Mechanisms Regulating the Production and Emission of Methanol in *Lycopersicon esculentum*
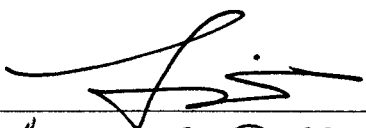
Patricia Yoshino Oikawa
Charlottesville, VA

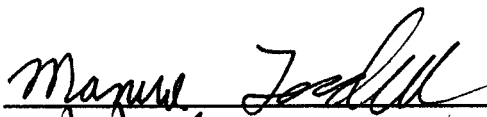Bachelor of Science, University of California, Santa Barbara, 2004

A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
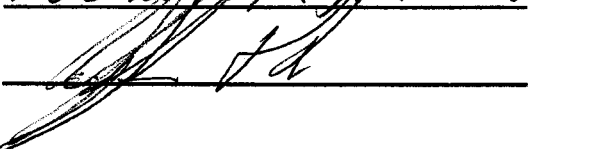Doctor of Philosophy

Department of Biology

University of Virginia
May, 2011

Abstract

Phytogenic methanol (MeOH) emission is the largest source of MeOH to the atmosphere, where it is the second most abundant organic compound, after methane. MeOH is believed to be a by-product of pectin methylesterase (PME) activity in cell walls. Immature leaves are known to have higher MeOH emission rates than mature leaves and are believed to produce MeOH within leaf tissue via PME activity. The source of MeOH from mature leaves, however, is unknown and has been suggested to be derived from below-ground sources. MeOH emissions are known to increase with stomatal conductance, light, and wounding. The mechanisms controlling MeOH production and emission, however, are not well understood. In my dissertation, I pursued questions concerning the source of MeOH emissions from mature leaves, the ability of PME activity to predict MeOH flux, the direct influence of light on MeOH production and emission, and the influence of mechanical damage on local and systemic MeOH production and emission. Results suggested that MeOH from mature and immature leaves was derived from the same biosynthetic pathway. Enriched isotopic signatures of MeOH emission relative to the isotopic signature of the pectin methoxyl pool, as well as unexplained variance in PME activity and concentrations of MeOH in plant tissue, suggested the presence of a MeOH sink (e.g. MeOH catabolism). PME activity was related to MeOH emission but was not a good predictor of MeOH emission. Results suggested that below-ground production of MeOH was not a significant source to mature leaves. Light did not directly influence MeOH production or emission over short time

scales. Mechanical damage significantly increased MeOH emission, but no significant changes in PME activity were detected. PME transcription was locally and systemically down-regulated in response to damage. My dissertation contributes to basic biological knowledge concerning the production and emission of MeOH, an important biogenic flux to the atmosphere.

Dedication

To my parents Sandy and Steve Oikawa,

Thank you for your unconditional love and support

Table of Contents

Preface

I have received assistance from many people throughout the course of my dissertation. Manuel Lerdau provided equipment, contributed to the study design, and assisted in the editing for every chapter of my dissertation. Chapter 2 has been accepted as "Oikawa P.Y., Giebel B.M., Sternberg L. da S.L., Li L., Timko M.P., Swart P.K., Riemer D.D., Mak J.E., Lerdau M.T. 2011. Leaf and root pectin methylesterase activity and $^{13}C/^{12}C$ stable isotopic ratio measurements of methanol emissions give insight into methanol production in *Lycopersicon esculentum*. *New Phytologist*." I wrote Chapter 2 and received editing assistance from all co-authors. I developed the ideas and study design for the project with the assistance of co-authors. I collected the majority of the data and ran the statistical analyses. Leo Sternberg provided stable carbon isotope ratio measurements of pectin. I collaborated with Peter Swart, Dan Riemer, and Brian Giebel during the collection of stable carbon isotopic signatures of live methanol emissions from *L. esculentum*. Funding for this project was provided by the University of Virginia and the University of Miami.

Chapter 3 has been accepted as "Oikawa P.Y., Li L., Timko M.P., Mak J.E., Lerdau M.T. 2011. Short term changes in methanol emission and pectin methylesterase activity are not directly affected by light in *Lycopersicon esculentum*. *Biogeosciences*." I wrote Chapter 3 and received editing assistance from all co-authors. Manuel Lerdau and I contributed equally to the study design. I collected the data and ran the statistical analyses. Funding for this project was provided by the University of Virginia.

Chapter 4 has been submitted for publication in the Journal of Experimental Botany as "Oikawa P.Y., Wu C., Li L., Timko M.P., Mak J.E., Lerdau M.T. 2011. Local and systemic methanol production and emission responses to wounding in wild-type and jasmonic acid insensitive (*jai1-1*) *Lycopersicon esculentum*." I wrote Chapter 4 and

received editing assistance from all co-authors. The ideas for this chapter were primarily my own. I collected and processed the majority of the data and ran the statistical analyses. Lei Li and Chengjun Wu also contributed ideas to this chapter and were instrumental in the collection and processing of molecular data. Manuel Lerdau played an important role in the development of ideas for the chapter, synthesis, and writing. Funding for this project was provided by the NSF-IGERT Biosphere-Atmosphere Research and Training (BART) program and the University of Virginia.

Acknowledgements

I have received support from many sources during my doctoral career. I want to first thank my advisor Manuel Lerdau. Manuel has been very influential and supportive. He taught me how to think like a plant physiological ecologist and has acted as a great source of knowledge on a wide range of subjects. He is not only an excellent scientist, but a true scholar and has always encouraged my academic enquiries, whether they were a part of my dissertation or not.

I also want to thank my committee. John Mak has supported me throughout my entire doctorate. As an excellent analytical chemist, John set a high standard for analytical measurements and I have him to thank for my success in the laboratory. Mike Timko was extremely helpful in both the development of analytical techniques and the writing of my dissertation. Mike has provided diverse pieces of advice, all of which have made me a better scientist. Lei Li was helpful on many fronts. He was my first reader and assisted in the development and writing of Chapter 4. He also invited me into his lab and reintroduced me to molecular biology, even though I had not run a gel since I was in high school. Laura Galloway provided helpful statistical advice. Laura always provided clear insight during committee meetings and was a constant source of support. Finally, Hank Shugart was an important and valued member of my committee. Hank is not only brilliant, but very friendly and approachable. Hank taught me to always have a "plan B". He taught me a lot about experimental design and challenged me to think rigorously about the questions I was asking and the interpretation of data. All of my committee members were generous and kind and I was honored to have them on my committee.

I have received technical assistance and support from many people. Kolby Jardine spent a lot of time helping me with my first measurements at Stony Brook. Kolby taught me how to be an analytical chemist and I am very grateful for his generosity

and patience. All of my labmates and fellow graduate students at SUNY at Stony Brook made my time in New York both successful and unforgettable. I want to thank the NSF-IGERT Biosphere-Atmosphere Research and Training (BART) administrators Mary Ann Carroll, Steve Bertman, and Dave Karowe for advising me throughout my dissertation, teaching me how to think about the "big picture", and being amazing role models. I also want to thank the entire BART community and, especially, my BART cohort who truly are family to me and provided much needed support during my summers at the University of Michigan Biological Station. At the University of Virginia, Wendy Crannage helped me keep my tomatoes alive. Aaron Mills provided a GC-FID system and lab space. Chengjun Wu took me under his wing and taught me all of the molecular techniques I needed for Chapter 4. Federico Brilli taught me how to use the PTR-MS. John Maben provided lab equipment and technical assistance. Deborah Lawrence, Howard Epstein, and their lab groups helped with early versions of my manuscripts and were a supportive and inviting group of researchers. Todd Scanlon, Clara Funk, and Ami Riscassi shared lab equipment and provided technical advice. Stephen Chan has been a very supportive labmate and answered many of my chemistry questions throughout the years. Megan McGroddy has been a constant source of support. I can always rely on Megan to tell it like it is. Brian Giebel was not only a collaborator and an excellent analytical chemist, but a friend. I hope to work with him again in the future. Dan Riemer, Peter Swart, and Leo Sternberg were all instrumental in my stable carbon isotope measurements.

I also want to thank David Chapman, my undergraduate advisor at the University of California, Santa Barbara. David inspired me to investigate trace gas emissions from plants and provided the support I needed to start my career in science.

I want to thank my family, who believed in me when I said I wanted to be a scientist. My dad encouraged my writing from a young age and brought me to the book

store whenever I needed a new book. He is a creative giant and a constant source of inspiration. My mom has provided unconditional love and support. She is the strongest woman I know and I am honored to be her daughter. My brother Paul has supported and watched out for me all these years. He is and always will be my best friend.

I want to thank Adam Zeilinger for being my partner and supporting me even when I don't deserve it. Adam provided advice, support, love and laughter throughout my dissertation.

Chapter 1

Introduction

Trace gas emissions from plants play major roles in Earth's chemistry, climate, and carbon balance. Over the past 60 years scientists have become aware of the significance of biogenic volatile organic compounds (BVOCs) for air pollution and climate change (Haagen-Smit, 1952; Fuentes *et al.*, 2000; Guenther *et al.*, 2000; Monson & Holland, 2001). Plants emit over a gigatonne of BVOCs annually (equal to 10^{15} g of carbon) with significant implications for biogeochemistry and the earth's radiative and chemical balance (Goldstein & Galbally, 2007). As BVOCs undergo oxidation in the atmosphere, they influence the concentration and lifetime of atmospheric constituents such as secondary organic aerosols (SOA) (Kanakidou et al., 2005), hydroxyl radicals, tropospheric ozone, and greenhouse gases (Atkinson & Arey, 1998; Atkinson, 2000; Lelieveld *et al.*, 2008).

Until recently, most BVOC research has focused on the reduced hydrocarbons isoprene and the mono- and sesquiterpenes (C_5H_8, $C_{10}H_{16}$, $C_{15}H_{24}$). In the last 15 years, BVOC research has expanded to include a broader range of phytogenic compounds. In particular, the study of oxygenated volatile organic compounds (OVOCs) has increased (Sharkey, 1996; Fall, 2003; Seco *et al.*, 2007). Compounds such as formaldehyde, acetaldehyde, acetone, methanol, ethanol, and formic and acetic acids have only recently been studied, aided by the development of new analytical techniques such as proton-transfer-reaction mass spectrometry (PTR-MS). These compounds are involved in

diverse metabolic pathways, and investigation of their emission has enhanced our understanding of basic plant biology. For example, OVOCs are involved in photorespiration, fatty acid metabolism, fermentation, cell wall expansion, wound response, the cyanogenic pathway, and catabolism (Fall & Benson, 1996; Kreuzwieser *et al.*, 1999; Gout *et al.*, 2000; Fall *et al.*, 2001; Kreuzwieser *et al.*, 2001; Downie *et al.*, 2004; von Dahl *et al.*, 2006; Seco *et al.*, 2007). The diverse metabolic pathways regulating the production and emission of OVOCs in addition to the functional significance of their emission has produced a rich field of inquiry for plant biologists.

Biogenic methanol (MeOH) is described as the simplest natural product derived from plants and is known to be a by-product of cell wall metabolism and produced by all plant species (Fall & Benson, 1996). The main source of MeOH in leaves is believed to be the demethylation of pectin by the enzyme pectin methylesterase (PME) (Nemecek-Marshall *et al.*, 1995; Frenkel *et al.*, 1998; Rose & Bennett, 1999; Galbally & Kirstine, 2002; Keppler *et al.*, 2004). Demethylation of pectin by PME facilitates cross-linking of pectin polymer chains and stabilizes the cell wall during expansion. Studies consistently show that immature expanding leaves emit greater amounts of MeOH than mature leaves, and that immature-leaf emissions are mainly derived from PME activity (Macdonald & Fall, 1993; Hüve *et al.*, 2007). One survey of MeOH emission rates across 11 plant species documented fluxes ranging from 1.5 - 61.3 $ug\ g^{-1}\ hr^{-1}$ (Macdonald & Fall, 1993). Predicting MeOH emission remains a challenge due to significant variation in emission rates measured at the scale of the leaf, plant, and ecosystem level.

The significance of phytogenic MeOH for atmospheric chemistry is only beginning to be understood. MeOH is the second most abundant organic gas in the

atmosphere, after methane, with plant-produced MeOH being the largest source to the atmospheric pool (Jacob *et al.*, 2005). Annual global budgets have estimated plant-produced MeOH sources to be anywhere from 75 to 280 Tg (teragrams, 10^{12} g) yr^{-1}, while emissions resulting from industrial processes range from only 4 to 8 Tg yr^{-1} (Singh *et al.*, 2000b; Galbally & Kirstine, 2002; Heikes *et al.*, 2002; Tie *et al.*, 2003; von Kuhlmann *et al.*, 2003a; von Kuhlmann *et al.*, 2003b; Jacob *et al.*, 2005). As these budgets are not well resolved, the significance of MeOH for atmospheric chemistry is still disputed and expected to vary depending on season and species composition. Regional impacts of MeOH on atmospheric chemistry, as opposed to global, have larger potential for influencing tropospheric chemistry. In some cases, particularly low-isoprene environments, canopy chemistry can be significantly influenced by MeOH. For example, in a conifer forest, MeOH was one of the main contributors to OH reactivity (Holzinger *et al.*, 2005) and OH-initiated HCHO production (Choi *et al.*, 2010). Recent studies have suggested that BVOCs, including MeOH, have an underestimated influence on the oxidative capacity of the troposphere. Large missing OH reactivity sinks within forest canopies (Di Carlo *et al.*, 2004; Sinha *et al.*, 2010; Wolfe *et al.*, 2011), as well as the recycling of OH in low NO environments (Lelieveld *et al.*, 2008), have been attributed to BVOC chemistry. The role of MeOH, and other BVOCs, in OH dynamics has yet to be fully determined. Improving MeOH budgets and ambient concentration measurements may help fill the missing OH reactivity sink and thereby improve our understanding of the influence of phytogenic MeOH on tropospheric chemistry.

MeOH emission dynamics differ from those of other well-studied BVOCs, such as isoprene, making mechanistic model development difficult. MeOH is highly soluble

(Henry's law constant at 25°C is 0.461 Pa m^3 mol^{-1}) and tends to be dissolved in the transpiration stream and within cells. Therefore, unlike isoprene emissions, foliar MeOH emissions are tightly regulated by stomatal conductance (g_s; the more open the stomata, the more MeOH emission) (Niinemets, Ü & Reichstein, M, 2003a). Another important way MeOH emissions differ from other BVOC emissions is by a lack of correlation with photosynthesis. Unlike BVOCs 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). MeOH emission instead depends on temperature, biosynthesis, catabolism, and g_s (Macdonald & Fall, 1993; Nemecek-Marshall *et al.*, 1995; Harley *et al.*, 2007; Hüve *et al.*, 2007). Increasing temperature increases the solubility of MeOH or the partitioning into vapor phase and may also stimulate MeOH production (Harley *et al.*, 2007). Plants are also known to catabolize MeOH (Cossins, 1964; Gout *et al.*, 2000), which could influence MeOH emissions. Therefore, temperature, catabolism, biosynthesis, and stomatal conductance affect the emission of MeOH from the intercellular air-space to the atmosphere. Currently there is no emission model that considers all of these processes and prediction of MeOH flux at the diurnal and seasonal scale is associated with significant uncertainty (Cossins, 1964; Harley *et al.*, 2007).

My goal was to elucidate the mechanisms regulating MeOH production and emission by relating gene transcription rates, enzyme activity, MeOH pools in plant tissue, and g_s to MeOH emission over a range of temporal scales. The dissertation consists of three studies focusing on (1) the factors regulating MeOH production and emission from mature leaves, (2) the influence of light on MeOH production and

emission over short time scales, and (3) the implications of wounding for MeOH

production and emission over multiple time scales.

Preliminary studies

In three preliminary studies, I investigated mechanisms regulating MeOH

emission from temperate trees in field and greenhouse settings. To provide context to the

subsequent chapters, I will summarize the preliminary data here and provide figures in

the Appendix in order to introduce my preliminary research interests in phytogenic

MeOH emission and how those interests inspired Chapters 2-4.

The first preliminary study focused on long-term MeOH emission response to

mechanical wounding in big tooth aspen *Populus grandidentata* and white pine *Pinus*

strobus. Both species were grown in the greenhouse at SUNY at Stony Brook, Stony

Brook, NY. MeOH fluxes were recorded before, 10 min post-, and 24 hrs post-treatment.

Although *P. strobus* showed no MeOH emission response to the treatment, significant

MeOH emission responses to wounding were observed in *P. grandidentata* (appendix

Fig. 1). This preliminary study demonstrated that mechanical damage can induce large

MeOH emission responses and provided helpful preliminary data for Chapter 4, in which

I investigated the mechanisms regulating MeOH emission response to mechanical

damage in *Lycopersicon esculentum*.

I conducted a second preliminary study comparing MeOH emission response to

mechanical damage and herbivory in *P. grandidentata*. This study was conducted in the

field at the University of Michigan's Biological Station, Pellston, MI. Gypsy moth

Lymantria dispar caterpillars were used for the herbivory treatments. Measurements

were conducted before, 10 min post- and 24 hrs post-treatment. No differences were detected between mechanical damage and herbivory treatments (appendix Fig. 2). However, due to generally low emission responses (possibly due to late-season sampling), interpretation of the data was difficult and the results were inconclusive.

The third preliminary study investigated seasonal variability in MeOH emissions in temperate forests. MeOH emissions from understory red maple *Acer rubrum* and white oak *Quercus alba* were measured on a weekly basis from 18 August-6 November, 2008 within the Virginia Forest Research Facility (37.92°N, 78.27°W). Due to sustained low light conditions (photosynthetic photon flux density was on average 59 μmol m^{-2} s^{-1}), MeOH emission rates were low throughout the sampling period and no seasonal trends were observed in the data (appendix Fig. 3). No relationships were observed between MeOH emissions and photosynthetic photon flux density (PPFD; appendix Fig. 4), temperature (appendix Fig. 5), or g_s (appendix Fig. 6). It was determined that low light and highly variable environmental conditions did not allow detection of relationships between MeOH flux and environmental parameters. This preliminary field study helped inform Chapter 3, which focused on the influence of light on MeOH emission under controlled conditions.

All of the preliminary studies demonstrated the need for more controlled physiological investigation of MeOH flux and greater biological understanding of MeOH production. Preliminary studies yielded highly variable data that were difficult to interpret, indicating that future MeOH emission experiments would require controlled conditions with few variables under consideration. Preliminary data were also difficult to interpret due to lack of basic biological knowledge concerning MeOH production and

emission in plants. For example, without understanding the factors regulating MeOH production, it is difficult to interpret the relationships between MeOH emission and environmental variables at the seasonal scale. In response to inconclusive preliminary experiments, the remainder of my dissertation investigated MeOH emission at a fine scale, involving measurements such as gene transcription and enzyme activity in leaf tissue. In an effort to remove variation, Micro Tom *Lycopersicon esculentum* clones were grown under greenhouse conditions. With the help of preliminary data, application of fine scale physiological measurements, and employment of model species *L. esculentum*, I was able to design and execute rigorous investigations of the mechanisms regulating phytogenic MeOH production and emission.

Research Questions

1. What is the source of MeOH emissions from mature leaves?

Although it is generally accepted that PME activity is the source of MeOH emissions from immature leaves (Fall & Benson, 1996; Frenkel *et al.*, 1998; Galbally & Kirstine, 2002), the role of PME activity in MeOH production in mature leaf tissue has remained unstudied. The fully expanded nature of mature leaves and a previous study indicating that some mature leaf MeOH is derived from below-ground production (Folkers *et al.*, 2008), suggests that MeOH emission from mature leaves may be mainly derived from PME activity in root tissue. The spatial heterogeneity of MeOH production in plants may therefore significantly influence MeOH emissions from mature leaves.

Emissions from mature leaves are generally much lower than from expanding leaves, however, the relative abundance of the two age groups integrated over the course

of the year makes emissions from mature leaves a significant source for biogenic MeOH at the ecosystem level. To predict MeOH emissions over long time scales, we need to understand the factors regulating MeOH production (Harley *et al.*, 2007). In Chapter 1, I investigated the source of MeOH emissions from mature and immature *Lycopersicon esculentum* leaves using stable carbon isotopic signatures of MeOH emissions and the pectin methoxyl pool. I also examined whether PME activity was a good predictor of MeOH emission and if below-ground production of MeOH was a significant source of MeOH to mature leaves.

2. Are short term changes in methanol emission and pectin methylesterase activity directly affected by light?

Although the majority of MeOH is not immediately derived from light-dependent photosynthesis, light may regulate MeOH production through the stimulation of leaf expansion. Previous studies have suggested that MeOH production may be directly influenced by light. The Niinemets-Reichstein model predicts MeOH emissions by assuming constant MeOH production while accounting for changes in g_s and gas- and liquid-phase MeOH pool sizes (Niinemets, Ü & Reichstein, M, 2003a; Niinemets, Ü & Reichstein, M, 2003b). Overestimations of nighttime MeOH emissions by the model suggested that light may directly influence MeOH production (Harley *et al.*, 2007). Folkers et al. (2008) clearly demonstrated the influence of light on MeOH emission, but variation in light was confounded with variation in g_s in their study.

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 (Loreto *et al.*, 1996a; Loreto *et al.*, 1996b; Ciccioli *et al.*, 1997) and isoprene synthase activity (Lehning et al., 1999) as predictor variables. Similar to previously studied VOCs, investigating the direct influence of light on MeOH production is an important step in understanding and modeling emission behavior. In Chapter 3, I investigated the direct effect of light on short term changes in MeOH production and emission in *Lycopersicon esculentum* by measuring PME activity and MeOH emission under conditions where light varied and changes in g_s and temperature were limited.

3. How does wounding influence local and systemic methanol production and emission?

Previous work has demonstrated that MeOH production and emission increase in response to wounding. Wounding is known to elicit a strong and rapid burst of MeOH, assumed to be the depletion of a stored aqueous pool of MeOH within the leaf (Fall, 2003; Loreto *et al.*, 2006). Long-term up-regulated MeOH emission (up to 24hrs) has also been documented in response to wounding and herbivory (Peñuelas *et al.*, 2005; von Dahl *et al.*, 2006). Previous studies have shown that PME transcription increases for up to 24 hrs post-herbivore attack (von Dahl et al., 2006) and herbivore-induced PME influences jasmonic acid (JA) signaling (Korner *et al.*, 2009). The mechanism for sustained PME activity and MeOH emission responses to wounding, however, is not known. The relationship between the JA pathway and PME activity is also not fully understood.

Damage-induced MeOH emissions can significantly increase local MeOH mixing ratios and are important for atmospheric chemistry (Karl *et al.*, 2001; Warneke *et al.*, 2002; Brunner *et al.*, 2007). Understanding the mechanisms underlying the connection between wounding and MeOH emission will improve our ability to predict the significance of MeOH flux to the atmosphere and give insight into plant physiological response to damage. In Chapter 3, local and systemic MeOH production and emission responses to wounding were investigated in *Lycopersicon esculentum*. PME transcription, PME activity, and MeOH flux were measured locally and systemically over multiple time scales. Transgenic *L. esculentum* with defective JA signaling (*jasmonic acid-insensitive1* [*jai1-1*]) was used to investigate the relationship between JA signaling and the PME pathway.

Chapter 2

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

Abstract

(1) Plant production of methanol (MeOH) is a poorly understood aspect of metabolism, and understanding MeOH production in plants is crucial for modeling MeOH emissions. I 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. I also investigated the significance of belowground MeOH production for mature leaf emissions.

(2) I present measurements of MeOH emission, PME activity, and MeOH concentration in mature and immature tissues of tomato (*Lycopersicon esculentum* L.). I also present stable carbon isotopic signatures of MeOH emission and the pectin methoxyl pool.

(3) The results suggest that belowground 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.

(4) The data show that MeOH production and emission is complex and cannot be predicted using PME activity alone. I 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 (teragrams, 10^{12} g) yr^{-1}, while anthropogenic emissions resulting from industrial processes range from only 4 to 8 Tg yr^{-1} (Singh *et al.*, 2000a; Galbally & Kirstine, 2002; Heikes *et al.*, 2002; Tie *et al.*, 2003; von Kuhlmann *et al.*, 2003a; von Kuhlmann *et al.*, 2003b; Jacob *et al.*, 2005). MeOH has an atmospheric lifetime of approximately 10 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 background concentrations of other volatile organic compounds (VOC) in the upper troposphere are low (Singh *et al.*, 2000a; Singh *et al.*, 2001; Tie *et al.*, 2003). MeOH is also believed to play an important role at the regional and canopy scales. In environments where the concentrations of more reactive VOCs are low, MeOH can significantly influence OH reactivity (Holzinger *et al.*, 2005), and therefore has implications for tropospheric ozone and air quality.

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 less than immature leaves (on average 3-4x less 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 LAI 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 methylesterase (PME) (Fall & Benson, 1996; Frenkel *et al.*, 1998; Galbally & Kirstine, 2002; Keppler *et al.*, 2004). Demethylation of pectin by PME facilitates cross-linking 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 due to low activity rates, e.g. 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, Ü & Reichstein, M, 2003a; Niinemets, Ü & Reichstein, M, 2003b), 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, I investigated three main questions: 1) Are MeOH emissions from mature and immature leaves derived from the same biosynthetic pathway? 2) Is PME activity a good predictor of MeOH emissions? 3) Do below-ground sources significantly contribute to MeOH emissions from mature leaves? I 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* 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. MeOH emissions from mature leaves of *L. esculentum*, *Fagus sylvatica*, and *Quercus robur* are on average 3.6, 0.77, 0.33 nmoles m^{-2} s^{-1} respectively (Folkers *et al.*, 2008). Plants were grown in the greenhouse 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 16hr 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.

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 GC-IRMS (Agilent 6800 GC-

Europa Scientific GEO 20-20 IRMS) capable of measuring $\delta^{13}C$ ratios of

oxygenated/biological volatile organic compounds (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

(1.86×10^{-2} μL/L) after dynamic dilution in zero and were ± 2.8‰ with an associated

error of 2.5% compared to 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 levels (peak irradiance of 665 nm and 470

nm), temperature, and relative humidity within the cuvette. Keeping light levels constant

(PPFD of 950 μmol photon $m^{-2} s^{-1}$), leaf-level measurements were only taken under

steady state conditions which were on average: leaf temperature 26.1 ±0.9°C, stomatal

conductance 0.14 ±0.05 mol H_2O m^{-2} s^{-1} mature and 0.20 ±0.06 mol H_2O m^{-2} s^{-1} immature, photosynthetic rates 9.0 ±2 μmol CO_2 m^{-2} s^{-1} mature and 12.6 ±3 μmol CO_2 m^{-2} s^{-1} immature, and relative humidity 53.1 ±4% mature and 56.0 ±5% immature (means ±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 due to the high solubility of MeOH (Niinemets, Ü & Reichstein, M, 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 leafs, 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 100-300 cm^3 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 cm^3 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 to the GC. Separated components in the eluant gas passed through a heated combustion column and 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}C to ^{12}C and reported in delta (δ) notation as a per mil (‰) difference of the sample compared to a working reference gas calibrated to the international standard 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 to the methanol peak to determine the $\delta^{13}C$ of methanol.

The stable carbon isotopic signature of pectin methoxyl groups was calculated from the isotopic signatures of untreated and de-methylated apple pectin (Apple pectin, ~70% methylated, Sigma-Aldrich). Alkaline hydrolysis (1 N NaOH) of pectin at 70°C generated de-methylated pectin as in Rosenbohm *et al.* (2003). Carbon isotope ratios were determined both in pectin and de-methylated pectin in an Isoprime isotope ratio mass spectrometer (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 de-methylated 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 (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 pre-concentration 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 ppt level. Air exiting the LI-6400 cuvette was routed to the PTR-MS inlet via ¼ 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 hrs. PTR-MS measurements were recorded for 20 cycles for a total sampling time of approximately 3 min. All measurements were taken under steady state conditions at photosynthetic photon flux density (PPFD) 750 µmol m^{-2} s^{-1}, leaf temperature 31 ±1.9°C, stomatal conductance 0.09 ±0.04 mol H$_2$O m^{-2} s^{-1} mature and 0.15 ±0.06 mol H$_2$O m^{-2} s^{-1} immature, photosynthetic rates 7.0 ±2 µmol CO$_2$ m^{-2} s^{-1} mature and 10.5 ±2 µmol CO$_2$ m^{-2} s^{-1} immature, and relative humidity 55.6 ±3% mature and 58.2 ±4% immature (means ± SD). Leaf surface area enclosed in the cuvette was measured using a LI-COR Leaf Area Meter (LI-COR Inc., Lincoln, NE, USA). 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 fresh weight basis (nmol g fwt^{-1} 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 lab (University of Miami) containing 3 ppmv (3 µl/l) ±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%. 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. 25µl 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). Sample solution pH was adjusted to 7.5 using small

amounts of 0.1 M NaOH (in 1-5µl). Once the solution dropped back down to pH 7, 1-5

µl 0.1 M NaOH was added until solution pH reached 7.3. Time for 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 fwt^{-1} 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 mid-stem and an adjacent

mature leaf. Tissues were weighed and ground in 5x equal weight

ethylenediaminetetraacetic acid (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 9

mature plants were measured for MeOH concentration.

Statistical analysis

Differences between mature and immature mean MeOH δ^{13}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 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., Cary, NC, USA). Differences between mature and immature PME activity 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 for MeOH concentration in leaf tissue (Proc GAM, SAS 9.1; SAS Institute Inc., Cary, NC, USA). 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., Cary, NC, USA). 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 investigate whether or not the stable carbon isotopic signatures of MeOH from mature and immature leaves are different. The measured δ^{13}C of MeOH emissions from mature and immature *L. esculentum* leaves were not significantly different (*t*=-1.08 df=8 *P*=0.31); mature and immature leaves were on average -19.0‰ and -21.5‰ respectively (Fig. 1). I interpret this result as support for 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 investigate whether or not the isotopic signature of the pectin methoxyl pool is different from the signature of MeOH emissions. I measured the $\delta^{13}C$ values of purified apple pectin and apple pectin methoxyl groups. The $\delta^{13}C$ of purified apple pectin (-26.2‰) was enriched in ^{13}C relative to the 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 $\delta^{13}C$ 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). I 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). I 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 concerning PME activity in fully expanded leaves (average±SE PME activity rates were 6.4±1.7 and 11.9±2.1 nmol g fwt^{-1} s^{-1} for mature and immature leaves, respectively; Fig. 4). 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 approximately 50% of immature leaf PME activity, while MeOH flux from mature leaves was approximately 33% of MeOH flux from immature leaves (average±SE MeOH flux were 0.03±0.01 and 0.09±0.02 nmol g fwt^{-1} s^{-1} for mature and immature leaves, respectively; Fig. 3). I 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 concentration of MeOH (P=0.28 Wilcoxon exact; average±SE MeOH concentration was 0.74±0.17 and 0.93±0.20 mg g fwt^{-1} for mature and immature leaves, respectively), indicating that although immature leaf MeOH emission was high, immature leaf MeOH concentration was not. I 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, I tested whether or not MeOH concentrations in stems could predict concentrations in leaves. In contrast to my hypothesis, concentrations of MeOH in stems were not good predictors for concentrations of MeOH in leaves (*Chi Square*=3.28 *P*=0.35 across leaf types; *Chi Square*=6.51 *P*=0.10 mature only; *Chi Square*=2.20 *P*=0.53 immature only; Fig. 4). I 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), I predicted that mature leaf MeOH would be mainly derived from PME activity in root tissue. I 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 my 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. I am not, however, aware of a mechanism for the partitioning of MeOH out of the transpiration stream and subsequent storage of MeOH in tissue. I 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. I, however, 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 the experiments did not explore the significance of nighttime root growth for early morning MeOH emissions.

Nighttime root growth could lead to the accumulation of MeOH in the transpiration stream, contributing to high MeOH emission rates which 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.

PME 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 to 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.

The results indicate that PME activity alone could not predict MeOH emissions from *L. esculentum*, and I 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. I hypothesize that PME substrate availability may be a good predictor of MeOH production. Cells are known to export 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, I 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. The 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). I 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 CO_2 or incorporated into amino acids (Cossins, 1964; Gout *et al.*, 2000). The isotopic data are congruent with this suggestion. While I 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, Ü & Reichstein, M, 2003a). Therefore, I 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 to mature leaves despite being in a rapidly expanding growth stage. Unfortunately, very little is known concerning rates of MeOH catabolism, therefore source-sink MeOH dynamics within plants requires further investigation. The 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 the 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 not significantly different, 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 the 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 non-PME 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. The 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 due to

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 galacturonic acid (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). I therefore do

not expect the isotopic signature of pectin to vary greatly among C3 plants. I also would

not expect the isotopic signatures of pectin and the pectin methoxyl pool to significantly

differ between mature and immature leaves as variation in carbon fractionation due to 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), I am confident that apple pectin is an appropriate substrate for the 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).

The 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 ±11.2‰ averaged across 11 species of C_3 plants. Similarly, Yamada *et al.* (2009) measured MeOH emissions to be on average -74.6 ± 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 discussion above).

An explanation for the discrepancy between the 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 to 18hrs 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 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. My method was designed for the measurement of MeOH emission from live, intact leaves as I measured emissions from individual *L. esculentum* leaves under standardized conditions. I was 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, the results may reflect a different plant-derived MeOH than has been previously measured. Similarly, the measured isotopic ratio of the pectin methoxyl pool deviated 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. I 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. I 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.

The investigation of mature leaf MeOH emission has implications for atmospheric chemistry and basic plant biology. I show that below-ground sources of MeOH were not a dominant source of MeOH to foliar *L. esculentum* emissions during the day. The work instead provides evidence that foliar PME activity is related to MeOH emissions. Isotopic signatures of mature and immature MeOH emissions were not significantly different, 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 MeOH production in plants must be understood (Harley *et al.*, 2007). I suggest that in order to understand and model MeOH emissions, there is a 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 require the concurrent measurement of multiple physiological variables, including not only PME activity, MeOH concentration, MeOH flux and stomatal conductance, but also PME substrate limitation and MeOH catabolism.
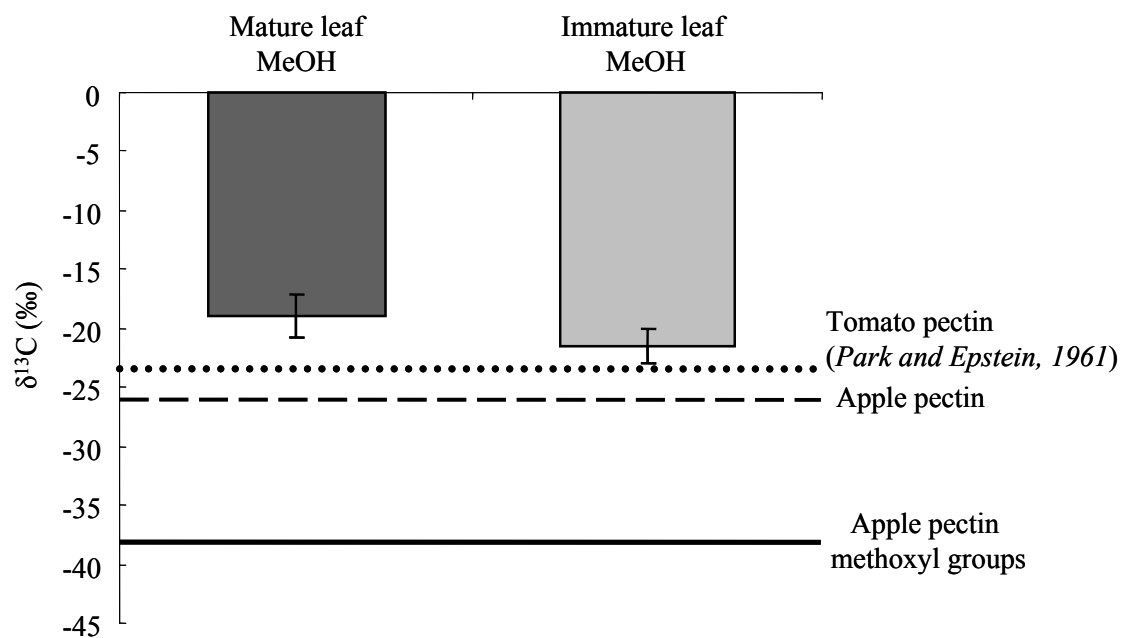
Acknowledgements

Figure 1. Average δ^{13}C values for MeOH emissions from mature (*n*=5) and immature

(*n*=6) *L. esculentum*, tomato pectin measured by Park and Epstein (1961), apple pectin,

and pectin methoxyl groups. Values are means ± standard error. No significant

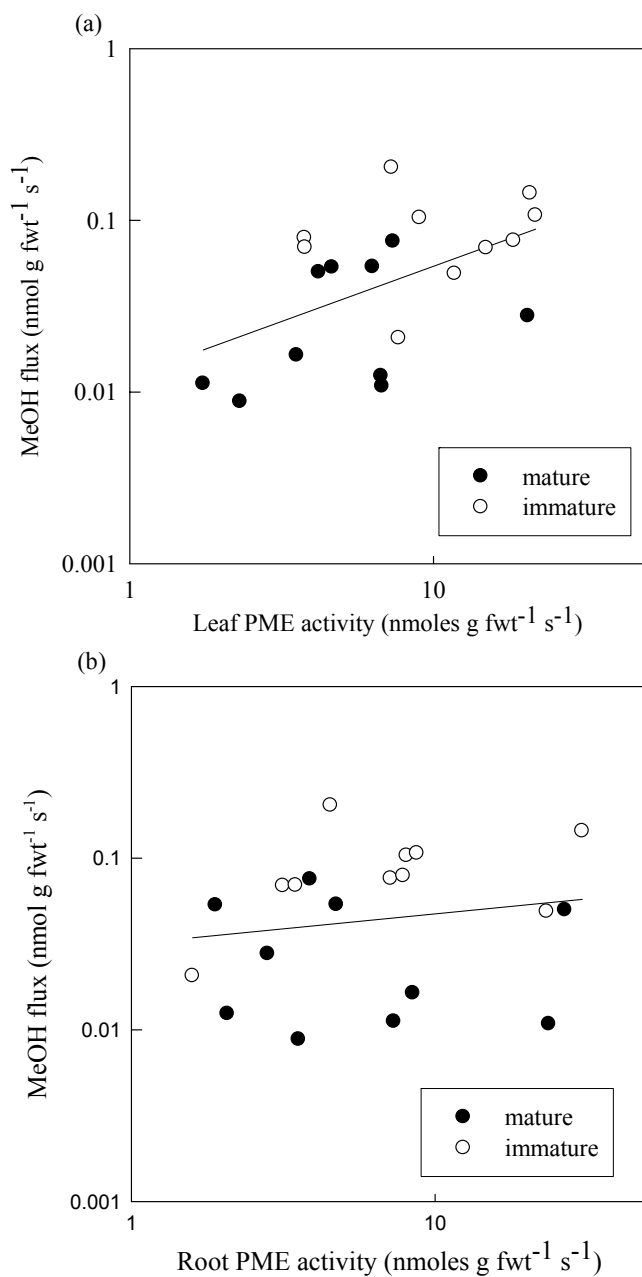differences were found between the 2 leaf types (*t*=-1.08 df=8 *P*=0.31).

(a)

(b)

Figure 2. The relationship between (a) leaf PME activity and leaf MeOH flux

(Regression; F=6.24, P=0.022 across leaf types; F=1.66, P=0.22 within leaf type) and (b)

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) in mature (n=10) and immature (n=10) *L. esculentum*.

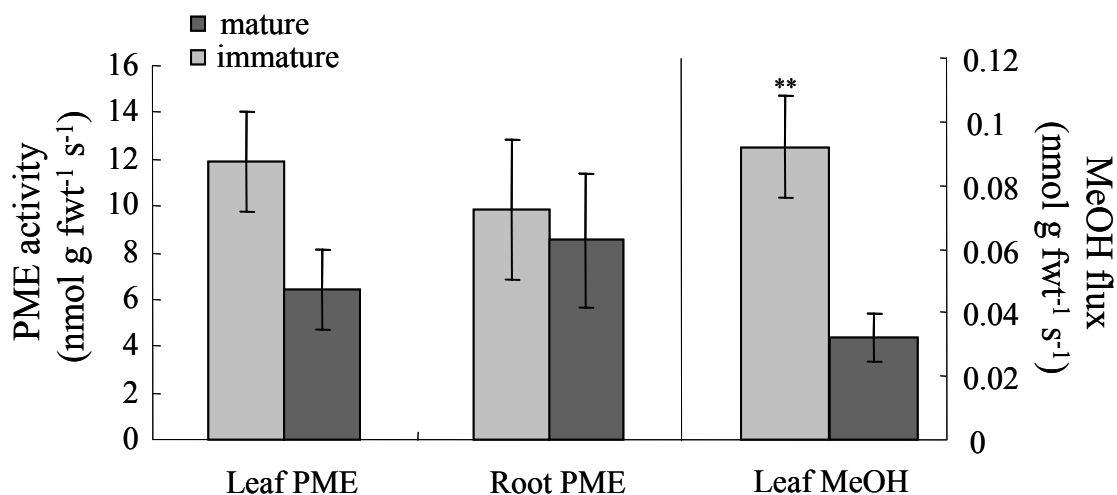Lines are fit to all data. Data are shown on a log-log scale.

Figure 3. Leaf PME activity, root PME activity and leaf MeOH flux for mature (*n*=10) and immature (*n*=10) *L. esculentum*. Values are means ± standard error. Immature leaves tended to have higher PME activity (*t*-test; df=18 *t*=2.03 *P*=0.057) and higher MeOH flux (*t*-test; df=18 *t*=3.35 *P*=0.0036) than mature leaves. An asterisk represents a significant difference between leaf types at *P*=0.05; two asterisks represent a difference at *P*<0.01. No significant difference was detected between PME activity in immature and mature root tissue (*t*-test; df=18 *t*=0.32 *P*=0.755).
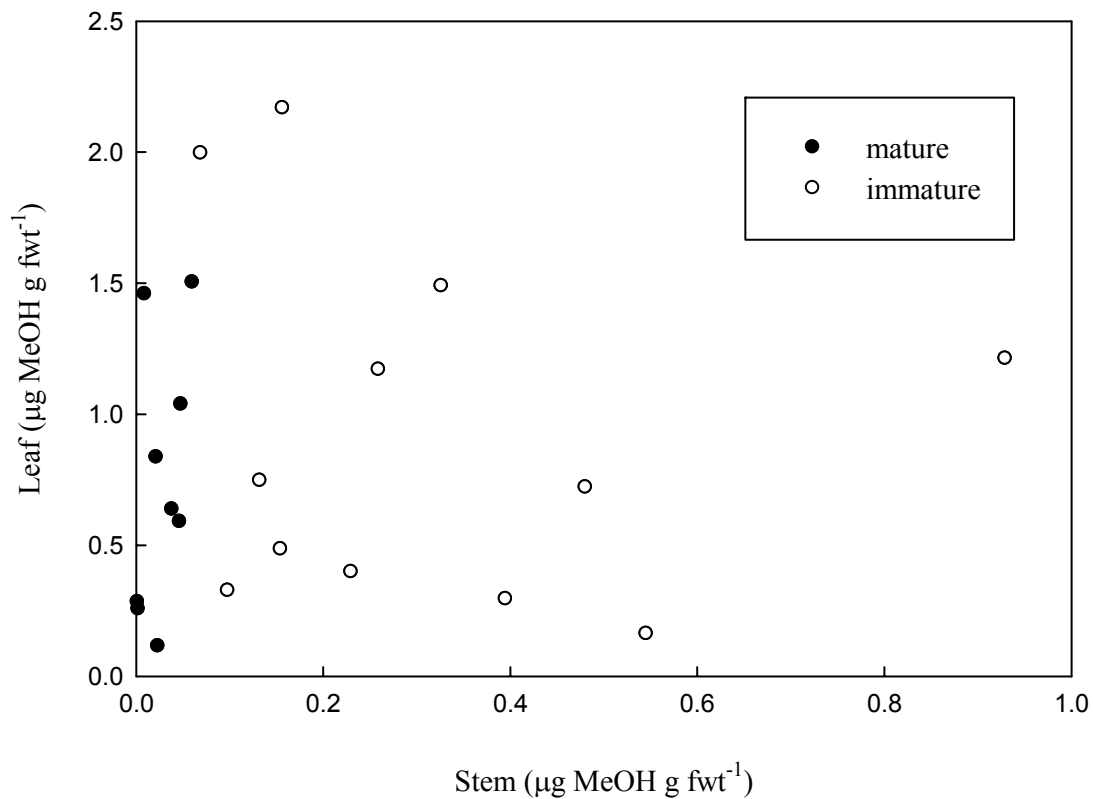
Figure 4. The relationship between MeOH concentrations in stem and leaf tissue from

mature (*n*=9) and immature (*n*=12) *L. esculentum* (Nonparametric regression; across leaf

type *Chi Square*=3.28 *P*=0.35; mature only *Chi Square*=6.51 *P*=0.10; immature only *Chi*

Square=2.20 *P*=0.53).

Chapter 3

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

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. The 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.

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.*, 2000b; Galbally & Kirstine, 2002; Heikes *et al.*, 2002; Tie *et al.*, 2003; von Kuhlmann *et al.*, 2003a; von Kuhlmann *et al.*, 2003b; 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.*, 2000b; Singh *et al.*, 2001; Tie *et al.*, 2003). MeOH is believed to play a larger role, however, at the canopy and regional scale, where the influence of MeOH varies depending on the season and species composition of the forest. In environments where the concentrations of more reactive VOCs are low, MeOH can significantly influence OH reactivity (Holzinger *et al.*, 2005) and therefore has implications for tropospheric O_3 production and air quality.

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 & Benson, 1996; Frenkel *et al.*, 1998; Rose & Bennett, 1999; Galbally & 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 & 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 & 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 & 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 & 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 my 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

& 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; , 2003b) 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, U & Reichstein, M, 2003a;

Niinemets, U & Reichstein, M, 2003b). 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. The

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; Loreto *et al.*, 1996b; 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, I 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, I can determine the direct relationship between light and MeOH production.

Methods

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 hr 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.

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 μmol 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 (μmol 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.

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 pre-concentration 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 ¼ 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 hrs. 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) $\pm$ 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 section 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.

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 & 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 &

Barrett, 2006). A total of 25 immature and 25 mature plants were assayed for PME

enzyme activity.

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.

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 ±1°C, photosynthetic rates 6.7 ±4 µmol

CO_2 m^{-2} s^{-1} mature and 10 ±4 µmol CO_2 m^{-2} s^{-1} immature, and relative humidity 55 ±5%

mature and 58 ±6% immature (means ± 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 ±1°C, g_s 0.08 ±0.02 mol H_2O m^{-2} s^{-1}, and relative humidity 58.3 ±7% (means ± 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 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 ±1°C, g_s 0.09 ±0.02 mol H_2O m^{-2} s^{-1}, and relative humidity 54.5 ±9% (means ± 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 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.

Discussion

Factors regulating MeOH production in plants are not well constrained in current

MeOH emission models (Galbally & 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, I 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 & Van Volkenburgh, 1999), and I 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 the 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 the 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; Harley *et al.*,

1997; Fuentes & Wang, 1999; Sharkey *et al.*, 1999; Geron *et al.*, 2000; Hanson & 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.

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.

Acknowledgements
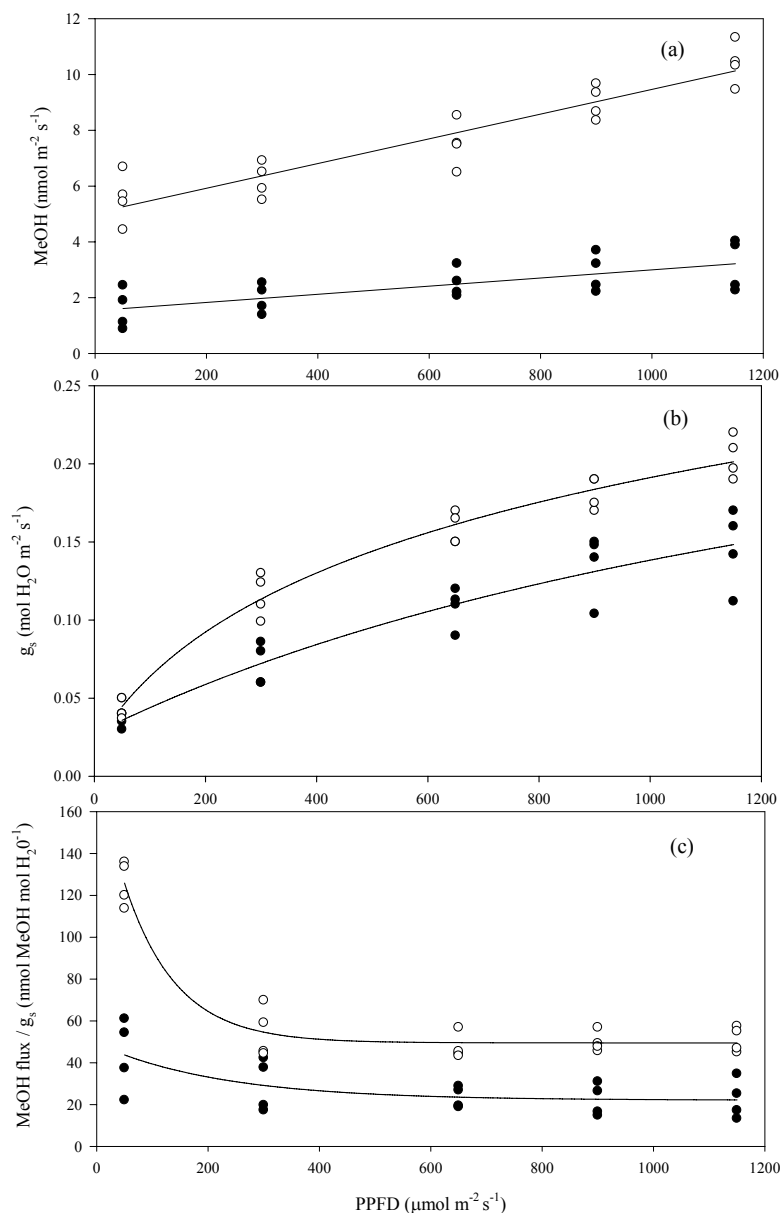
Figures 1a, b, and c. (a) MeOH emission, (b) stomatal conductance (g_s), and (c) MeOH emission normalized for g_s from mature (n=4; black circles) and immature (n=4; open circles) *L. esculentum* over five light levels at constant temperature (29 ±1°C). MeOH emission (slope = 0.0015, t=4.09, P=0.001 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).
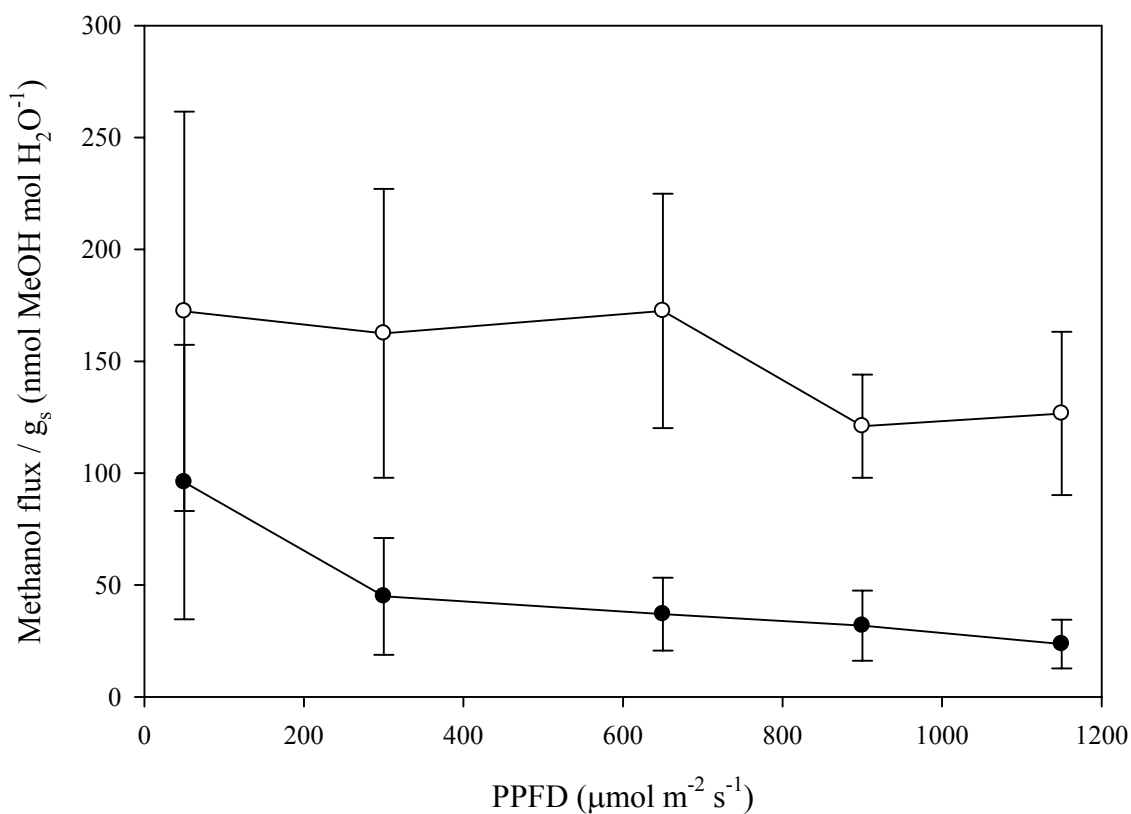

Figure 2. Average MeOH flux values normalized for stomatal conductance (g_s) over five light levels for n=5 mature (black circles) and immature (white circles) *L. esculentum* (each plant was measured 5x; standard error bars shown). PPFD had no significant effect on normalized MeOH flux for either leaf type (F=2.96; df=1,19; P=0.10 for mature; F=0.6; df=1,19; P=0.45 for immature).
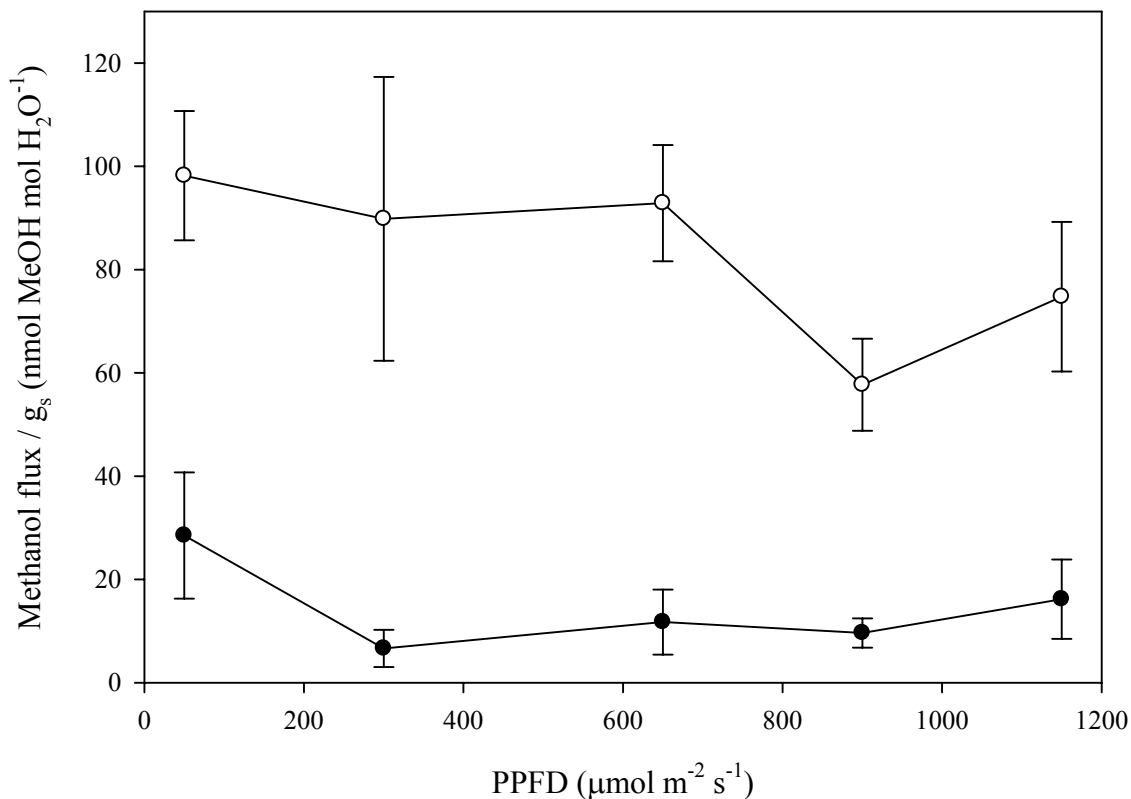
Figure 3. Average MeOH flux values normalized for stomatal conductance (g_s) over five light levels for n=5 mature (black circles) and immature (white circles) *L. esculentum* (standard error bars shown). PPFD had no significant effect on normalized MeOH flux (F=0.81 df=4 P=0.53). Plant type had a significant effect on normalized MeOH emission overall (F=79.43 df=1 P<0.0001).
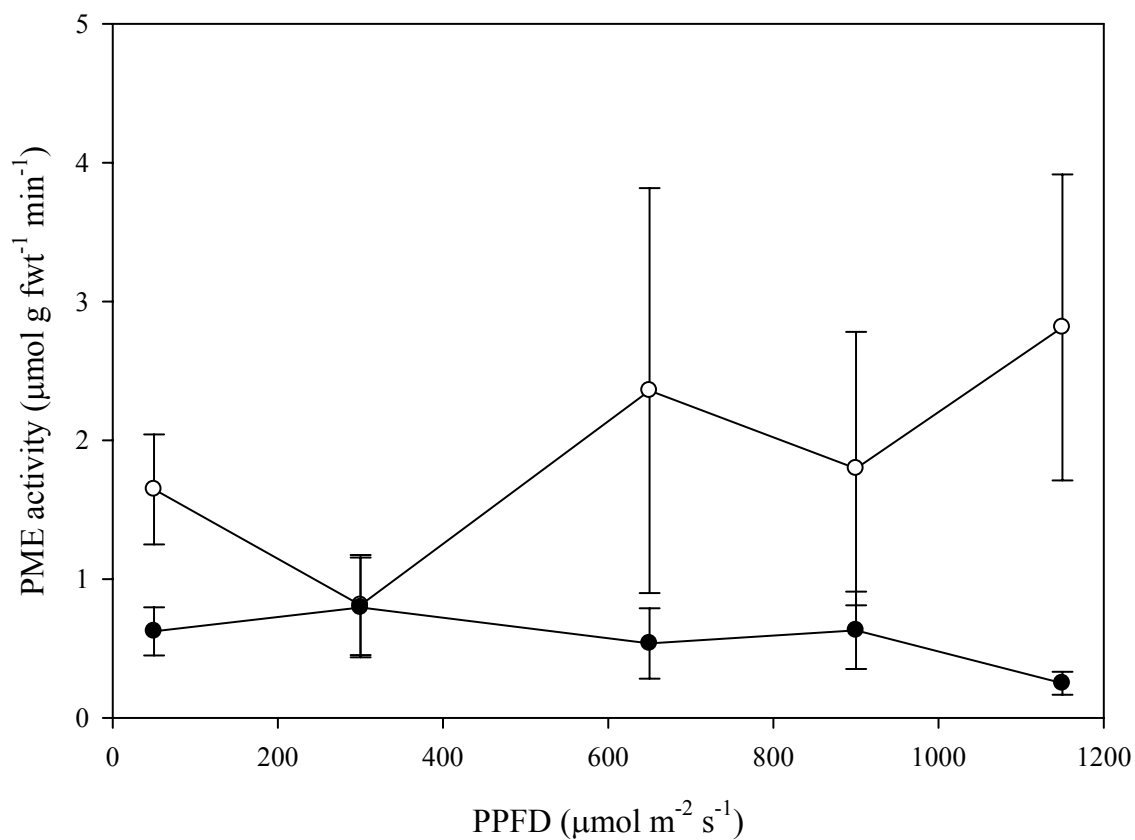
Figure 4. Average PME activity rates over five light levels for *n*=5 mature (black circles) and immature (white circles) *L. esculentum* (standard error bars shown). PPFD had no significant effect on enzyme activity (*F*=0.40 df=4 *P*=0.81). Plant type had a significant effect on PME activity overall (*F*=14.46 df=1 *P*=0.0005).

Chapter 4

Local and systemic methanol production and emission responses to wounding in wild-type and jasmonic acid insensitive (*jai1-1*) *Lycopersicon esculentum*

Abstract

MeOH is believed to be a by-product of the demethylation of pectin by enzyme pectin methylesterase (PME). PME activity and MeOH emission are known to be influenced by wounding and herbivory for up to 24 hrs. PME activity has also been shown to be important for JA accumulation and other wound responses. The mechanisms regulating long-term changes in MeOH production and emission in response to damage, however, are not known. Additionally, the influence of jasmonic acid (JA) signaling for local and systemic MeOH emission responses to wounding has not been investigated. I investigated the role of JA signaling for local and systemic MeOH emission, PME activity, and PME transcription responses to wounding across multiple time scales. Transgenic tomato, (*Lycopersicon esculentum* Mill.) with a deficient JA signaling system, was used to test whether or not local and systemic PME responses to wounding were JA mediated. Elevated MeOH emissions observed 10 min and 1 hr post wounding were attributed to decreased diffusive barriers resulting from damaged leaf tissue. These results document, for the first time, local and systemic down-regulation of certain PME candidate genes 1 hr and 24 hrs after wounding. Long-term and systemic PME transcription responses to wounding were not JA-dependent. Additional study is needed in order to identify the mechanisms regulating PME transcription and PME activity and

how these processes translate into MeOH emission an important biogenic flux for atmospheric chemistry.

Introduction

Methanol (MeOH) production by plants is an interesting yet understudied metabolic process, and MeOH emissions from plants play important roles in atmospheric chemistry. Plant-derived MeOH fluxes account for 75% of the global annual MeOH flux to the atmosphere (Singh *et al.*, 2000a; Galbally & Kirstine, 2002; Heikes *et al.*, 2002; Tie *et al.*, 2003; von Kuhlmann *et al.*, 2003a; von Kuhlmann *et al.*, 2003b; Jacob *et al.*, 2005). MeOH has a relatively long atmospheric lifetime of 10 days (Jacob *et al.*, 2005), which allows it to be transported to the upper troposphere where concentrations of other, more reactive, volatile organic compounds (VOC) are low. Once in the upper troposphere, MeOH plays a significant role in lowering hydroxyl radical concentrations and elevating ozone and carbon monoxide concentrations (Singh *et al.*, 2000a; Singh *et al.*, 2001; Tie *et al.*, 2003). Phytogenic MeOH, however, is believed to play a larger role at the canopy and regional scales, depending on the time of year and species composition of the forest. In environments where the concentrations of other more reactive VOCs are low, MeOH can play a significant role in OH reactivity (Holzinger *et al.*, 2005), thereby influencing tropospheric O_3 production and air quality.

Despite the large quantities of MeOH produced by plants, the regulation of MeOH production and emission is not yet well understood. Previous work has indicated that MeOH is primarily produced as a by-product during the demethylation of pectin by enzyme pectin methylesterase (PME) (Fall & Benson, 1996; Frenkel *et al.*, 1998;

Galbally & Kirstine, 2002; Keppler *et al.*, 2004). The demethylation of pectin by PME allows pectin polymer chains to crosslink and stabilize the cell wall. Plants are known to have numerous isoforms of PME, with some plant species containing up to 89 PME protein-encoding regions of DNA (Pelloux *et al.*, 2007). The biology of MeOH production through the PME pathway is therefore complex as it involves the regulation of multiple PME isoforms.

Previous work has demonstrated that MeOH production and emission increases in response to wounding. Wounding is known to elicit a strong and rapid burst of MeOH (von Dahl *et al.*, 2006), assumed to be the depletion of a stored aqueous pool of MeOH within the leaf and not the result of *de novo* MeOH production (Fall, 2003; Loreto *et al.*, 2006). MeOH is highly water soluble and is therefore under stomatal control. As a result, MeOH can accumulate within the leaf and is known to burst out of the leaf when diffusive barriers are decreased (Harley *et al.*, 2007). Although short-term MeOH emission responses to damage may simply result from the release of accumulated MeOH within the leaf, induced production of MeOH through the PME pathway may also contribute to short-term emission responses. A recent study demonstrated that the induction of PME activity can increase MeOH emission responses to herbivory on the time scale of 10-20 min post treatment (von Dahl *et al.*, 2006). Therefore, it is not clear whether MeOH emissions in response to damage are purely the release of stored MeOH or if induced MeOH production through the PME pathway also plays a role.

Long-term elevated MeOH production and emission (up to 24 hrs) in response to herbivory have also been observed. For example, MeOH emission responses were significantly elevated 24 hrs after herbivore attack in *Succisa pratensis* (Peñuelas *et al.*,

2005). Another study documented increased transcription of a PME candidate gene in tobacco, *NaPME*, for 4-24 hrs following herbivore attack (von Dahl *et al.*, 2006). Silencing *NaPME* led to significantly decreased PME activity and MeOH emission responses to herbivory (Korner *et al.*, 2009). Although long-term MeOH emission responses to damage may be associated with anti-herbivore or re-growth responses, the mechanism for long-term PME activity and MeOH emission response to wounding has not been investigated. Examination of the variety of PME isoform responses to damage is also needed to understand long-term MeOH emission and PME response to damage.

One mechanism that may influence long-term MeOH production and emission responses to damage is jasmonic acid (JA) signaling. Wound response pathways are known to alter plant metabolism for multiple days following attack (Foggo, 1996; Kessler & Baldwin, 2002). Therefore, it is possible that feedback between JA and PME could sustain long-term PME responses to damage. Previous studies have demonstrated a relationship between PME, cell wall structure, JA biosynthesis, and stress response (Ellis *et al.*, 2002; Ko *et al.*, 2006; Pelloux *et al.*, 2007). Korner *et al.* (2009) showed that plants without a functioning *NaPME* gene had reduced levels of jasmonic acid (JA), altered levels of salicylic acid (SA), and reduced levels of defense compound trypsin proteinase inhibitor (TPI). Therefore, certain PME isoforms not only increase activity in response to damage, but also play a role in the proliferation of wound response. The role of JA signaling, however, for long-term PME responses to wounding is not known.

Mechanical damage of plant tissue associated with the harvesting of crops can lead to extreme MeOH flux events where mixing ratios of MeOH above an agricultural field can reach up to 75 ppbv (Karl *et al.*, 2001; Warneke *et al.*, 2002; Brunner *et al.*,

2007). Although damage-induced MeOH emissions are important for atmospheric

chemistry, the mechanisms regulating these responses are not yet well understood. In

this study, the influence of JA signaling for MeOH production and emission responses to

wounding was investigated in tomato (*Lycopersicon esculentum* Mill.). First, the

influence of PME activity on short-term (10 min) MeOH emission responses to wounding

was investigated. The influence of PME transcript expression and PME activity on

medium- (1 hr) and long-term (24 hrs) MeOH emission responses to wounding were also

investigated. Finally, transgenic *L. esculentum* with defective JA signaling (*jasmonic

acid-insensitive1* [*jai1-1*]) were used to investigate the relationship between JA signaling

and the PME pathway. PME transcription, PME activity and MeOH emission were

measured both locally and systemically in WT and *jai1-1* plants to test if JA signaling is

important for whole-plant responses to damage. It was hypothesized that short-term (10

min) MeOH emission responses to wounding would be the result of released MeOH

stored within the leaf and independent of PME activity. In contrast, medium- (1 hr) and

long-term (24 hrs) responses to wounding were hypothesized to involve elevated MeOH

emission, PME activity, and PME transcript expression. Finally, systemic MeOH

emission and production responses were predicted to be lower in transgenic tomato, as

systemic wound responses are mediated by JA signaling.

Methods

Experimental design and plant materials preparation

    Lycopersicon esculentum Mill. individuals were Micro Tom clones, a dwarf

variety of tomato (Meissner *et al.*, 1997). Plants were grown in the greenhouse at the

University of Virginia in Charlottesville, VA (38°N, 78°W). Pots were placed in flats

filled with 1 inch of water and were illuminated during a 16 hr 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

were kept insect-free using a variety of insecticides. All leaves were immature and

sampled three weeks past germination. Wounded leaves were crushed along the midvein

using metal forceps. The leaf adjacent to the wounded leaf was measured for systemic

responses.

Multiple measurements were conducted on each leaf in order to examine the

effect of wounding on local and systemic MeOH emission, PME activity and PME

transcript expression in WT and *jai1-1* leaves. Gas exchange and MeOH emission

measurements were conducted on two leaves per plant (local and systemic) for both WT

and *jai1-1* plant types. Measurements were conducted 10 min, 1 hr and 24 hrs post

treatment (n=5 for each treatment; 60 plants total). Transcription levels of PME-like

genes were only investigated 1 hr and 24 hrs post-treatment as transcription rates of

PMEs were not expected to significantly change within 10 min of wounding. Directly

following gas exchange and MeOH emission measurements, leaves were fast frozen in

liquid nitrogen. Each leaf was assayed for PME activity. Leaf tissue (n=5) from each

treatment was pooled for RNA isolation and PME transcription analysis.

Identification of jai1-1

Homozygous *jai1-1* plants (sterile mutants of Micro Tom *L. esculentum* that are

defective in JA signaling) were selected as described previously (Li *et al.*, 2004). Seeds

were germinated in closed Tupperware containers containing water-saturated filter

paper in the dark at room temperature. After 4 to 5 days, the filter paper was re-saturated

with a solution of 1 mM methyl jasmonate (MeJA). Seedlings were allowed to grow for

an additional 1-2 days before being evaluated for sensitivity to MeJA on the basis of

previously described phenotypes (Li *et al.*, 2004). Seedlings were then planted and later

screened using a PCR-based assay to distinguish the *jai1-1* deletion allele from the WT

allele. Genomic DNA of fresh leaves was extracted by grinding a 10 mg leaf disc in 0.5

N NaOH solution. The ground tissue was incubated in a boiling water bath for 1 min and

centrifuged at 10000 rpm for 30 sec. The 10 μl supernatant was transferred into 490 μl of

100 mM Tris (pH 8.0). The 5 μl diluted DNA extracting solution was used as a template

to perform PCR in 25 μl volume. For screening *jai1-1* mutant, the PCR primers were 5'-

GTG GAG ACG ATA TGT TGA GAC TAA-3' and 5'-GTG GTC ACG TCA GAG

CCC TCT ATT-3' (777 bp). PCR was performed on a C1000 thermal cycler (BIO-RAD,

INC. USA) for an initial step of 3 min at 94ºC, followed by 35 cycles of 30 s at 94ºC, 30

s at 56ºC, 60s at 72ºC, and a final 10 min extension step at 72ºC.

Gas exchange and 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 pre-concentration 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 ¼ inch Teflon tubing with a T-fitting in order to release extra flow. Flow rates through the cuvette ranged from 225 to 275 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 1330 hrs. PTR-MS measurements were recorded for 20 cycles for each sample. 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). All measurements were taken at PPFD 950 µmol m^{-2} s^{-1}. Leaves were allowed to stabilize for 10 min prior to taking the MeOH emission measurement with the exception of recently wounded leaves which were measured approximately 1 min after being inserted into the cuvette. All unwounded leaf measurements were taken under steady state conditions at leaf temperature 28 ±0.4°C, stomatal conductance 0.12 ±0.04 mol H_2O m^{-2} s^{-1}, photosynthetic rates 12.2 ±4 µmol CO_2 m^{-2} s^{-1}, and relative humidity 58 ±6% (means ± SD). Leaf surface area was quantified using a LI-COR Leaf Area Meter (LI-COR Inc., Lincoln, NE, USA). MeOH emission rates are expressed on a per unit area basis (nmol m^{-2} 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 lab (University of Miami) containing 3 ppmv (3 µl/l) ± 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%.

PME enzyme activity rates

PME activity was assayed using 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. 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 & Barrett, 2006).

RNA extraction, Reverse transcription and Amplification of DNA

Total RNA was extracted using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA), and 1.0 µg RNA was used to synthesize cDNA by Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) in a 20 µl reaction volume. The cDNA reactions were diluted to 40 µl, and 2 µl was used as a template for standard PCR. Three *L. esculentum*

genes, described as *PME* genes, were selected for the analysis (LEFL2023F06

abbreviated as *HTC1*, LEFL1053AH11 abbreviated as *HTC2*, and *PMEU1* abbreviated as

U1). Transcription of proteinase inhibitor ii (*PI-II*) gene was measured as a wound

response control. The housekeeper gene tomato translation initiation factor *elF4A* was

used as an expression control.

Accession Numbers

The GenBank accession numbers for the PME-like genes are AK327162.1

(*HTC1*), AK323293.1 (*HTC2*), and AY046596.1 (*U1*). The control genes were

proteinase inhibitor ii K03291 (*PI-II*) and initiation factor Al484542 (*elF4A*).

Statistical analysis

MeOH emission and PME activity measurements were examined in a two-way

MANOVA where the effect of plant type (WT, *jai1-1*), leaf type (control local, control

systemic, wound local, wound systemic), and time (10 min, 1 hr, 24 hrs) on MeOH

emission and PME activity was analyzed (Proc GLM, SAS 9.1; SAS Institute Inc., Cary,

NC, USA). Multiple comparisons of the least square means using Tukey-Kramer's

method were conducted to examine differences within each time period for MeOH

emission data (Proc GLM, SAS 9.1; SAS Institute Inc., Cary, NC, USA). PME data were

log transformed and MeOH emission data were inverse square root transformed to meet

normality and homogeneity of variance assumptions.

Results

Comparing overall PME and MeOH emission responses between WT and jai1-1 *plants*

In order to assess the influence of JA signaling on MeOH production and emission, local and systemic MeOH emission and PME activity were measured in wounded and control leaves of WT and *jai1-1* mutants. Measurements were conducted at three time scales in order to encompass short (10 min), medium (1 hr), and long-term (24 hrs) responses to damage. Across all time periods and treatments, WT and *jai1-1* plants had similar MeOH emission rates (F=2.26; df=1; P=0.13; Fig.1A) and PME activity (F=0.53; df=1; P=0.47; Fig.1B), indicating that JA signaling did not significantly influence overall MeOH production or emission.

PME and MeOH emission responses 10 min post wounding

The role of PME activity in short-term MeOH emission responses to wounding was investigated 10 min post treatment. Local and systemic PME activity and MeOH emission rates were measured in both WT and *jai1-1* plants. Wounded WT and *jai1-1* leaves had significantly elevated MeOH flux 10 min post wounding (least square means, Tukey-Kramer test, P<0.05; Fig.2A), but PME activity showed no response to the treatment in either plant type (Fig.2B). On average, WT plants had a stronger MeOH emission response to wounding (average WT and *jai1-1* MeOH emissions ±SE were 32.5 ±6 and 20.8 ±5 nmol m^{-2} s^{-1} respectively), but no significant difference was detected between plant types at this time scale. Wounded leaves emitted on average six times more MeOH than controls. No systemic MeOH emission responses were detected.

PME and MeOH emission responses 1 hr post wounding

In addition, local and systemic MeOH production and emission responses to wounding 1 hr post treatment were investigated. WT and *jai1-1* plants were used to test if the JA pathway is important in mediating MeOH production and emission responses to damage. Although wounded WT leaves had significantly elevated MeOH flux 1 hr post wounding (least square means, Tukey-Kramer test, $P<0.05$; Fig.3A), PME activity was not elevated (Fig.3B). Wounded *jai1-1* leaves did not have significantly different MeOH flux from control or WT leaves 1hr post wounding (average WT and *jai1-1* MeOH emissions ±SE were 9.6 ±2 and 7.6 ±2 nmol m^{-2} s^{-1} respectively). Similarly to WT plants, PME activity in wounded *jai1-1* leaves did not change in response to the treatment (Fig.3B). No systemic MeOH emission (Fig.2A) or systemic PME activity response (Fig.3B) to wounding was detected in either plant type.

PME and MeOH emission responses 24 hr post wounding

Finally, long-term (24 hrs) PME and MeOH emission responses to wounding were investigated in *jai1-1* and WT plants to test for sustained changes in local and systemic MeOH production and emission. It was hypothesized that the JA pathway regulates long-term MeOH production and emission responses to wounding. Contrary to the hypothesis, MeOH emission responses from wounded WT and *jai1-1* leaves had returned to control levels (average wounded WT and *jai1-1* MeOH emissions ±SE were 2.2 ±0.3 and 3.3 ±0.5 nmol m^{-2} s^{-1} respectively; Fig.4A) and no changes in PME activity were observed 24 hrs post wounding (Fig.4B). Similarly to short- and medium-term

responses, no systemic MeOH emission (Fig.4A) or systemic PME activity (Fig.4B)

responses were observed in either plant type.

Transcriptional responses to wounding

In order to test if the mechanical damage treatment elicited the wound response

pathway, the expression of proteinase inhibitor II (*PI-II*) in leaf tissues 1 hr and 24 hrs

post treatment was analyzed. *PI-II* was locally up-regulated in WT plants both 1 hr and

24 hrs post wounding (Fig.5). No systemic up-regulation was observed in WT plants at

either time period. Transgenic *jai1-1* plants did not express *PI-II* at any time period for

either control or wounded leaves, confirming that *jai1-1* mutants have a deficient JA

signaling system.

The influence of wounding on the expression of individual PME genes was

investigated both locally and systemically 1 hr and 24 hrs post wounding. Transcription

levels of three PME candidate genes were measured in WT and *jai1-1* plants in order to

test if JA signaling influences PME transcription rates. While overall PME activity did

not show significant responses to wounding, the transcription levels of two PME-like

genes were wound-responsive. Locally and systemically expressed PME candidate genes

HTC1 and *HTC2* were both down-regulated 1 hr and 24 hrs post wounding in WT plants

(Fig.5). Local responses 1 hr post wounding were not detected in *jai1-1* plants, however

HTC1 and *HTC2* were down-regulated systemically. *Jai1-1* plants also down-regulated

HTC1 and *HTC2* in response to wounding both locally and systemically 24 hrs post

wounding. Overall, PME transcription responses to wounding were similar in WT and

jai1-1 plants, suggesting that the transcription responses of the three PME candidate

genes were not JA-dependent. PME-like gene *U1* showed no response to treatment at either time period, indicating that *U1* was not influenced by wounding (Fig.5).

Discussion

The measurements reported here were undertaken to elucidate the mechanisms regulating PME transcription, PME activity, and MeOH emission responses to wounding. Results showed that MeOH emissions from locally wounded leaves of WT and *jai1-1* plants were elevated 10 min post treatment. PME activity was not up-regulated 10 min post wounding, a result in agreement with the hypothesis that short-term emissions reflect the release of a stored pool of MeOH and not PME response to damage. Similarly, MeOH emissions were significantly elevated 1 hr post wounding in WT plants. *Jai1-1* emissions were similar to WT emission 1 hr post wounding, but were not significantly different from controls (Fig.3A). PME activity was also not up-regulated in either plant type 1 hr post treatment. Wounded leaf tissue may not have fully healed 1 hr post wounding, resulting in decreased diffusive barriers or "leaky" leaf tissue. Similar emission responses from WT and *jai1-1* plants suggested that JA signaling did not play a significant role in the regulation of short- and medium-term MeOH emission responses to damage. Overall, the results suggest that short- and medium-term MeOH emission responses to wounding were not the result of elevated PME activity or MeOH production. The size of the MeOH pool stored within leaves appears to be the most significant factor regulating short-term emission responses to wounding. Estimating the amount of stored MeOH would therefore be helpful in predicting the size and significance of large-scale wounding events, such as crop harvesting, for atmospheric chemistry.

Long-term MeOH production and emission responses to mechanical damage were not detected. By 24 hrs, wounded WT and *jai1-1* MeOH emissions and PME activity levels were the same as controls. Surprisingly, transcription rates of two PME-like genes were down-regulated 24 hrs post wounding. Changes in the expression of PMEs, however, did not have a significant effect on overall PME activity or MeOH emission. It is important to note that the up-regulation of certain PME isoforms could have occurred, but were not strong and/or prolonged enough to be detected in the total PME activity assay. Korner *et al.* (2009) similarly reported that while certain herbivore-inducible genes were up-regulated in response to simulated herbivory, overall PME activity did not show a significant response. The measurement of every PME isoform may therefore be necessary in order to understand how the regulation of PME influences MeOH production and emission.

The lack of long-term MeOH emission and overall PME response in our system may be due to the purely mechanical damage treatment. Mechanical damage in tomato is known to elicit the JA pathway and lead to the accumulation of proteinase inhibitors (Green & Ryan, 1972; Li *et al.*, 2002). As MeOH emission and PME response are involved in the wound response pathway, mechanical damage was expected to be a strong elicitor. However, previous work has shown that applying herbivore oral secretions to the wound site induces greater PME transcript expression and MeOH emission responses compared to wounding alone (von Dahl *et al.*, 2006). The down-regulation of certain PME isoforms observed in this study may therefore be a response to mechanical damage while the up-regulation of other isoforms, such as *NaPME* as observed in Korner *et al.* (2009), may be a response to herbivore cues. In other words,

PMEs may vary in the type of response (i.e. increase or decrease transcription) as well as the duration of response (i.e. from hours to days) depending on the chemical and physical cues resulting from the damage. In addition, wound-responsive *HTC1* and *HTC2* may belong to a separate clade of PME genes distally related to genes such as *NaPME* which may help explain why they are differentially regulated. Using BLAST alignment (Altschul *et al.*, 1990) to compare the sequences of *HTC1* and *HTC2* with *NaPME*, we found that *HTC1* and *HTC2* had little to no significant overlap with *NaPME* (2% and 0% for *HTC1* and *HTC2*, respectively). A complete phylogenetic analysis of these PME candidate genes, however, has yet to be conducted. The specific MeOH emission and production responses to damage versus herbivory are therefore not well understood and need further investigation.

While systemic responses in MeOH emission and PME activity were not detected, systemic changes in the transcription levels of PMEs were influenced by the wounding treatment. In contrast to previous studies (Schmidt *et al.*, 2005; von Dahl *et al.*, 2006), long-term down-regulation of *HTC1* and *HTC2* was observed locally and systemically for both WT and *jai1-1* plants. Many systemic responses to damage are primarily mediated by wound signaling compound JA (Stratmann, 2003). The results, however, suggest that JA does not play a role in regulating systemic PME responses to damage. The observed down-regulation may instead be a response to whole plant metabolic responses to damage or non-JA wound signaling compounds. Damage to leaf tissue is known to induce a range of physiological responses such as changes in photosynthetic rate, carbon storage, and growth (Baldwin & Preston, 1999). Although not always the case, herbivory is generally thought to decrease the transcription of photosynthesis-related genes in order to

direct resources to secondary metabolism (Schmidt *et al.*, 2005). Down-regulation of expansins, proteins associated with cell wall loosening, is also known to occur in order to divert resources away from growth and towards defense (Downie *et al.*, 2004). Similarly, down-regulation of PME genes may be the result of diverting resources away from growth. Additionally, non-JA signaling compounds may influence the expression of *HTC1* and *HTC2*. Microarray analyses have indicated that PME transcript levels respond to wound signaling compounds such as ethylene (De Paepe *et al.*, 2004) and oligogalacturonides (Moscatiello *et al.*, 2006). However, the regulation of transcription of PMEs is not well understood and preliminary studies are only beginning to shed light on these processes (Pelloux *et al.*, 2007). It is clear that further studies are necessary to resolve the above speculations.

Interestingly, PME candidate gene *U1* did not respond to the treatment. Using sequence alignment in BLAST (Altschul *et al.*, 1990), *HTC1* and *HTC2* were found to have 48% overlap (max score 217), while *HTC1* and *HTC2* had no significant overlap with *U1*. As *U1* is more distally related to *HTC1* and *HTC2*, it may be differentially regulated and belong to a separate clade of PME genes. The transcription levels of PME candidate genes *HTC1*, *HTC2* and *U1* demonstrate that PME response to damage is not uniform. Further investigation of the functional significance and expression of PME isoforms is necessary in order to understand how plant cell walls respond to damage and how those responses influence MeOH production and emission.

Damage-induced MeOH emissions can significantly increase local MeOH mixing ratios and are important for atmospheric chemistry (Karl *et al.*, 2001; Warneke *et al.*, 2002; Brunner *et al.*, 2007). Therefore, it is valuable to understand the dynamics of

MeOH production and emission in response to mechanical damage. The results showed that short-term emission responses to wounding reflect the release of a stored pool of MeOH resulting in emissions six times higher than control levels. Medium-term emission responses are likely the result of decreased diffusive barriers due to unhealed leaf tissue. This study shows, for the first time, local and systemic down-regulation of PME transcription in response to damage. While the roles of PMEs in wound-response are not well constrained, it is clear that PMEs are influenced by wounding and herbivory in different ways depending on the PME isoform. Although the results show that this response is not JA dependent, the mechanism for PME response to wounding remains unknown. More information on the regulation of PMEs in plants is needed in order to understand how wounding influences cell wall metabolism and MeOH production in plants. Greater understanding of the PME pathway will also lead to improved predictive abilities for modeling MeOH production and emission.
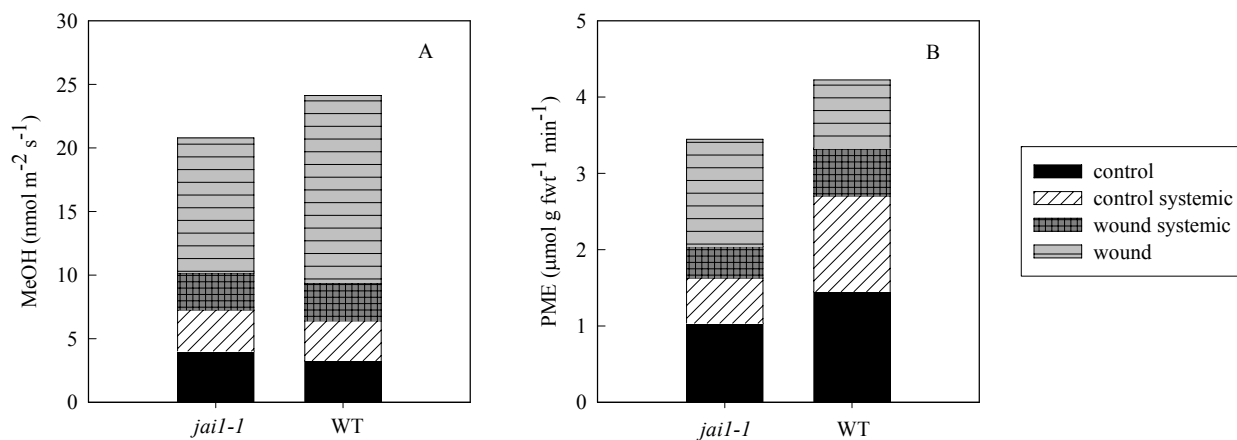
Acknowledgements

Figure 1. Leaf MeOH emission and PME activity represented as stacked bars averaged across all time scales for *jai1-1* and WT *L. esculentum*. Measured leaves were wounded, adjacent to wounded leaves (wound systemic), adjacent to control leaves (control systemic), and control (*n*=5 for each treatment). WT and *jai1-1* plants were similar overall in MeOH emission (*F*=2.26; df=1; *P*=0.13) and PME activity rates (*F*=0.53; df=1; *P*=0.47).
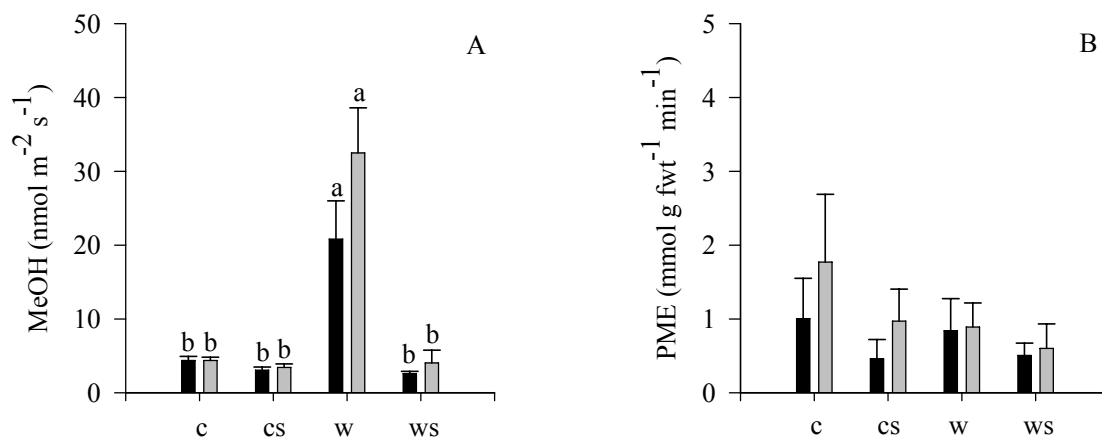
Figure 2. Mean (±SE) leaf MeOH emission (A) and PME activity (B) for WT (black

bars) and *jai1-1* (gray bars) *L. esculentum* 10 min post treatment. Leaves were

mechanically wounded (w) and systemically wounded (leaf adjacent to wounded leaf;

ws). Control leaves (c) and leaves adjacent to controls (cs) remained untreated (*n*=5 for

each treatment). Wounded WT and *jai1-1* leaves had significantly higher MeOH flux

relative to controls. PME activity did not respond to treatment in either plant type.

Significant differences between means are marked with different letters (least square
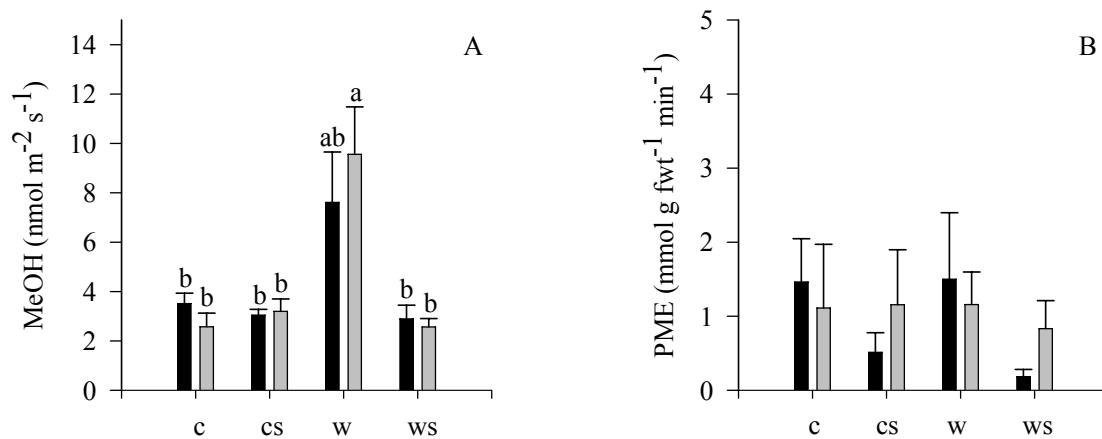
means, Tukey-Kramer, *P*<0.05).

Figure 3. Mean (±SE) leaf MeOH emission (A) and PME activity (B) for WT (black bars) and *jai1-1* (gray bars) *L. esculentum* 1 hr post treatment. Leaves were mechanically wounded (w) and systemically wounded (leaf adjacent to wounded leaf; ws). Control leaves (c) and leaves adjacent to controls (cs) remained untreated (n=5 for each treatment). WT MeOH emissions were significantly elevated relative to controls, while *jai1-1* plants were not significantly different from controls. PME activity did not respond to treatment in either plant type. Significant differences between means are marked with different letters (least square means, Tukey-Kramer, $P<0.05$).
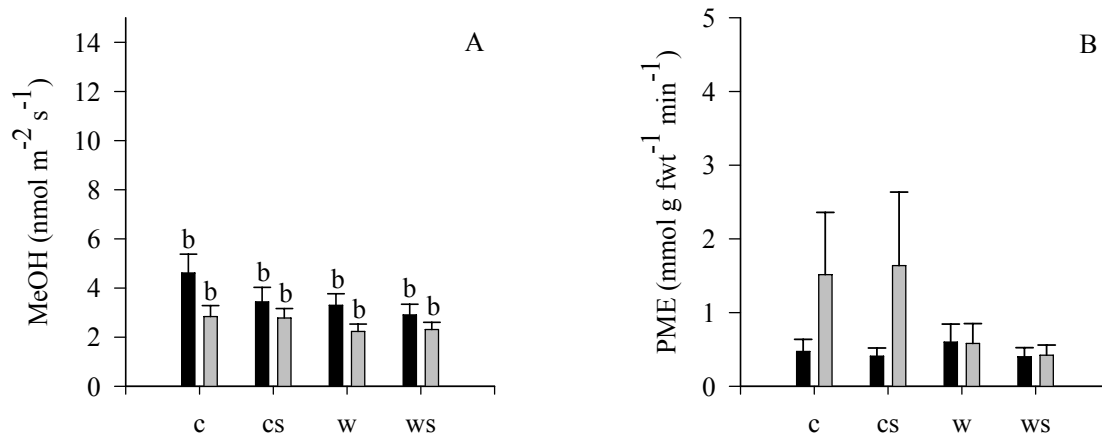
Figure 4. Mean (±SE) leaf MeOH emission (A) and PME activity (B) for WT (black bars) and *jai1-1* (gray bars) *L. esculentum* 24 hrs post treatment. Leaves were mechanically wounded (w) and systemically wounded (leaf adjacent to wounded leaf; ws). Control leaves (c) and leaves adjacent to controls (cs) remained untreated (*n*=5 for each treatment). MeOH emissions from WT and *jai1-1* plants were not significantly different from control plants. PME activity did not respond to treatment in either plant type. Significant differences between means are marked with different letters (least square means, Tukey-Kramer, *P*<0.05).
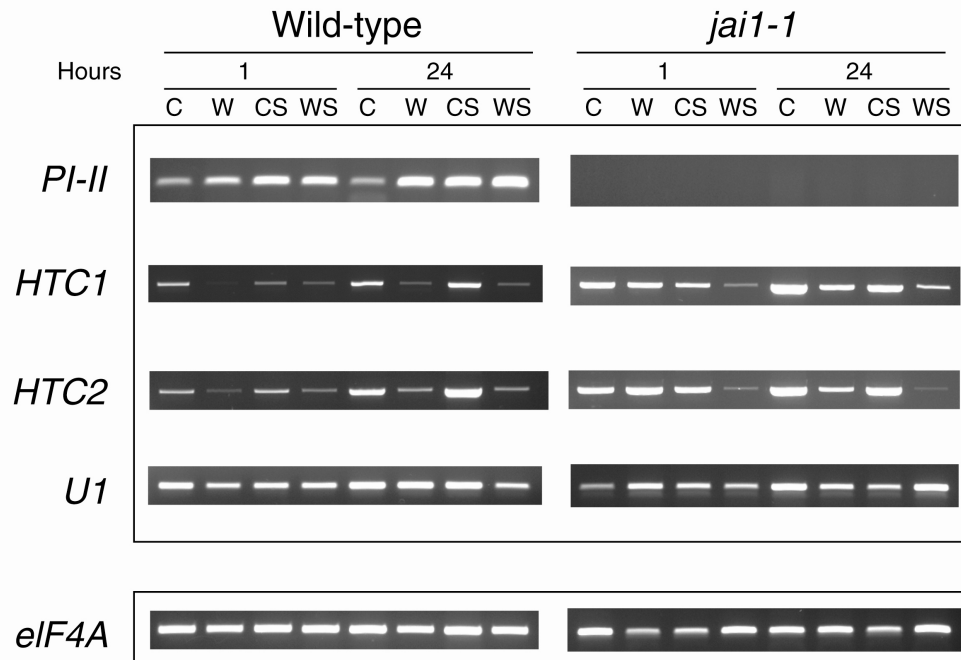
Figure 5. WT and *jai1-1* plants were sampled 1 hr and 24 hrs post treatment (c, control; w, wounded; cs; adjacent to control; ws; adjacent to wounded). Leaves from each treatment were pooled for RNA extraction (*n*=5 for each treatment). RNA gel blots were hybridized to cDNA probes representing 3 PME-like genes (*HTC1*, *HTC2* and *U1*). Blots were also hybridized to proteinase inhibitor II (*PI-II*) and, as a loading control, *elF4A*.

Chapter 5

Conclusion

Summary

In Chapters 2-4, I investigated the mechanisms regulating MeOH production and emission. Chapter 2 investigated the source of MeOH in mature leaves. Results from Chapter 2 suggested that MeOH emissions from mature and immature leaves were derived from the same pathway. The data also suggested that below-ground MeOH production did not significantly contribute to mature leaf emission. Finally, leaf PME activity was related to MeOH emission but could only explain a fraction of the variation in MeOH flux. Chapter 3 investigated the direct effect of light on short-term changes in MeOH production and emission. After controlling for stomatal conductance and temperature, light did not directly influence PME activity or MeOH emission over short time scales. Chapter 4 investigated the influence of mechanical damage on MeOH production and emission over multiple time scales and compared responses between WT and JA-deficient plants. Neither PME transcription nor MeOH emission responses to wounding were JA dependent, suggesting that JA signaling does not play a significant role in MeOH production and emission responses to wounding. In contrast with previously observed PME transcription responses to herbivory (von Dahl *et al.*, 2006), local and systemic PME transcription decreased post-wounding, suggesting that herbivory and wounding may elicit different MeOH production and emission responses.

The results from Chapters 2-4 have increased our understanding of phytogenic MeOH production and emission and have implications for plant biology and atmospheric chemistry. My results provide basic plant biological information concerning the influence of leaf ontogeny, spatial heterogeneity of PME activity, light environment, mechanical damage, and hormone signaling for MeOH production and emission. The results also have implications for atmospheric chemistry as they evaluated the feasibility of using PME activity for predicting MeOH emission, the ontogenetic influences on stable carbon isotopic signatures of MeOH, the environmental regulation of short-term changes in MeOH emission, and the influence of mechanical damage for MeOH production and emission. Although multiple hypotheses were tested in Chapters 2-4, many lingering questions concerning the regulation of MeOH flux remain.

Future directions

Future research should explore which physiological variables are needed in order to predict changes in MeOH production and emission. Specifically, there are three variables that deserve further investigation: MeOH catabolism, PME substrate limitation, and transcript regulation of PMEs. First, we need to explore the significance of MeOH catabolism for the regulation of MeOH emission. Unexplained variation in foliar PME activity and MeOH concentration in leaves, as well as enriched isotopic signatures of MeOH emissions relative to the pectin methoxyl pool, suggest the presence of a MeOH sink. Although studies have shown that plant cells catabolize MeOH (Cossins, 1964; Gout *et al.*, 2000), rates of MeOH catabolism in vivo have not been measured. To understand the significance of catabolism for MeOH emission, plants could be exposed to

isotopically labeled MeOH and later measured for the proportion of labeled MeOH remaining in the leaf, the proportion that is catabolized, and the proportion that is emitted. Comparing rates of catabolism between young and mature leaves could help determine how ontogeny influences a leaf's tendency to catabolize MeOH. Additional experimentation with ambient CO_2 concentrations and temperature could influence carbon demand and give insight into the environmental factors regulating rates of methanol catabolism. A previous study showed that incorporation of methanol-derived carbon into tissues increased when the tissues were incubated in oxygen compared to air (Cossins, 1964), suggesting that carbon-starved cells are more likely to catabolize methanol. Experiments such as these could lead to a better understanding of how to predict rates of MeOH catabolism and the influence of catabolism on emission rates. Additionally, activity measurements of enzymes responsible for catabolizing MeOH, such as NAD-dependent formaldehyde dehydrogenase (FALD) and NAD-dependent formate dehydrogenase (FDH), could be evaluated in relation to MeOH emission rates. More information on the pathway for MeOH catabolism may be required to carry out experiments using enzyme activity as a proxy for catabolism. A model assuming that all MeOH produced within a plant is emitted, such as Galbally and Kirstine's global methanol model (2002), may overestimate phytogenic MeOH flux. If physiological studies can describe rates of MeOH catabolism, models such as the Galbally and Kirstine model (2002) could incorporate a catabolism term and improve the accuracy of their predictions.

PME substrate availability is another variable to consider for mechanistic model development. Chapter 1 showed that PME activity alone cannot predict emissions,

suggesting that PME may be substrate limited. Enzyme activity may only be a strong

predictor when PME is not substrate limited. Similarly, isoprene synthase activity has

been shown to be a sufficient predictor of isoprene emissions when isoprene synthase is

not substrate limited (Logan *et al.*, 2000). Chemical analysis of methylesterification of

galacturonic acid (GA) has been conducted in various plant tissues (McFeeters &

Armstrong, 1984; Femenia *et al.*, 1998), but has yet to be related to MeOH emission.

Measuring the amount of available methylesterified GA in leaves in conjunction with

measurements of PME activity, MeOH concentration, g_s, and MeOH emission could

provide critical insight for mechanistic model development.

Investigating the influence of PME transcription on MeOH production and

emission could also be valuable. The down-regulation of PME candidate genes measured

in Chapter 4 were not associated with down-regulated MeOH emission. As there are

numerous isoforms of PME in plants (Pelloux *et al.*, 2007), a comprehensive

investigation of PME transcription must be conducted in order to understand how

transcript regulation of PME influences MeOH emission. In order to gain more

information about the diverse set of PME genes, I propose a comprehensive phylogenetic

analysis of PME genes. If the major clades of PME are identified in multiple species, the

functional significance of each PME clade could be identified. The transcription rate

responses of each major clade of PME could be measured in response to stimuli such as

light environment, temperature, herbivory, and mechanical damage. Comparing PME

transcription between mature and immature leaves could reveal how ontogenetic factors

regulate PME transcription. These experiments would reveal the transcriptional control

of MeOH production and emission and also give insight into the regulation of cell wall metabolism.

MeOH production and emission remains an understudied process despite its implications for cell wall metabolism and atmospheric chemistry. Future efforts involving the investigation of MeOH catabolism, PME substrate limitation, and PME transcription are intriguing avenues for future research and will serve as new and exciting ways to link plant physiology to atmospheric science.

Literature Cited

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**(3): 403-410.

Anthon GE, Barrett DM. 2006. Characterization of the Temperature Activation of Pectin Methylesterase in Green Beans and Tomatoes. *Journal of Agricultural and Food Chemistry* **54**(1): 204-211.

Atkinson R. 2000. Atmospheric Chemistry of Vocs and Nox. *Atmospheric Environment* **34**(12-14): 2063-2101.

Atkinson R, Arey J. 1998. Atmospheric Chemistry of Biogenic Organic Compounds. *Accounts of Chemical Research* **31**(9): 574-583.

Badeck F-W, Tcherkez G, Nogués S, Piel C, Ghashghaie J. 2005. Post-Photosynthetic Fractionation of Stable Carbon Isotopes between Plant Organs—a Widespread Phenomenon. *Rapid Communications in Mass Spectrometry* **19**(11): 1381-1391.

Baldwin IT, Preston CA. 1999. The Eco-Physiological Complexity of Plant Responses to Insect Herbivores. *Planta* **208**(2): 137-145-145.

Bordenave M, Goldberg R. 1994. Immobilized and Free Apoplastic Pectinmethylesterases in Mung Bean Hypocotyl. *Plant Physiology* **106**(3): 1151-1156.

Brunner A, Ammann C, Neftel A, Spirig C. 2007. Methanol Exchange between Grassland and the Atmosphere. *Biogeosciences* **4**(3): 395-410.

Choi W, Faloona IC, Bouvier-Brown NC, McKay M, Goldstein AH, Mao J, Brune WH, LaFranchi BW, Cohen RC, Wolfe GM, Thornton JA, Sonnenfroh DM, Millet DB. 2010. Observations of Elevated Formaldehyde over a Forest Canopy Suggest Missing Sources from Rapid Oxidation of Arboreal Hydrocarbons. *Atmospheric Chemistry and Physics* **10**(18): 8761-8781.

Ciccioli P, Fabozzi C, Brancaleoni E, Cecinato A, Frattoni M, Loreto F, Kesselmeier J, Schafer L, Bode K, Torres L, Fugit JL. 1997. Use of the Isoprene Algorithm for Predicting the Monoterpene Emission from the Mediterranean Holm Oak *Quercus Ilex* L.: Performance and Limits of This Approach. *J. Geophys. Res-Atmos.* **102**(D19): 23319-23328.

Cossins EA. 1964. The Utilization of Carbon-1 Compounds from Plants. *Canadian Journal of Biochemistry* **42**: 1793-1802.

De Paepe A, Vuylsteke M, Van Hummelen P, Zabeau M, Van Der Straeten D. 2004. Transcriptional Profiling by Cdna-Aflp and Microarray Analysis Reveals Novel Insights into the Early Response to Ethylene in Arabidopsis. *The Plant Journal* **39**(4): 537-559.

Di Carlo P, Brune WH, Martinez M, Harder H, Lesher R, Ren X, Thornberry T, Carroll MA, Young V, Shepson PB, Riemer D, Apel E, Campbell C. 2004. Missing Oh Reactivity in a Forest: Evidence for Unknown Reactive Biogenic Vocs. *Science* **304**(5671): 722-725.

Doronina NV, Ivanova EG, Suzina NE, Trotsenko YA. 2004. Methanotrophs and Methylobacteria Are Found in Woody Plant Tissues within the Winter Period. *Microbiology* **73**(6): 702-709.

Downie A, Miyazaki S, Bohnert H, John P, Coleman J, Parry M, Haslam R. 2004. Expression Profiling of the Response of Arabidopsis Thaliana to Methanol Stimulation. *Phytochemistry* **65**(16): 2305-2316.

Ellis C, Karafyllidis I, Wasternack C, Turner JG. 2002. The Arabidopsis Mutant Cev1 Links Cell Wall Signaling to Jasmonate and Ethylene Responses. *Plant Cell* **14**(7): 1557-1566.

Elzenga JTM, Staal M, Prins HBA. 1997. Calcium-Calmodulin Signalling Is Involved in Light-Induced Acidification by Epidermal Leaf Cells of Pea, *Pisum Sativum* L. *J. Exp. Bot.* **48**(12): 2055-2061.

Fall R. 2003. Abundant Oxygenates in the Atmosphere: A Biochemical Perspective. *Chemical Reviews* **103**(12): 4941-4951.

Fall R, Benson AA. 1996. Leaf Methanol - the Simplest Natural Product from Plants. *Trends in Plant Science* **1**(9): 296-301.

Fall R, Custer TG, Kato S, Bierbaum VM. 2001. New Directions: The Biogenic Acetone-Hcn Connection. *Atmospheric Environment* **35**(9): 1713-1714.

Fall R, Wildermuth MC. 1998. Isoprene Synthase: From Biochemical Mechanism to Emission Algorithm. *Journal of Geophysical Research* **103**(D19): 25599-25609.

Femenia A, Garosi P, Roberts K, Waldron KW, Selvendran RR, Robertson JA. 1998. Tissue-Related Changes in Methyl-Esterification of Pectic Polysaccharides in Cauliflower (*Brassica Oleracea* L. Var. *Italica*) Stems. *Planta* **205**(3): 438-444.

Foggo A. 1996. Long- and Short-Term Changes in Plant Growth Following Simulated Herbivory: Adaptive Responses to Damage? *Ecological Entomology* **21**(2): 198-202.

Folkers A, Hüve K, Ammann C, Dindorf T, Kesselmeier J, Kleist E, Kuhn U, Uerlings R, Wildt J. 2008. Methanol Emissions from Deciduous Tree Species: Dependence on Temperature and Light Intensity. *Plant Biology* **10**(1): 65-75.

Frenkel C, Peters JS, Tieman DM, Tiznado ME, Handa AK. 1998. Pectin Methylesterase Regulates Methanol and Ethanol Accumulation in Ripening Tomato (*Lycopersicon Esculentum*) Fruit. *Journal of Biological Chemistry* **273**(8): 4293-4295.

Fuentes JD, Gu L, Lerdau M, Atkinson R, Baldocchi D, Bottenheim JW, Ciccioli P, Lamb B, Geron C, Guenther A, Sharkey TD, Stockwell W. 2000. Biogenic Hydrocarbons in the Atmospheric Boundary Layer: A Review. *Bulletin of the American Meteorological Society* **81**(7): 1537-1575.

Fuentes JD, Wang D. 1999. On the Seasonality of Isoprene Emissions from a Mixed Temperate Forest. *Ecological Applications* **9**(4): 1118-1131.

Galbally IE, Kirstine W. 2002. The Production of Methanol by Flowering Plants and the Global Cycle of Methanol. *Journal of Atmospheric Chemistry* **43**(3): 195-229.

Geron C, Guenther A, Sharkey T, Arnts RR. 2000. Temporal Variability in Basal Isoprene Emission Factor. *Tree Physiology* **20**(12): 799-805.

Ghashghaie J, Badeck F-W, Lanigan G, Nogués S, Tcherkez G, Deléens E, Cornic G, Griffiths H. 2003. Carbon Isotope Fractionation During Dark Respiration and Photorespiration in C3 Plants. *Phytochemistry Reviews* **2**(1): 145-161.

Giebel BM, Swart PK, Riemer DD. 2010. Δ^{13}c Stable Isotope Analysis of Atmospheric Oxygenated Volatile Organic Compounds by Gas Chromatography-Isotope Ratio Mass Spectrometry. *Analytical Chemistry* **82**(16): 6797-6806.

Goldberg R, Morvan C, Jauneau A, Jarvis MC 1996. Methyl-Esterification, De-Esterification and Gelation of Pectins in the Primary Cell Wall. In: J. VisserA. G. J. Voragen eds. *Pectins and Pectinases*. Amsterdam: Elsevier Science, 151-172.

Goldstein AH, Galbally IE. 2007. Known and Unexplored Organic Constituents in the Earth's Atmosphere. *Environmental Science & Technology* **41**(5): 1514-1521.

Gout E, Aubert S, Bligny R, Rebeille F, Nonomura AR, Benson AA, Douce R. 2000. Metabolism of Methanol in Plant Cells. Carbon-13 Nuclear Magnetic Resonance Studies. *Plant Physiology* **123**(1): 287-296.

Gray DW, Goldstein AH, Lerdau MT. 2005. The Influence of Light Environment on Photosynthesis and Basal Methylbutenol Emission from *Pinus Ponderosa*. *Plant Cell Environ.* **28**(12): 1463-1474.

Gray DW, Goldstein AH, Lerdau MT. 2006. Thermal History Regulates Methylbutenol Basal Emission Rate in *Pinus Ponderosa*. *Plant Cell Environ.* **29**(7): 1298-1308.

Green TR, Ryan CA. 1972. Wound-Induced Proteinase Inhibitor in Plant Leaves: A Possible Defense Mechanism against Insects. *Science* **175**(4023): 776-777.

Guenther A, Geron C, Pierce T, Lamb B, Harley P, Fall R. 2000. Natural Emissions of Non-Methane Volatile Organic Compounds; Carbon Monoxide, and Oxides of Nitrogen from North America. *Atmospheric Environment* **34**(12-14): 2205-2230.

Haagen-Smit AJ. 1952. Chemistry and Physiology of Los Angeles Smog. *Industrial & Engineering Chemistry* **44**(6): 1342-1346.

Hanson DT, Sharkey TD. 2001. Effect of Growth Conditions on Isoprene Emission and Other Thermotolerance-Enhancing Compounds. *Plant Cell Environ.* **24**(9): 929-936.

Harley P, Greenberg J, Niinemets U, Guenther A. 2007. Environmental Controls over Methanol Emission from Leaves. *Biogeosciences* **4**(6): 1083-1099.

Harley P, Guenther A, Zimmerman P. 1996. Effects of Light, Temperature and Canopy Position on Net Photosynthesis and Isoprene Emission from Sweetgum (*Liquidambar Styraciflua*) Leaves. *Tree Physiology* **16**(1-2): 25-32.

Harley P, Guenther A, Zimmerman P. 1997. Environmental Controls over Isoprene Emission in Deciduous Oak Canopies. *Tree Physiology* **17**(11): 705-714.

Heikes BG, Chang WN, Pilson MEQ, Swift E, Singh HB, Guenther A, Jacob DJ, Field BD, Fall R, Riemer D, Brand L. 2002. Atmospheric Methanol Budget and Ocean Implication. *Global Biogeochemical Cycles* **16**(4).

Holzinger R, Lee A, Paw KT, Goldstein AH. 2005. Observations of Oxidation Products above a Forest Imply Biogenic Emissions of Very Reactive Compounds. *Atmospheric Chemistry and Physics* **5**: 67-75.

Hüve K, Christ MM, Kleist E, Uerlings R, Niinemets U, Walter A, Wildt J. 2007. Simultaneous Growth and Emission Measurements Demonstrate an Interactive Control of Methanol Release by Leaf Expansion and Stomata. *Journal of Experimental Botany* **58**(7): 1783-1793.

Jacob DJ, Field BD, Li QB, Blake DR, de Gouw J, Warneke C, Hansel A, Wisthaler A, Singh HB, Guenther A. 2005. Global Budget of Methanol: Constraints from Atmospheric Observations. *Journal of Geophysical Research-Atmospheres* **110**(D8).

Kanakidou M, Seinfeld JH, Pandis SN, Barnes I, Dentener FJ, Facchini MC, Van Dingenen R, Ervens B, Nenes A, Nielsen CJ, Swietlicki E, Putaud JP, Balkanski Y, Fuzzi S, Horth J, Moortgat GK, Winterhalter R, Myhre CEL, Tsigaridis K, Vignati E, Stephanou EG, Wilson J. 2005. Organic Aerosol and Global Climate Modelling: A Review. *Atmospheric Chemistry and Physics* **5**: 1053-1123.

Karl T, Guenther A, Jordan A, Fall R, Lindinger W. 2001. Eddy Covariance Measurement of Biogenic Oxygenated Voc Emissions from Hay Harvesting. *Atmospheric Environment* **35**(3): 491-495.

Karl T, Guenther A, Spirig C, Hansel A, Fall R. 2003. Seasonal Variation of Biogenic Voc Emissions above a Mixed Hardwood Forest in Northern Michigan. *Geophys. Res. Lett.* **30**(23).

Keppler F, Harper DB, Rockmann T, Moore RM, Hamilton JTG. 2005. New Insight into the Atmospheric Chloromethane Budget Gained Using Stable Carbon Isotope Ratios. *Atmospheric Chemistry and Physics* **5**: 2403-2411.

Keppler F, Kalin RM, Harper DB, McRoberts WC, Hamilton JTG. 2004. Carbon Isotope Anomaly in the Major Plant C-1 Pool and Its Global Biogeochemical Implications. *Biogeosciences* **1**(2): 123-131.

Kessler A, Baldwin IT. 2002. Plant Responses to Insect Herbivory: The Emerging Molecular Analysis. *Annual Review of Plant Biology* **53**(1): 299-328.

Ko J-H, Kim JH, Jayanty SS, Howe GA, Han K-H. 2006. Loss of Function of Cobra, a Determinant of Oriented Cell Expansion, Invokes Cellular Defence Responses in Arabidopsis Thaliana. *Journal of Experimental Botany* **57**(12): 2923-2936.

Korner E, von Dahl CC, Bonaventure G, Baldwin IT. 2009. Pectin Methylesterase Na*pme1* Contributes to the Emission of Methanol During Insect Herbivory and to the Elicitation of Defence Responses in *Nicotiana Attenuata*. *Journal of Experimental Botany* **60**(9): 2631-2640.

Kreuzwieser J, Harren FJM, Laarhoven LJJ, Boamfa I, te Lintel-Hekkert S, Scheerer U, Huglin C, Rennenberg H. 2001. Acetaldehyde Emission by the Leaves of Trees - Correlation with Physiological and Environmental Parameters. *Physiologia Plantarum* **113**(1): 41-49.

Kreuzwieser J, Scheerer U, Rennenberg H. 1999. Metabolic Origin of Acetaldehyde Emitted by Poplar (*Populus Tremula* X *P-Alba*) Trees. *Journal of Experimental Botany* **50**(335): 757-765.

Leavitt SW, Long A. 1985. Stable-Carbon Isotopic Composition of Maple Sap and Foliage. *Plant Physiology* **78**(2): 427-429.

Leegood RC. 1993. *Carbon Metabolism Photosynthesis and Production in a Changing Environment*: Chapman and Hall.

Lehning A, Zimmer I, Steinbrecher R, Bruggemann N, Schnitzler JP. 1999. Isoprene Synthase Activity and Its Relation to Isoprene Emission in *Quercus Robur* L-Leaves. *Plant Cell Environ.* **22**(5): 495-504.

Lehning A, Zimmer W, Zimmer I, Schnitzler JP. 2001. Modeling of Annual Variations of Oak (*Quercus Robur* L.) Isoprene Synthase Activity to Predict Isoprene Emission Rates. *J. Geophys. Res-Atmos.* **106**(D3): 3157-3166.

Lelieveld J, Butler TM, Crowley JN, Dillon TJ, Fischer H, Ganzeveld L, Harder H, Lawrence MG, Martinez M, Taraborrelli D, Williams J. 2008. Atmospheric Oxidation Capacity Sustained by a Tropical Forest. *Nature* **452**(7188): 737-740.

Lerdau M 1991. Plant Function and Biogenic Terpene Emission. In: T. D. Sharkey, E. A. HollandH. A. Mooney eds. *Trace Gas Emissions by Plants*: Academic Press, Inc., 121-134.

Li L, Li C, Lee GI, Howe GA. 2002. Distinct Roles for Jasmonate Synthesis and Action in the Systemic Wound Response of Tomato. *PNAS* **99**(9): 6416-6421.

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA. 2004. The Tomato Homolog of Coronatine-Insensitive1 Is Required for the Maternal Control of Seed Maturation, Jasmonate-Signaled Defense Responses, and Glandular Trichome Development. *Plant Cell* **16**(1): 126-143.

Lindinger W, Hansel A, Jordan A. 1998. Proton-Transfer-Reaction Mass Spectrometry (Ptr-Ms): On-Line Monitoring of Volatile Organic Compounds at Pptv Levels. *Chemical Society Reviews* **27**(5): 347-354.

Logan BA, Monson RK, Potosnak MJ. 2000. Biochemistry and Physiology of Foliar Isoprene Production. *Trends in Plant Science* **5**(11): 477-481.

Loreto F, Barta C, Brilli F, Nogues I. 2006. On the Induction of Volatile Organic Compound Emissions by Plants as Consequence of Wounding or Fluctuations of Light and Temperature. *Plant Cell and Environment* **29**(9): 1820-1828.

Loreto F, Ciccioli P, Cecinato A, Brancaleoni E, Frattoni M, Fabozzi C, Tricoli D. 1996a. Evidence of the Photosynthetic Origin of Monoterpenes Emitted by *Quercus Ilex* L. Leaves by ^{13}c Labeling. *Plant Physiol.* **110**(4): 1317-1322.

Loreto F, Ciccioli P, Cecinato A, Brancaleoni E, Frattoni M, Tricoli D. 1996b. Influence of Environmental Factors and Air Composition on the Emission of [Alpha]-Pinene from *Quercus Ilex* Leaves. *Plant Physiol.* **110**(1): 267-275.

Macdonald RC, Fall R. 1993. Detection of Substantial Emissions of Methanol from Plants to the Atmosphere. *Atmospheric Environment Part a-General Topics* **27**(11): 1709-1713.

McFeeters RF, Armstrong SA. 1984. Measurement of Pectin Methylation in Plant Cell Walls. *Analytical Biochemistry* **139**(1): 212-217.

Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashri A, Elkind Y, Levy A. 1997. A New Model System for Tomato Genetics. *Plant Journal* **12**(6): 1465-1472.

Monson RK, Holland EA. 2001. Biospheric Trace Gas Fluxes and Their Control over Tropospheric Chemistry. *Annual Review of Ecology and Systematics* **32**: 547-576.

Moscatiello R, Mariani P, Sanders D, Maathuis FJM. 2006. Transcriptional Analysis of Calcium-Dependent and Calcium-Independent Signalling Pathways Induced by Oligogalacturonides. *Journal of Experimental Botany* **57**(11): 2847-2865.

Nemecek-Marshall M, Macdonald RC, Franzen FJ, Wojciechowski CL, Fall R. 1995. Methanol Emission from Leaves - Enzymatic Detection of Gas-Phase

Methanol and Relation of Methanol Fluxes to Stomatal Conductance and Leaf Development. *Plant Physiology* **108**(4): 1359-1368.

Niinemets U, Reichstein M. 2003a. Controls on the Emission of Plant Volatiles through Stomata: A Sensitivity Analysis. *J. Geophys. Res-Atmos.* **108**(D7).

Niinemets U, Reichstein M. 2003b. Controls on the Emission of Plant Volatiles through Stomata: Differential Sensitivity of Emission Rates to Stomatal Closure Explained. *J. Geophys. Res-Atmos.* **108**(D7): 17.

Niinemets Ü, Reichstein M. 2003a. Controls on the Emission of Plant Volatiles through Stomata: A Sensitivity Analysis. *Journal of Geophysical Research* **108**(D7): 4211.

Niinemets Ü, Reichstein M. 2003b. Controls on the Emission of Plant Volatiles through Stomata: Differential Sensitivity of Emission Rates to Stomatal Closure Explained. *Journal of Geophysical Research* **108**(D7): 4208.

Park R, Epstein S. 1961. Metabolic Fractionation of C13 & C12 in Plants. *Plant Physiology* **36**(2): 133-138.

Pelloux J, Rusterucci C, Mellerowicz EJ. 2007. New Insights into Pectin Methylesterase Structure and Function. *Trends in Plant Science* **12**(6): 267-277.

Peñuelas J, Filella I, Stefanescu C, Llusia J. 2005. Caterpillars of *Euphydryas Aurinia* (Lepidoptera : Nymphalidae) Feeding on *Succisa Pratensis* Leaves Induce Large Foliar Emissions of Methanol. *New Phytologist* **167**(3): 851-857.

Petron G, Harley P, Greenberg J, Guenther A. 2001. Seasonal Temperature Variations Influence Isoprene Emission. *Geophys. Res. Lett.* **28**(9): 1707-1710.

Quay P, Stutsman J, Wilbur D, Snover A, Dlugokencky E, Brown T. 1999. The Isotopic Composition of Atmospheric Methane. *Global Biogeochemical Cycles* **13**(2): 445-461.

Refregier G, Pelletier S, Jaillard D, Hofte H. 2004. Interaction between Wall Deposition and Cell Elongation in Dark-Grown Hypocotyl Cells in Arabidopsis. *Plant Physiol.* **135**(2): 959-968.

Rose JKC, Bennett AB. 1999. Cooperative Disassembly of the Cellulose-Xyloglucan Network of Plant Cell Walls: Parallels between Cell Expansion and Fruit Ripening. *Trends Plant Sci.* **4**(5): 176-183.

Rosenbohm C, Lundt I, Christensen T, Young NWG. 2003. Chemically Methylated and Reduced Pectins: Preparation, Characterisation by H-1 Nmr Spectroscopy, Enzymatic Degradation, and Gelling Properties. *Carbohydrate Research* **338**(7): 637-649.

Schmidt DD, Voelckel C, Hartl M, Schmidt S, Baldwin IT. 2005. Specificity in Ecological Interactions. Attack from the Same Lepidopteran Herbivore Results in Species-Specific Transcriptional Responses in Two Solanaceous Host Plants. *Plant Physiol.* **138**(3): 1763-1773.

Seco R, Penuelas J, Filella I. 2007. Short-Chain Oxygenated Vocs: Emission and Uptake by Plants and Atmospheric Sources, Sinks, and Concentrations. *Atmospheric Environment* **41**(12): 2477-2499.

Sharkey TD. 1996. Emission of Low Molecular Mass Hydrocarbons from Plants. *Trends in Plant Science* **1**(3): 78-82.

Sharkey TD, Singsaas EL, Lerdau MT, Geron CD. 1999. Weather Effects on Isoprene Emission Capacity and Applications in Emissions Algorithms. *Ecological Applications* **9**(4): 1132-1137.

Singh H, Chen Y, Staudt A, Jacob D, Blake D, Heikes B, Snow J. 2001. Evidence from the Pacific Troposphere for Large Global Sources of Oxygenated Organic Compounds. *Nature* **410**(6832): 1078-1081.

Singh H, Chen Y, Tabazadeh A, Fukui Y, Bey I, Yantosca R, Jacob D, Arnold F, Wohlfrom K, Atlas E, Flocke F, Blake D, Blake N, Heikes B, Snow J, Talbot R, Gregory G, Sachse G, Vay S, Kondo Y. 2000a. Distribution and Fate of Selected Oxygenated Organic Species in the Troposphere and Lower Stratosphere over the Atlantic. *Journal of Geophysical Research-Atmospheres* **105**(D3): 3795-3805.

Singh H, Chen Y, Tabazadeh A, Fukui Y, Bey I, Yantosca R, Jacob D, Arnold F, Wohlfrom K, Atlas E, Flocke F, Blake D, Blake N, Heikes B, Snow J, Talbot R, Gregory G, Sachse G, Vay S, Kondo Y. 2000b. Distribution and Fate of Selected Oxygenated Organic Species in the Troposphere and Lower Stratosphere over the Atlantic. *J. Geophys. Res-Atmos.* **105**(D3): 3795-3805.

Sinha V, Williams J, Lelieveld J, Ruuskanen TM, Kajos MK, Patokoski J, Hellen H, Hakola H, Mogensen D, Boy M, Rinne J, Kulmala M. 2010. Oh Reactivity Measurements within a Boreal Forest: Evidence for Unknown Reactive Emissions. *Environmental Science & Technology* **44**(17): 6614-6620.

Smirnoff N. 1996. The Function and Metabolism of Ascorbic Acid in Plants. *Annals of Botany* **78**(6): 661-669.

Somerville C, Bauer S, Brininstool G, Facette M, Hamann T, Milne J, Osborne E, Paredez A, Persson S, Raab T, Vorwerk S, Youngs H. 2004. Toward a Systems Approach to Understanding Plant Cell Walls. *Science* **306**(5705): 2206-2211.

Stahlberg R, Van Volkenburgh E. 1999. The Effect of Light on Membrane Potential, Apoplastic Ph and Cell Expansion in Leaves of *Pisum Sativum* L. Var. Argenteum. *Planta* **208**(2): 188-195.

Stratmann JW. 2003. Long Distance Run in the Wound Response - Jasmonic Acid Is Pulling Ahead. *Trends in Plant Science* **8**(6): 247-250.

Terwilliger VJ. 1997. Changes in the Delta C-13 Values of Trees During a Tropical Rainy Season: Some Effects in Addition to Diffusion and Carboxylation by Rubisco? *American Journal of Botany* **84**(12): 1693-1700.

Tie X, Guenther A, Holland E. 2003. Biogenic Methanol and Its Impacts on Tropospheric Oxidants. *Geophysical Research Letters* **30**(17).

Trotsenko YA, Ivanova EG, Doronina NV. 2001. Aerobic Methylotrophic Bacteria as Phytosymbionts. *Microbiology* **70**(6): 623-632.

Van Volkenburgh E. 1999. Leaf Expansion - an Integrating Plant Behaviour. *Plant Cell Environ.* **22**(12): 1463-1473.

von Dahl CC, Havecker M, Schlogl R, Baldwin IT. 2006. Caterpillar-Elicited Methanol Emission: A New Signal in Plant-Herbivore Interactions? *Plant Journal* **46**(6): 948-960.

von Kuhlmann R, Lawrence MG, Crutzen PJ, Rasch PJ. 2003a. A Model for Studies of Tropospheric Ozone and Nonmethane Hydrocarbons: Model Description and Ozone Results. *Journal of Geophysical Research-Atmospheres* **108**(D9): 22.

von Kuhlmann R, Lawrence MG, Crutzen PJ, Rasch PJ. 2003b. A Model for Studies of Tropospheric Ozone and Nonmethane Hydrocarbons: Model Evaluation of Ozone-Related Species. *Journal of Geophysical Research-Atmospheres* **108**(D23): 26.

Warneke C, Luxembourg SL, de Gouw JA, Rinne HJI, Guenther AB, Fall R. 2002. Disjunct Eddy Covariance Measurements of Oxygenated Volatile Organic Compounds Fluxes from an Alfalfa Field before and after Cutting. *Journal of Geophysical Research-Atmospheres* **107**(D7-8).

Weilacher T, Gleixner G, Schmidt HL. 1996. Carbon Isotope Pattern in Purine Alkaloids a Key to Isotope Discriminations in C-1 Compounds. *Phytochemistry* **41**(4): 1073-1077.

Whiticar MJ. 1999. Carbon and Hydrogen Isotope Systematics of Bacterial Formation and Oxidation of Methane. *Chemical Geology* **161**(1-3): 291-314.

Willats WGT, McCartney L, Mackie W, Knox JP. 2001. Pectin: Cell Biology and Prospects for Functional Analysis. *Plant Molecular Biology* **47**(1-2): 9-27.

Wolfe GM, Thornton JA, Bouvier-Brown NC, Goldstein AH, Park J-H, McKay M, Matross DM, Mao J, Brune WH, LaFranchi BW, Browne EC, Min K-E, Wooldridge PJ, Cohen RC, Crounse JD, Faloona IC, Gilman JB, Kuster WC, de Gouw JA, Huisman A, Keutsch FN. 2011. The Chemistry of Atmosphere-Forest Exchange (Cafe) Model - Part 2: Application to Bearpex-2007 Observations. *Atmospheric Chemistry and Physics* **11**(3): 1269-1294.

Xin JY, Cui JR, Niu JZ, Hua SF, Xia CG, Li SB, Zhu LM. 2004. Production of Methanol from Methane by Methanotrophic Bacteria. *Biocatalysis and Biotransformation* **22**(3): 225-229.

Yamada K, Hattori R, Ito Y, Shibata H, Yoshida N. 2009. Carbon Isotopic Signatures of Methanol and Acetaldehyde Emitted from Biomass Burning Source. *Geophysical Research Letters* **36**: 5.
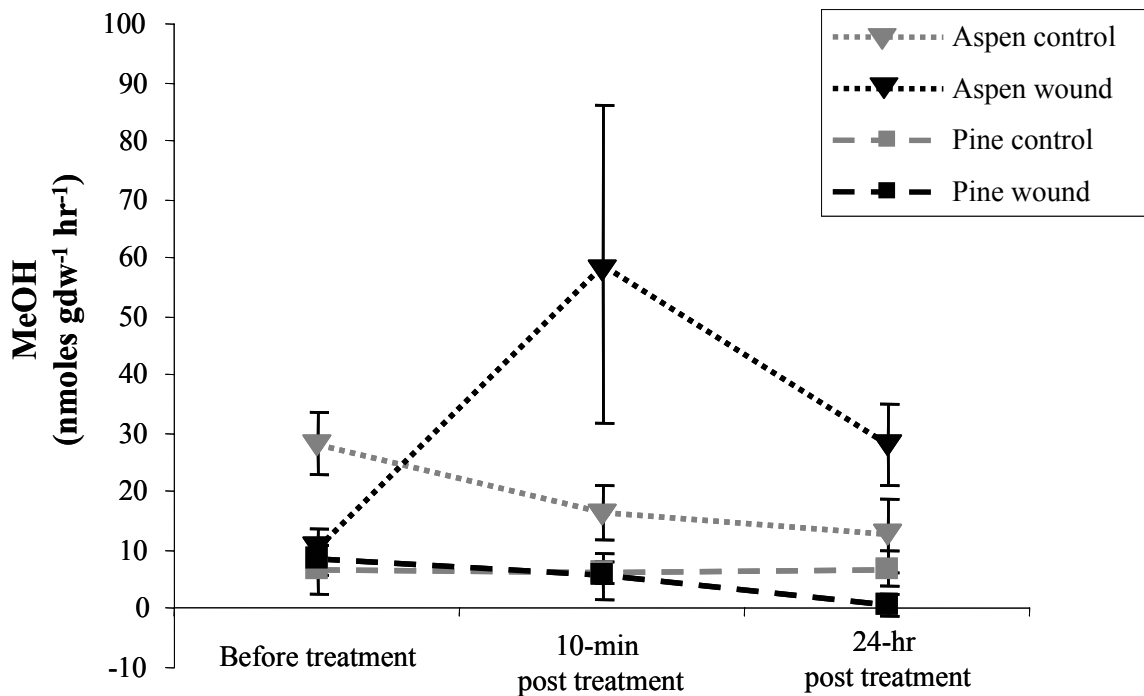
Appendix: Preliminary data

Figure 1. MeOH flux for big tooth aspen *Populus grandidentata* (*n*=6) and white pine *Pinus strobus* (*n*=6). Values are means ± standard error. *P. grandidentata* leaves had overall higher MeOH flux than *P. strobus* needles (Wilcoxon rank sum; *P*<0.0001). MeOH emissions from *P. grandidentata* were significantly elevated relative to controls 10 min post wounding (Wilcoxon rank sum; *P*=0.02), but returned to control levels 24 hrs post wounding (Wilcoxon rank sum; *P*=0.27).
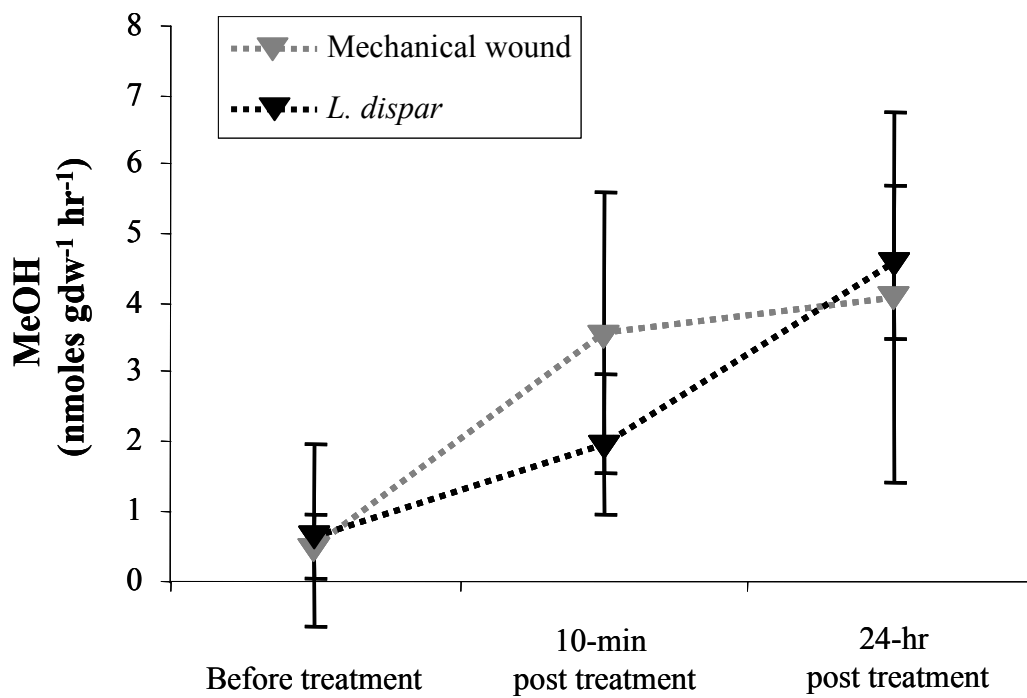
Figure 2. MeOH flux from big tooth aspen *Populus grandidentata* in response to mechanical wounding (*n*=6) and herbivory by gypsy moth *Lymantria dispar* (*n*=6). Values are means ± standard error. No differences were detected between mechanical wound and herbivory treatments (*F*=0.28 *P*=0.61).
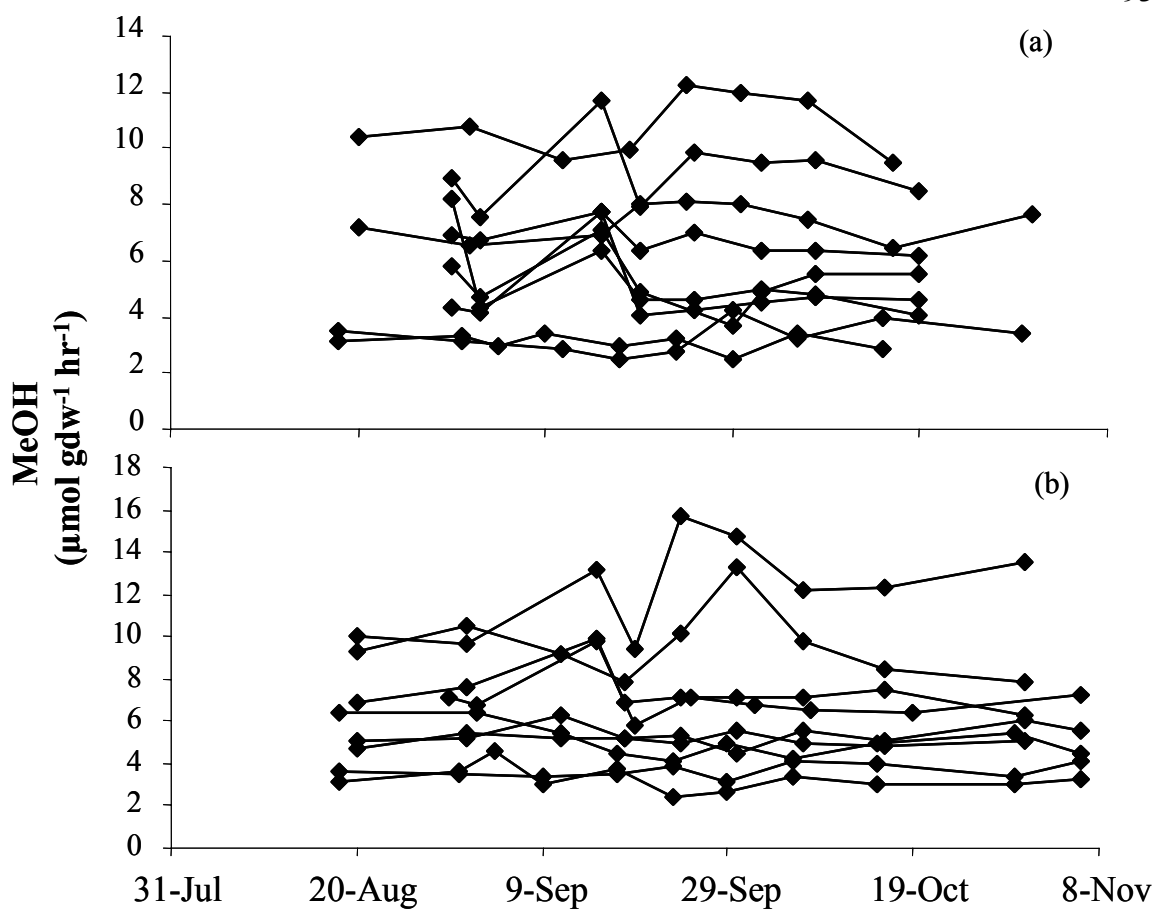
Figure 3. MeOH flux measurements for (a) red maple *Acer rubrum* (*n*=9) and (b) white oak *Quercus alba* (*n*=9). Individuals were sampled on a weekly basis between 18 August-6 November, 2008. No seasonal trends in MeOH emission were detected for either species. Each line represents one individual.
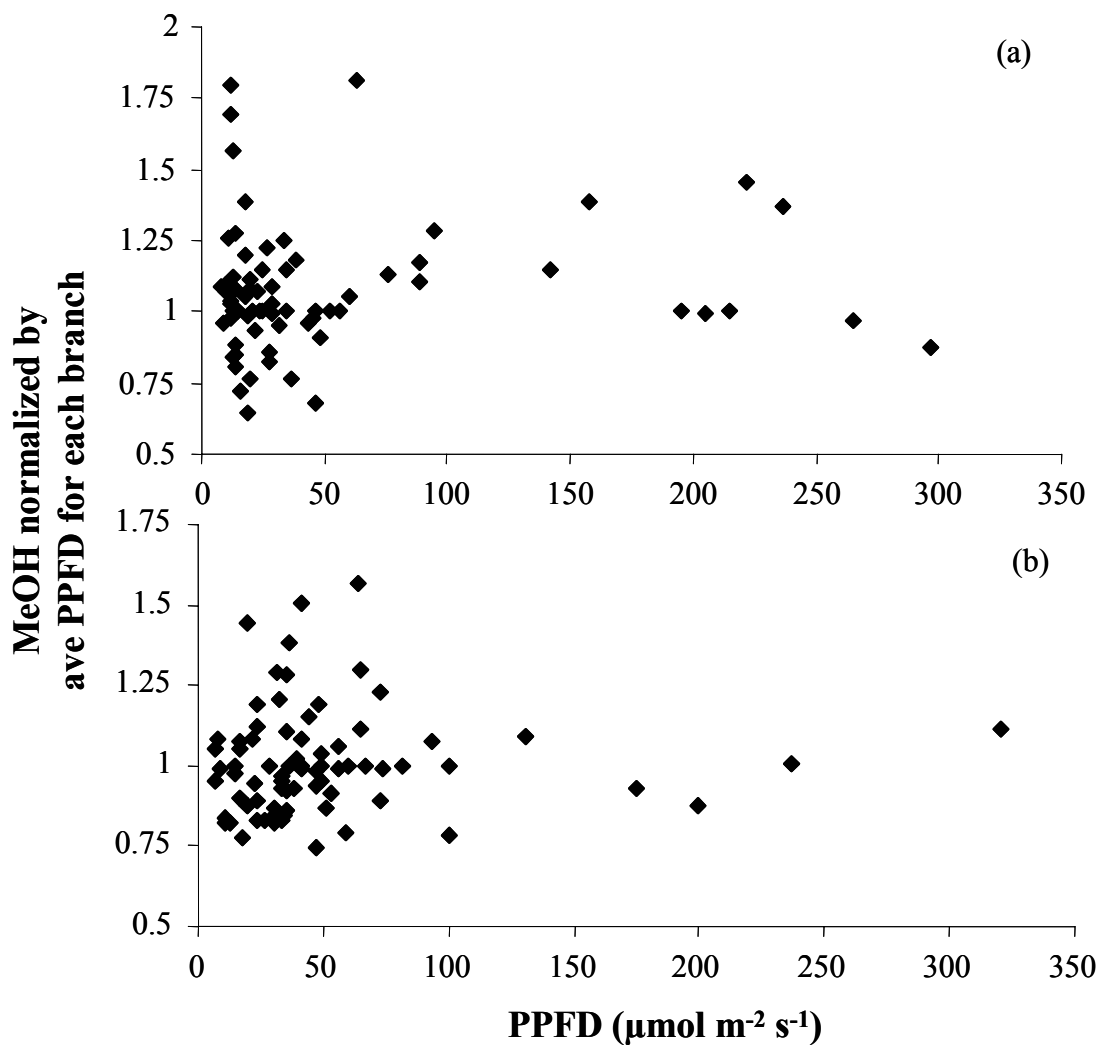
Figure 4. MeOH emission normalized for average photosynthetic photon flux density (PPFD) for individual (a) red maple *Acer rubrum* (*n*=9) and (b) white oak *Quercus alba* (*n*=9). Data were collected on a weekly basis between 18 August-6 November, 2008. No relationship was detected between normalized MeOH and PPFD for either species (Spearman's rank correlation coefficient= 0.017, *P*=0.12 for white oak; Spearman's rank correlation coefficient= 0.018, *P*=0.12 for red maple).
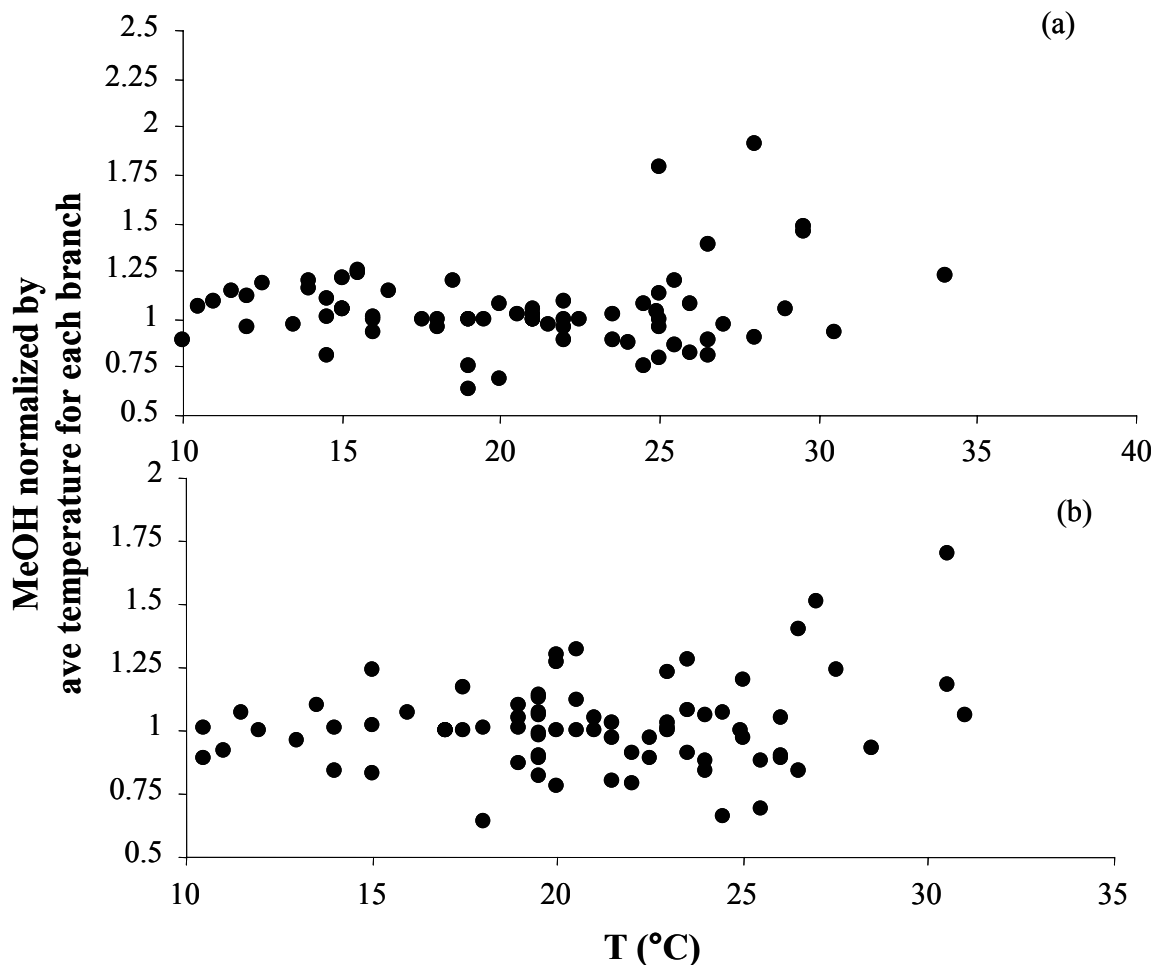
Figure 5. MeOH emission normalized for average temperature for individual (a) red maple *Acer rubrum* (*n*=9) and (b) white oak *Quercus alba* (*n*=9). Data were collected on a weekly basis between 18 August-6 November, 2008. No relationship was detected between normalized MeOH and temperature for either species. (Spearman's rank correlation coefficient= -0.08, *P*=0.45 for white oak; Spearman's rank correlation coefficient= -0.19, *P*=0.09 for red maple).
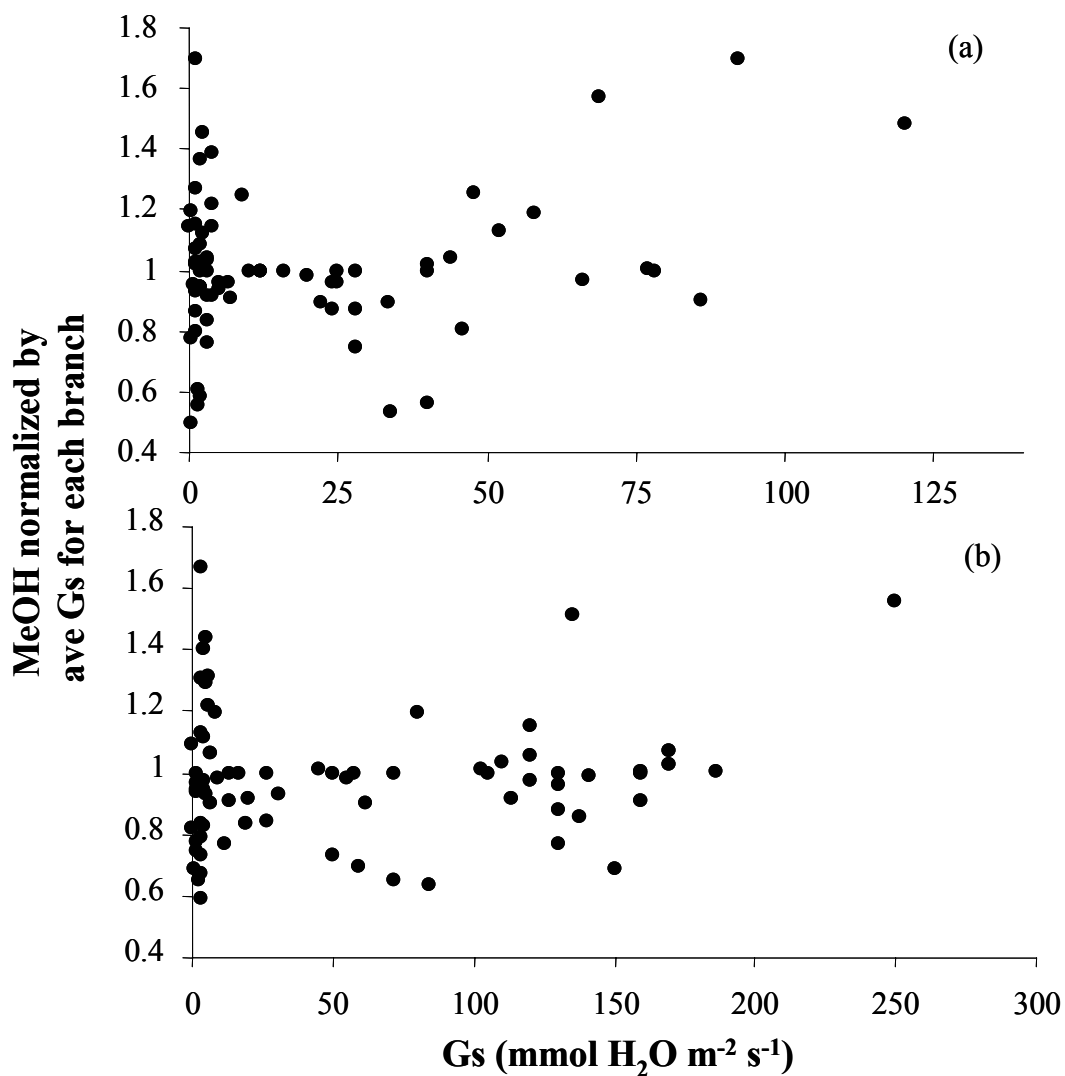
Figure 6. MeOH emission normalized for average stomatal conductance (g_s) per individual (a) red maple *Acer rubrum* (*n*=9) and (b) white oak *Quercus alba* (*n*=9). Data were collected on a weekly basis between 18 August-6 November, 2008. No relationship was detected between normalized MeOH and g_s for either species. (Spearman's rank correlation coefficient= -0.01, *P*=0.93 for white oak; Spearman's rank correlation coefficient= -0.04, *P*=0.71 for red maple).