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RESEARCH PAPER

Carbon isotope analysis of acetaldehyde emitted from leaves following mechanical stress and anoxia

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ABSTRACT

Although the emission of acetaldehyde from plants into the atmosphere following biotic and abiotic stresses may significantly impact air quality and climate, its metabolic origin(s) remains uncertain. We investigated the pathway(s) responsible for the production of acetaldehyde in plants by studying variations in the stable carbon isotope composition of acetaldehyde emitted during leaf anoxia or following mechanical stress. Under an anoxic environment, C3 leaves produced acetaldehyde during ethanolic fermentation with a similar carbon isotopic composition to C3 bulk biomass. In contrast, the initial emission burst following mechanical wounding was 5–12‰ more depleted in ^{13}C than emissions under anoxia. Due to a large kinetic isotope effect during pyruvate decarboxylation catalysed by pyruvate dehydrogenase, acetyl-CoA and its biosynthetic products such as fatty acids are also depleted in ^{13}C relative to bulk biomass. It is well known that leaf wounding stimulates the release of large quantities of fatty acids from membranes, as well as the accumulation of reactive oxygen species (ROS). We suggest that, following leaf wounding, acetaldehyde depleted in ^{13}C is produced from fatty acid peroxidation reactions initiated by the accumulation of ROS. However, a variety of other pathways could also explain our results, including the conversion of acetyl-CoA to acetaldehyde by the esterase activity of aldehyde dehydrogenase.

INTRODUCTION

The emission of acetaldehyde from plant canopies to the atmosphere may significantly influence air quality and climate. Although its metabolic origin remains uncertain, acetaldehyde is emitted into the atmosphere from plants after a wide variety of stresses and may play important roles in plant defence against stress. Acetaldehyde is a potent antibiotic and its emission from damaged tissue may help prevent infections (Utama et al. 2002) and activate the expression of plant defence genes (Tadege et al. 1998). Enhanced acetaldehyde emissions have been observed following mechanical wounding, desiccation, freeze–thaw events, herbivore attack, ozone fumigation, high light, high temperature, and many other biotic and abiotic stresses (Kimmerer & Kozlowski 1982; de Gouw et al. 1999; Fall et al. 1999; Karl et al. 2001a,b, 2005;

Cojocariu et al. 2005; Loreto et al. 2006). Although a small amount of this acetaldehyde can be attributed to what was present in the transpiration stream, another source is likely (Fall 2003).

The production of acetaldehyde and ethanol in plants by pyruvate decarboxylase (PDC) and alcohol dehydrogenase during ethanolic fermentation induced by anoxia is well established (Kimmerer & MacDonald 1987; Vartapetian & Jackson 1997; Vartapetian et al. 1997). In anoxic conditions, ethanol produced from ethanolic fermentation in roots is transported to leaves via the transpiration stream, where it is oxidised to acetaldehyde (Kreuzwieser et al. 2004). This is a potentially significant source of acetaldehyde to the atmosphere, for example during the wet season in the Amazon forest, where roots are regularly flooded (Rottenberger et al. 2008). Karl et al. (2002) reported that bursts of acetaldehyde emissions occurred

following light–dark transitions and argued that these conditions enhance the rate of the PDC reaction in the cytoplasm due to the transient accumulation of pyruvate. They demonstrated that inhibitors of mitochondrial respiration and pyruvate transport, which are expected to increase pyruvate concentrations in the cytoplasm, induced acetaldehyde emissions, and they termed this process the pyruvate overflow mechanism. Using excised branches, Jardine (2008) suggested that the full ethanolic fermentation pathway may be active in leaves under aerobic conditions. Emission rates of ethanol, acetaldehyde and acetic acid from excised poplar branches were light/temperature dependent. As reviewed by Tadege *et al.* (1999), there is also evidence emerging that various stresses increase ethanolic fermentation rates and that a switch from respiration to ethanolic fermentation can accompany stress under aerobic conditions. For example, the expression of several genes involved in ethanolic fermentation increased dramatically under abiotic stress in *Arabidopsis* (Dolferus *et al.* 1994a,b). However, the ubiquitous production of acetaldehyde from plants under stress cannot be used to conclusively demonstrate the switch to ethanolic fermentation since other pathways for producing acetaldehyde may exist.

Halliwell & Gutteridge (1999) suggested that free radical peroxidation of membrane fatty acids may lead to the production of acetaldehyde, although, to our knowledge, no experimental evidence has come forward to support this hypothesis. However, several prior studies have observed emissions of both acetaldehyde and C-6 volatiles (*e.g.* hexenal and hexanal) under a variety of stresses. C-6 volatiles are produced during the stress-induced activation of the octadecanoid pathway in plants from the lipo-oxygenase-catalysed oxidation of membrane fatty acids (Hatanaka *et al.* 1987). For example, following mechanical wounding and exposure to high light, Loreto *et al.* (2006) observed strong temporal correlations between acetaldehyde and C-6 volatile emissions from *Phragmites* leaves. In addition, no ^{13}C was incorporated into the emitted acetaldehyde when exposing a wounded leaf or a leaf disc to a $^{13}CO_2$ atmosphere. This indicates that the wound-induced acetaldehyde emissions are not derived from recent photoassimilated carbon. Graus *et al.* (2004) also observed a strong temporal correlation between C-6 volatiles and acetaldehyde emissions following light–dark transitions. They suggested that acetaldehyde emissions under these conditions are related to leaf responses to wounding.

Knowledge of the intermolecular distribution of carbon isotopes among organic compounds is a powerful emerging tool in the area of biosphere–atmosphere interactions. As discussed by Augusti & Schleucher (2007), changes in the relative abundance of the stable carbon isotopes in plant metabolites can be directly related to their metabolic origin. In 1961, Park & Epstein discovered that the lipid fraction of plants was significantly depleted in ^{13}C when compared with the bulk biomass. Deniro & Epstein (1977) demonstrated that, due to a large kinetic isotope effect, pyruvate decarboxylation by pyruvate dehydrogenase (PDH) was the major cause of the ^{13}C depletion in lipids. Compounds produced from PDH-derived acetyl-CoA, such as fatty acids, are depleted in ^{13}C relative to the bulk biomass. For example, Ballentine *et al.* (1998) found that fatty acids from C3 plants are on average 6.5‰ more depleted in ^{13}C than the bulk biomass, where isotopic ratios are reported on the Vienna-Pee Dee Belemni (V-PDB) scale and calculated as $\delta^{13}C$ (‰) = $(R_{sample} - R_{standard})/R_{standard} \times 1000$‰, with R_{sample} = $^{13}C/^{12}C$ and with $R_{standard}$ set to 0.0112372 (molar ratio). In contrast, compounds that are produced from ethanolic fermentation, such as acetaldehyde and ethanol, are not as depleted in ^{13}C. When Hobbie & Werner (2004) surveyed the literature on ethanolic fermentation by C3 plants, they found that the ethanol produced during fermentation was between 1‰ and 2‰ depleted in ^{13}C relative to the parent carbohydrate. For example, in Europe, when the $\delta^{13}C$ values of sugars from grape must and ethanol in the corresponding wine were measured, the ethanol was between 1.3‰ and 1.7‰ more depleted in ^{13}C than the sugars (Rossmann *et al.* 1996).

In this study, we performed stable carbon isotope ratio measurements of gas phase acetaldehyde emitted from various C3 tree species under anoxic conditions, on mechanical wounding, as well as during leaf decay. We hypothesised that if acetaldehyde is produced from ethanolic fermentation following mechanical wounding, then it should have a similar carbon isotope composition to acetaldehyde emitted from leaves under anoxia. However, if the acetaldehyde emitted following mechanical wounding is depleted in ^{13}C relative to that under anoxia, then it would indicate its production from a carbon source having similar ^{13}C depletion such as fatty acids or acetyl-CoA.

MATERIALS AND METHODS

Plants

Potted poplar (*Populus deltoides,* clone #ST109) individuals were obtained from the Stony Brook University greenhouse (Stony Brook, NY, USA). Plants were potted into 4-l plastic pots containing a commercial potting mix (MiracleGro) with Osmocote slow release fertilisers. White oak (*Quercus rubra*), red maple (*Acer rubrum*) and sassafras (*Sassafras albidum*) branches were obtained from plants in the forest surrounding the Marine Science Research Center at Stony Brook University. Post-senescent red maple leaves were obtained from the forest floor in early December.

Acetaldehyde $\delta^{13}C$ measurements

$\delta^{13}C$ measurements of gas phase acetaldehyde were made without the need for derivitisation by coupling traditional cryogenic pre-concentration of volatile organic compounds (VOCs) from ambient air (Greenberg & Zimmer-

man 1984) to a gas chromatograph–combustion–isotope ratio mass spectrometer (GC–C–IRMS) system. A custom VOC pre-concentration system was built in order to pre-concentrate gas phase acetaldehyde as well as separate it from N_2, O_2, Ar and trace species including H_2O and CO_2. All tubing and traps were composed of fused silica-lined stainless steel tubing (Restek, Bellefonte, PA, USA) or Teflon. Stainless steel switching valves (VICI, Houston, TX, USA) were heated to 100 °C, and tubing through which the sample passed was heated to 200 °C. The sample was drawn into the pre-concentration system by a diaphragm pump (Pfeiffer Vacuum, Nashua, NH, USA) connected to a mass flow controller (Omega, Stamford, CT, USA) at 250 ml·min^{-1} for 10 min, resulting in a sampled volume of 2.5 l. The air sample first passed through a 3-foot coil of 1/8″ O.D. Teflon tubing held at 0 °C in order to condense a large percentage of the water vapour. In contrast to alternate drying methods using solid adsorbents, this technique did not affect the concentration of acetaldehyde. We assume that carbon isotope fractionation during the partitioning of acetaldehyde into the condensed water during this step is insignificant, by analogy with the results of Johnson & Dawson (1993), who found that the equilibrium carbon fractionation associated with the partitioning of gaseous formic acid into an aqueous phase is negligible. They concluded that the carbon isotope signature of oxygenated organics is not affected by wet deposition. Following drying, the sample was passed through a hydrocarbon trap [1/4″ O.D. × 9″ Silcosteel tube packed with TENAX-TA (Sigma-Aldrich)] held at −50 °C. Hydrocarbon trap temperatures above 0 °C were shown to strongly fractionate acetaldehyde due to incomplete trapping efficiency (acetaldehyde breakthrough).

The concentrated sample was then injected directly onto the GC column (0.53 mm × 30 m: RTX-624) by rapidly heating the hydrocarbon trap to 200 °C for 5 min while back-flushing with 1.0 ml·min^{-1} UHP helium. After the injection, the pre-concentration system was continuously back-flushed with ultra high purity (UHP) helium at a flow rate of 25 ml·min^{-1} until the next sample was ready for collection. In order to improve peak separation and shape, we employed a cryofocusing trap consisting of two small loops at the beginning of the GC column that were immersed in liquid nitrogen. The cryofocusing trap was rapidly heated by removing the liquid nitrogen bath. Following sample injection, the GC oven (6890 GC; Agilent Technologies, Santa Clara, CA, USA) was maintained at 25 °C for 10 min and then rapidly heated to 200 °C for 2 min to 'bake out' the GC column. Eluents of the GC column then underwent oxidation to water and carbon dioxide in an alumina tube held at 950 °C and packed with CuO, NiO and Pt wires. The oxidation products then passed through a Nafion drying membrane (Permapure Inc., Toms River, NJ, USA), and chromatographic peaks were introduced into an IRMS (Finnigan Delta Plus; Finnigan MAT, Bremen, Germany) through an open split system providing ∼0.5 ml·min^{-1} to the ion source. Prior to the arrival of the acetaldehyde peak

at the IRMS, three injections of a carbon dioxide standard of known isotopic composition (−42.7‰) were performed in order to calibrate the $\delta^{13}C$ measurement. The pressure of the reference carbon dioxide was adjusted prior to the run to approximately match the expected peak height of the sample. Peaks were identified based on retention time relative to calibration standards (acetaldehyde, methanol, acetone, isoprene). Isotope data are expressed using the conventional δ notation in units of per mil (‰):

$$\delta^{13}C = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000$$

where R is the ratio of the peak area for m/z 45 ($^{13}CO_2$) to m/z 44 ($^{12}CO_2$) for sample and standard (V-PDB-CO_2). The $\delta^{13}C$ values were calculated using the ISODAT NT 2.0 software and a manual correction based on a linear dependence of $\delta^{13}C$ on the difference in peak heights between the reference carbon dioxide peak and the VOC-derived carbon dioxide peak was applied when necessary. The precision of the instrument was between 0.2‰ and 1.0‰ (V-PDB) determined by multiple measurements of a 30-ppbv acetaldehyde standard diluted in zero air with a dynamic dilution system. Due to losses at the split (50%) and IRMS sensitivity, the minimum acetaldehyde concentration needed to produce a 1.0-volt peak for an accurate $\delta^{13}C$ measurement was determined to be ∼10 ppbv. Peaks smaller than 1.0 V were not included in the analysis.

Leaf treatments

Three to five excised leaves from the four species examined were mechanically wounded by ripping them in half by hand, and then placing them in 15-l polyethylene bags filled with UHP hydrocarbon-free air. The enclosure was immediately connected to the GC–C–IRMS instrument and $\delta^{13}C$ measurements of the acetaldehyde emitted were performed. Control experiments were also performed with empty polyethylene bags filled with a similar volume of UHP zero air. The experiment was repeated 11, 11, 9 and 14 times for red maple, white oak, poplar and sassafras respectively.

For anoxic treatments, three to five excised leaves from a single species were placed in a 15-l polyethylene bag filled with UHP helium to induce ethanolic fermentation. The enclosure was immediately connected to the GC–C–IRMS instrument and $\delta^{13}C$ measurements of the acetaldehyde emitted were made approximately every 30 min. Control experiments were also performed with empty polyethylene bags filled with a similar volume of UHP helium. The experiment was repeated three times for red maple and white oak leaves.
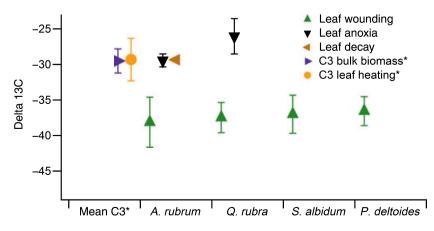
Approximately 50 post-senescent red maple leaves were collected from the forest floor during December, placed in a polyethylene bag and incubated for ∼1 h in order to accumulate acetaldehyde in the headspace sufficient for carbon isotope analysis by GC–C–IRMS. Five replicate experiments were performed.

RESULTS AND DISCUSSION

A summary of the δ^{13}C values of acetaldehyde emitted from the C3 leaves following leaf wounding, anoxia or decay is presented in Fig. 1. For the two species in which both leaf anoxia and leaf wounding were performed (*A. rubrum* and *Q. rubra*), the acetaldehyde emitted following wounding was depleted in ^{13}C by 5–12‰ relative to the acetaldehyde emitted during leaf anoxia. In addition, the δ^{13}C values of acetaldehyde emitted from all four C3 species following wounding had a similar range, which suggests that the production of acetaldehyde depleted in ^{13}C is a common feature of mechanically wounded C3 leaves. For the single species in which both leaf decay and leaf anoxia were investigated (*A. rubrum*), the δ^{13}C values of acetaldehyde emitted from both treatments had similar values. This suggests that the emissions are derived from sources with similar carbon isotope signatures. During C3 leaf heating experiments at 200 °C in Keppler *et al.* (2004), measured δ^{13}C values of acetaldehyde emissions were found to have similar values to those of average bulk C3 biomass, which is close to −28‰ for C3 plants. These observations support the current understanding that acetaldehyde emitted from decaying litter is derived from the decomposition of bulk biomass (carbohydrates and proteins) and that under leaf anoxia, ethanolic fermentation of carbohydrates gives rise to acetaldehyde production. In addition, the results presented here imply that during localised mechanical wounding, a metabolic pathway exists that produces acetaldehyde from a source of carbon depleted in ^{13}C. There is a large carbon isotope fractionation during the production of acetyl-CoA from pyruvate by PDH (Deniro & Epstein 1977), and because fatty acids are derived from acetyl-CoA via the acetogenic pathway, they are also depleted in ^{13}C. For example, individual fatty acids isolated from the C3 plant eucalyptus were 5–11‰ more depleted in ^{13}C relative to bulk biomass (Ballentine *et al.* 1998). The similar range of ^{13}C depletion for acetaldehyde emitted from wounded leaves and fatty acids leads us to hypothesise that fatty acids are a source of acetaldehyde emissions from stressed plants.

Plant phospholipids contain polyunsaturated fatty acids derived from acetyl-CoA, such as linoleic and linolenic acids, which are highly susceptible to oxidation due to the presence of multiple double bonds between carbon atoms. Wagner *et al.* (1994) concluded that the oxidisability of lipids increases linearly with their extent of unsaturation. The generation of large oxidative bursts is known to be a general response of plants to stresses such as ozone damage, high light, desiccation, pathogen attack and mechanical damage (Cazale *et al.* 1998; Langebartels *et al.* 2002; Kotchoni & Gachomo 2006). Interestingly, these same stresses also induce the emission of acetaldehyde. The rapid increase in the concentrations of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, singlet oxygen and the hydroxyl radical may have direct and indirect roles in the stress response, such as acting as an antibiotic agent and controlling gene expression (Kotchoni & Gachomo 2006). Many oxygenated VOCs important in atmospheric chemistry such as formaldehyde, acetone and acetaldehyde are major products of lipid peroxidation by ROS (Dennis & Shibamoto 1990; DeZwart *et al.* 1997; Enoiu *et al.* 2000; Orhan *et al.* 2006; Shibamoto 2006). Although extensively used as biomarkers for lipid oxidation in animals, including humans, their production in stressed plants via this mechanism has not previously been demonstrated. To our knowledge, this study provides the first experimental evidence indicating that acetaldehyde is produced via different metabolic pathways during wounding or anoxia. Similar δ^{13}C values between wound-induced acetaldehyde and membrane fatty acids are consistent with the previously described hypothesis that acetaldehyde is derived from fatty acid oxidation (Halliwell & Gutteridge 1999), and satisfies three constraints. The source of acetaldehyde is only active during stress events; it is depleted in ^{13}C relative to the bulk biomass, and constitutes a pool sufficiently large to sustain the observed emission rates. In model plant membrane systems, Barclay & McKersie (1994) found that increased concentrations of free linoleic acid and linolenic acid, as opposed to the corresponding membrane-associated phospholipids, increased aldehyde production during lipid peroxidation reactions with ROS. Since stress can induce the massive release of fatty acids from plant membranes as a part of the octadecanoid pathway (Farmer & Ryan 1992), the potential for peroxi-

Fig. 1. Summary of δ^{13}C values of acetaldehyde emitted from C3 leaves of the four tree species following leaf wounding, anoxia or decay. For comparison, also plotted are: average δ^{13}C values of acetaldehyde emitted from C3 leaf heating experiments and δ^{13}C values of C3 bulk biomass from Keppler *et al.* (2004).

dation reactions is large. The role of fatty acid peroxidation in producing acetaldehyde immediately following mechanical wounding is supported by many studies that found that a very rapid burst of oxidants accompanies stress. For example, within 30 s of stem cutting, leaves experienced a ROS burst that lasted for over 3 min (Dong & Xu 2006). In another study, the cutting of leaf blades induced an immediate ROS burst (Le Deunff et al. 2004). However, the wound-induced production of acetaldehyde depleted in ^{13}C could also be derived from other pathways such as the conversion of acetyl-CoA to acetaldehyde by aldehyde dehydrogenase (ALDH), as suggested by Graus et al. (2004). While consistent with the requirement of being depleted in ^{13}C relative to bulk biomass, it is not yet clear if this reaction occurs in higher plants.

In high concentrations, acetaldehyde can have dangerous effects on cellular processes by releasing zinc from zinc-containing enzymes (Hao & Maret 2006). Additional sinks of acetaldehyde are likely to exist in plants due to its high reactivity. For example, acetaldehyde readily forms adducts with proteins, phospholipids and DNA (Niemela et al. 1995; Shibamoto 2006). In addition, the presence of acetaldehyde itself in excessive amounts leads to the production of ROS and lipid peroxidation (Zhang et al. 1996; Novitskiy et al. 2006), which is a major cause of liver damage in alcoholic humans. Excessive damage due to the accumulation of ROS and toxic lipid peroxidation products such as aldehydes is prevented by ALDH enzymes. The expression of several ALDH enzymes is induced under stresses such as wounding, osmotic stress, dehydration, etc. (Kirch et al. 2004). These are known as 'turgor responsive' or 'stress' ALDH enzymes. For example, Kotchoni et al. (2006) showed that the Arabidopsis thaliana genes ALDH3I1and ALDH7B4 are transcriptionally activated by abiotic stress and protect plants against oxidative damage and lipid peroxidation during abiotic stress events. Both chloroplastic ALDH3I1 and cytoplasmic ALDH7B4 enzymes help protect against damage by reducing both ROS and aldehydes produced during lipid peroxidation reactions following plant stresses. The simultaneous occurrence of ROS, lipid peroxidation products such as aldehydes, and their detoxification by ALDH enzymes under stress supports the hypothesis presented here that lipid peroxidation leads to acetaldehyde production in stressed plants. In future experiments, we plan to test this hypothesis by determining if the wound-induced emission of ^{13}C depleted acetaldehyde is dependent on the presence of oxygen. While ethanolic fermentation will be stimulated under anoxia, the absence of ^{13}C depleted acetaldehyde following wounding would provide strong support for our hypothesis.

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