

Leaf and root pectin methylesterase activity and $^{13}\text{C}/^{12}\text{C}$ stable isotopic ratio measurements of methanol emissions give insight into methanol production in *Lycopersicon esculentum*

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Summary

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Key words: biogenic volatile organic compounds (BVOC), *Lycopersicon esculentum* (tomato), methanol, pectin methylesterase (PME), stable carbon isotopes.

- Plant production of methanol (MeOH) is a poorly understood aspect of metabolism, and understanding MeOH production in plants is crucial for modeling MeOH emissions. Here, we have examined the source of MeOH emissions from mature and immature leaves and whether pectin methylesterase (PME) activity is a good predictor of MeOH emission. We also investigated the significance of below-ground MeOH production for mature leaf emissions.
- We present measurements of MeOH emission, PME activity, and MeOH concentration in mature and immature tissues of tomato (*Lycopersicon esculentum*). We also present stable carbon isotopic signatures of MeOH emission and the pectin methoxyl pool.
- Our results suggest that below-ground MeOH production was not the dominant contributor to daytime MeOH emissions from mature and immature leaves. Stable carbon isotopic signatures of mature and immature leaf MeOH were similar, suggesting that they were derived from the same pathway. Foliar PME activity was related to MeOH flux, but unexplained variance suggested PME activity could not predict emissions.
- The data show that MeOH production and emission are complex and cannot be predicted using PME activity alone. We hypothesize that substrate limitation of MeOH synthesis and MeOH catabolism may be important regulators of MeOH emission.

Introduction

Emission of methanol (MeOH) from plants is ubiquitous and plays major roles in atmospheric chemistry. MeOH is the second most abundant organic gas after methane. Annual global budgets of phytogenic MeOH emissions are estimated to be anywhere from 75 to 280 Tg yr⁻¹ (Tg = teragrams = 10¹² g), while anthropogenic emissions resulting from industrial processes range from only 4 to 8 Tg yr⁻¹ (Singh *et al.*, 2000; Galbally & Kirstine, 2002; Heikes *et al.*, 2002; von

Kuhlmann *et al.*, 2003a,b; Tie *et al.*, 2003; Jacob *et al.*, 2005). MeOH has an atmospheric lifetime of *c.* 10 d (Jacob *et al.*, 2005). This long lifetime allows MeOH to move into the upper troposphere where it can substantially lower hydroxyl radical concentrations as background concentrations of other volatile organic compounds (VOCs) in the upper troposphere are low (Singh *et al.*, 2000, 2001; Tie *et al.*, 2003).

Studies have consistently shown that young expanding leaves emit greater amounts of MeOH than mature leaves

(Macdonald & Fall, 1993; Hüve *et al.*, 2007). Although mature leaf MeOH emissions are significantly lower than immature leaves (on average three to four times lower across species), they are still substantial and should not be ignored in modeling efforts (Macdonald & Fall, 1993; Nemecek-Marshall *et al.*, 1995; Harley *et al.*, 2007). Mature leaf MeOH emission can be just as significant as immature leaf emission on an annual scale. For example, after accounting for changes in leaf area index and length of time spent at each ontogenetic stage, mature and immature leaves of deciduous trees contribute approximately equal amounts to annual MeOH flux. Therefore, mature leaf MeOH emission has significant implications for atmospheric chemistry and deserves attention.

Currently, the dominant biosynthetic pathway for MeOH production in mature leaves is unknown. MeOH production in immature leaves, on the other hand, is believed to be derived from the demethylation of pectin by the enzyme pectin methyltransferase (PME) (Fall & Benson, 1996; Frenkel *et al.*, 1998; Galbally & Kirstine, 2002; Keppler *et al.*, 2004). Demethylation of pectin by PME facilitates crosslinking of pectin polymer chains and stabilizes the cell wall during expansion. As a by-product of cell growth, cumulative daily MeOH flux is known to strongly correlate with leaf expansion (Hüve *et al.*, 2007). The PME pathway has also been directly linked to MeOH production and emission in two studies in which silencing PME genes led to significantly decreased MeOH production in tomato fruit (Frenkel *et al.*, 1998) and significantly decreased MeOH emission response to herbivory (Korner *et al.*, 2009). MeOH production resulting from the demethylation of DNA and protein repair pathways is believed to be small because of low activity rates; for example, PME activity rates are at least six orders of magnitude higher than protein repair enzyme activity rates (Fall & Benson, 1996). Although it is generally accepted that PME activity is the source of MeOH emissions from immature leaves, the relationship between PME activity and MeOH emissions has yet to be described.

In addition, the role of PME activity in MeOH emissions from mature leaves is unknown. As mature leaves are fully expanded, foliar PME activity is expected to be low. Mature cell walls are known to have lower degrees of methyl esterification than immature cell walls and therefore have lower potential for MeOH production via the PME pathway. Alternatively, production of MeOH in other areas of the plant may be supplying mature leaves with MeOH via the transpiration stream. Previous work has suggested that MeOH emissions from mature leaves are derived from below-ground MeOH production (Folkers *et al.*, 2008). Experimentation with the cooling of roots, thereby decreasing metabolic activity in root tissue, indicated that some MeOH emitted from mature deciduous tree leaves is derived from MeOH production below ground (Folkers

et al., 2008). The spatial heterogeneity of MeOH production in plants may therefore significantly influence MeOH emissions from mature leaves and deserves further investigation.

While the instantaneous flux of MeOH from leaves is a function of leaf MeOH concentration and stomatal conductance (Niinemets & Reichstein, 2003a,b), an outstanding challenge for the field of biogenic VOC emission studies is to develop models that can predict the leaf concentration of individual VOCs (Lerdau, 1991). Such models for MeOH concentration do not yet exist. In order to help develop mechanistic MeOH emission models and address the uncertainty surrounding the role of PME activity in MeOH production, we investigated three main questions. Are MeOH emissions from mature and immature leaves derived from the same biosynthetic pathway? Is PME activity a good predictor of MeOH emissions? Do below-ground sources significantly contribute to MeOH emissions from mature leaves? We addressed the three research questions using stable carbon isotope analysis, PME activity assays, MeOH flux measurements, and MeOH extractions from mature and immature *Lycopersicon esculentum*.

Materials and Methods

Study species

All *Lycopersicon esculentum* L. individuals were Micro Tom clones, a dwarf variety of tomato (Meissner *et al.*, 1997). *L. esculentum* was chosen as a model plant because of its rapid growth and high MeOH emission behavior. MeOH emissions from mature leaves of *L. esculentum*, *Fagus sylvatica*, and *Quercus robur* are on average 3.6, 0.77, 0.33 nmol m⁻² s⁻¹, respectively (Folkers *et al.*, 2008). Plants were grown in the glasshouse at the University of Virginia in Charlottesville (38°N, 78°W). Pots were placed in flats filled with 1 inch of water and illuminated during a 16 h period with natural light supplemented with high-pressure sodium lamps. Plants were fertilized every 2 wk (Scotts 20% N, 20% P, 20% K; Scotts Miracle-Gro Company, Marysville, OH, USA) and kept insect-free using a variety of insecticides. Immature leaves were sampled 3 wk past germination and mature leaves 6 wk past germination. Leaf size was measured regularly with calipers to ensure that immature leaves were rapidly expanding and mature leaves were fully expanded.

Stable carbon isotope measurements

Gaseous MeOH released from immature ($n = 6$) and mature ($n = 5$) leaves of *L. esculentum* were measured by coupling a Li-Cor LI-6400 portable gas exchange system (Li-Cor, Inc., Lincoln, NE, USA) to a heavily modified gas chromatography isotope ratio mass spectrometer

(GC-IRMS; Agilent 6800 GC-Europa Scientific GEO 20-20 IRMS) capable of measuring $\delta^{13}\text{C}$ ratios of oxygenated/biological VOCs (O/BVOCs) in air samples (Giebel *et al.*, 2010). Measurement precisions for MeOH using this method were evaluated using a gravimetrically prepared gas-phase standard yielding a final mixing ratio of 18.6 ppbv ($1.86 \times 10^{-2} \mu\text{l l}^{-1}$) after dynamic dilution in zero and were $\pm 2.8\%$ with an associated error of 2.5% compared with the raw material used to make the calibrant gas. A detailed description of the GC-IRMS system and method is available (Giebel *et al.*, 2010); a brief review and additional details, however, are provided here.

The LI-6400 enabled leaf-level gas measurements to be standardized for multiple photosynthetic variables by controlling light intensities (peak irradiance of 665 and 470 nm), temperature, and relative humidity within the cuvette. Keeping light intensities constant (photosynthetic photon flux density (PPFD) of $950 \mu\text{mol photon m}^{-2} \text{s}^{-1}$), leaf-level measurements were only taken under steady-state conditions which were, on average, as follows: leaf temperature, $26.1 \pm 0.9^\circ\text{C}$; stomatal conductance, $0.14 \pm 0.05 \text{ mol H}_2\text{O m}^{-2} \text{s}^{-1}$ (mature) and $0.20 \pm 0.06 \text{ mol H}_2\text{O m}^{-2} \text{s}^{-1}$ (immature); photosynthetic rates, $9.0 \pm 2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ (mature) and $12.6 \pm 3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ (immature); and relative humidity, $53.1 \pm 4\%$ (mature) and $56.0 \pm 5\%$ (immature) (means \pm SD). Isotopic measurements were taken at temperatures similar to those previously reported for phytogenic MeOH (Keppler *et al.*, 2004; Yamada *et al.*, 2009). Steady-state conditions were important because MeOH emissions are tightly regulated by stomatal conductance as a result of the high solubility of MeOH (Niinemets & Reichstein, 2003a).

Air was supplied to the LI-6400 at a rate of 1.0 l min^{-1} by a zero-air generator. The zero-air generator contained a catalytic converter which removed all hydrocarbons, including MeOH, from the air stream; however, carbon dioxide and water were unaffected. Individual leaves, with an area between 4 and 6 cm^2 , were placed in the cuvette of the LI-6400 and allowed to reach steady state over a period of 10–20 min before sampling. Outflow from the cuvette, with a leaf in place, was between 100 and $300 \text{ cm}^3 \text{ min}^{-1}$ and connected directly to a custom-made preconcentration system located on the GC inlet. Approximately 1.0 l volumes were sampled directly from the cuvette outflow to the preconcentration system and controlled at a rate of $50 \text{ cm}^3 \text{ min}^{-1}$. After sampling, the adsorbent trap was purged and subsequently back-flushed with helium carrier gas while the trap was resistively heated. Volatized MeOH was cryofocused in liquid nitrogen before being injected into the GC. Separated components in the eluant gas passed through a heated combustion column and were transferred to the open split and ion source of the IRMS (Giebel *et al.*, 2010).

For carbon, the stable isotopic composition of a sample is expressed as a ratio (R) of $^{13}\text{C} : ^{12}\text{C}$ and reported in delta

(δ) notation as a per-mil (‰) difference of the sample compared with a working reference gas calibrated to the international standard Vienna PeeDee Belemnite (V-PDB). MeOH derived CO_2 , and that used for reference (0.1% CO_2 , 41.9‰), was delivered through the open split to the ion source of the IRMS. Six working reference gas injections were made during each chromatographic run and compared with the methanol peak to determine the $\delta^{13}\text{C}$ of methanol.

The stable carbon isotopic signature of pectin methoxyl groups was calculated from the isotopic signatures of untreated and demethylated apple pectin (Apple pectin, *c.* 70% methylated; Sigma-Aldrich). Alkaline hydrolysis (1 N NaOH) of pectin at 70°C generated demethylated pectin as in Rosenbohm *et al.* (2003). Carbon isotope ratios were determined in both pectin and demethylated pectin in an Isoprime IRMS (Elementar, Hanau, Germany) connected to a Eurovector elemental analyzer (Milan, Italy). The stable carbon isotopic signature of the methoxyl groups (representing 10% of all carbon in pectin) was calculated from the isotopic signature of untreated pectin (a reflection of 100% of all carbon in pectin, including both glucose and methoxyl groups) and the isotopic signature of demethylated pectin (a reflection of 90% of all carbon in pectin).

Gas exchange and MeOH emission measurements

Leaf-level MeOH emissions were quantified with a Li-Cor LI-6400 portable gas exchange system coupled with a proton-transfer-reaction mass spectrometer (high sensitivity PTR-MS; Ionicon Analytik, Innsbruck, Austria). PTR-MS has been described in detail elsewhere (Lindinger *et al.*, 1998). PTR-MS requires no preconcentration or chromatography of VOCs. Instead, the air flows directly to the drift tube where VOCs undergo chemical ionization via proton-transfer reaction with H_3O^+ . Protonated VOCs are then counted by the ion detector and can be measured down to the parts-per-trillion (ppt) level. Air exiting the LI-6400 cuvette was routed to the PTR-MS inlet via 1/4 inch Teflon tubing with a T-fitting in order to release extra flow. Flow rates through the cuvette ranged from 150 to $350 \mu\text{mol s}^{-1}$. Despite typically stable concentrations of MeOH in ambient air throughout the sampling periods, empty cuvette measurements were coupled with each leaf measurement in order to control for fluctuations in background MeOH. All measurements were taken between 10:00 and 16:00 h. PTR-MS measurements were recorded for 20 cycles for a total sampling time of *c.* 3 min. All measurements were taken under steady-state conditions at a PPFD of $750 \mu\text{mol m}^{-2} \text{s}^{-1}$, a leaf temperature of $31 \pm 1.9^\circ\text{C}$, stomatal conductances of $0.09 \pm 0.04 \text{ mol H}_2\text{O m}^{-2} \text{s}^{-1}$ (mature) and $0.15 \pm 0.06 \text{ mol H}_2\text{O m}^{-2} \text{s}^{-1}$ (immature), photosynthetic rates of $7.0 \pm 2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ (mature) and $10.5 \pm 2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ (immature), and relative humidities of $55.6 \pm 3\%$ (mature) and $58.2 \pm 4\%$ (immature)

(means \pm SD). The leaf surface area enclosed in the cuvette was measured using a Li-Cor Leaf Area Meter. The portion of leaf enclosed in the cuvette was weighed directly after being removed from the plant. MeOH emission rates are expressed on a per unit FW basis (nmol g^{-1} FW s^{-1}). Four point calibrations were made regularly throughout the sampling period with dilutions of a gravimetrically prepared MeOH gas standard provided by the Riemer laboratory (University of Miami) containing 3 ppmv ($3 \mu\text{l l}^{-1}$) \pm 2% MeOH in nitrogen gas. The accuracy of MeOH measurements was estimated to be *c.* 20% (based on the accuracy of calibration measurements) and reproducibility of *c.* 10%. MeOH emission measurements were made on 10 immature and 10 mature *L. esculentum* leaves.

PME enzyme activity rates

Directly following MeOH emission measurement, sampled leaves were excised and frozen in liquid nitrogen. A portion of the sampled plant's root mass was rinsed and also frozen in liquid nitrogen. Frozen samples were assayed for PME enzyme activity via a titration technique previously developed for *L. esculentum* (Anthon & Barrett, 2006). Plant tissue was ground in a mortar and pestle to a fine powder, weighed, and mixed in equal weight with a solution composed of 50% 2 M NaCl and 50% 10 mM phosphate buffer (pH 7.5). Samples were then centrifuged at 8000 *g* for 5 min. A 25 μl quantity of plant supernatant was added to 2.5 ml of pectin solution containing 0.5% pectin, 0.2 M NaCl, 0.1 mM phosphate buffer (pH 7.5). The sample solution pH was adjusted to 7.5 using small amounts of 0.1 M NaOH (in 1–5 μl). Once the solution dropped back down to pH 7, 1–5 μl 0.1 M NaOH was added until the solution pH reached 7.3. The time for the solution to drop back down to pH 7 was recorded. The demethylation of pectin by PME acidifies the solution. PME activity is therefore expressed in nmol g^{-1} FW s^{-1} based on the change in pH for a given amount of fresh tissue over time. Measuring change in pH over time is a proxy for PME activity and not a direct measurement of enzyme activity, but this change in pH has been shown to be a highly repeatable proxy for enzyme activity (Anthon & Barrett, 2006). A total of 10 immature and 10 mature *L. esculentum* were assayed for PME enzyme activity.

MeOH extractions

MeOH extraction was conducted on stem and leaf *L. esculentum* tissue. Whole plants were frozen in a liquid nitrogen bath before removal of the midstem and an adjacent mature leaf. Tissues were weighed and ground in 5 \times equal weight EDTA with a mortar and pestle (Leegood, 1993; Nemecek-Marshall *et al.*, 1995). Samples were then centrifuged at 3000 *g* for 4 min before removing the top

layer and neutralizing with NaOH. Samples were then injected into a gas chromatograph coupled with a flame ionization detector (GC-FID). A three-point calibration was made with dilutions of pure MeOH in deionized water. An additional calibration curve was made with aliquots of pure MeOH added to plant extract, which produced a standard equation similar to the DI water calibration curve. MeOH concentration was measured with an uncertainty of 4%. Tissues from 12 immature and nine mature plants were measured for MeOH concentration.

Statistical analysis

Differences between mature and immature mean MeOH $\delta^{13}\text{C}$ values were examined with a *t*-test (Proc TTEST, SAS 9.1; SAS Institute Inc., Cary, NC, USA). Linear regression was used to assess how well the independent variables leaf enzyme activity, root enzyme activity, and leaf type (mature and immature) predict MeOH emissions (Proc GLM, SAS 9.1; SAS Institute Inc.). Differences between mature and immature PME activities in root and leaf tissue were examined with *t*-tests. Differences between mature and immature MeOH emission rates were also examined with a *t*-test. Data used in regression analyses and *t*-tests were log-transformed to meet normality and homogeneity of variance assumptions. Nonparametric regression was used to determine whether or not MeOH concentration in stem tissue was a good predictor of MeOH concentration in leaf tissue (Proc GAM, SAS 9.1; SAS Institute Inc.). A Wilcoxon two-sample exact test was used to compare MeOH concentrations measured in leaf tissue between leaf types (Proc NPAR1WAY, SAS 9.1; SAS Institute Inc.). Three outlier points were detected according to Cook's D influence statistic and were removed from the analysis.

Results

Stable carbon isotope analysis of MeOH emissions was used to test the hypothesis that the stable carbon isotopic signatures of MeOH from mature and immature leaves are similar. The measured $\delta^{13}\text{C}$ of MeOH emissions from mature and immature *L. esculentum* leaves were not significantly different ($t = -1.08$, $\text{df} = 8$, $P = 0.31$); the values for mature and immature leaves were, on average, -19.0 and -21.5‰ , respectively (Fig. 1). We interpret this similarity to support the hypothesis that the dominant biosynthetic pathway for MeOH production in plants, PME activity, is conserved as leaves develop. Stable carbon isotope analysis of pectin and the pectin methoxyl pool was used to test the hypothesis that the isotopic signature of the pectin methoxyl pool is similar to the signature of MeOH emissions. We measured the $\delta^{13}\text{C}$ values of purified apple pectin and apple pectin methoxyl groups. The $\delta^{13}\text{C}$ of purified apple pectin (-26.2‰) was enriched in ^{13}C relative to the

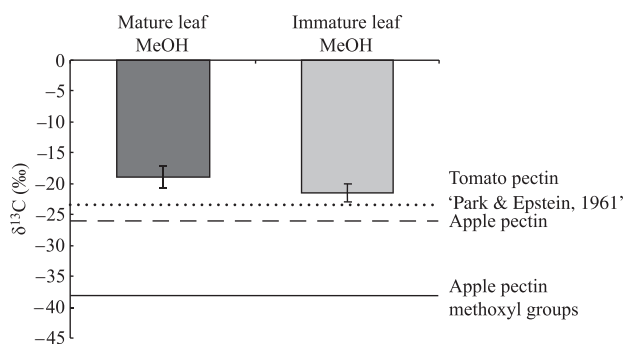


Fig. 1 Average $\delta^{13}\text{C}$ values for methanol (MeOH) emissions from mature ($n = 5$) and immature ($n = 6$) *Lycopersicon esculentum*, tomato pectin measured by Park & Epstein (1961), apple pectin, and pectin methoxyl groups. Values are means \pm SE. No significant differences were found between the two leaf types ($t = -1.08$, $df = 8$, $P = 0.31$).

pectin methoxyl groups (-38‰ ; Fig. 1). The depletion of the ^{13}C pectin methoxyl pool is biosynthetically reasonable because the methyl donor to pectin is *S*-adenosyl-methionine (SAM), which has a $\delta^{13}\text{C}$ of -39.2‰ (as measured in caffeine by Weilacher *et al.*, 1996). The apple pectin methoxyl pool was isotopically distinct from apple pectin, previously measured tomato pectin (Park & Epstein, 1961), and MeOH emissions from tomato (Fig. 1). We interpret the difference in isotopic signature between the pectin methoxyl pool and MeOH emissions as evidence that an enrichment process (e.g. MeOH catabolism) may occur during the production and emission of MeOH in plants.

Enzyme activity is known to be a good predictor for mechanistic VOC emission models (Fall & Wildermuth, 1998; Logan *et al.*, 2000). Flux measurements were taken in conjunction with enzyme activity rate measurements in leaves to test if PME activity in leaves and roots were good predictors of MeOH emission. Foliar PME activity was significantly related to MeOH emission across both leaf types ($F = 6.24$, $P = 0.022$; Fig. 2a), but only explained a small amount of the variance in MeOH emission ($R^2 = 0.26$). Additionally, no significant relationship between PME activity and MeOH emission was detected within leaf type ($F = 1.66$, $P = 0.22$; Fig. 2a). We interpret these results as evidence that, although foliar PME activity was related to MeOH emission, other factors must also be considered when predicting MeOH emission. Root PME activity did not correlate with MeOH emission across leaf types ($F = 0.52$, $P = 0.48$) or within leaf type ($F = 0.25$, $P = 0.63$), indicating that below-ground PME activity was not related to foliar MeOH emission (Fig. 2b). These data were graphed on log scale plots as they were log-transformed for statistical analysis (Fig. 2a,b).

Mature leaf PME activity was higher than expected based on our knowledge of PME activity in fully expanded leaves (average \pm SE PME activity rates were 6.4 ± 1.7 and 11.9 ± 2.1 $\text{nmol g}^{-1} \text{FW s}^{-1}$ for mature and immature

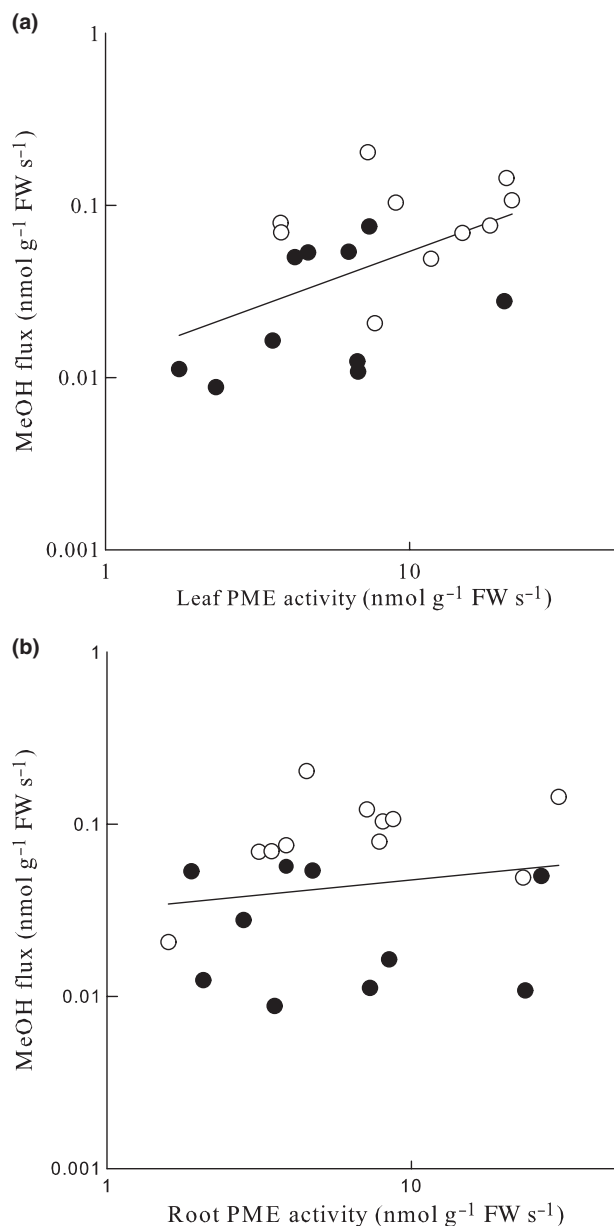


Fig. 2 The relationship between leaf pectin methylesterase (PME) activity and leaf methanol (MeOH) flux (regression; $F = 6.24$, $P = 0.022$ across leaf type; $F = 1.66$, $P = 0.22$ within leaf type) (a) and root PME activity and leaf MeOH flux (regression; $F = 0.52$, $P = 0.48$ across leaf type; $F = 0.25$, $P = 0.63$ within leaf type) (b) in mature ($n = 10$) and immature ($n = 10$) *Lycopersicon esculentum*. (a, b) Closed circles, mature; open circles, immature. Lines are fitted to all data. Data are shown on a log–log scale.

leaves, respectively; Fig. 3). Based on mean PME activity rates measured in mature and immature leaves, average mature leaf MeOH flux was lower than expected. Mature leaf PME activity was *c.* 50% of immature leaf PME activity, while MeOH flux from mature leaves was *c.* 33% of MeOH flux from immature leaves (average \pm SE MeOH flux rates were 0.03 ± 0.01 and 0.09 ± 0.02 $\text{nmol g}^{-1} \text{FW s}^{-1}$ for

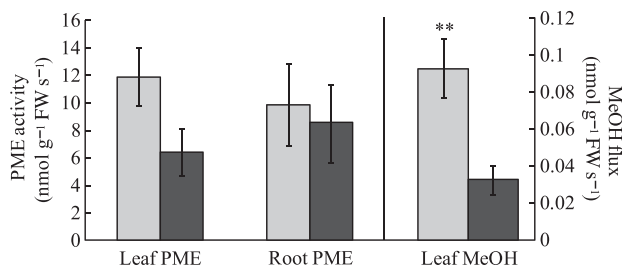


Fig. 3 Leaf pectin methylesterase (PME) activity, root PME activity and leaf methanol (MeOH) flux for mature (black bars; $n = 10$) and immature (gray bars; $n = 10$) *Lycopersicon esculentum*. Values are means \pm SE. Immature leaves tended to have higher PME activity (t -test; $t = 2.03$, $df = 18$, $P = 0.057$) and higher MeOH flux (t -test; $t = 3.35$, $df = 18$, $P = 0.0036$) than mature leaves. **, significant difference at $P < 0.01$. No significant difference was detected between PME activity in immature and mature root tissues (t -test; $df = 18$, $t = 0.32$, $P = 0.76$).

mature and immature leaves, respectively; Fig. 3). We interpret relatively high PME activity and low MeOH emission from mature leaves as possibly indicative of a MeOH sink. Mature and immature leaves did not have significantly different concentrations of MeOH ($P = 0.28$, Wilcoxon exact; average \pm SE MeOH concentrations were 0.74 ± 0.17 and 0.93 ± 0.20 mg g^{-1} FW for mature and immature leaves, respectively), indicating that although immature leaf MeOH emission was high, immature leaf MeOH concentration was not. We interpret relatively high MeOH emissions without high MeOH concentrations in immature leaves as also congruent with a MeOH sink.

In order to assess whether the transpiration stream was the dominant contributor of MeOH to leaves, we tested whether or not MeOH concentrations in stems could predict concentrations in leaves. In contrast to our hypothesis, concentrations of MeOH in stems were not good predictors

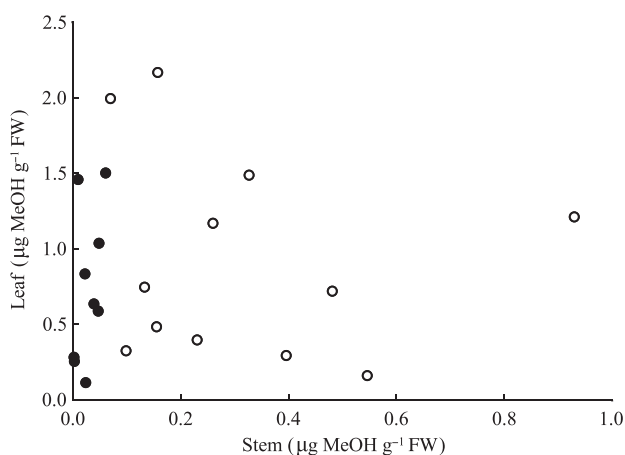


Fig. 4 The relationship between methanol (MeOH) concentrations in stem and leaf tissue from mature (closed circles; $n = 9$) and immature (open circles; $n = 12$) *Lycopersicon esculentum* (nonparametric regression; across leaf type, $\chi^2 = 3.28$, $P = 0.35$; mature only, $\chi^2 = 6.51$, $P = 0.10$; immature only, $\chi^2 = 2.20$, $P = 0.53$).

for concentrations of MeOH in leaves ($\chi^2 = 3.28$, $P = 0.35$, across leaf types; $\chi^2 = 6.51$, $P = 0.10$, mature only; $\chi^2 = 2.20$, $P = 0.53$, immature only; Fig. 4). We interpret this result as evidence that MeOH transported in the transpiration stream was likely not the dominant source of MeOH to leaves.

Discussion

Although it is believed that MeOH emission from immature leaves is derived from the PME pathway, the relationship between PME activity and immature leaf MeOH emission has not previously been described. Furthermore, the role of PME activity in MeOH production in mature leaf tissue has remained unstudied. Because of the fully expanded nature of mature leaves and a previous study indicating that some mature leaf MeOH is derived from below-ground MeOH production (Folkers *et al.*, 2008), we predicted that mature leaf MeOH would be mainly derived from PME activity in root tissue. We also hypothesized that if the dominant source of MeOH in mature leaves is below-ground production, the concentrations of MeOH in stems would predict concentrations in leaves. In contrast to our hypothesis, root PME activity was not related to MeOH flux, and MeOH extractions from mature stem and leaf tissue showed that MeOH in the transpiration stream could not predict MeOH in leaf tissue. These results provide strong evidence that below-ground MeOH production through the PME pathway was unlikely to be the dominant source of MeOH to *L. esculentum* leaves. We are not, however, aware of a mechanism for the partitioning of MeOH out of the transpiration stream and subsequent storage of MeOH in tissue. We cannot, therefore, exclude the possibility that leaves extracted MeOH from the transpiration stream. Additionally, it is possible to have significant transport of MeOH from roots to leaves without significant correlation between MeOH concentrations in stems and leaves. This could occur, for example, if flow rates of MeOH in stems and leaves were not constant. However, we assumed constant flow rates of MeOH in *L. esculentum* as the plants were grown and maintained in well-watered and stable environmental conditions. It may be that other species have high rates of MeOH transport from roots to leaves and that this root-derived MeOH is significant in these taxa. It is also important to note that our experiments did not explore the significance of night-time root growth for early morning MeOH emissions. Night-time root growth could lead to the accumulation of MeOH in the transpiration stream, contributing to high MeOH emission rates that have been observed during stomatal opening (Harley *et al.*, 2007). Modeling studies considering morning MeOH emission bursts should take this into consideration. A full understanding of the role of roots as MeOH suppliers to leaves would require a separate study.

Pectin methylesterase enzyme assays revealed that mature leaves of *L. esculentum* maintained surprisingly high foliar PME activity despite their fully expanded nature. This high activity may be the result of PME's involvement in plant development (both cell wall expansion and cell wall turnover) and stress response (i.e. in response to cold temperature, ethylene, wounding and herbivory, and wound-signaling compounds such as oligogalacturonides) (Pelloux *et al.*, 2007). Plant PMEs are known to belong to large multigene families yielding numerous isoforms of PME (e.g. 66 PME protein-encoding regions of DNA have been identified in Arabidopsis), which may be differentially regulated as leaves mature (Bordenave & Goldberg, 1994; Willats *et al.*, 2001; Pelloux *et al.*, 2007). A previous study found PME activity to be higher in mature cells of mung bean hypocotyl tissue compared with immature cells with relative abundances of PME isoforms differing between cell types (Bordenave & Goldberg, 1994). Therefore, certain isoforms of PME involved in cell wall turnover and/or environmental stress response may be more active in mature leaf tissue and may thus explain the surprisingly high PME activity rates measured in mature *L. esculentum* leaves.

Our results indicate that PME activity alone could not predict MeOH emissions from *L. esculentum*, and we hypothesize that other mechanisms, such as PME substrate limitation and MeOH catabolism, may contribute to MeOH emission regulation. Although foliar PME activity was significantly related to MeOH emission across leaf types, foliar PME activity was not a good predictor of MeOH emission ($R^2 = 0.26$). As PME activity was not a good predictor of MeOH emission, investigation of additional predictor variables should be pursued. We hypothesize that PME substrate availability may be a good predictor of MeOH production. Cells are known to export galacturonic acid (GA) to the cell wall with differing degrees of methylesterification (Goldberg *et al.*, 1996), thereby limiting available substrate for MeOH production through the PME pathway. Previous studies have demonstrated that under saturating conditions, isoprene synthase activity can explain isoprene emissions (Logan *et al.*, 2000), but when conditions are no longer saturating, isoprene synthase activity can only account for some of the variation in isoprene emissions. Similarly, for MeOH production, PME activity may only be a strong predictor under saturating conditions. PME substrate availability is therefore an important area for future investigation.

In addition to PME substrate limitation, we suspect that MeOH catabolism may contribute to MeOH emission regulation. Although a pathway for MeOH catabolism in plants is known (Cossins, 1964; Gout *et al.*, 2000), the significance of that pathway for MeOH emission has not been investigated. Our results suggest that a MeOH sink, such as MeOH catabolism, may influence MeOH storage and emission. While PME activity rates in mature leaves

were only slightly lower than in immature leaves ($P = 0.057$), MeOH emissions from mature leaves were significantly lower than immature leaf emissions ($P = 0.0036$; Fig. 3). We hypothesize that MeOH within the leaf that is not emitted may be catabolized or exported for catabolism in other tissues. MeOH in leaves is known to be oxidized to CO₂ or incorporated into amino acids (Cossins, 1964; Gout *et al.*, 2000). Our isotopic data are congruent with this suggestion. While we hypothesize the presence of a MeOH sink, there are several additional processes that may also explain low MeOH emissions from mature leaves despite relatively high PME activity. PME may be substrate-limited in mature leaves, resulting in overall lower MeOH production rates. In addition, the physical resistances between mature and immature leaves may differ. Leaf architecture, however, plays a minor role in dictating emission rates for compounds such as MeOH (Niinemets & Reichstein, 2003a). Therefore, we would not expect mesophyll resistance to play a significant role in inhibiting fluxes from mature leaves. Slightly lower stomatal conductance in mature leaves may inhibit emission and lead to accumulation of MeOH within the leaf. Differences in stomatal conductance, however, were not sufficient to explain differences in flux between mature and immature leaves (stomatal conductance in mature leaves was, on average, 60% of immature leaf stomatal conductance, while MeOH flux from mature leaves was, on average, 30% of immature leaf MeOH flux). Another result that suggested the presence of a MeOH sink concerned MeOH extractions from leaf tissue showing that mature and immature leaves had surprisingly similar concentrations of MeOH. MeOH catabolism could lower the concentrations of MeOH in immature leaves and explain why immature leaves had similar concentrations as mature leaves despite being in a rapidly expanding growth stage. Unfortunately, very little is known concerning rates of MeOH catabolism, and therefore source–sink MeOH dynamics within plants requires further investigation. Our results raise the issue of MeOH catabolism as a significant sink for MeOH in leaves which could influence the amount of MeOH that is stored and transported within plant systems and complicate our ability to use MeOH production alone as a proxy for MeOH emission.

The isotopic signatures of MeOH emitted from mature and immature *L. esculentum* leaves were similar, indicating that the dominant biosynthetic pathway for MeOH production, PME activity in cell walls, was most likely conserved as leaves developed. Enriched isotopic signatures of MeOH emissions relative to our measurements of a pectin methoxyl pool suggest that an enrichment process, such as MeOH catabolism, may have strongly influenced the isotopic signature of MeOH emissions. Although the fractionation factor associated with MeOH catabolism has not been measured, previous investigation has shown that MeOH is first oxidized to formaldehyde and formate,

which could entail a substantial kinetic isotope effect (Cossins, 1964; Gout *et al.*, 2000). Therefore, the observed difference in isotopic signature could be the result of preferential catabolism of lighter MeOH leading to isotopically heavier emissions, an effect that is amplified in mature leaves. The process of enrichment of carbon emissions is known to occur if the catabolic product being produced and retained within the plant is preferentially made from light carbon (Ghashghaie *et al.*, 2003). As there is no other known sink beyond MeOH production for the methyl groups released from pectin by PME in the cell wall, it is unlikely that alternate sinks for the methoxyl groups are influencing the isotopic signature of MeOH emissions. Given the possibility of small nonPME sources of MeOH (e.g. demethylation of DNA and protein repair pathways) and mass-dependent catabolism of MeOH, it is impossible to definitively constrain a complete source–sink model. Our data are congruent, though, with the argument that PME is most likely the dominant contributor to MeOH production in plants and that SAM is likely the dominant source to the pectin methoxyl pool. It is unlikely that variation in isotopic signature as a result of interspecific variation and environmental conditions can account for the large difference observed between our measurements of the apple pectin methoxyl pool and MeOH emissions from tomato leaves. Production of GA (the backbone of pectin) from glucose (Smirnoff, 1996) and methylation of GA by pectin methyltransferase (PMT) (Goldberg *et al.*, 1996) are conserved processes across plant species. Although numerous isotopic measurements of pectin have not been conducted, other compounds such as cellulose and starch have been measured multiple times with low variability in isotopic signature across species (up to 4–5‰ across 107 and 34 measurements for cellulose and starch, respectively) (Badeck *et al.*, 2005). We therefore do not expect the isotopic signature of pectin to vary greatly among C_3 plants. We also would not expect the isotopic signatures of pectin and the pectin methoxyl pool to significantly differ between mature and immature leaves as variations in carbon fractionation as a result of changes in leaf age are known to be relatively small (Leavitt & Long, 1985; Terwilliger, 1997). After determining that the $\delta^{13}C$ values of apple pectin and tomato pectin (Park & Epstein, 1961) were similar (Fig. 1), we are confident that apple pectin is an appropriate substrate for our investigation of the isotopic signature of the pectin methoxyl carbon pool. Future investigations will be required in order to understand how the isotopic signature of MeOH emissions changes in relation to the pectin methoxyl pool under conditions of low and high MeOH catabolic rates. This type of study is highly desired as plants are believed to be the main contributor to atmospheric MeOH and the isotopic signature of that source could be a useful tool in balancing the global MeOH budget (Quay *et al.*, 1999; Keppler *et al.*, 2005).

Our measured isotopic signature of MeOH differed greatly from previously measured isotopic signatures of MeOH emissions from plants (Keppler *et al.*, 2004; Yamada *et al.*, 2009). MeOH emissions from fresh plant tissue measured by Keppler *et al.* (2004) were $-68.2 \pm 11.2\%$ averaged across 11 species of C_3 plants. Similarly, Yamada *et al.* (2009) measured MeOH emissions to be, on average, $-74.6 \pm 1.2\%$ for *Ligustrum japonicum*. Although neither of these previous studies sampled *L. esculentum*, there is no reason to expect tomato to differ so significantly from the 12 C_3 species previously surveyed (see earlier discussion).

An explanation for the discrepancy between our measured isotopic values for MeOH and previously measured values could be differences in analytical methods. Both Keppler *et al.* (2004) and Yamada *et al.* (2009) sampled MeOH emissions by destructively sampling multiple leaves from a plant, sealing the leaves in a small vial for 12–18 h at room temperature, and measuring the MeOH accumulated in the vial via GC-IRMS. Sampling wounded plant tissue removed from the transpiration stream is very different from live plant emission sampling as plant metabolism is significantly altered during destructive sampling and broken tissues are exposed to oxygen and have suffered cell death. These conditions could lead to the production of MeOH derived from a variety of sources, such as from anaerobic metabolism. Methanotrophs and methylobacteria are ubiquitous in the phyllosphere and are known to produce and consume MeOH, respectively (Trotsenko *et al.*, 2001; Doronina *et al.*, 2004; Xin *et al.*, 2004). Large fractionation factors which discriminate against the heavier C isotope are associated with these processes and could have a significant impact on MeOH sampled from incubated plant material (Whiticar, 1999). Giebel *et al.* (2010) also employed an incubation technique when sampling MeOH emissions from *Citrus sinensis* and *Quercus geminata* and reported $\delta^{13}C$ values for MeOH that were nearly as depleted as those reported by Keppler *et al.* (2004). As the same analytical system was used for Giebel *et al.* (2010) and the data presented here, it appears that the gas sampling technique, not the isotopic analytical method, may be the determining factor for detecting a highly depleted isotopic signature for phytogenic MeOH. These results provide evidence that incubating plant material may alter the isotopic signature of MeOH. Our method was designed for the measurement of MeOH emission from live, intact leaves as we measured emissions from individual *L. esculentum* leaves under standardized conditions. We were therefore able to account for leaf-to-leaf variation, intraspecific variation via use of clones, and variation in stomatal conductance, which is known to regulate MeOH emissions and influence fractionation of gases during diffusion into and out of the leaf. Therefore, our results may reflect a different plant-derived MeOH than has been previously measured. Similarly, our measured isotopic ratio of the pectin methoxyl pool devi-

ated from the value reported by Keppler *et al.* (2004). Keppler *et al.* (2004) measured the MeOH released from incubated freeze-dried biomass by alkaline hydrolysis. We believe the range in isotopic signatures for the pectin methoxyl pools measured by Keppler *et al.* (2004) may be attributed to variation resulting from incubation methods. We measured the signature of purified pectin before and after alkaline hydrolysis and then calculated the signature of the pectin methoxyl pool. Therefore, differences in analytical methods could account for the differences in reported isotopic values for pectin methoxyl pools.

Our investigation of mature leaf MeOH emission has implications for atmospheric chemistry and basic plant biology. We show that below-ground sources of MeOH were not a dominant source of MeOH to foliar *L. esculentum* emissions during the day. Our work instead provides evidence that foliar PME activity is related to MeOH emissions. Isotopic signatures of mature and immature MeOH emissions were similar, suggesting that the dominant pathway for MeOH production in plants, PME activity, was likely conserved as leaves developed. No new pathway for MeOH production in plants therefore needs to be added to mechanistic models in order to predict phytogenic MeOH flux to the atmosphere. However, PME activity alone was not sufficient to predict MeOH emission, and other factors, such as PME substrate availability and MeOH catabolism, should be considered for mechanistic model development. In order to successfully model long-term MeOH emission dynamics, not only MeOH emission, but also MeOH production in plants, must be understood (Harley *et al.*, 2007). We suggest that in order to understand and model MeOH emissions, we need to learn more about the factors, in addition to PME activity, that regulate MeOH production and the factors that contribute to MeOH consumption within leaves. Additional studies will need to measure multiple physiological variables concurrently, including not only PME activity, MeOH concentration, MeOH flux, and stomatal conductance, but also PME substrate limitation and MeOH catabolism.

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