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Short term changes in methanol emission and pectin methylesterase activity are not directly affected by light in *Lycopersicon esculentum*

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Abstract. Plants are an important source of atmospheric methanol (MeOH), the second most abundant organic gas after methane. Factors regulating phytogenic MeOH production are not well constrained in current MeOH emission models. Previous studies have indicated that light may have a direct influence on MeOH production. As light is known to regulate cell wall expansion, it was predicted that light would stimulate MeOH production through the pectin methylesterase (PME) pathway. MeOH emissions normalized for stomatal conductance (g_s) did not, however, increase with light over short time scales (20–30 min). After experimentally controlling for g_s and temperature, no light activation of PME activity or MeOH emission was observed. The results clearly demonstrate that light does not directly influence short-term changes in MeOH production and emission. Our data suggest that substrate limitation may be important in regulating MeOH production over short time scales. Future investigation of the long-term impacts of light on MeOH production may increase understanding of MeOH emission dynamics at the seasonal time scale.

1 Introduction

Plants play a dominant role in the global production of methanol (MeOH), an important feature of atmospheric chemistry. Annual global emissions of MeOH range from 75–280 Tg (teragrams, 10^{12} g) yr^{-1}, with plants contributing approximately 75% of this flux, and the remainder coming from industrial processes, plant decay, biomass burning, and in situ atmospheric production (Singh et al., 2000; Galbally and Kirstine, 2002; Heikes et al., 2002; Tie et al., 2003; von Kuhlmann et al., 2003a, b; Jacob et al., 2005). In contrast to other volatile organic compounds (VOCs), such as isoprene, which have atmospheric lifetimes on the order of tens of minutes, the lifetime of MeOH is approximately ten days (Jacob et al., 2005). This long lifetime allows MeOH to move into the upper troposphere where it can substantially lower hydroxyl radical concentrations as well as increase concentrations of ozone and formaldehyde (Singh et al., 2000, 2001; Tie et al., 2003).

The dominant biosynthetic pathway for MeOH production in leaves is believed to be the demethylation of pectin by the enzyme pectin methylesterase (PME) (Nemecek-Marshall et al., 1995; Fall and Benson, 1996; Frenkel et al., 1998; Rose and Bennett, 1999; Galbally and Kirstine, 2002; Keppler et al., 2004). Rapidly growing cells export highly methylesterified chains of galacturonic acid (GA; the backbone of pectin) to cell walls (Goldberg et al., 1996). In an ester hydrolysis reaction, GA is demethylated by PME, allowing chains of GA to cross-link and stabilize the cell wall. The methyl groups cleaved from the GA chain form MeOH, which accumulates in the leaf and is released through stomatal openings. As a by-product of this growth-related process, cumulative daily MeOH flux is known to strongly correlate with leaf expansion (Hüve et al., 2007) and studies have consistently shown that young expanding leaves emit greater amounts of MeOH than mature leaves (Macdonald and Fall, 1993; Hüve et al., 2007). Although expanding leaves are associated with higher rates of MeOH production, it is important to note that PMEs are also active in mature tissue (Bordenave and Goldberg, 1994). Plant PMEs are known to belong to large multigene families yielding numerous isoforms of PME, each of which has activities that are regulated by pH (Bordenave and

Goldberg, 1994; Goldberg et al., 1996; Willats et al., 2001; Pelloux et al., 2007). The regulation of PME isoforms helps maintain a more flexible cell wall in expanding cells and a rigid cell wall in mature non-expanding cells. Therefore, both mature and immature leaves have active PMEs that produce MeOH at different rates.

Unlike other VOCs such as monoterpenes and isoprene that are directly linked to photosynthesis, less than 10% of MeOH is produced from recently assimilated carbon (Folkers et al., 2008). Although the majority of MeOH is not immediately derived from light-dependent photosynthesis, light may regulate MeOH production through the stimulation of leaf expansion. Light is known to lower the pH of growing cell walls, thereby increasing extensibility of the wall and allowing expansion (Van Volkenburgh, 1999). The process of light-induced apoplastic acidification and cellular growth occurs on the scale of seconds to minutes (Elzenga et al., 1997; Stahlberg and Van Volkenburgh, 1999). Light-induced acidification of the cell wall may influence the activity of certain isoforms of PME, thereby increasing production of MeOH. Additionally, the expansion of the cell wall may increase substrate availability for PME activity, again leading to increased MeOH production. To our knowledge, the direct response of PME activity and MeOH production to short-term changes in light has not been measured.

MeOH emissions are tightly regulated by stomatal conductance (g_s) (Macdonald and Fall, 1993; Nemecek-Marshall et al., 1995; Hüve et al., 2007). In order to account for stomatal effects on VOC emission, Niinemets and Reichstein (2003a, b) developed a model incorporating Henry's law constants (H, $Pa\,m^3\,mol^{-1}$), which describe a compound's tendency to partition between gas- and liquid-phases. The emission of compounds with large H values, such as isoprene, is not controlled by stomata as the compound will tend to partition and accumulate in the gas-phase, thereby overcoming stomatal closure. Compounds such as MeOH with low H values are highly soluble in water and are therefore under stomatal control (Niinemets and Reichstein, 2003a, b). Harley et al. (2007) applied the Niinemets-Reichstein model to predict MeOH emissions from several species, assuming temperature-regulated MeOH production while accounting for changes in g_s and gas- and liquid-phase MeOH pool sizes. Overestimations of nighttime MeOH emissions by the model suggested that light may directly influence MeOH production. The model's inability to account for changes in MeOH production due to leaf expansion and/or light conditions resulted in significant discrepancies between measurements and model predictions. Similarly, Folkers et al. (2008) could not differentiate the effects of increased MeOH production and increased g_s on MeOH emission responses to light. Our understanding of the effects of light on MeOH is therefore confounded by MeOH's dependence on g_s.

Light induction of VOC production and emission has been demonstrated for VOCs such as isoprene and monoterpenes. As isoprene and monoterpene biosynthesis are directly linked to photosynthesis, isoprene and non-stored monoterpene emissions are successfully modeled using light as a predictor variable (Loreto et al., 1996a, b; Ciccioli et al., 1997). Isoprene synthase activity is also correlated with light (Lehning et al., 1999). Similarly to previously studied VOCs, investigating the direct influence of light on MeOH production is an important step in understanding and modeling emission behavior.

In this study, we look at the effects of light on short term changes in MeOH production and emission in *Lycopersicon esculentum* under conditions where potential confounding factors such as temperature variation and changes in g_s are limited. In this manner, we can determine the direct relationship between light and MeOH production.

2 Materials and methods

2.1 Study species

All *Lycopersicon esculentum* Mill. individuals were Micro Tom clones, a dwarf variety of tomato (Meissner et al., 1997). *L. esculentum* was chosen as a model plant due to its rapid growth and high MeOH emission behavior. Plants were grown in the greenhouse at the University of Virginia in Charlottesville (38° N, 78° W). Pots were placed in flats filled with one inch of water and were illuminated during a 16 h period with natural light supplemented with high-pressure sodium lamps. Plants were fertilized every two weeks (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 three weeks past germination and mature leaves six weeks past germination. Leaf size was measured regularly with calipers to ensure that immature leaves were rapidly expanding and mature leaves were fully expanded.

2.2 Gas exchange measurements

Leaf-level gas exchange measurements were made with a LI-COR LI-6400 portable gas exchange system (LI-COR Inc., Lincoln, NE, USA). Temperature was regulated within the cuvette using thermoelectric (Peltier) coolers (LI-COR). Photosynthetic photon flux density (PPFD) within the cuvette was controlled with a set of red and blue light-emitting diodes (LI-COR). Depending on the treatment, leaves were exposed to five light levels ranging from low to high light conditions (50, 300, 650, 900, 1150 $\mu mol\,m^{-2}\,s^{-1}$). Leaf surface area enclosed in the cuvette was measured using a LI-COR Leaf Area Meter (LI-COR Inc., Lincoln, NE, USA). Photosynthetic rates (P_s) and stomatal conductance (g_s) are expressed on a per unit leaf area basis ($\mu mol\,CO_2\,m^{-2}\,s^{-1}$ and $mol\,H_2O\,m^{-2}\,s^{-1}$ for P_s and g_s, respectively). Relative

humidity and leaf temperature were also recorded during gas exchange measurements.

2.3   MeOH emission measurements

Leaf-level MeOH emissions were quantified with a LI-COR LI-6400 portable gas exchange system (LI-COR Inc., Lincoln, NE, USA) 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 VOC. 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 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 μmol 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 1000 and 1600 h. Leaves were allowed to stabilize at each light level for 20–30 min prior to taking the MeOH emission measurement. PTR-MS measurements were recorded for 20 cycles for each sample. MeOH emission rates are expressed on a per unit leaf area basis (nmol m^{-2} s^{-1}). MeOH emissions were divided by g_s in order to normalize emission rates for changes in g_s. Normalized MeOH emissions are expressed in nmol MeOH per mol of H_2O. Four point calibrations were made regularly throughout the sampling period with dilutions of a gravimetrically prepared MeOH gas standard provided by the Riemer lab (University of Miami) containing 3 ppmv (3 μl l^{-1}) $\pm$2% MeOH in nitrogen gas. Accuracy of MeOH measurements was estimated to be around 20% (based on accuracy of calibration measurements) and reproducibility of around 10%.

Three sets of MeOH emission measurements were conducted. All measurements were taken under steady-state conditions. First, repeated MeOH emission measurements were conducted on four immature and four mature *L. esculentum* in which each leaf was exposed to five light levels while keeping temperature constant and allowing g_s to change. Second, repeated MeOH emission measurements were conducted on five immature and five mature *L. esculentum* in which each leaf was exposed to five light levels while temperature and g_s were held constant. Using 12 g LI-COR CO_2 cartridges, g_s was kept relatively constant by changing CO_2 concentrations within the cuvette. The third set of MeOH emission measurements were made on leaves that were destructively sampled for enzyme assay analysis directly following the emission measurement (see Sect. 2.4). Emission measurements were collected at five light levels, where a different group of plants ($n = 5$) were sampled at

each level totaling 25 immature and 25 mature *L. esculentum*. Temperature and g_s were held constant.

2.4   PME enzyme activity assay

For the third set of MeOH emission measurements, sampled leaves were excised and frozen in liquid nitrogen directly following the emission measurement. Frozen samples were assayed for PME enzyme activity via a titration technique previously developed for *L. esculentum* (Anthon and 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. 25 μl of plant supernatant was added to 2.5 ml of pectin solution containing 0.5% pectin, 0.2 M NaCl, and 0.1 mM phosphate buffer (pH 7.5). 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 of 0.1 M NaOH was added until solution pH reached 7.3. Time for solution to drop back down to pH 7 was recorded. PME activity is expressed in μmol g fwt^{-1} min^{-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 and Barrett, 2006). A total of 25 immature and 25 mature plants were assayed for PME enzyme activity.

2.5   Statistical analysis

Repeated measurements collected in the first MeOH emission dataset, where leaves were exposed to five light levels under constant temperature, were analyzed using a mixed model ANOVA (Proc MIXED, SAS 9.1; SAS Institute Inc., Cary, NC, USA). Strength of association between MeOH emission and PPFD was determined by the significance of the slope of the mixed model. Normalized MeOH emission data in the first dataset exhibited a decreasing exponential relationship with PPFD and were examined with a generalized linear model with a negative binomial distribution (Proc GENMOD, SAS 9.1; SAS Institute Inc., Cary, NC, USA). The relationship between g_s and PPFD was analyzed using a repeated-measures ANOVA model (Proc GLM, SAS 9.1; SAS Institute Inc., Cary, NC, USA). Linear and non-linear regression lines were fit using SigmaPlot 9.0 (Systat software, Inc. Point Richmond, California, USA).

The second set of repeated MeOH emission measurements, where leaves were exposed to five light levels under constant temperature and g_s, was analyzed using a mixed model ANOVA with MeOH emission data normalized for g_s (Proc MIXED, SAS 9.1; SAS Institute Inc., Cary, NC, USA). Again, strength of association between normalized MeOH

emission and PPFD was determined by the significance of the slope of the mixed model.

In the third set of MeOH emission measurements, leaves were destructively sampled for PME activity assays under conditions of constant temperature and g_s. This third dataset was examined in a two-way MANOVA where the effect of plant type and PPFD on normalized MeOH emission and PME activity was analyzed (Proc GLM, SAS 9.1; SAS Institute Inc., Cary, NC, USA). Data used in the analysis were Log and square root transformed to meet normality and homogeneity of variance assumptions.

3   Results

In the first set of measurements, the relationship between g_s and MeOH emission was investigated under conditions where light varied and temperature was held constant. MeOH emission and g_s were measured at five light levels. Stomatal conductance ranged from 0.03–0.17 mol H_2O m^{-2} s^{-1} and 0.04–0.22 mol H_2O m^{-2} s^{-1} for mature and immature leaves, respectively. Measurements of mature and immature leaves were taken under steady-state conditions at leaf temperature $29 \pm 1\,°C$, photosynthetic rates $6.7 \pm 4\,\mu$mol CO_2 m^{-2} s^{-1} mature and $10 \pm 4\,\mu$mol CO_2 m^{-2} s^{-1} immature, and relative humidity $55 \pm 5\%$ mature and $58 \pm 6\%$ immature (means $\pm$ SD). MeOH emissions increased with light ($F = 16.69$ $df = 1$, 15 $P = 0.001$ for mature; $F = 110.59$ $df = 1$, 15 $P < 0.0001$ for immature; Fig. 1a). Slopes were positive and significant for both leaf types (slope $= 0.0015$ $t = 4.09$ $P = 0.001$ for mature; slope $= 0.0044$ $t = 10.52$ $P < 0.0001$ for immature). Light also had an overall significant effect on g_s for both leaf types (Fig. 1b; Wilk's lambda $F = 77.78$ $df = 4$, 3 $P = 0.002$). When MeOH flux values were normalized by g_s, the influence of light disappeared. The relationship between normalized MeOH and light was not positive (regression coefficient $= -0.0006$, $z = -5.53$, $P < 0.0001$ for mature leaves; regression coefficient $= -0.0008$, $z = -50.20$, $P < 0.0001$ for immature leaves; Fig. 1c). It is important to note that the calculated negative relationship between normalized MeOH and PPFD is almost certainly an artifact of the normalization of MeOH and not a biological phenomenon.

As an extension to the first set of measurements, g_s and temperature were experimentally controlled before making repeated MeOH flux measurements across five light levels. Measurements of mature and immature leaves were taken under steady state conditions at leaf temperature $29 \pm 1\,°C$, g_s 0.08 ± 0.02 mol H_2O m^{-2} s^{-1}, and relative humidity $58.3 \pm 7\%$ (means $\pm$ SD). Although changes in g_s were small across the 5 light levels, MeOH emissions were normalized for g_s in order to remove all influence of g_s on MeOH emission. Non-normalized emission data were also analyzed and found to behave similarly to the normalized emission data (data not shown). Under constant temperature and g_s no significant
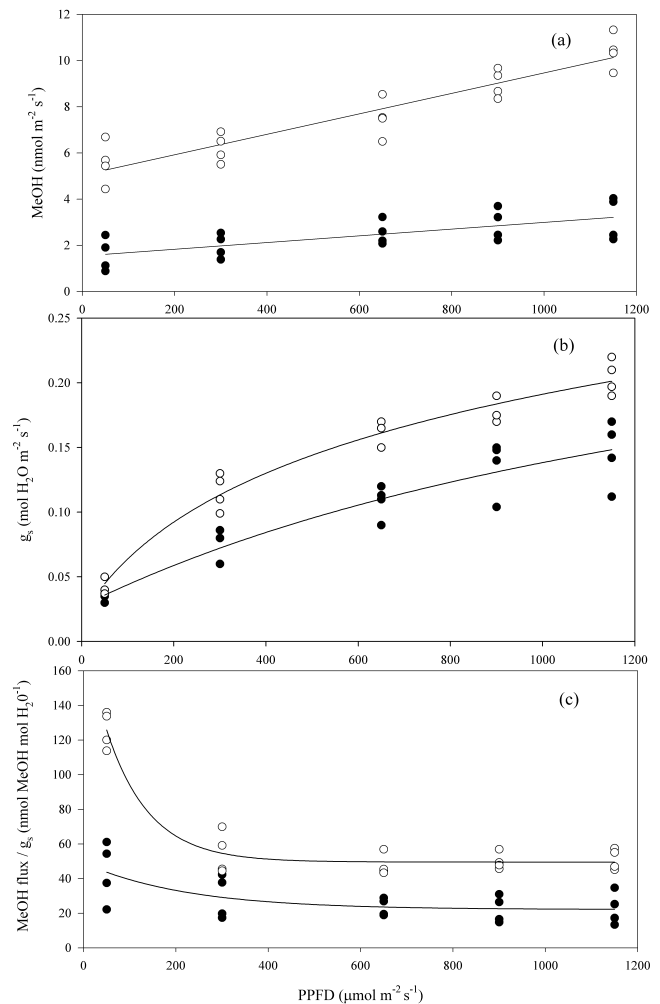

Fig. 1. (a) MeOH emission, **(b)** stomatal conductance (g_s), and **(c)** MeOH emission normalized for g_s from mature ($n = 4$; black circles) and immature ($n = 4$; open circles) *L. esculentum* over five light levels at constant temperature ($29 \pm 1\,°C$). MeOH emission (slope $= 0.0015$, $t = 4.09$, $P = 0.001$ for mature; slope $= 0.0044$, $t = 10.52$, $P < 0.0001$ for immature) and g_s ($F = 112.94$ $P < 0.0001$ $R^2 = 0.86$ for mature; $F = 19.06$ $P = 0.004$ $R^2 = 0.96$ for immature) significantly increased with PPFD. Normalized MeOH emissions were negatively associated with PPFD (regression coefficient $= -0.0006$, $z = -5.53$, $P < 0.0001$ for mature; regression coefficient $= -0.0008$, $z = -50.20$, $P < 0.0001$ for immature).

effect of light on normalized MeOH emissions for mature ($F = 2.96$ $df = 1$, 19 $P = 0.10$) or immature leaves ($F = 0.6$ $df = 1$, 19 $P = 0.45$) was observed (Fig. 2). The results agreed with those from the first set of measurements in that light did not directly stimulate MeOH emission.

In the third set of MeOH emission measurements, the direct influence of light on MeOH production was investigated by assaying PME activity at each light level. Measurements of mature and immature leaves were taken under steady state conditions at leaf temperature $29 \pm 1\,°C$, g_s
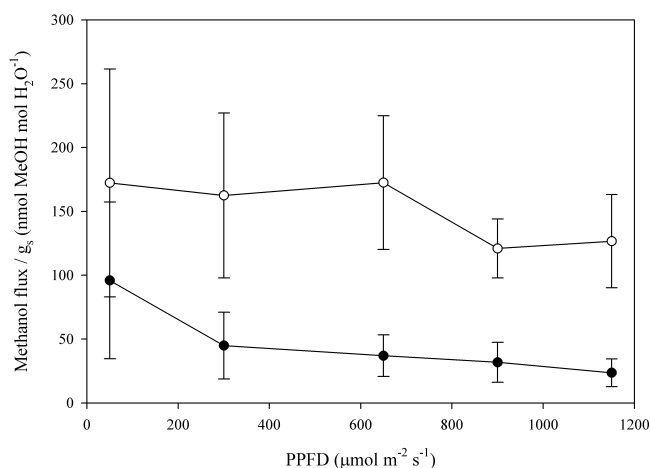
Fig. 2. Average MeOH flux values normalized for stomatal conductance (g_s) over five light levels for $n = 5$ mature (black circles) and immature (white circles) *L. esculentum* (each plant was measured 5x; standard error bars shown). PPFD had no significant effect on normalized MeOH flux for either leaf type ($F = 2.96$; $df = 1, 19$; $P = 0.10$ for mature; $F = 0.6$; $df = 1, 19$; $P = 0.45$ for immature).
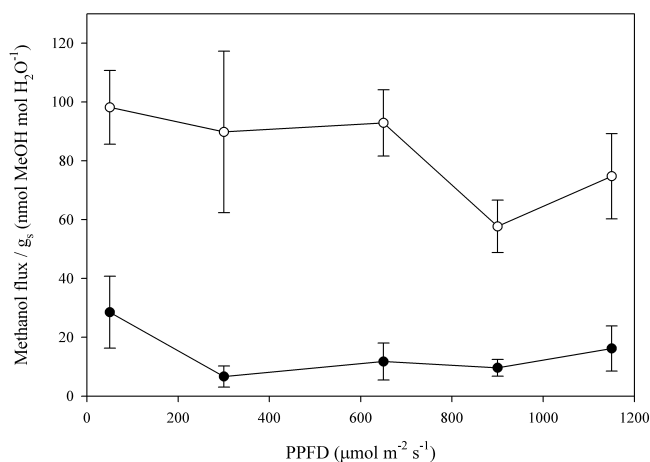

Fig. 3. Average MeOH flux values normalized for stomatal conductance (g_s) over five light levels for $n = 5$ mature (black circles) and immature (white circles) *L. esculentum* (standard error bars shown). PPFD had no significant effect on normalized MeOH flux ($F = 0.81$ $df = 4$ $P = 0.53$). Plant type had a significant effect on normalized MeOH emission overall ($F = 79.43$ $df = 1$ $P < 0.0001$).

0.09 ± 0.02 mol H_2O m^{-2} s^{-1}, and relative humidity $54.5 \pm 9\%$ (means $\pm$ SD). Immature leaves had significantly greater MeOH emission and PME activity overall (Wilk's lambda $F = 45.47$ $df = 2, 38$ $P < 0.0001$). Again, MeOH emissions were normalized for g_s in order to remove all influence of g_s on MeOH emission. Non-normalized data were analyzed and found to behave similarly to the normalized emission data (data not shown). Light had no significant effect on either PME activity or normalized MeOH emission under relatively
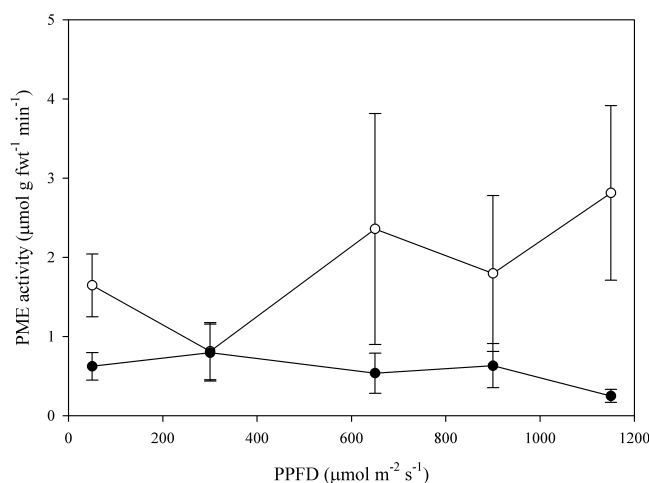

Fig. 4. Average PME activity rates over five light levels for $n = 5$ mature (black circles) and immature (white circles) *L. esculentum* (standard error bars shown). PPFD had no significant effect on enzyme activity ($F = 0.40$ $df = 4$ $P = 0.81$). Plant type had a significant effect on PME activity overall ($F = 14.46$ $df = 1$ $P = 0.0005$).

constant temperature and g_s (Figs. 3 and 4; Wilk's lambda $F = 0.57$ $df = 8, 76$ $P = 0.80$). Light was therefore not observed to stimulate MeOH production through the PME pathway on the time scale of 0–30 min.

4 Discussion

Factors regulating MeOH production in plants are not well constrained in current MeOH emission models (Galbally and Kirstine, 2002; Karl et al., 2003; Harley et al., 2007). Previous studies have suggested that light directly stimulates MeOH production (Harley et al., 2007). As light is known to regulate cell wall expansion on the scale of minutes, we predicted that short-term increases in light would stimulate PME activity and MeOH emission in immature leaves. The results, however, did not bear out these predictions. MeOH emissions normalized for g_s suggested that changes in g_s were capable of explaining changes in MeOH emission in response to light. It is important to note that if light influenced g_s and MeOH emission similarly, then the normalization of MeOH emission by g_s would have resulted in the removal of the effect of light on MeOH emission as well. Further experimentation, however, demonstrated that light did not directly influence MeOH emission as it does g_s. Data from the second and third sets of MeOH emission measurements demonstrated that short-term changes in light do not influence MeOH emission if g_s and temperature are experimentally controlled. In accord with previous studies, immature leaves had significantly higher MeOH emission (Nemecek-Marshall et al., 1995; Hüve et al., 2007), as well as higher PME activity, than mature leaves. Despite being in a rapidly expanding growth phase, PME activity in immature leaves

was not stimulated by light over time scales of 20–30 min. It is clear that MeOH emission and production do not behave similarly to other VOCs such as isoprene which are known to increase production and emission in response to short term changes in light. Light does not stimulate PME activity or MeOH emission in either mature or immature leaves on short time scales.

MeOH production is regulated not only by PME activity, but also by multiple interacting factors, such as cell wall pH, substrate availability, and PME gene expression (Goldberg et al., 1996; Pelloux et al., 2007). The acidification of the cell wall in response to light-induced growth is known to occur on the scale of seconds to minutes (Elzenga et al., 1997; Stahlberg and Van Volkenburgh, 1999), and we hypothesized that light-induced changes in cell wall pH and cell expansion would influence MeOH production. The results, however, suggested that the fast-acting effects of light on the acidification of the cell wall did not significantly alter overall PME activity. It is likely that our PME assay was insensitive to rapidly reversible post-translational changes in PME activity. Additionally, individual PME activity rates may have been stimulated by the light treatment but went undetected, as our assay measured total PME activity. Therefore, changes in PME activity could have occurred, but were not strong and/or prolonged enough to be detected. As PME activity is dependent on cell wall pH, it is surprising that PME activity did not change across a wide spectrum of light intensity. This lack of change in PME activity and MeOH emission response to light suggests that PME may, at times, be substrate limited. Studies have shown that cell expansion is not immediately accompanied by cell wall synthesis. Rapidly growing cells are known to stretch cell walls thin due to the lack of cell wall components, such as pectin polysaccharides (Refregier et al., 2004). The addition of methylated pectin to the cell wall during 20–30 min of light-induced growth can therefore not be assumed. Although pectin synthesis is generally known to be under developmental control and also induced by mechanical damage, pathogenesis, and cell-cell interactions, understanding of the transcriptional control of cell wall polysaccharides is incomplete (Somerville et al., 2004). In addition to pectin synthesis, the regulation of PME gene expression could also limit MeOH production. Unfortunately, little is known concerning the gene expression of pectin and PMEs and how they regulate MeOH production in plants. Despite the lack of knowledge, future research should focus on measuring PME substrate limitation and gene regulation, with the possibility of linking these processes with MeOH production.

Investigation of long-term changes in MeOH production and emission in response to light may be valuable for modeling efforts. Factors such as PME activity, PME substrate limitation, and PME gene regulation are not easily incorporated into MeOH emission models operating over long time scales (hours to days) and greater spatial scales (canopy to regional). Hüve et al. (2007) reported a strong relationship between leaf expansion and MeOH emission over several days.

Therefore, factors such as light, light-induced growth, and leaf developmental stage may be most relevant for predicting emissions from greater spatial and temporal scales. Previous work has shown that long term effects of light and temperature are important for estimating the production and emission of VOCs such as isoprene and methylbutenol (Harley et al., 1996, 1997; Fuentes and Wang, 1999; Sharkey et al., 1999; Geron et al., 2000; Hanson and Sharkey, 2001; Lehning et al., 2001; Petron et al., 2001; Gray et al., 2005). Incorporating variables that account for light and thermal history have improved VOC model performance, particularly at the seasonal time scale (Gray et al., 2006). The development of a light history term for MeOH emission models may expand modeling capabilities to the seasonal time scale.

5 Conclusions

In order to accurately predict MeOH emissions, there is a need to understand the factors regulating MeOH production. This study contributes to that effort as it demonstrates that light does not stimulate PME activity or MeOH emission over short time scales. Future investigation of PME substrate limitation and gene regulation may improve understanding of the short-term factors regulating MeOH production. Although light did not regulate MeOH emission over short time scales, long-term effects of light on MeOH production and emission may be important for predicting emissions on the seasonal time scale.

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