C-labelled isotopologues increased while emissions of the fully unlabelled isotopologue began to decline. After 25 min
Fig. 3. PTR-MS time series plot showing isoprene $^{13}$C-labelling dynamics under $^{13}$CO$_2$ (top panel) and GC-MS relative ion intensities of isoprene and its five putative oxidation products from a mango branch during photosynthesis under $^{12}$CO$_2$ and $^{13}$CO$_2$ (lower panels). The branch was placed in the empty enclosure at 10.27 a.m. and isoprene isotopologue concentrations were monitored by PTR-MS while four sequential TD tubes were collected for GC-MS analysis. The $^{13}$C-labelling pattern of isoprene followed $^{13}$CO$_2$ assimilation, which initially increased and then decreased as the stomata opened and closed (typical response of detached mango branches). Note that, relative to assimilation under natural abundance CO$_2$ ($^{12}$CO$_2$), strong $^{13}$C labelling was observed in isoprene and its five putative oxidation products.
of photosynthesis under $^{13}$CO$_2$ (10:52 a.m.), emissions of the fully $^{13}$C-labelled isoprene isotopologue ($m/z$ 74) dominated emissions. During this period, photosynthesis was also increasing and reached a maximum after 53 min (11:20 a.m.), the same approximate time that emissions of the fully labelled isotopologue of isoprene ($m/z$ 74) peaked and emissions of the unlabelled isotopologue of isoprene ($m/z$ 69) reached a minimum. This was followed by a decline in net photosynthesis and emissions of fully labelled isoprene ($m/z$ 74) and a corresponding increase in unlabelled isoprene emissions ($m/z$ 69). Thus, the temporal dynamics of fully unlabelled isoprene emissions ($m/z$ 69) and the fully labelled isoprene emissions ($m/z$ 74) were anti-correlated as the $^{13}$C content of the isoprene precursor pools filled and then declined. The increase in unlabelled isoprene after the peak in emissions indicated a carbon source switch for isoprene, with photosynthesis dominating during the first part and an increased contribution from non-photosynthetic sources (e.g. glycolysis) during the decline of photosynthesis. Similar switches in carbon source usage have been observed previously in plants under stress (Loreto and Delfine, 2000; Funk et al., 2004).

Due to the acquisition of multiple $^{13}$C atoms during $^{13}$CO$_2$-labelling experiments, extensive overlap between the PTR-MS signals corresponding to the labelled isotopologues of isoprene and its putative oxidation products exists (e.g. isoprene $m/z$ 69–74 and MVK+MACR $m/z$ 71–75). Therefore, we relied on GC-MS analysis of the enclosure air to assess the $^{13}$C-labeling pattern of putative isoprene oxidation products with a total concentration estimated to be less than 5–10 ppbv. Thus, isoprene oxidation products accounted for only a few per cent of PTR-MS signals for isoprene isotopologues. GC-MS analysis of enclosure air during the $^{13}$C-labelling experiment (four enclosure air samples sequentially collected and analysed) revealed that, relative to emissions under natural abundance CO$_2$ (four enclosure air samples sequentially collected and analysed from a separate mango branch under $^{12}$CO$_2$), a strong $^{13}$C-labeling pattern was observed for both isoprene and its five putative oxidation products, with all carbon atoms acquiring a $^{13}$C atom in most cases (average relative ion abundances across the four GC-MS samples for each shown in Fig. 3, lower panels). Note that, for isoprene, the GC-MS relative mass spectra indicated labelling of all five carbon atoms. However, $^{13}$C labelling of the $m/z$ 67 fragment ion interfered with the $^{13}$C-labelling pattern of the molecular ion ($m/z$ 68), resulting in the dominance of the $m/z$ 72 ion rather than the $m/z$ 73 ion. Nevertheless, a similar $^{13}$C-labelling pattern observed for isoprene by PTR-MS was also observed by GC-MS.

**Abiotic stress**

In order to investigate further the hypothesis that the five oxidized volatiles (MVK, MACR, 3-MF, 3-MT, and MBO) derived from the oxidation of isoprene within plants, we conducted a series of abiotic stress treatments known to induce oxidative damage in plants. These experiments included high light/temperature stress, freeze-thaw stress, and mechanical wounding stress. Upon placing the detached mango branches in the enclosure with 3.0 slpm of hydrocarbon-free air flowing through and normal temperature/light conditions, slightly elevated enclosure concentrations of the putative isoprene oxidation products were observed. However, upon induction of high temperature/light stress, the emission rates increased with elevated concentrations of 3-MF (0.3–3.7 ppbv), MACR (2.1–6.8 ppbv), MBO (0.7–2.4 ppbv), and MVK (0.4–0.6 ppbv) (Fig. 4a). Only a trace amount of 3-MT could be detected following the heat stress (0.02–0.16 ppbv); levels remained very low and no clear pattern could be observed. For the mango branch shown in Fig. 4, which was monitored for 4.5 h following the application of high temperature/light stress, MVK increased transiently (concentrations doubled to 0.4 ppbv) and then plateaued and sharply declined towards the end of the experiment to low levels (0.02 ppbv). The MBO enclosure concentrations followed a similar pattern but with a higher magnitude. Peaking relatively rapidly following the stress (concentrations slightly more than doubled to 0.9 ppbv), plateauing and then declining slightly towards the end of the experiment (0.6 ppbv). 3-MF enclosure concentrations peaked more slowly, increasing to a maximum of 3.7 ppbv, approximately 18.5-fold higher than before the stress. However, similar to MVK concentrations, a sharp decline was observed towards the end of the experiment such that concentrations were very low by the end of the experiment (0.01 ppbv). In contrast, MACR concentrations showed a very different pattern. Upon application of the high temperature/light stress, MACR concentrations increased gradually in the enclosure over time, reaching 2.1 ppbv at the end of the experiment (13-fold increase) and displayed an inverse pattern to isoprene concentrations. To investigate further the possible connections between the proposed isoprene oxidation products and the isoprene emissions of the mango plants, the non-isoprene-emitting tropical plant soursop was utilized as a control. The same high temperature/light stress experiments under air were performed following the same procedure as was used for the mango branches. As shown in Fig. 4b, enhanced emissions of the putative isoprene oxidation products were not observed from soursop branches following high temperature/light stress (enclosure concentrations <0.2 ppbv). Due to issues in branch enclosures including shading, variations in total leaf area, and different leaf temperatures, we utilized a leaf photosynthesis system (LI-6400XT) to evaluate the relationship between leaf temperature, stomatal conductance to water vapour, and isoprene emissions from mango leaves (Fig. 5). Similar to previous leaf level studies on mango leaves (Harley et al., 2004), the maximum temperature for isoprene emissions (37.5 °C) was higher than that of the maximum temperature for photosynthesis and stomatal conductance (32 °C).

Of all of the abiotic stress treatments, branch freeze-thaw/desiccation treatment resulted in the highest enclosure concentrations of the MACR and MVK products (Fig. 6, left panel). However, levels of 3-MF and MBO were similar to those seen in the high-temperature/light-stress experiment, and 3-MT remained low (<0.2 ppbv) during these experiments. For example, during the freeze-thaw/desiccation experiment...
shown in Fig. 6, MACR concentrations within the enclosure were 0.0 ppbv when the frozen branch was initially placed into the enclosure, and increased to 16.7 ppbv after 5h. 3-MF concentrations peaked early, reaching 3.3 ppbv within 38 min and then declined sharply after they peaked and remained low (<0.2 ppbv) for the remainder of the experiment. MVK and MBO concentrations both increased more slowly, reaching peaks of 3.0 and 0.6 ppbv, respectively, after 70 min and declined thereafter. Net photosynthesis was recorded as zero during the thawing process; consequently, isoprene concentrations in the enclosure were low. For example, in the branch shown in Fig. 6, isoprene concentrations peaked at 5.9 ppbv and declined to 1.3 ppbv after 5h. Isoprene emission levels were essentially inversely correlated with MACR levels, similar to the high-temperature/light-stress experiment shown in Fig. 4. During identical branch freeze–thaw experiments with the non-isoprene-emitting plant soursop, concentrations of the putative isoprene oxidation products remained less than 0.2 ppbv (Fig. 6b).

The final abiotic stress utilized for potential stimulation of isoprene oxidation product emissions involved mechanical leaf wounding. During these experiments, elevated enclosure concentrations of MACR and MVK were observed from two out of three mango branches investigated in response to the leaf wounding (Fig. 7a). However, elevated concentrations of MBO, 3-MF, and 3-MT were not observed. MACR levels showed a small increase after wounding (0.3–0.6 ppbv) and declined thereafter. MVK emissions showed a higher increase after wounding (0.8–0.9 ppbv), also declining thereafter. Identical leaf-cutting experiments on soursop leaves did not lead to elevated enclosure concentrations of MACR and MVK (Fig. 7b).

**Discussion**

In this study, we verified previous observations that isoprene emissions become uncoupled from photosynthesis (Keller and Lerdau, 1999) and stomatal conductance (Fall and
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Monson, 1992) at high temperatures. We also showed that, under high light/temperature stress, branches emit MVK and MACR directly to the atmosphere (Jardine et al., 2010a, 2012). We extended this analysis to include additional abiotic stresses (freeze–thaw and mechanical wounding) as well as novel putative isoprene oxidation products (MBO, 3-MF, and 3-MT) (Fig. 1). A substantial effort was made to validate the presence of these compounds in branch enclosure air and minimize the possibility of artifacts in the analytical systems. Enclosure GC-MS and PTR-MS measurements of the empty branch enclosures showed small (<0.2 ppbv) to negligible background concentrations of the compounds of interest, and GC-MS mass spectra of the compound peaks from enclosure air with mango branches matched those of the NIST mass spectral library as well as retention time and mass spectra of authentic standards. A careful calibration procedure of each analytical system with authentic standards allowed a direct comparison between enclosure concentration estimates by PTR-MS and GC-MS. During mango abiotic stress experiments, agreeable quantitative data between PTR-MS and GC-MS concentration measurements of MVK+MACR was generally found. However, PTR-MS concentration estimates for MBO, 3-MF, and 3-MT generally exceeded those determined by GC-MS, probably due to the
presence of C₅ and C₆ GLVs, which are known to be emitted from plants under stress (Fall et al., 1999, 2001). Thus, the results from our study demonstrated that PTR-MS based on quadrupole mass spectrometers possess the capability to accurately quantify MVK+MACR emissions under abiotic stress, but that chromatography is needed to accurately identify and quantify 3-MF, 3-MT, and MBO. In all abiotic stress experiments, enclosure concentrations of 3-MT were very low (<0.2 ppbv). Additional research is therefore needed to verify its presence and establish its relevance to atmospheric and plant processes.

The lack of a strong enhancement in MBO, MVK, MACR, 3-MF, and 3-MT enclosure concentrations from the non-isoprene-emitting plant soursop under abiotic stress provided additional indirect evidence that isoprene oxidation within the plant cell is largely responsible for their production in mango leaves. Moreover, the rapid incorporation of photo-assimilated ¹³CO₂ into both isoprene and the oxidized product emissions provided new evidence that these compounds derive from the direct oxidation of isoprene within mango plants. Although atmospheric oxidation of isoprene is known to produce 3-MF as a minor product (4.2–5.4%) (Atkinson and Arey, 2003), we observed 3-MF as a major emission component from high-temperature/light-stressed mango branches (Fig. 4) and freeze–thaw/desiccation (Fig. 6). On average, MACR dominated emissions of putative isoprene oxidation products from high-temperature/light and freeze–thaw stress treatments of mango branches (Fig. 4). However, mechanical wounding resulted in the stimulation of higher MVK emissions than MACR (Fig. 7). The differences seen between abiotic stress factors might be attributed to different oxidation mechanisms within plants, including the generation of various ROS involved in the oxidation reactions and different downstream metabolism pathways of the various isoprene oxidation products. As ROS was not quantified in leaf tissues, the mechanism of isoprene oxidation remains speculative, as enzymatic mechanisms for isoprene oxidation remains a possibility. For example, a cytochrome P450 enzyme in mice and rats has been shown to efficiently metabolize high levels of isoprene (Bogaards et al., 2001).

To date, all studies on isoprene synthase enzymes have reported only isoprene as a product (Fall and Wildermuth, 1998; Datukishvili et al., 2001), while a recent in vitro study on MBO synthase revealed that the enzyme produces MBO to isoprene at a ratio of 90:1 (Gray et al., 2011). Both isoprene and MBO are produced from the common precursor dimethylallyl diphosphate with the difference in product formation related to the exclusion (isoprene) or entry (MBO) of water into the active site of the respective synthase (Gray et al., 2011). Thus, although MBO emissions from mango were shown in this study to have a similar ¹³C-labelling pattern to that of isoprene, MBO (as well as the other oxidized compounds studied) may be produced from the oxidation of isoprene by ROS within plants or simply share the same precursor as isoprene (dimethylallyl diphosphate).

During high-temperature/light and freeze–thaw stress experiments, we generally observed two distinct patterns of putative isoprene oxidation product emissions from mango branches (e.g. Figs 4 and 5). During the first period following the initiation of stress, MBO and 3-MF emissions increased and then declined, while MACR emissions continued to increase throughout the experiment and dominated emissions near the end. If isoprene oxidation serves as the main in vivo source of these compounds, these observations suggest that a different isoprene oxidation mechanism occurs during early-stage versus late-stage stress. When the magnitude of the enclosure concentrations of putative isoprene oxidation products were compared between the abiotic stress treatments on mango branches, freeze–thaw/desiccation (MACR up to 17 ppbv), high light/temperature (MACR up to 4 ppbv), and mechanical wounding (MACR up to 0.4 ppbv) were ranked from highest to lowest. These observations remain consistent with the idea that freeze–thaw/desiccation may be the harshest form of abiotic stress that we applied due to the fact that most of the plant cells in the enclosed mango branch were probably damaged by this treatment. Harsh freeze–thaw stress dramatically damages membrane structures (Hincha et al., 1987) and results in a massive accumulation of ROS (Heidarvand and Amiri, 2010), leading to substantial lipid peroxidation (Tajvar et al., 2011). Hence, while isoprene levels were seen as relatively low after the freeze–thaw experiment, a very high pool of oxidizing species was probably available for its oxidation.

Due to high global emission rates from vegetation and rapid oxidation rates within the atmosphere, isoprene remains the most intensively investigated biogenic volatile organic compound in the Earth system. Isoprene oxidation yields a variety of first-order oxidation products including MVK, MACR, and 3-MF previously assumed to derive exclusively from atmospheric oxidation. While oxidative reactions in the atmosphere play a central role in determining air quality and climate (Monson, 2002; Pacifico et al., 2009), oxidation within vegetation may protect plants from oxidative damage during stress through direct depletion of the oxidant pool and through signalling processes (Vickers et al., 2009a). While once considered a toxic byproduct of aerobic metabolism, ROS signalling is now recognized as an integral component of plant response to abiotic and biotic stress as well as regulation of growth, development, and programmed cell death (Mittler et al., 2011; Suzuki et al., 2011). However, these oxidation and signalling processes remain poorly understood, in part because the quantification of ROS within plant tissues remains extremely difficult (Bournonville and Diaz-Ricci, 2011). Owing to their high reactivity and unstable nature, ROS have not been directly quantified. Instead, detection of ROS relies on quantifying products that are formed when they react with various natural or applied substances. In addition to their destructive sampling and labour-intensive nature, many existing techniques suffer from problems including the requirement to deliver cytotoxic chemicals that cause stress to tissues, poor cellular permeability of applied chemicals, and other major artifacts (Shulaev and Oliver, 2006). As a result, the majority of methods for ROS quantification in plants are restricted to model plants (due to the presence of interferences and inhibitors) or provide only qualitative or semi-quantitative information (Shulaev and Oliver, 2006).
In this study, we configured a gas analysis system with high selectivity and sensitivity for isoprene and its putative oxidation products. The potential power of our approach may stem from the possibility that isoprene oxidation reactions leave unique volatile biomarkers behind that can be quantified in the gas phase. These volatile profiles could potentially be observed across a wide range of temporal (seconds to years) and spatial (cells to global) scales. By comparison with established techniques for determining oxidative damage in plants such as the thiobarbituric acid-reactive substances lipid peroxidation assay (Draper and Hadley, 1990), we suggest that emissions of isoprene oxidation biomarkers from plants could potentially be used as quantitative indicators of oxidative stress in plants. With changes in land use and climate change, plants are exposed to increasing levels of stress (e.g. high and low temperatures and mechanical wounding) that can result in extensive oxidative damage (Allen and Ort, 2001; Leon et al., 2001; Kotak et al., 2007). Development of the techniques described here may therefore help researchers understand how plants respond to oxidative stress and consequently increase our ability to predict and perhaps mitigate some of the resulting oxidative damage. Moreover, isoprene production and oxidation dynamics within plants may be important for overall forest response to climate change including expected shifts in species composition, as isoprene-producing species may be favoured as more robust responders to stresses associated with climate, air quality, and land-use changes (Harley et al., 1999).

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Simplified diagram of experimental setup used to identify and quantify emissions of isoprene and its putative oxidation products from mango branches using a coupled GC-MS, PTR-MS, and photosynthesis (LI-7000) system.

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References


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