Running title: Isoprene measurement

Title: Isoprene Measurements to Assess Plant Hydrocarbon Emissions and the Methylerythritol Pathway

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Definition: Methods and strategies for measuring isoprene produced by plants and bacteria

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Abstract

Isoprene is the most abundant non-methane hydrocarbon emitted to the atmosphere and a target of biotechnology. Measurements of the amount of isoprene or the rate of production of isoprene are important for atmospheric chemistry, evaluating biotechnology processes, and can provide information on the capacity and regulation of the methyl erythritol 4-phosphate pathway found in plants and bacteria. In this chapter we discuss techniques, and their strengths and weaknesses, of methods in common use for measuring isoprene. There are many sources of isoprene for measurements including emissions from leaves and head space analysis of reactions involving recombinant enzymes or bacterial or fungal cultures. Similarly, there are a

variety of detection methods including several mass spectrometer methods that are useful for examining rates of labeling of isoprene when carbon isotopes are used.

Contents

1. Introduction

Isoprene is the root member of the isoprenoid family of compounds. Isoprenoids (also known as terpenoids) have one to many isoprene units, a five-carbon branched chain. Isoprene in the strict sense is 2-methyl 1,3-butadiene but is not the precursor to higher order isoprenoids.

53 The primary precursor of isoprenoids is dimethylallyl diphosphate (DMADP) with one- to
54 many- isopentenyl diphosphate (IDP) molecules added. For reasons not apparent to us,
55 molecules with two isoprenoid units are called monoterpenes, three units = sesqui-, four = di-.
56 Therefore, isoprene is a hemiterpene, half of a monoterpene. Other hemiterpenes are
57 isoamylene (2-methyl-2-butene) and 2-methyl-3-buten-2-ol, the latter being emitted from
58 several evergreen conifers (Lehnert et al., 2020; Schade et al., 2000).

59 Isoprene is made from DMADP by isoprene synthase (IspS) (Miller et al., 2001; Silver
60 & Fall, 1991). The enzyme found in angiosperm (flowering) plants is related to monoterpene
61 synthases in the TpsB family, especially β-ocimene synthase (Li et al., 2017; Sharkey et al.,
62 2005). Plants other than angiosperms do not have TpsB genes so the isoprene synthases
63 responsible for significant isoprene emissions from ferns and mosses are unknown. Some
64 bacteria make isoprene, especially *Bacillus* species. In at least one case isoprene is made by
65 hydroxymethylbutenyl diphosphate (HMBDP) reductase (also known as IspH and LytB).
66 Normally this enzyme converts HMBDP to a mix of DMADP and IDP but there is a report that
67 the enzyme can also convert HMBDP directly to isoprene (and convert DMADP to isoamylene)
68 (Ge et al., 2016). Humans and other animals exhale isoprene in their breath (~40 mg day^{-1} for
69 an average human) but the mechanism is unknown (Karl et al., 2001; Miekisch et al., 2001;
70 Mochalski et al., 2011; Sharkey, 1996; Trovarelli et al., 2001).

71 In plants, DMADP for isoprene synthesis is made by the methyl erythritol 4-phosphate
72 (MEP) pathway. There is significant interest in engineering the MEP pathway in part because
73 it is more efficient than the mevalonic acid pathway used by animals to make DMADP.
74 Protocols for measuring metabolites of the MEP pathway have recently been published
75 (González-Cabanelas et al., 2016). Isoprene measurements can supplement LC-MS/MS
76 methods for measuring DMADP and another key pathway intermediate methylerythritol
77 cyclodiphosphate (MEcDP) using post-illumination isoprene emission characteristics.

Recombinant isoprene synthase can convert all DMADP in a sample to isoprene, which can then be measured by one of several methods described below. Adding isopentenyl diphosphate will ensure conversion of all DMADP and IDP to isoprene and allow separate determination of IDP and DMADP (Zhou et al., 2013). Here we focus on how measuring isoprene can be used to gain insight into the MEP pathway. Other methods for measuring these compounds will not be presented here.

2. Methods for detecting and measuring isoprene

There are many methods for measuring isoprene (Cao & Hewitt, 1995) but we will cover three methods in common usage in biology laboratories. Isoprene is highly volatile and so is handled in the gas phase for most measurements. It is generally stable and does not partition into water (and buffers etc.) to a great degree (Niinemets et al., 2010). It also does not stick to walls of containers as much as many other isoprenoids.

2.1 Gas chromatography

Isoprene-containing gas samples can be introduced into a gas chromatograph (GC). Typically, some form of cryofocusing is used in which air is passed through a cold trap, which is then heated to quickly release all the isoprene into the GC. This can be done external to the GC making use of a six-way valve (Hills et al., 1992; Loreto & Sharkey, 1990). This allows very large air samples to be processed increasing the system sensitivity. On-column cryofocusing is often used when samples are introduced using solid phase microextraction systems (SPME). Cartridges are often used with GC (and other detection methods). Some care is required when using cartridges to ensure that the packing material will retain the isoprene at ambient temperature and release it upon moderate heating (Niinemets et al., 2011). Gas chromatography relies on the chromatography to separate isoprene from other gases. Detection

102 can be by flame ionization (FID), photoionization detection (PID), or mass spectrometry (MS).
103 PID is typically more sensitive than FID and modern mass spectrometers are even more
104 sensitive. Both FID and PID are very quantitative and discreet measurements can be made
105 rapidly (less than 3 minutes per sample). MS detectors provide the mass spectrum of individual
106 compounds and as such provide a benchmark method for confirming the identity of the given
107 compound. FID and PID cannot separate among interfering compounds that have similar
108 retention time as isoprene in widely used GC columns for biogenic volatile separation.

109

110 2.2 Fast Isoprene Sensor

111 An instrument designed to measure isoprene using chemiluminescence is sold
112 commercially by Hills Scientific and is called the Fast Isoprene Sensor (FIS) (Hills et al., 1992;
113 Hills & Zimmerman, 1990) (http://hills-scientific.com/). This instrument combines a flow of
114 oxygen with very high ozone content with air being pulled through the instrument with a small
115 air pump. Isoprene in the air reacts with the ozone to make a chemiluminescent product in front
116 of a photomultiplier tube. The selectivity for isoprene depends on the wavelength of emitted
117 light and the timing of the reaction. This instrument was developed for measurements related
118 to atmospheric chemistry but is easily adapted to laboratory measurements. It has some
119 sensitivity to water vapor that is problematic when air humidity is varying during the
120 measurements as is often the case with plant measurements. The problem with water vapor
121 sensitivity can be handled by passing the air through an ice trap to maintain a constant low
122 humidity in the gas stream going into FIS or humidify the air to a constant humidity (Rasulov
123 et al., 2009). FIS also has some cross-sensitivity to other hydrocarbons, for example propene
124 (Hills & Zimmerman, 1990), but little cross reactivity to hydrocarbons likely to be present in
125 isoprene air samples. It has an especially strong cross-sensitivity to some sulfur gases. This
126 limits how much dithiothreitol can be used in enzyme assays when head space analysis using

an FIS is planned. Thus, regularly checking for the magnitude of other hydrocarbons by gas-chromatography is recommended. The FIS has a very wide dynamic range and is very sensitive to isoprene (Cao & Hewitt, 1995; Toda & Dasgupta, 2008). One of the key advantages of using the FIS to measure isoprene is the fact that it provides high time resolution (as fast as 0.1 sec sampling time).

2.3 Proton-transfer-reaction mass-spectrometry

Proton transfer reaction mass spectrometry (PTR-MS) uses chemical ionization of volatile molecules with protonated water vapor (H_3O^+) followed by subsequent detection of protonated molecules with a mass spectrometer (Hansel et al., 1995; Jordan, Haidacher, Hanel, Hartungen, Märk, et al., 2009; Lindinger et al., 1998). Currently, two principal spectrometric detectors are in use, quadrupole mass spectrometer (QMS, PTR-QMS) and time-of-flight mass spectrometer (TOF-MS, PTR-TOF-MS). PTR-MS measurements are not confined to isoprene, but all volatiles with a proton affinity greater than that of water vapor (691 kJ mol^{-1}, (Hunter & Lias, 1998)) can be measured. Several PTR-MS models also include selective reagent ion (SRI) option that allows use of additional reagent ions, NO^+, O_2^+, NH_4^+, thereby extending the range of volatile compounds that can be measured (Jordan, Haidacher, Hanel, Hartungen, Herbig, et al., 2009; Lehnert et al., 2020). The time resolution of PTR-MS systems is on the order of 0.1 s. However, for the highest sensitivity, the sampling rate may need to be decreased. In practice, in laboratory measurements with a certain ambient air background isoprene concentration, a time resolution of 0.5-1 s is typically used to measure plant isoprene emissions (Rasulov et al., 2019).

In the case of PTR-QMS, the protonated masses measured are defined a priori before the measurements, and they are measured by the QMS in sequence. Thus, the time-resolution of the instrument depends on the number of compounds measured. In contrast, all protonated

masses present can be measured simultaneously with PTR-TOF-MS. The detection limit of PTR-QMS systems is on the order of 0.1-0.5 ppb (Warneke et al., 2015). Modern PTR-TOF-MS instruments are characterized by superior sensitivity, typically a few ppt (Jordan, Haidacher, Hanel, Hartungen, Märk, et al., 2009), and the sensitivity of the newest instruments even extends to less than 0.1 ppt (FUSION PTR-TOF, www.ionicon.com). Furthermore, PTR-TOF-MS has a superior mass resolution, better than 0.1 amu that is important for distinguishing among compounds with similar molecular mass.

PTR-MS systems only measure protonated ion concentrations and isoprene is detected as a protonated parent mass (*m/z*) of 69^+ (69.1^+ for PTR-TOF-MS). There are no typical plant volatiles that could provide the same parent ion. However, several plant species are significant constitutive emitters of another C5 DMADP pathway compound 2-methyl-3-buten-2-ol (MBO Gray et al., 2011; Gray et al., 2006). The protonated parent ion of MBO has a *m/z* of 87^+, however, even upon soft ionization, it fragments, yielding a main fragment ion with *m/z* of 69^+ identical to isoprene (Karl et al., 2013). In addition, stressed plants might emit the C5 green leaf volatile pentenol (e.g. 1-penten-3-ol) (Fisher et al., 2003; Rasulov et al., 2019) and some plants species also emit significant amounts of another C5 volatile pentanone (Jardine et al., 2010) that both also partly fragment to the ion with *m/z* 69^+. Thus, for compound identification, regular checks with GC-MS are advisable, especially when starting experiments with new species or conducting experiments with stressed plants. Furthermore, in addition to the ion *m/z* 69^+, it is important to simultaneously monitor the ions of relevant parent ions (e.g. *m/z* 87^+ for MBO) that could yield fragments ions *m/z* 69^+, and also confirm the identity of detected compounds by GC-MS. Provided there is only one interfering compound in the plant volatile mixture, the share of the ion *m/z* 69^+ between isoprene and the interfering compound can be estimated based on the degree of fragmentation of the interfering volatile. For example, in the case of MBO, the share of the ion *m/z* 69^+ is ca. 75% of total (Karl et al., 2013), while it is on

the order of 1% for 3-pentanone (Malásková et al., 2019). However, simultaneous emission of both isoprene and MBO by a C5 isoprenoid-emitting species is very rare (Lehnert et al., 2020). Thus, unless heavily stressed plants are measured, interference due to other volatiles is typically not a major issue in isoprene emission measurements by PTR-MS.

Compared with other methods of isoprene detection, a major advantage of PTR-MS measurements is that it allows conduction of real-time quantitative isoprene ^{13}C-labelling experiments (Karl et al., 2002). Upon $^{13}CO_2$-feeding, isoprene molecules become progressively enriched with ^{13}C, starting from *m/z* 70^+ (one ^{13}C atom and four ^{12}C atoms) to *m/z* 74^+ (fully ^{13}C-labelled). Such measurements provide detailed insight into relationships among photosynthesis, use of alternative carbon sources and MEP pathway activity (Sharkey et al., 2020).

3. Strategies for isoprene measurements

Isoprene is measured for several reasons and specific measurement systems are better for some strategies than others. On the other hand, there is a great deal of flexibility in matching sample generation with detection methods.

3.1 Continuous measurements

A common reason to measure isoprene is to determine how fast plant leaves are emitting isoprene. This is typically done with continuous measurements. Both the FIS and PTR-MS continuously sample air to provide real-time, continuous data for isoprene emission. This is particularly helpful for observing transients in isoprene emission, especially from leaves. Continuous sampling is also used by the atmospheric chemistry community together with

198 sensitive wind measurements to measure isoprene emission from forests by an eddy covariance
199 technique, but these methods will not be covered here.

200 3.2 Discrete measurements

201 In many instances it is best to make discreet measurements of isoprene, for example
202 when sampling head space above an enzyme assay or bacterial production assay. To assess
203 candidate genes for improving the capacity for the MEP pathway or isoprene synthase, the
204 amount of isoprene accumulating in the head space above a closed culture can be measured at
205 a specific time point. However, this may miss important information about how quickly gene
206 expression is induced. A better measure is to leave the bacterial culture open (or sparged with
207 desired gas composition, for example with low oxygen or no oxygen). Then at specific time
208 points a small amount of the culture is removed and put into a sealed vial. This is incubated at
209 a specific temperature for a specified time and then the head space is sampled for isoprene.
210 This provides a measure of the rate of isoprene production by the culture.

211

212 4. Examples

213 Some examples will be described here but detection methods can be varied. Most
214 flexible is detection by PTR-MS because it can make continuous or discreet measurements,
215 can distinguish among isotopologues, and can measure other molecules in the gas sample. Least
216 flexible is GC-FID. Example protocols for using these methods applied to specific questions
217 are given here.

218 4.1 Analyzing isoprene production by bacterial cultures using GC-MS

219 When a mass-selective detector is used, both the chromatography and mass selection
220 are used to identify the isoprene signal. In addition, various isotopologues (differences in ^{13}C

amount) can be distinguished (Sharkey et al., 2020). Below is a protocol for measuring isoprene production from a purified protein by GC-MS using a SPME fiber to introduce the sample into the GC. This is a good method when the goal is to determine the degree of labeling. For quantitation an alternative, making use of an FIS for detection, is presented.

4.1.1 Materials required:

- Purified protein
- Dimethylallyl diphosphate (DMADP) (Echelon Biosciences)
- Borosilicate glass vials (we use 2 mL volume vials)
- Aluminum crimp top with red rubber septa
- 20 mm Kebby Standard Crimper (20001-00-C01A) for aluminum seals
- Water bath
- Solid-phase microextraction (SPME) fused-silica fiber coated with Carboxen/ Polydimethlysiloxane (Cat # 57318, Supelco, PA)
- SPME fiber holder (Cat # 577330-U, Supelco, PA)
- Ring stand
- Agilent 7010B Triple Quadrupole GC/MS (Agilent, CA)
- EZ guard column (VF5 CP9013, Agilent, 30 m length, 10 m guard length, EZ Guard, 7 in cage, 0.25 mm inner diameter)

4.1.2 Procedure:

1. Before sampling, the SPME fiber needs to be conditioned. After the GC oven reaches 230°C place the SPME fiber holder in the GC injection port and carefully push the fiber into the port. Make sure to lock the fiber in place.

2. Let the fiber condition for 20 minutes. Then retract the fiber and remove it from the GC injection port.
3. Prepare the assay buffer that contains 50 mM HEPES buffer (pH 8.0), 10 mM $MgCl_2$, 20 mM KCl, 2 mM DTT, and 1 mM EDTA.
4. Put buffer into the glass vials, add DMADP to make the desired concentration, then start the assay by adding the desired amount of enzyme solution. Adjust the amount of buffer added to make the total volume 300 µl.
5. Crimp seal the vial with an aluminum crimp top immediately and then vortex the vial to ensure complete mixing.
6. Put the vial in 40°C water bath for 10 minutes.
7. Take out the vial from the water bath after 10 minutes.
8. Insert the needle at the end of the SPME fiber holder through the rubber septum of the crimp top.
9. Insert the SPME fiber into the headspace making sure it doesn't touch the liquid and lock it in place. Allow the fiber to absorb the analytes in the headspace for 10 minutes.
10. Clip the upper part of the SPME fiber holder to a ring stand to keep it steady.
11. At the end of 10 minutes, retract the fiber and immediately insert it into the GC injection port.
12. Allow it to desorb for 2 minutes at 230°C. During this process, isoprene is collected in a cryotrap in the GC cooled to -10°C using CO_2.
13. At the end of 2 minutes, start the Agilent QQQ/MassHunter program (which also warms the cryotrap) and run it for 6.75 minutes. Isoprene should elute around 1.4 minutes.

14. At the end of the program, retract the SPME fiber and remove it from the GC injection port.

15. Repeat the procedure for the rest of the gas samples.

4.1.3 Alternative

Instead of analyzing with GC-MS, the amount of isoprene can be analyzed with an FIS. Starting at step 8, the head space gas is displaced into a one mL syringe by simultaneously withdrawing the plunger of an empty syringe and injecting an equal amount of water to keep the pressure inside the vial constant (**Figure 1**) (Weise et al., 2013). This one mL sample is then injected into the air stream of an FIS instrument. The peak of isoprene is integrated by summing the counts from the FIS for 15 seconds before and 15 seconds after the peak (background) and subtracting that from the 30 second period that encompasses the signal from the injection. This is compared to a standard curve to determine the amount of isoprene in the head space. The standard curve can be established by mixing different concentrations of isoprene in air or nitrogen and injecting one mL of these standards into the FIS air stream. We have used glass flasks with a septum to mix standards (e.g. https://chemglass.com/gas-sampling-tubes-ptfe-stopcocks-with-sampling-portand) or Tedlar bags (e.g. ESS GD0707-7000 Sampling Bags With Combination Valve, 1L, from Cole Parmer).

Isoprene dissolves in water better than some monoterpenes (Copolovici & Niinemets, 2005) and this can be accounted for using the Henry's constant (7780 Pa m^3 L^{-1}at 25°C) (Copolovici & Niinemets, 2005; Weise et al., 2013). An example of this calculation is shown below.

Example –In the gas phase:

$$I = \chi_I \cdot \frac{v_H}{v_M}$$

where I is the number of moles of isoprene, χ_I is the mole fraction of isoprene in the headspace air (mole fraction is interchangeably nmol isoprene/mol air or nPa isoprene/Pa air), v_H is the volume of the head space and v_M is the molar volume (22.4 L/mole corrected for temperature and pressure). For example, in a 2 mL vial with 200 µL liquid medium containing reactants, 1.8 mL gas phase, and assuming the FIS returns a reading of 100 ppb, and the temperature is 25°C

$$100 \cdot 10^{-9} \frac{\text{mol}}{\text{mol}} \cdot 1.8 \cdot 10^{-3} \text{L} \cdot \frac{\text{mol}}{22.8 \text{ L}} = 7.89 \cdot 10^{-12} \text{mol isoprene} .$$

In the liquid phase:

$$I = \chi_I \cdot \frac{v_l \cdot P_H}{H}$$

where v_l is the volume of the liquid phase, P_H is the pressure in the head space, assumed to be 1 atmosphere or 101 kPa, and H is the Henry's constant in units of Pa m^3 mol^{-1}. In the 2 mL vial with 200 µL liquid phase

$$100 \cdot 10^{-9} \frac{\text{Pa}}{\text{Pa}} \cdot \frac{200 \cdot 10^{-6} \text{L} \cdot 101 \cdot 10^{3} \text{Pa}}{7780 \text{ Pa m}^3} \cdot \frac{\text{mol}}{10^3 \text{ L}} \cdot \frac{\text{m}^3}{} = 0.26 \cdot 10^{-12} \text{mol isoprene} .$$

Thus 3.3% (0.26/7.89) of the isoprene is in the liquid phase and the total isoprene in the vial is 7.89 + 0.26 = 8.15 pmol. This can then be expressed relative to the incubation time and amount of material (e.g., amount of protein) in the reaction.

4.2 Analyzing isoprene emission from leaves using an FIS

The rate of isoprene emission can be used as a proxy to measure the activity of the MEP pathway and its regulation. An FIS coupled with the LI-6800 Portable Photosynthesis System

309 (LI-COR Biosciences, Lincoln, NE) can be used to measure isoprene and photosynthesis rates
310 simultaneously in leaves detached from plants **(Figure 2)** or intact leaves **(Figure 3)**.

311

312 *4.2.1 Materials and equipment*

313 - A plant

314 - Bev-a-line tubing (BEV-IV 1/8" ID X1/4" OD, EW-06490-12 and 1/2" ID x 5/8" OD
315 EW-06490-19). Alternatives include Teflon tubing. Teflon is more inert but stiffer and
316 a little more difficult to handle. The differences are minor for isoprene but can be
317 significant if larger terpenoids are also being measured. The high ozone tube from the
318 ozonizer to the FIS must be Teflon.

319 - Swagelock connectors

320 - LI-6800 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE). We most
321 often use a Multiphase FlashTM Fluorometer (6800- 01A) chamber but other chambers
322 work fine.

323 - A Fast Isoprene Sensor (FIS) with ozone generator

324 - Ultra-high purity oxygen

325

326 *4.2.2. Procedure*

327 *Setting up the FIS*

328 First, turn on the oxygen supply to the FIS, and power up the FIS and the ozonizer
329 following the manufacturer's instructions. After the ozone has been flowing for several minutes,
330 turn on the PMT and open the LabVIEW software program. We normally select the 5 second
331 averaging period, but a higher sampling rate is used when following transients. Start the

program. A file name is suggested that includes the date and time and can be modified so that you can organize your data. The FIS has a flow controller that allows mixing in known amounts of an isoprene standard gas (we use a 3 PPM standard from Airgas). Plot the photon counts per ppb of isoprene. You will need this slope to analyze data from leaf measurements. It is important to use the same flow rate during the calibration that you will use during the measurements.

Setting up the LI-6800

Switch on the LI-6800 following the manufacturer's instructions. Go to the 'Environment' window and set environmental conditions. It is very helpful to synchronize the clocks on the FIS and LI-COR so that gas exchange data and isoprene emission data can be correlated.

Standard environmental conditions for isoprene measurements are: leaf chamber flow rate[1] - 500 μmol s^{-1}; temperature - 30°C; sample CO_2 concentration - 420 μmol mol^{-1}; light intensity - 1000 μmol m^{-2} s^{-1} is the preferred default.

The LI-COR 6800 instrument allows two methods for sampling the air that has passed over the leaf. At the back of the head there is an outlet that allows the user to split the air leaving the leaf change between the LI-COR analyzer and an external analyzer such as an FIS or PTR-MS. At the front, users can take all the air that has flowed over the leaf and through the analyzer. When sampling from the front it is important to impose the least possible resistance to flow since this can change the pressure in the analyzer and introduce errors. To measure in the exhaust air, assemble a sample-exhaust line using Bev-A-Line IV or Teflon tubing, and a

[1] Flow rate in the LI-COR is expressed in μmol s^{-1} while flow meters in the FIS report standard cubic centimeters per minute (SCCM), also expressed as mL min^{-1}. There are 22.4 standard liters per mole (do not adjust for temperature and pressure because the flow meter has already made this adjustment). One SCCM = 0.744 μmol s^{-1}.

353 Swagelok union tee-joint **(Figure 2A** shows a leaf detached from the plant while **Figure 3**
354 shows a leaf still attached to the plant**)**. The details shown in Figure 2B and C are the same for
355 the attached plant system shown in Figure 3.

356 Tee-joint-1 **(Figure 2A)** connects the LI-6800 sample-exhaust port A **(Figure 2A, B)**
357 with the 'sample in' port at the back of the FIS. We typically use a total flow rate of the FIS of
358 1200 mL min^{-1} and the O_2 flow rate of 800 mL min^{-1}, and so the sample flow rate into the FIS
359 is 400 mL min^{-1} **(Figure 2C)**. The LI-6800 sample flow rate when set at 500 μmol s^{-1} is 672
360 mL min^{-1}). The excess flow from the leaf chamber will vent from the 'tube for excess flow out'
361 at the tee-joint **(Figure 2A, C)**. A length of tubing on this exhaust will prevent back diffusion
362 which would dilute the air entering the FIS, but it should not be too long to avoid back pressure
363 on the LI-COR analyzers. The FIS pulls in 400 mL min^{-1} or ~295 μmol s^{-1} and so consumes
364 295/500 or approximately 60% of the air flow out of the LI-COR (but see below for the effect
365 of transpiration on total flow rate exiting the chamber). The excess flow protects against the
366 FIS pulling in room air. Increasing the flow to the FIS increases the signal but runs the risk of
367 pulling in room air, which would spoil the measurement and would require a recalibration at
368 the new flow rates.

369 *Measuring isoprene*

370 Clamp a leaf in the LI-6800 leaf chamber and close the chamber **(Figure 2A and 3)**.
371 Zero readings can be made by switching the LI-COR to "Match" mode. There is little drift in
372 sensitivity of the FIS but significant zero drift. The background reading taken while in Match
373 mode will account for the baseline signal of the FIS, any isoprene in the supply air, and any
374 contaminating/cross reacting components of the air supplied to the LI-COR. It is important to
375 make the zero reading frequently, especially early in the day.

To measure isoprene and photosynthesis simultaneously, log photosynthesis on the 6800 manually or use the auto-program feature in the LI-6800. Record time units in the FIS and the LI-6800 so that the FIS reading can be matched with the corresponding photosynthesis measurements. Once isoprene and photosynthesis reach steady state, record start and end time in the FIS for a one-minute period during which isoprene measurement is stable. For each sample, record the start and stop times of the sampling period.

If using a large leaf that covers the entire area of the LI-6800-01A leaf chamber, the area is 6 cm^2. If the leaf is smaller, photographing the section of the leaf that was inserted to the leaf chamber, followed by analysis using imaging software will have to be carried out to accurately estimate leaf area.

Data analysis

The slope (counts / isoprene ppb) from the calibration data of the FIS will be needed for data analysis. Subtract the average number of photon counts of the background from the sample. Using the slope (counts/isoprene ppb) of the calibration curve, divide the number of counts by the calibration factor (slope) to give the mole fraction of isoprene in ppb (nanomoles of isoprene per mole of air). Multiply the mole fraction of isoprene in the air exiting the chamber by the flow out of the leaf chamber. This will be different from the flow entering the leaf chamber reported by the LI-COR because transpiration is adding gas to the total flow. This is the same effect that affects gas exchange measurements of photosynthesis (von Caemmerer & Farquhar, 1981). Niinemets et al. (2011) provides the following equation that should be used:

$$F_{out} = F_{in} + \lambda \cdot A$$

where F is flow out or flow in, λ is transpiration rate and A is leaf area. With large leaf areas, high transpiration rates, or slow flow rates the flow rate out can be 20% more than that reported by the LI-COR but the effect is more typically 2 to 5% (Niinemets et al., 2011). In East Lansing we sometimes use an ice trap before the FIS because of minor water sensitivity and this will also affect the conversion of ingoing chamber flow rate to outgoing flow rate.

$$F_{out} = F_{in} \cdot (1 + W_{out} - W_{in}).$$

where W is the mole fraction of water vapor in the air entering the chamber (in) or leaving the chamber (out). With an ice trap in place, W_{out} will be 0.006 Pa/Pa and if the dew point of the ingoing air is 15ºC then W_{in} is 0.016 and F_{out} (flow into the FIS) will be 1% less than the flow reported by the LI-COR.

Use the concentration of isoprene in mol isoprene mol^{-1} air and the LI-COR flow (corrected for water vapor) (mol air s^{-1}) to calculate the rate of isoprene emission in mol isoprene s^{-1}. Then divide by leaf area. Typical values are 1 to 60 nmol isoprene m^{-2} s^{-1}. Niinemets et al. (2011) provides much additional information on measuring isoprene (and other terpenes).

4.3 Labeling isoprene by feeding $^{13}CO_2$

4.3.1. Method To feed $^{13}CO_2$ to observe the rate and degree of labeling of isoprene by photosynthesis, the LI-COR console is set to provide CO_2 free air to the LI-COR head. Different isotopes are then fed through mass flow controllers and switched using a four-way valve **(Figure 4 A, B, and C)**. Most CO_2 analyzers have much reduced sensitivity to $^{13}CO_2$ compared to $^{12}CO_2$ so it is not possible to measure the rate of photosynthesis during feeding.

Also, it is necessary to calculate air flows to set the rate of flow of $^{13}CO_2$ during the feeding. This should be set so that the concentration of CO_2 does not change regardless of which isotope is being fed. The flow out of the chamber can be collected in a Tedlar bag (for example "ESS GD0707-7000 Sampling Bags With Combination Valve" from Cole-Parmer). Be careful not to impose back pressure on the LI-COR.

The degree of label in isoprene can be determined by GC-MS using a SPME fiber or PTR-MS **(Figure 5D and E**). A PTR-MS provides real time readings and simplifies measuring time courses.

Many publications have assumed that labeling to less than 100% indicates an alternative carbon source for isoprene emission and hence the MEP pathway, but this assumes that the Calvin Benson cycle labels to 100%. It does not (Sharkey et al., 2020). Isoprene, and so the MEP pathway, label to the same degree as the Calvin-Benson cycle intermediates and so labeling of isoprene can provide a window on photosynthetic carbon metabolism.

4.4 Measuring DMADP and MEcDP using post-illumination isoprene measurements

In vivo estimation of DMADP and MEcDP pools in plants leaves of isoprene-emitting species rests on the observation that when light is switched off isoprene emission continues for about 10 min. at the expense of MEP pathway metabolites synthesized during the previous light period (Li et al., 2011; Rasulov et al., 2009; Rasulov et al., 2011; Weise et al., 2013). The post-illumination isoprene emission is biphasic. The first phase continues for about 300 s after switching off the light, followed by a second rise of isoprene emission between about 300-1000 s. The first phase is used to estimate DMADP pool size and the second phase to estimate MEcDP pool size. The intermediate pool sizes estimated by the in vivo method are in very good agreement with separate destructive chemical measurements of the pool sizes (Rasulov et al., 2009; Weise et al., 2013).

For in vivo estimation of isoprene precursor pool size, a real-time isoprene sensor is needed (either FIS or PTR-MS), whereas the protocol of measurements depends on the system response time, which depends on the chamber volume and air flow rate. For fast systems, the measurements do not need to consider the chamber response time (as e.g. in Rasulov et al., 2016), whereas for slow systems, the system response time should be separately estimated (as e.g. in Rasulov et al., 2009). For example, the Rasulov et al. (2016) ultra-fast gas-exchange system has a chamber volume of 2.4 mL and flow rate of 0.67 L min^{-1}, yielding a system half-time (τ) of only 0.15 s and time to reach a steady state (4τ) of 0.6 s, whereas the volume of LI-6400 standard 2 cm x 3 cm (6 cm^2 window area) chamber is 80 mL, and for the same flow rate, 4τ is 20 s, and for the conifer chamber of LI-COR (volume of 155 mL) 4τ is 39 s (Niinemets, 2012).

The gas exchange systems can have one (measurements switched between reference measurement and sample measurement) or two measurement lines (reference and sample measured continuously). For one-line systems, the time for switching and stabilization of gas flows between the reference and sample measurement can further add to the whole system response time.

4.4.1. Use of a fast gas-exchange system for estimation of intermediate pool sizes

Here we provide a sample protocol with the ultrafast gas-exchange system of Rasulov et al. (2016) that includes two identical parallel gas lines that allow independent measurement of the background and chamber isoprene concentrations. Isoprene concentration can be measured by both FIS (see above for settings), or PTR-MS as explained here.

4.4.1.1. Materials

- A plant

- A PTR-TOF-MS
- An ultra-fast gas-exchange system with a 2.4 mL circular leaf chamber (3 mm height, 32 mm diameter) (Rasulov et al., 2016)
- Isoprene calibration standard (3.43 ppm isoprene in N_2)

4.4.1.2. Procedure

Setting up PTR-TOF-MS

Use the following PTR-TOF-MS setup through the measurements: the inlet and drift chamber temperature: 60 °C, inlet flow 100: mL min^{-1}, flow of water vapor: 5.0 mL min^{-1}, ion current: 4 mA, drift chamber pressure: 2.1 mbar, drift tube field density ratio: 140 Td, pressure of the TOF-MS module: $2.4 \cdot 10^{-7}$ mbar (Rasulov et al., 2019). Calibrate the PTR-MS-TOF instrument using the flow of the isoprene standard into the leaf measurement chamber.

Setting up the gas exchange system

In vivo MEP pool sizes can be estimated for any combination of environmental drivers. First, enclose the leaf in the chamber and establish the desired environmental conditions in the chamber (e.g., leaf temperature of 30 °C, air humidity of 60%, CO_2 concentration of 410 mol mol^{-1} and light intensity of 1000 μmol m^{-2} s^{-1}, air flow rate of 0.67 L min^{-1}). Establish identical ingoing gas concentrations in both lines of the gas exchange system.

Measurement procedure

Measure isoprene background concentration in the reference line with the PTR-TOF-MS at 400 ps sample interval and record averages at 1 Hz interval. Switch the channels and measure isoprene concentration in the gas exchange chamber exhaust air. Wait until leaf isoprene emission reaches a steady state, typically for 10-20 min after leaf enclosure. Switch back to reference line and measure again isoprene background concentration. Simultaneously switch

494 to the sample line and switch off the light. Measure isoprene emission through both phases of
495 post-illumination kinetics (**Figure 5A**) for about 10-15 min after switching off the light. Switch
496 back to the reference line and measure again the background isoprene concentration. Calculate
497 isoprene emission rates through the post-illumination kinetics as explained above. Establish
498 the baseline through the post-illumination measurements using the reference line
499 measurements at different time points. Estimate the baseline for the first phase of the post-
500 illumination decay curve by extrapolating from the start of the emission rise to reference line
501 (between ca. 570 s to 200 s in **Fig. 5A**). Integrate the first phase of the dark decay kinetics
502 above the baseline (between ca. 200 to 410 s in **Fig. 5A**) and the second phase using the
503 trapezoidal rule. The first integral is the DMADP pool size and the second MEcDP pool size
504 supporting the isoprene emission rate prior to leaf darkening.

505

506 *4.4.2. Use of a slower gas-exchange system for estimation of intermediate pool sizes*

507 In the case of the slower system, all measurements are carried out identically to fast system
508 measurement. The only difference is the need to consider system delay effects. At the end of
509 the measurement, the leaf or plant is removed from the chamber and a flow of isoprene is fed
510 into the carrier air flow or directly into the leaf chamber **(Fig. 5B)** (Li et al., 2011). Once a
511 stable isoprene flow from the chamber is achieved, isoprene flow into the chamber is stopped
512 and isoprene flow from the chamber is measured until it reaches the background level. The
513 artificial isoprene release kinetics is scaled such that the value of isoprene release prior to
514 stopping isoprene flow is scaled to the level corresponding to steady-state isoprene emission
515 rate. The artificial isoprene kinetics is superimposed on the plant measurements and the
516 baseline for the first phase of the post-illumination kinetics is established as shown in **Fig. 5B**.
517 For plant measurements, the two peaks of isoprene emission are integrated as explained in 4.4.1
518 and the isoprene release without plant is also integrated. The DMADP pool size is the

519 difference between the integral of the first peak of plant measurements minus the integral of
520 isoprene release without the plant (**Fig. 5B**).

521

522 Safety considerations and standards

523 Many of these techniques require handling pressurized gases and care must be exercised
524 in handling gas tanks. At low concentrations in air, isoprene is not toxic (humans generally
525 exhale air with 25 to 100 ppb isoprene) but higher concentrations in air should be avoided. If
526 liquid isoprene is stored, it should be in an explosion-proof container and refrigerated because
527 the boiling point is 32°C. Care should be taken to avoid contact with liquid isoprene. Long-
528 term storage of liquid isoprene can lead to formation of impurities due to condensation
529 reactions, including formation of monoterpenes.

530

531 Summary

532

533 Measuring isoprene in air contributes to studies in atmospheric chemistry, plant physiology,
534 biotechnology, and biochemistry. There are many methods used to measure isoprene and
535 different methods of detection. Isoprene measurements can be made with bacterial cultures,
536 isolated enzymes, and attached or detached leaves. Isotopic labeling can add to the utility of
537 isoprene measurements. The Proton Transfer Reaction mass spectrometers are the most
538 versatile instruments for detection but also the most expensive (Table 1). Isoprene is relatively
539 easy to handle, it is relatively stable and does not stick to tubing and chamber walls as much as
540 other terpenes. It also is emitted from leaves as soon as it is made allowing insight into the
541 working of the methyl erythritol 4-phosphate pathway, the pathway in plants responsible for
542 isoprene emission.

543

544 Table 1. Pros and cons of the methods discussed here.

	Pros	**Cons**
Continuous	Can follow transients	Requires PTR-MS to follow isotopologues
Discrete	Flexibility, simplicity	Difficult to follow transients
Detection		
GC-PID	High sensitivity, good quantitation Easily adapted to large samples Other gases can be measured in single run	Less selective Discreet measurements only
GC-MS (SPME)	Good selectivity Other gases can be measured in single run	Discreet measurements only Poor quantitation
FIS	Large dynamic range Good quantitation High temporal resolution	Specialized instrument for isoprene measurements
PTR-MS (both quadrupole and time-of-flight)	High sensitivity and time resolution Many other gases can be measured simultaneously Continuous monitoring Isotopologue-specific	Expensive to acquire Some compound fragments might interfere with isoprene detection

545

546

547

548

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recombinant isopentenyl diphosphate isomerase and isoprene synthase. *Analytical Biochemistry*, *440*(2), 130-136. https://doi.org/10.1016/j.ab.2013.05.028

Figure legends

Figure 1. Measuring isoprene production by bacterial cultures. A bacterial culture is grown until a desired concentration is achieved. If required, the culture is induced to express enzymes of interest. At any time point 200 μL of culture are removed and put into a 2 mL crimp seal vial. The vial is incubated for a set time (we often use 10 min) and then the headspace is removed with a 1 mL syringe while water is injected to prevent formation of a vacuum. The sample is then injected into the airstream of a Fast Isoprene Sensor instrument (a PTR-MS either quadrupole or time-of-flight would also work well). We often use an ice bath to bring the water vapor in the gas sample to 0°C dew point. Because these are discreet measurements gas chromatographs would also work.

Figure 2. System for measuring isoprene emission from a leaf. In Panel A, a leaf detached from the plant is shown. This allows feeding poisons such as fosmidomycin or potential substrates. Panel B shows a closeup of the leaf chamber and the sample exhaust that is used for measurements as shown in panel C. PTR-MS instruments would also work well but gas chromatographs would be less convenient.

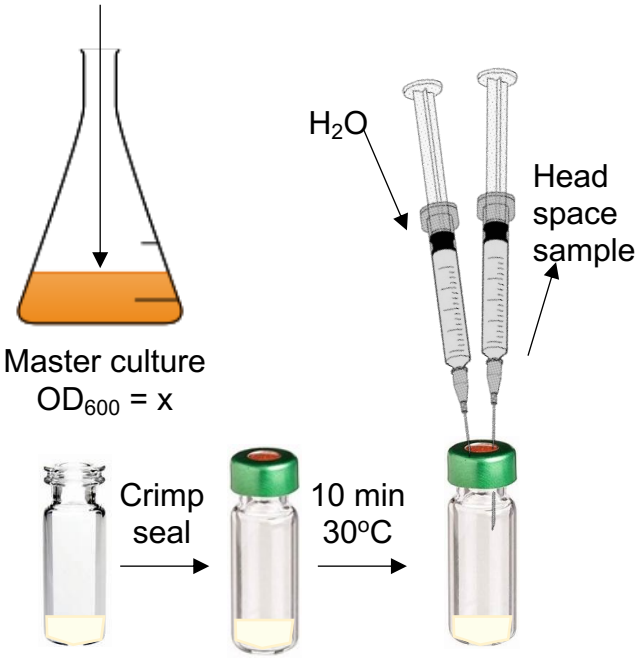
Figure 3. Measuring isoprene emitted from a leaf attached to the plant. This allows for repeated measurements in the days following a treatment. Many plants show little effect of detaching leaves but using attached leaves circumvents any possible problem associated with cutting the leaf off them plant. The flow path is the same as shown in Figure 2C.

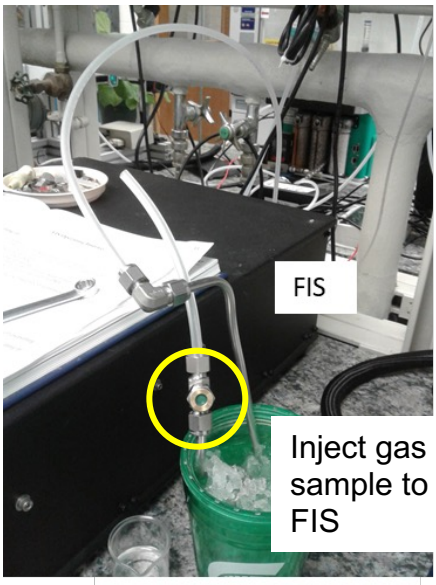
Figure 4. Measuring labeling of isoprene when feeding $^{13}CO_2$. A system for rapid switching between $^{12}CO_2$ and $^{13}CO_2$ at the back of the LI-COR head is shown in Panel A. Panel B shows the mass flow controllers that are used to set the concentration of the two isotopes so that there is no change in CO_2 concentration when switching from one isotope to the other. Panel C shows exposing a Solid Phase Micro Extraction (SPME) element to a sample in a Tedlar bag and Panel D shows desorbing the sample into a GC-MS. This measurement is easier with a PTR-MS and the high resolution of a PTR-TOF-MS can help ensure that isoprene is being measured.

Figure 5. Measurements of the pool sizes of isoprene precursors DMADP and MEcDP using a two-channel ultra-fast gas exchange system (Rasulov et al., 2016) (chamber volume 2.4 mL, flow rate 0.67 L min^{-1}, system half-time 0.15 s) that does not require consideration of system delay effects (A) and a slower system where the system delay (amount of isoprene present within the chamber and system gas lines) needs to be taken into account (Rasulov et al., 2009) (chamber volume 1 L, flow rate 1.5 L min^{-1}, system half-time 28 s). In panel A, the leaf was first stabilized until steady-state conditions were reached, at time a, the reference line was measured (leaf switched from line 1 to line 2), and at time b, the leaf was switched from line 2 to line 1 and light was simultaneously switched off and the dark decay kinetics of isoprene release was followed until the emission reached to the background level. In B, the measurement protocol was similar. After measurement of the dark decay kinetics of isoprene release, the plant was removed from the chamber, and a stable isoprene flow was established through a calibrated capillary. After reaching a stable value, the supply of isoprene was interrupted and the artificial "isoprene release" was recorded again. This line was superimposed on the post-illumination emission kinetics. The baselines for DMADP pool sizes were determined as shown in the figure. In (a), the DMADP pool size was estimated as the integral of the first peak and the MEcDP pool size as the integral of the second peak. In (b), the DMADP pool size was

estimated as the integral of the first peak of the plant emissions minus the integral of artificial isoprene release, and the MEcDP pool size as the integral of the second peak with plant emissions.

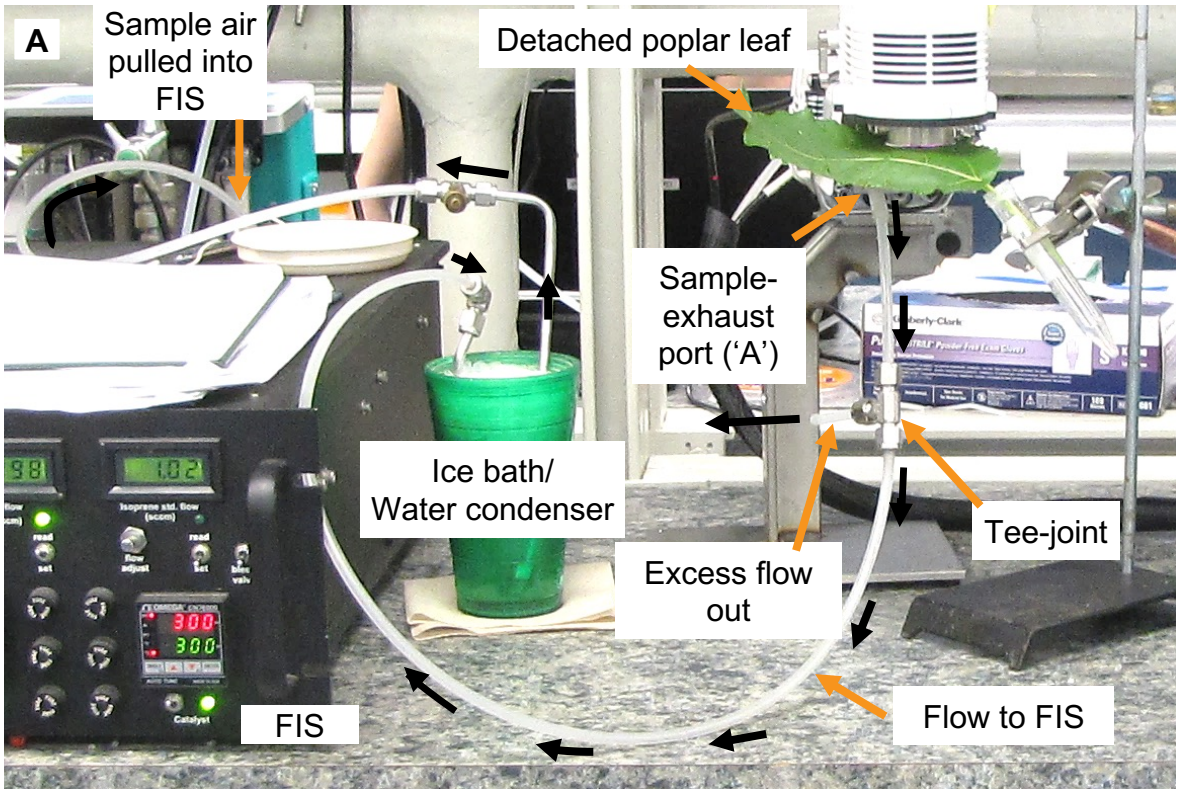
Figure 1

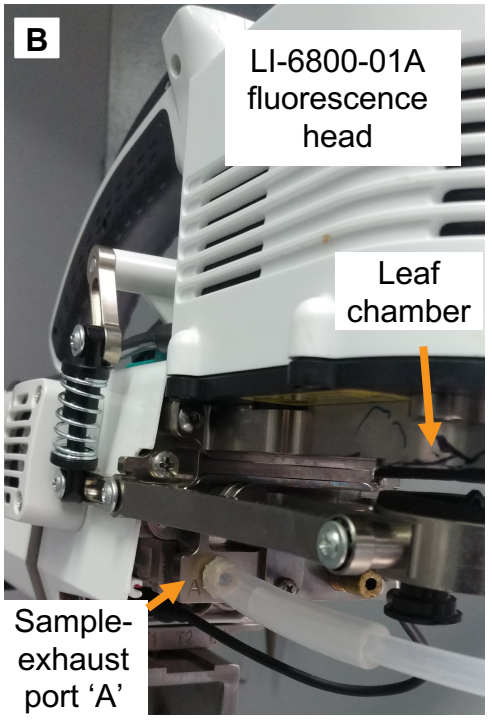

H_2O
Head space sample
Master culture
OD_{600} = x
Crimp seal
10 min 30°C


FIS
Inject gas sample to FIS


New FIS

Figure 2


A
Sample air pulled into FIS
Detached poplar leaf
Sample-exhaust port ('A')
Ice bath/ Water condenser
Tee-joint
Excess flow out
Flow to FIS
FIS


B
LI-6800-01A fluorescence head
Leaf chamber
Sample-exhaust port 'A'

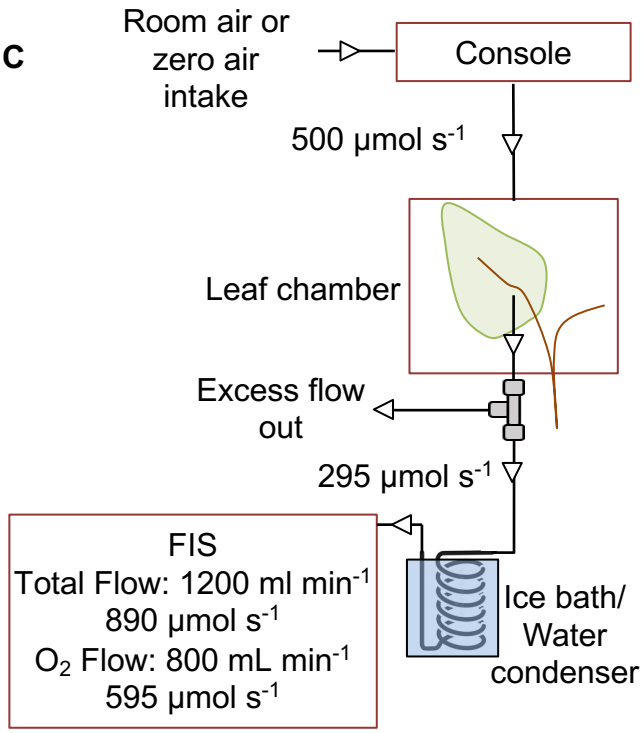
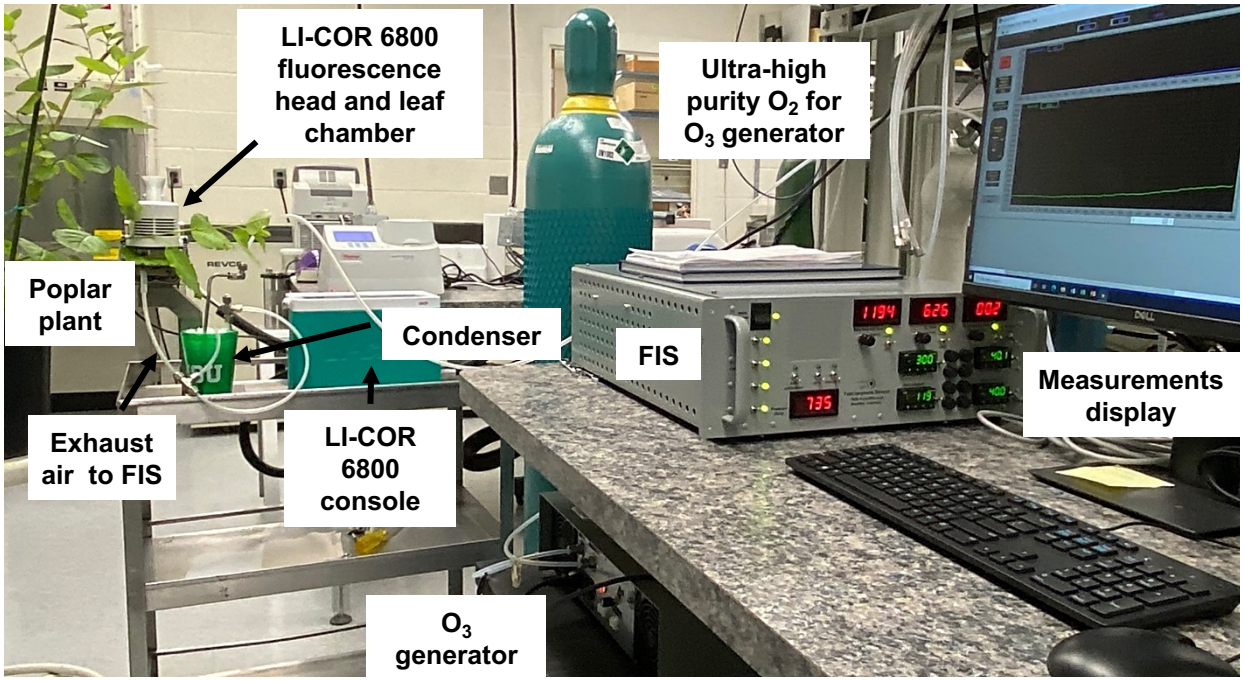

C
Room air or zero air intake
Console
500 µmol s-1
Leaf chamber
Excess flow out
295 µmol s-1
FIS
Total Flow: 1200 ml min-1
890 µmol s-1
O2 Flow: 800 mL min-1
595 µmol s-1
Ice bath/ Water condenser

Figure 3


LI-COR 6800 fluorescence head and leaf chamber
Ultra-high purity O_2 for O_3 generator
Poplar plant
Condenser
FIS
Measurements display
Exhaust air to FIS
LI-COR 6800 console
O_3 generator

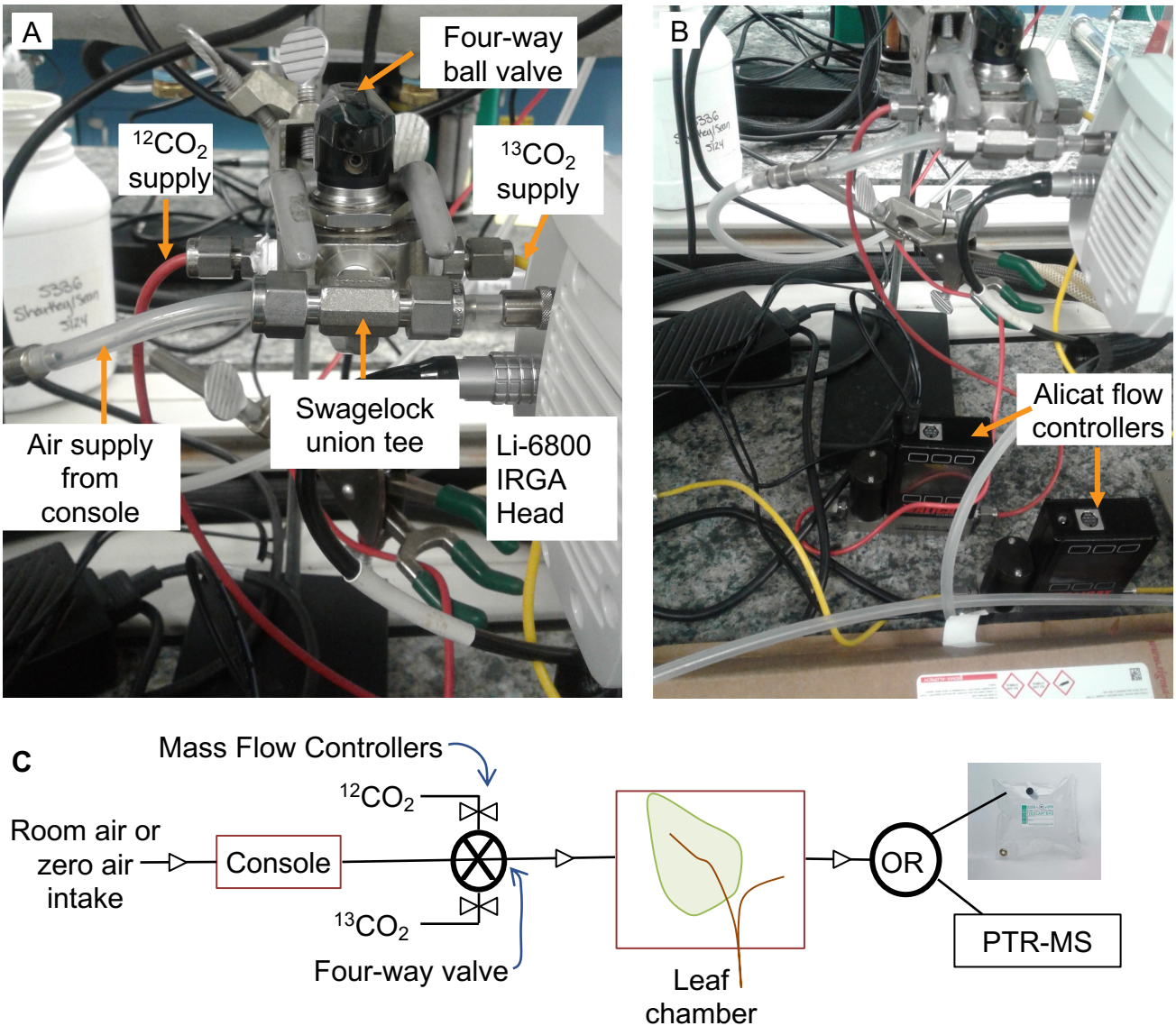
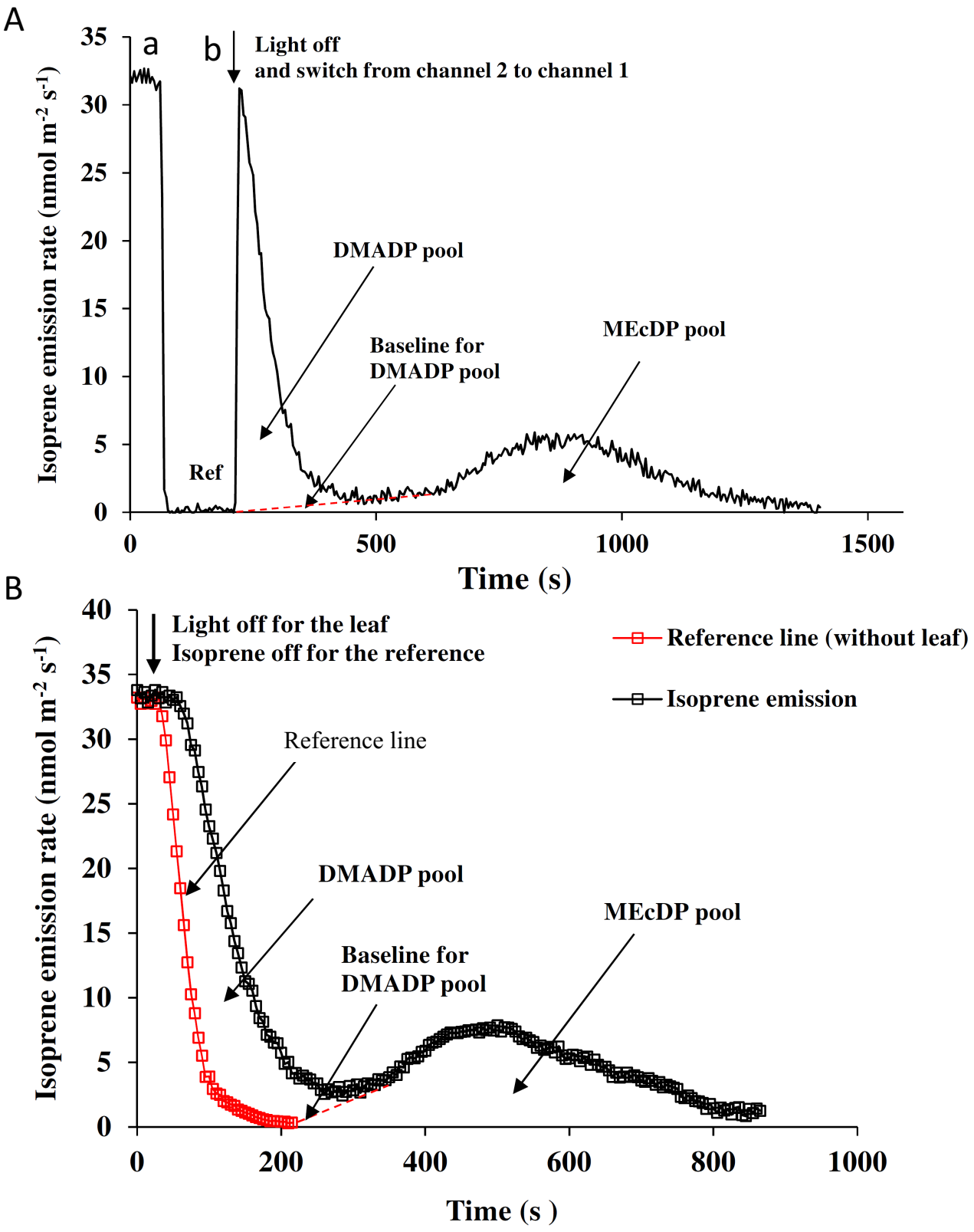

A
Four-way ball valve
$^{12}CO_2$ supply
$^{13}CO_2$ supply
Air supply from console
Swagelock union tee
Li-6800 IRGA Head
B
Alicat flow controllers
C
Mass Flow Controllers
$^{12}CO_2$
Room air or zero air intake
Console
$^{13}CO_2$
Four-way valve
Leaf chamber
OR
PTR-MS
D
Analyte adsorption onto SPME fiber
Ring stand
SPME fiber holder
Tedlar gas sampling bag
E
Desorption into GC
SPME fiber inserted into GC
GC injection port

Figure 4

Figure 5


A
a
b
Light off
and switch from channel 2 to channel 1
DMADP pool
Baseline for
DMADP pool
MEcDP pool
Ref
Isoprene emission rate (nmol m-2 s-1)
Time (s)
B
Light off for the leaf
Isoprene off for the reference
Reference line (without leaf)
Isoprene emission
Reference line
DMADP pool
MEcDP pool
Baseline for
DMADP pool
Isoprene emission rate (nmol m-2 s-1)
Time (s)