

METABOLOMIC PROFILING OF C₄ GRASS RESPONSES TO ENVIRONMENTAL
STRESS

BY

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DISSERTATION

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ABSTRACT

Over half of the world's grass species, including some of the most productive food and energy crops, use C₄ photosynthesis. However, the responses of C₄ crops to global atmospheric change, specifically rising carbon dioxide ([CO₂]) and ozone ([O₃]) concentrations, have been examined disproportionately less than responses of C₃ crops. This thesis uses metabolomics approaches, along with integrated 'omic' technologies, to achieve a greater understanding of C₄ grass responses to abiotic stresses associated with global atmospheric change.

Rising temperatures will have a significant effect on major food and forage crops across the globe. Although the impact is expected to be greatest in tropical regions, the impacts of climate change have been poorly studied in those regions. In Chapter 2, Guinea grass (*Panicum maximum* Jacq.) was exposed to the individual and combined effects of elevated [CO₂] and temperature, and transcript and metabolite profiles were examined. Samples were collected at a combined Free Air CO₂ Enrichment and Temperature Controlled Enhancement (Trop-T-FACE) facility in Sao Paulo, Brazil. Field transcriptomics and metabolomics revealed that elevated temperature and [CO₂] altered transcript and metabolite profiles associated with an environmental response, secondary metabolism and stomatal function. These metabolic responses were consistent with greater growth and leaf area production under elevated temperature. The tropical C₄ grass had an unpredicted response to global climate change, with canopy warming during a cool growing season, enhancing growth and alleviating stress.

Tropospheric O₃ is the most damaging air pollutant to crops. Exposure to O₃ causes oxidative stress to vegetative and reproductive tissues which can affect the abundance of transcripts, proteins and metabolites, leading to accelerated senescence and decreased yield. In Chapter 3, two diverse maize (*Zea mays*) inbred lines and the hybrid cross were exposed to season-long elevated [O₃] (~100 ppb) in the field based FACE facility modified for O₃. The metabolomic profile of leaves was sampled over the course of leaf senescence to achieve an understanding of the biochemical response during this developmental stage. The hybrid line, B73 x Mo17, showed an acceleration of chlorophyll loss under elevated [O₃] accompanied by a significant change in the metabolite profile. In contrast, the metabolite profile of the two inbred lines (B73 and Mo17) was not different in ambient and elevated [O₃] treatments. Levels of secondary metabolites were increased in B73 x Mo17 leaves as they aged and to a significantly

greater degree in elevated [O₃] stress. Untargeted metabolomic profiling revealed that inbred and hybrid lines of maize differ in key metabolic responses to elevated O₃ pollution.

The specific reactive oxygen species initially formed after O₃ exposure and the antioxidants involved in apoplastic detoxification vary among plant species and even genotypes within a species. Very little is known about how C₄ grasses respond to acute O₃ exposure. In Chapter 4, maize and foxtail millet (*Setaria viridis*) were exposed to an acute O₃ dose (200 – 400 nL L⁻¹) for 24 hours. Leaf material from the youngest fully expanded leaf was taken for nuclear magnetic resonance (NMR) for identification of antioxidants and additional metabolites, and electron paramagnetic resonance (EPR) for ROS identification. EPR results showed the concentration of hydroxyl and superoxide radicals in leaves were higher after acute O₃ exposure. Untargeted metabolomics performed with ¹H-NMR showed altered amino acid content following initial O₃ exposure, especially isoleucine and alanine. These experiments established the potential for EPR analysis of O₃ response in live tissues and lay the foundation for further work to identify the specific molecular signature of the initial O₃ response.

This dissertation research provides insight into the metabolomic mechanisms behind the response of C₄ grasses to elevated [CO₂], temperature, and [O₃]. Metabolomics approaches can be used for high-throughput phenotyping of diverse metabolites and their responses to stress. While field metabolomics can be challenging, the results identified novel metabolic pathways of response to global atmospheric change.

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CHAPTER I: GENERAL INTRODUCTION

The global atmospheric concentration of carbon dioxide ($[\text{CO}_2]$) has risen from a pre-industrial concentration of 280 ppm to the current concentration exceeding 410 ppm, with approximately half of that increase within the past 50 years. In addition to CO_2 , anthropogenic greenhouse gas (GHG) emissions of methane (CH_4), nitrous oxide (N_2O), and halocarbons contribute significantly to global surface warming. GHGs in the atmosphere absorb a proportion of the infrared radiation emitted from the surface directly warming the earth. Current anthropogenic emissions of CO_2 are responsible for most of the global warming with two main sources from land-use change and fossil fuel consumption (Fig.1.1). The overall CO_2 burden is partially offset by the oceans and land sinks, but the remainder stays in the atmosphere contributing to warming (Fig.1.1). In 2015 the UN Paris Agreement agreed to limit global warming to 2°C above pre-industrial levels. Rising global average surface temperatures are “virtually certain” to result in more frequent and extended heat waves (Masson-Delmotte *et al.* 2018). Even if the target 2°C is met, extreme heatwave days are projected to reach $3\text{--}4^\circ\text{C}$ and up to 6°C nighttime temperatures (Masson-Delmotte *et al.* 2018). To achieve this target, rapid development and adoption of negative emission technologies are required (Anderson 2015). The development of such technologies will depend on the socio-economic development and population growth within individual countries (Masson-Delmotte *et al.* 2018). However, rising $[\text{CO}_2]$ is a global problem requiring a concerted effort to prevent emissions from exceeding 1000 Pg of CO_2 from 2011 to 2100 (Anderson 2015).

Tropospheric ozone (O_3) is a secondary pollutant formed as a by-product of the photochemical oxidation of NO_x in the presence of carbon monoxide (CO), methane (CH_4) and non-methane hydrocarbons (Myhre *et al.* 2013; Fig. 1.1). The increased amount of tropospheric O_3 from 1750 to 2011 has contributed to a radiative forcing of $+0.40 \text{ W}\cdot\text{m}^{-2}$, making O_3 the third most important contributor to anthropogenic warming following CO_2 and CH_4 (Myhre *et al.* 2013). It is estimated that global production of tropospheric O_3 is $\sim 5.29 \text{ Pg}\cdot\text{year}^{-1}$, produced primarily from chemical production ($4.96 \text{ Pg}\cdot\text{year}^{-1}$) with a smaller contribution from stratospheric exchange (Hu *et al.* 2017). Chemical loss of O_3 accounts for most of the O_3 destruction in the troposphere, but dry deposition of O_3 to the terrestrial surface is also a significant sink for O_3 , accounting for $\sim 18\%$ of removal from the troposphere (Hardacre *et al.* 2015). While Figure 1.1 shows the global average O_3 budget, surface O_3 concentrations are

highly dynamic, and depend on temporal, seasonal and spatial factors. Concentrations typically peak in the late afternoon, with high [NO_x] and sunlight levels, and are seasonally variable with low concentrations in cold winter months and high concentrations in warm summer months in temperate regions. The average daytime concentrations of surface O₃ are largely dependent on the primary air pollutant levels within the region, ranging from 10 parts per billion (ppb) over the Pacific Ocean to more than 100 ppb down-wind of large industrial emitting sources (Myhre *et al.* 2013). Precursors can travel far from the source on the regional, intercontinental and hemispheric scales before reacting (Cooper & Derwent 2013). Increasing O₃ precursors in East Asia have been identified as major drivers of rising spring and summer background [O₃] in the western United States over the past decades, despite control of local emissions (Cooper *et al.* 2010; Lin *et al.* 2017).¹

Past trends and future estimates of surface [O₃] depend on legislation and control of precursor emissions in large part because O₃ is a short-lived molecule, lasting for only 22-24 days in the tropospheric (Hu *et al.* 2017; Young *et al.* 2013). Thus, regulations can drastically improve O₃ pollution in a short period of time. From 1990 to 2013, global mean [O₃] increased by 8.9% (Brauer *et al.* 2016), but concentrations decreased in parts of the United States and Western Europe. The United States enacted more stringent NO_x regulations with the U.S. National Ambient Air Quality Standard (NAAQS) in 1997 (EPA 1997) and subsequently [O₃] decreased over the past two decades (Brauer *et al.* 2016). In stark contrast, surface [O₃] in East Asia increased 25-50 ppb over the past 25 years, with a tripling of NO_x emissions over that time (Lin *et al.* 2017). Under the IPCC emissions scenarios, future [O₃] will only decrease with the most optimistic, low emission scenario (RCP 2.6), and will either remain at current levels or increase under the other scenarios (Eyring *et al.* 2013). Future climate change could also increase the frequency of summer droughts and heatwaves, conditions that favor high [O₃] in some regions (Meehl *et al.* 2018).¹

Today C₄ grass species are responsible for a large majority of the world's food production. C₄ plants represent only a small proportion of the diversity within plant species;

¹These sections appeared in their entirety in the journal of *Plant Biology* and is referred to later in the dissertation as "Ainsworth *et al.* 2020" **Ainsworth EA, Lemonnier P, Wedow JM. 2020.** The influence of rising tropospheric carbon dioxide and ozone on plant productivity. *Plant biology* (Stuttgart, Germany) 22 Suppl 1, 5-11. This section is open access and printed with permission of the publisher and is available online at <https://onlinelibrary.wiley.com/using/doi/10.1111/plb.12973>.

however, they contribute disproportionately to global plant productivity (Sage 2004). These plants contribute to the global food supply both directly as grain and indirectly as feed and forage grasses. By concentrating CO₂ around the fixation enzyme, Rubisco, C₄ plants repress photorespiration and achieve up to 40% greater carbon fixation rates than C₃ plants (Taylor *et al.* 2010). Additionally, C₄ plants have increased water and nitrogen use efficiency, which could enable them to be better adapted to a changing environment. Understanding the response of C₄ crops to climate change is a first step towards adapting agriculture to a changing environment.

Fewer experiments have examined the effects of elevated [CO₂] on C₄ crops compared to C₃ crops (Leakey 2009). Studies of maize and sorghum found that C₄ photosynthesis was not directly stimulated by elevated [CO₂], and only under water stress conditions did growing in a high [CO₂] environment benefit crop productivity (Leakey *et al.* 2006; Wall *et al.* 2001). Despite the lack of direct stimulation of photosynthesis under elevated [CO₂], the combination of high [CO₂] with any number of stress conditions might change the C₄ response. The effects of increasing [O₃] on global plant productivity has also received less attention than rising [CO₂], in part because of the complexity of plant response to O₃ pollution, and because understanding is less complete (Ainsworth *et al.* 2020). This is especially true for C₄ crops, that were until recently thought to be less susceptible to O₃ (Heagle *et al.* 1988; Miller 1988). Experimental studies have shown C₄ plants to be sensitive to O₃ in maize (Leitao *et al.* 2007; Yendrek *et al.* 2017a; Yendrek *et al.* 2017b), sugarcane (Moura *et al.* 2018), and switchgrass (Li *et al.* 2019).

Free Air Concentration Enrichment (FACE) systems were developed to fumigate plant ecosystems with elevated [CO₂] or [O₃], under realistic field conditions, and can be deployed in combination with other abiotic stresses (Long *et al.* 2004). The majority of FACE experiments have been located in the Northern Hemisphere, studying crops and ecosystems that are important to temperate regions (Leakey *et al.* 2012). Tropical and subtropical regions have not been studied in depth, and management and edaphic conditions in tropical regions are very different from temperate regions. Therefore, extrapolating conclusions from FACE studies done in the temperate regions of the world to the tropics may result in a misrepresentation in the state of future food security (Leakey 2009). Additionally, very few FACE experiments are performed with O₃ around the world (Ainsworth 2017). An individual FACE ring is a vital tool to simultaneously screen hundreds of genotypes for O₃ tolerance (Ainsworth *et al.* 2014). The

information gained from FACE technology cannot be matched with any other experimental method making them an essential tool in understand plant responses to environmental stress.

Research objectives

The aims of my thesis were to better understand the metabolomic signatures of C₄ plant response to environmental stresses, including rising [CO₂], temperature, and [O₃]. Metabolomic approaches can be used for high-throughput phenotyping of diverse metabolites and their responses to environmental stresses, providing potential targets to improve crop productivity in an increasingly changing world. The first objective of my thesis was to determine the metabolomic and transcriptomic response of Guinea grass (*Panicum maximum* Jacq.) to elevated [CO₂] and temperature stress. The study presented in Chapter 2 uses a FACE experiment located in a sub-tropical region, Brazil, and studies an economically important C₄ grass species. The second objective of my thesis was to understand the metabolomic response of diverse maize genotypes in response to season-long chronic [O₃]. Chapter 3 used untargeted metabolomic profiling to examine metabolic responses of maize to elevated [O₃] over the course of leaf senescence. The final chapter of my thesis aimed to understand the initial biochemical response of two C₄ species to acute O₃ exposure. The studies presented in Chapter 4 combined two metabolomic approaches to characterize the antioxidant and reactive oxygen species composition within the plant cell. Each chapter shows the power of metabolomic profiling to understand the biochemical responses of plants to environmental stress, and compliment additional physiological and molecular analysis.

FIGURE

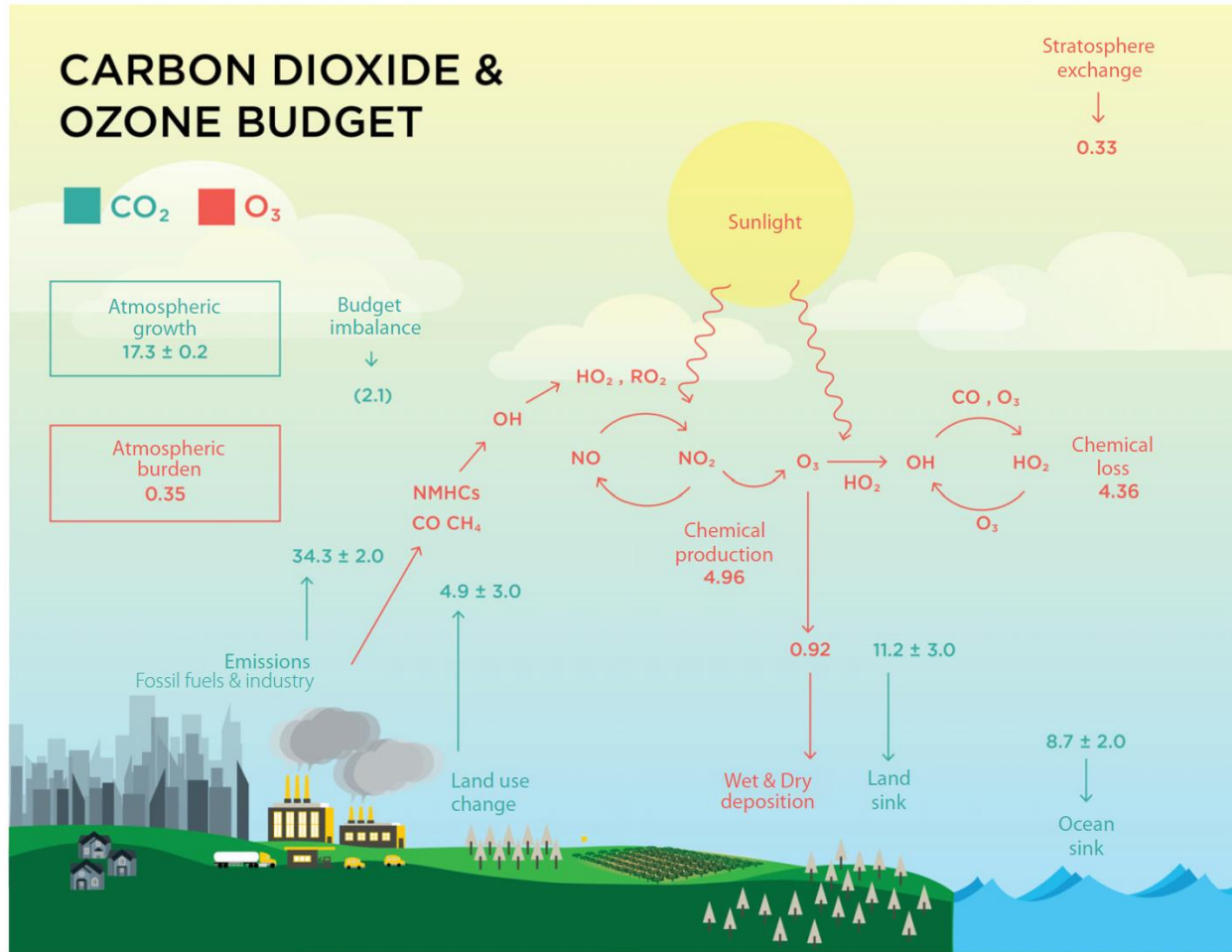


Figure 1.1. Schematic of the current global carbon dioxide and ozone budgets (Pg CO₂ or O₃). Pools and fluxes of CO₂ (in green) are taken from the Global Carbon Project (Le Quere *et al.* 2018). Pools and fluxes of O₃ (in red) are taken from Hu *et al.* (2017). Figure is taken from Ainsworth, Lemonnier & Wedow (2020).

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CHAPTER II: METABOLITE AND TRANSCRIPT PROFILING OF GUINEA GRASS (*PANICUM MAXIMUM* JACQ.) RESPONSE TO ELEVATED [CO₂] AND TEMPERATURE²

Abstract

By mid-century, global atmospheric carbon dioxide concentration ([CO₂]) is predicted to reach 600 $\mu\text{mol mol}^{-1}$ with global temperatures rising by 2 °C. Rising [CO₂] and temperature will alter the growth and productivity of major food and forage crops across the globe. Although the impact is expected to be greatest in tropical regions, the impact of climate-change has been poorly studied in those regions. This experiment aimed to understand the effects of elevated [CO₂] (600 $\mu\text{mol mol}^{-1}$) and warming (+ 2°C), singly and in combination, on *Panicum maximum* Jacq. (Guinea grass) metabolite and transcript profiles. We created a *de novo* assembly of the *Panicum maximum* transcriptome. Leaf samples were taken at two time points in the Guinea grass growing season to analyze transcriptional and metabolite profiles in plants grown at ambient and elevated [CO₂] and temperature, and statistical analyses were used to integrate the data. Elevated temperature altered the content of amino acids and secondary metabolites. The transcriptome of Guinea grass shows a clear time point separation, with the changes in the elevated temperature and [CO₂] combination plots. Field transcriptomics and metabolomics revealed that elevated temperature and [CO₂] result in alterations in transcript and metabolite profiles associated with environmental response, secondary metabolism and stomatal function. These metabolic responses are consistent with greater growth and leaf area production under elevated temperature and [CO₂]. These results show that tropical C₄ grasslands may have unpredicted responses to global climate change, and that warming during a cool growing season enhances growth and alleviates stress.

²This chapter appeared in its entirety in the journal of *Metabolomics* and is referred to later in the dissertation as “Wedow *et al.* 2019” **Wedow JM, Yendrek CR, Mello TR, Creste S, Martinez CA, Ainsworth EA. (2019). Metabolite and transcript profiling of Guinea grass (*Panicum maximum* Jacq.) response to elevated CO₂ and temperature. Metabolomics 15, 13.** This article is open access and available for reprint with proper citation of the authors and is online at <http://link.springer.com/> using doi:10.1009/s11306-019-1511-8.

Introduction

Atmospheric carbon dioxide concentrations ($[CO_2]$) have risen by 40% since pre-industrial times, with approximately half of that increase within the past 40 years. The International Panel on Climate Change (IPCC) has predicted that $[CO_2]$ will rise to 600 ppm and mean global temperature will increase 1.1 to 2.6 °C by the end of the century, with a moderate climate change projection scenario (IPCC 2014). Although the combined effects of rising atmospheric $[CO_2]$ and rising temperatures have been studied in field experiments in temperate forests (Sun *et al.* 2017; Svensson *et al.* 2018), croplands (Ruiz-Vera *et al.* 2015; Cai *et al.* 2016), and grasslands (Ryan *et al.* 2015), there have been fewer field studies of the combined effects of rising $[CO_2]$ and temperature in tropical and sub-tropical ecosystems. Differences in climate and nutrient limitation in tropical regions may fundamentally alter how tropical ecosystems respond to global climate change compared to well-studied temperate ecosystems (Leakey *et al.* 2012). Therefore, the paucity of data from tropical regions presents a significant challenge for an accurate understanding of global responses to climate change. Adding to this challenge is the realization that responses of vegetation to combined elevated $[CO_2]$ and temperature treatments are different from single-factor experiments, and that additive effects are rare (Dieleman *et al.* 2012). Thus, there is a need for multi-factor global change experiments, especially in tropical regions.

Pastures cover about 30% of the terrestrial surface in tropical regions and play an important role in carbon sequestration (Boval & Dixon 2012; Ramankutty *et al.* 2008). In tropical regions, C_4 grasses dominate those pastures (Still *et al.* 2003). *Panicum maximum* Jacq. (Guinea grass) is a perennial C_4 grass species native to tropical Africa, which has been adapted in Brazil as a forage and range grass (Pedreira *et al.* 2015). Approximately 90% of Brazilian cattle are raised on pastureland (Abmael *et al.* 2016), and perennial C_4 grasses such as Guinea grass are principle species for the Brazilian feedstock industry. Despite the economic importance of Guinea grass to this industry, very little is known about how it and other C_4 forage grasses will respond to rising atmospheric $[CO_2]$ and temperature. To address this knowledge gap, a Temperature Free Air CO_2 Enrichment (T-FACE) experiment was established in Ribeirão Preto, Brazil to expose Guinea grass to elevated $[CO_2]$ (600 ppm) and increased canopy temperatures of 2°C (Britto de Assis Prado *et al.* 2016).

‘Omic’ technologies, including transcriptomics and metabolomics, provide a functional analysis to connect physiological and molecular responses to genetic and phenotypic information

(Langridge & Fleury 2011). Such technologies can be used to better understand the mechanisms underpinning plant responses to global climate change and can reveal targets for improving plant performance in the future. In recent years there has been an increasing number of studies investigating transcriptomic and metabolomic responses of C₄ grasses to a variety of abiotic stresses imposed in greenhouses and/or chambers (e.g., Sicher *et al.* 2012, Meyer *et al.* 2014, Sui *et al.* 2015; De Souza *et al.* 2015). However, there is an increasing need to investigate and understand plant transcriptional and metabolic responses to global change or abiotic stress in the field where responses can be dramatically different from controlled environments (Lovell *et al.* 2016). The combined use of FACE and ‘omic’ technologies provides a powerful approach for gaining new insight into plant molecular responses to global climate change in the field. Combining metabolomic and transcriptomic studies allowing for a holistic view of the plant’s response to stress.

Our study combines the power of FACE experiments for growing plants under elevated [CO₂] and temperature in fully open-air conditions with transcriptomic and metabolomics profiling. The aims are to 1) understand the biochemical responses of Guinea grass to rising [CO₂] and temperature when applied individually and in combination; 2) understand how gene expression is altered in each treatment; and 3) integrate the metabolomic and transcriptional responses.

Methods

*Experimental design and tissue collection*³

The field experiment was conducted in a 2500 m² field at the University of São Paulo in Ribeirão Preto, São Paulo Brazil. Guinea grass was planted in a completely replicated design with four ambient temperature and [CO₂] plots (C), four elevated [CO₂] (600 µmol mol⁻¹) and ambient temperature plots (eC), four ambient [CO₂] and elevated temperature [+2°C] plots (eT), and four combined treatment (elevated [CO₂] and elevated temperature) plots (eCeT). Each plot was 2-m in diameter; [CO₂] was controlled using Free Air CO₂ Enrichment, and canopy warming was provided by infrared ceramic heaters as described by Britto de Assis Prado *et al.* (2016). Treatment was started on April 22, 2014.

³ Data collected and RNA extraction by Tathyana Mello and Silvana Creste

Leaf tissue was collected for transcriptomic and metabolomic analyses on two dates: May 22, 2014 (time point A – 30 d of treatment), and June 14, 2014 (time point B – 50 d of treatment). Six leaves per experimental plot were collected at each time point and then pooled for RNA extraction and metabolomics analysis. At the time of sampling, tissue was immediately quenched in liquid nitrogen. RNA was extracted in Brazil using the protocol described by Bilgin and colleagues (2009), and subsequently shipped in ethanol to the U.S. for transcriptomic analysis. Lyophilized leaf tissue from the same leaves was sent for metabolite analysis. Leaves were sampled for biomass, area, and specific leaf area on August 22 and 29th, 2014 from 60 tillers per treatment as described by Britto de Assis Prado *et al.* (2016).

Transcriptional analysis⁴

RNA samples that were shipped from Brazil were re-suspended by washing with sodium acetate (pH 5.5) and glycerol. RNA quantity and quality were re-assessed using a spectrophotometer (Nanodrop 1000, Thermo Fisher) and a microfluidic visualization tool (Bioanalyzer, Agilent Technologies). 32 samples (16 per time point including four from each [CO₂] and temperature treatment) were submitted for library construction and sequencing at the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana-Champaign. RNAseq libraries were prepared with Illumina's TruSeq Standard RNA sample prep kit according to the manufacturer's instruction (Illumina, San Diego, CA, USA). No public reference genome for Guinea grass was available at the time of the sequencing. Therefore, a combination of MiSeq and HiSeq was used to generate sequences of different lengths for *de novo* genome assembly.

For MiSeq analysis, 50 µg of total RNA per sample was pooled for library preparation without RNA fragmentation. The MiSeq libraries were quantified by qPCR and sequenced on one MiSeq flowcell for 301 cycles using paired-end sequencing. HiSeq paired-end sequencing was done with four quantified libraries per treatment which were pooled in equimolar concentration and sequenced on two lanes for 161 cycles. The final read lengths for MiSeq and HiSeq were 300 nt and 160 nt in length. A total of 635,649,277 reads were assembled from the MiSeq/HiSeq

⁴ RNASeq and Transcriptome assembly were done by Criag Yendrek

pools. Quality control for reads generated from sequencing was performed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Quality reads were used to perform *de novo* transcriptome assembly using Trinity. The initial assembly consisted of 187,216 genes. A filter was applied to keep only those genes that had at least 10 reads (across the 4 replicates) for an individual treatment. The resulting transcriptome contained 45,073 genes and reads. Functional annotation of the genes was done by using BLAST against *Arabidopsis thaliana*, *Zea mays*, and *Setaria italica*.

Differential gene expression analysis was performed using the R package, LIMMA (Ritchie *et al.* 2015). Within each time point, individual experimental treatments were compared to the control (ambient [CO₂], ambient temperature). Following post ‘voom’ normalization, extreme outliers were removed, and 39,208 genes were assessed for differential expression analysis. Final normalized reads were log₂ transformed for differential gene expression analysis. DESeq2 (Love *et al.* 2014) was also run for comparison with the LIMMA approach and results were found to be similar but less conservative (Appendix B Supplemental Fig. B.1). A classical multidimensional scaling plot (MDS) was created using the R base package with normalized and transformed reads. MDS plots were used to probe the relationships among samples in multidimensional space using a dissimilarity measure of each pairwise comparison.

Metabolic profiling by GC-MS

Untargeted metabolite analysis was performed with pre-weighed lyophilized tissue (12.67- 19.93 mg, average 16.93±1.92 mg) according to protocol described in Ulanov and Widholm (2010). For quality control (QC) 10 µl of leaf extract was taken from each sample and pooled, then run and analyzed after every 9 biological samples. Samples and QC were dried under vacuum and derivatized with 75 µl methoxyamine hydrochloride (Sigma-Aldrich, MO, USA) (40 mg ml⁻¹ in pyridine) for 90 min at 50°C, then with 125 µl MSTFA+1%TMCS (Thermo, MA, USA) at 50°C for 120 min followed by an additional 2-hour incubation at room temperature. An internal standard (30 µL hentriacontanoic acid) was added to each sample prior to derivatization. Samples were analyzed on a gas chromatography/ mass spectroscopy (GC/MS) system (Agilent Inc, Palo Alto, CA, USA) consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 mass selective detector, and a HP 7683B autosampler. Gas chromatography was performed on a ZB-5MS capillary column (Phenomenex, Torrance, CA, USA). The inlet and MS interface

temperatures were 250°C, and the ion source temperature was adjusted to 230°C. An aliquot of 1 µl was injected with the split ratio of 10:1. The helium carrier gas constant flow rate was 2.4 ml min⁻¹. The temperature program was: 5 min isothermal heating at 70°C, followed by an oven temperature increase of 50°C min⁻¹ to 310°C, and a final 10 min at 310°C. The mass spectrometer was operated in a positive electron impact mode at 69.9 eV ionization energy in m/z 50-800 scan range. The spectra of chromatogram peaks were compared with electron impact mass spectrum libraries NIST08 (NIST, MD, USA), WILEY08 (Palisade Corporation, NY, USA), and a custom library. To allow comparison among samples, data were normalized to the internal standard (hentriacontanoic acid, SIGMA, USA) and standardized to the dry weight of the individual sample. The chromatograms and mass spectra were evaluated using the AMDIS 2.71 (NIST, Gaithersburg, MD, USA) program, using a custom-built database. All known artificial peaks were identified and removed with prior data mining. The instrument variability was within the standard acceptance limit of 5%.

194 metabolites were identified with GC/MS. Technical replicates were averaged across samples. Normalized data was log₁₀ transformed and univariate analysis was performed using SAS (SAS/ STAT v9.4, SAS Institute, Inc.) to identify outliers. The MDS plot was created using the R base package with normalized and transformed reads. The transformed and normalized metabolomics dataset was analyzed using a general linear model (ANOVA) with SAS PROC GLM where time point was determined non-significant (p>0.01) and dropped from the model. Means separation tests were done using a two-tailed Dunnett, comparing each treatment to the control. Metabolites were considered significantly different from control if p < 0.01 (Table 2.1).

Weighted Gene Correlations Network Analysis (WGCNA)

WGCNA was run separately for each time point (Langfelder and Horvath, 2008). A signed network was performed with a power of 8 and 7 for time point A and B respectively. Metabolites were correlated to each module per time point, and physiological data was correlated for time point B. Time point B data did not perform well in the WGCNA network due to the variation within the data, this analysis was dropped. The genes present in each module were correlated with metabolites that were found significant in the ANOVA (p<0.01), Supplemental File 1 (Appendix A) shows the modules with DEG for time point A. The GOSlim-Plant was selected

for the gene ontology classification with a Benjamini & Hochberg FDR correction of 0.01 annotated to *Arabidopsis thaliana*.

Integration of metabolite and transcriptional data

Linear correlation analysis was performed in R, with the significantly differentially expressed transcripts and metabolites from the second time point (B), along with the physiological data collected and previously reported by Britto de Assis Prado et al. (2016). Linear correlation was selected to highlight possible relationships between the metabolite and transcript abundance. Additionally, the approach taken in time point A, using WGCNA, was not appropriate for the second time point because of high variation within replicates of a given treatment and because far fewer transcripts were significantly different among treatments (see results). Correlations with an adjusted p-value (False Discovery rate) of 0.05 or less, and a correlation coefficient of 0.6 or greater were considered significant in this analysis.

Results

Transcriptome analysis

Sequencing produced a total of 635,649,777 paired-end reads, which were initially assembled into 187,216 contigs. Using a filter that kept only transcripts with at least 10 reads across the 4 replicates resulted in a final transcriptome containing 45,073 contigs with an average read length of 400 bp. Annotation of those contigs identified 27,502 unigenes with 11,538 BLASTX hits in the NCBI database. The transcriptome was classified into GO slim terms describing cellular components, molecular functions and biological processes (Appendix B Supplemental Fig. B.2). The highest abundance terms for cellular components, molecular functions, and biological processes were “cell”, “catalytic activity”, and “metabolic process” respectively, which was similar to another *Panicum maximum* transcriptome previously published (Toledo-Silva *et al.* 2013).

Multidimensional scaling (MDS) analysis of leaf transcripts showed a clear time point separation and clustering of treatments within each time point (Fig. 1.1a). The treatment separation was less apparent in time point B and the variability within the control and eC plots was also greater (Fig. 1.1a). Differential transcript expression from time point A identified 666 transcripts that were altered in the eCeT treatment when compared to control ($p < 0.1$, Appendix

A Supplemental File 2). No transcripts were differentially expressed in the eC treatment, when compared to control, and only 3 transcripts were significantly altered in the eT treatment. GO slim terms were assigned to the 666 transcripts altered in the eCeT treatment. Overrepresentation tests were run to identify GO categories with increased occurrence within the differentially expressed genes, with a false discovery rate of 0.05. The most overrepresented GO categories were cellular component “ribosome”, molecular function “structural constituent of ribosome”, and biological process “protein folding” (Appendix B Supplemental Fig. B.3). Time point B showed the same trend as time point A, but with fewer transcripts significantly changing; only 71 total in eCeT, 2 in eT, and 0 in eC ($p < 0.1$, Appendix A Supplemental File 2). The relaxed p value for DEG allowed a larger set of transcripts to test for correlation with metabolite content and/ or leaf physiological traits. No GO categories were overrepresented within the significant transcripts in time point B.

Metabolite analysis

Untargeted metabolomics was used to investigate biochemical changes in Guinea grass exposed to eC, eT and eCeT. Although 194 total metabolites were identified, only 125 metabolites were consistently present in the different treatments and those were used for analysis. MDS analysis revealed a separation of the eT treatment (red symbols) from the C plots (black symbols), but no clear separation between sampling dates (Fig. 2.1b). Additionally, the time points were not significantly different in the ANOVA ($p > 0.01$). A general linear model with a Dunnett’s test was run to test for significant differences in abundance among treatments, and 34 metabolites were significantly altered in at least one treatment compared to the C plots ($p \leq 0.01$, Table 2.1). Only 1-benzylglucopyranoside and isoleucine were significantly different in each of the treatments compared to the C plots.

Overall the metabolome showed the greatest change in the eT treatment compared to C with 30 metabolites significantly changing (Table 2.1). All of the metabolites increased in abundance in eT relative to ambient, with the exception of 2-methylmalic acid and oxalic acid (Table 2.1). Melibiose showed the largest increase in eT amongst sugars, with a 1.94-fold change; arabinose, fructose, galactose, glucose maltose, ribose, and xylose were also significantly increased in eT. Notably sucrose was not altered under any treatment. Many amino acids showed significant increases under eT compared to ambient with Val Thr, and Phe

increasing the most (1.64, 1.38, 1.27-fold; Table 2.1). Organic acids, including neochlorogenic acid and sinapic acid, which are involved in secondary metabolism and specifically monolignol metabolism showed significant increases under the eT treatment compared to C (Table 2.1).

The eC treatment did not affect the metabolome as much as eT, with only 2 metabolites showing a $\log_2\text{-fold} \geq |1|$ and $p \leq 0.01$. 2-Methylmalic acid (citramalate) had a large decrease (-3.02-fold) in the eC plots, and this compound decreased across all treatments. Additionally, α -ketoglutaric acid had a large decrease (-2.15-fold) in eC. Ile was the only amino acid impacted in the eC plots, and was moderately increased by 0.81 fold (Table 2.1). Quinic acid, an important intermediate of the lignin pathway, increased in the eC plots (0.92-fold). 1-Benzylglucopyranoside had 0.95 $\log_2\text{-fold}$ change in the eC plots.

The combination of eC and eT showed similar trends to the eT plots alone, but with a dampened effect (Table 2.1). All metabolites that had a significant change in the eCeT treatment were also significantly altered in the eT treatment. In both eT and eCeT, α -tocopherol increased significantly with a $\log_2\text{-fold} > 1$. Many compounds that were significantly increased in eT were not significantly altered in eCeT. For example, Ser and Thr were significantly increased in the eT treatment, but were not significantly impacted in eCeT, with a fold-change trend of decreasing in relative leaf content. Additionally, many sugars and sugar alcohols including; arabinose, fructose, galactose, glucose, and inositol increased in the eT plots but not in eCeT.

Integration of metabolite and transcript data – Timepoint A

Weighted gene correlation network analysis (WGCNA) was used to construct a signed network of clustered genes from the time point A (sampled after 30 days of treatment). Using 9802 transcripts, expression data were clustered into 9 unique modules. GO enrichment was performed for each module to identify the overrepresented biological functions within each grouping (Appendix A Supplemental Table 1). Module 1 contained the largest number of transcripts (1719 total), but only 2 were differentially expressed in the eCeT treatments. Module 3 contained 948 transcripts, 84 of which were differentially expressed in eCeT, and had significant enrichment in regulation of salicylic acid biosynthesis process, defense to oomycetes, cell surface receptor signaling, plant-type hypersensitive response, protein phosphorylation, defense response to bacterium, and oxidation-reduction process. In addition, module 3 had a significant negative correlation with melibiose (Appendix A Supplemental Table 1, Appendix B Supplemental Fig.

B.4). Module 5 contained 484 transcripts, 35 of which were differently expressed in eCeT treatments, with significant enrichment in oxylipin biosynthesis, terpenoid biosynthesis, response to wounding, hormone biosynthesis, secondary metabolite biosynthesis, defense response, and oxidation-reduction process.

Within module 3, four transcripts were strongly correlated with melibiose content ($r \geq |0.8|$, $p < 0.01$; Fig 2.2). Melibiose showed significant negative correlations with the expression of two guard cell transcripts. The first, At4g23160, is annotated as cysteine-rich receptor-like protein kinase 8 (CRK8) and shows biological activity involved in defense response to bacterium and protein phosphorylation. The second was At3g48300, a cytochrome P450 (CYP71A23) involved in oxidation-reduction processes. The strongest relationship was identified for transcript ID, At1g54960, which was annotated as a subfamily of mitogen-activated protein kinase kinase kinase 2 (MAP3K) known as Arabidopsis Nucleus- and Phragmoplast-Localized Kinase1 (NPK1) – related protein kinases (ANPs) specifically ANP2, in the *Arabidopsis* genome. The final gene with a negative linear relationship with melibiose was identified as transcript ID, At5g54160, a caffeic acid/ 5-hydroxyferulic acid O-methyltransferase 1 (COMT) with functions in lignin and flavonoid biosynthesis.

Integration of metabolite and transcript data – Timepoint B

Several significant relationships were identified within the metabolite and transcript datasets, with a $r \geq |0.75|$ and an adjusted $p \leq 0.01$ (Table 2.2). All significant linear correlations between DEG and metabolites are shown in Supplemental File 3 (Appendix A). Xylose had the most significant linear correlations, 10 positive and one negative. The strongest relationship was between gene ID At5g19440 ($r=0.87$) (Fig. 2.3). The BlastX annotation hit for *Arabidopsis thaliana* identified At5g19440 as a NAD(P)-binding protein, located in the cytosol, and involved in cinnamyl-CoA reductase (CCR) activity in a variety of biological processes including lignin biosynthesis and response to cold. This sequence was highly similar to the annotated CCR1 in the *Z. mays* genome (LOC100382657) and predicated CCR1 in *S.italica* genome (LOC101786254).

The second strongest relationship between a transcript and xylose was transcript At5g56870 ($r= 0.84$). This transcript was annotated as β -galactosidase 4 (BGAL4) in the *Arabidopsis* genome. This gene is localized to the cytoplasm and functions in rRNA N-

glycosylase activity and defense response, also acting as a negative regulator of translation. Along with the strong linear correlation to xylose, BGAL4 was also significantly correlated with α -tocopherol, stigmasterol, and O-acetylsalicylic acid ($r=0.80, 0.76, 0.75$, respectively; Appendix A Supplemental File 3). Two additional transcripts, At5g03840 ($r=0.81$) and At1g36160 ($r= -0.81$), showed significant linear correlations with xylose content. Transcript ID At5g03840 was annotated as terminal flower 1 (TFL1) controlling inflorescence meristem identity within the *Arabidopsis* genome and showed 73% homolog with the *Zea mays* ZCN12 (*Zea centroradialis* (ZCN)) (NM_001112779.2). This gene functions in many biological roles including acting as a negative regulator of cell aging and is expressed during growth and development stages from flowering to leaf senescence. ZCN12 was also found to have strong correlations with sinapic acid and valine (Appendix A Supplemental File 3). Unlike the first three gene correlations, At1g36160, showed a negative linear correlation with xylose and sinapic acid ($r = -0.74$). This gene was annotated in the *Arabidopsis* genome as acetyl-CoA carboxylase 1 (ACC1), which regulates the initial step in the fatty acid biosynthesis pathway of acetyl-CoA to malonyl-CoA.

In addition to the strong associations with xylose (Fig. 2.3e), transcript ID At1g65480 was correlated with 4 additional metabolites. The BlastX annotation hit for *Arabidopsis thaliana* identified this transcript as flowering locus T (FLT) functioning in phosphatidylethanolamine binding with biological processes in flower development. The Blast annotation for *Zea mays* had only 52% homology with TWIN SISTER of FT (NM_001309850.1) and predicted *Setaria italica* FLT (XM_004960683.3) with a homology of 53%. Positive linear correlations were identified between FLT and valine, sinapic acid, O-acetylsalicylic acid, β - sitosterol, and xylose ($r = 0.84, 0.82, 0.78, 0.77, 0.75$) respectively (Appendix B Supplemental Fig. B.5).

Discussion

This study investigated the transcriptional and metabolite response of Guinea grass to growth at eC, eT and the combination of eCeT under open-air field conditions. Previous physiological analysis of the plants grown at this FACE experiment revealed that growth at eT increased leaf biomass and leaf area, but there was considerable variation within each treatment (Appendix B Supplemental Fig. B.6) (Britto de Assis Prado *et al.* 2016). The transcript and metabolite data also showed significant variation within each treatment, revealed by MDS (Fig. 2.1), but different groupings were apparent in the transcript and metabolite data. The transcriptome

showed a clear separation among sampling dates (30 or 50 days of treatment), while the metabolome data showed a clear separation of treatments, most clearly C and eT (Fig. 2.1). The transcriptome also showed separation of treatments at the first sampling date (Timepoint A), but not the second (Timepoint B) (Fig. 2.1a). The within-treatment variability observed in the data may be due to environmental and edaphic properties of the field site (i.e., soil characteristics and/or slope), which could not be accounted for in the analysis, or from the relatively low sample size (n=4). However, the fact that no transcripts were significantly altered in response to eC alone is consistent with previous experiments done with other C₄ grasses, which showed that in the absence of drought stress, there is no direct effect of elevated [CO₂] on C₄ metabolism (Wall *et al.* 2001; Leahey *et al.* 2006). On the other hand, eT stimulated the growth of *P. maximum* in this study (Appendix B Supplemental Fig. B.6), yet only a few genes were significantly differentially expressed in eT alone. Optimal growth for *P. maximum* is estimated to be at least 21°C, and background temperature in ambient conditions during this experiment was only 17.0 ± 0.06°C, so the eT treatment moved the plants closer to optimal growth temperatures (Araujo *et al.* 2013). In the combination (eCeT) treatment, significant changes in the transcriptome were observed, most notably transcripts involved in protein translation and assembly.

A key aim of this work was to integrate the transcript and metabolite data sets. Because of the clear differences in the transcript data between time points A and B (Fig. 2.1a), integration was done within each time point and not across time points. In addition, time point B offered the potential to integrate leaf physiological data with the transcript and metabolite datasets. Because of the large level of variability within individual treatments for all of the parameters, linear correlation analysis was used for data integration. For time point A WGCNA networks were built to group genes responding in similar ways together to form a larger network. These networks were then correlated with the metabolite data to identify interactions of interest. Because there were fewer DEG within time point B, WGCNA was not possible, and so for time point B, simple linear correlations between the DEG, significant metabolites, and leaf physiological data were used to identify correlations of interest.

Integration of metabolomic and transcript data – Time point A

Two modules (3 and 5) from the WGCNA analysis contained significant numbers of DEG (Appendix A Supplemental Table 2). The DEG identified within module 3 were enriched in

salicylic acid (SA) biosynthesis (p 0.016) and general defense response to microbes, suggesting greater stress in the eCeT treatment compared to control. The enrichment of SA biosynthesis transcripts has previously been identified to be part of the heat-stress response in plants. Salicylic acid stabilizes heat shock transcription factors increasing their binding to heat shock elements and thus promoting heat shock-related genes (Wahid *et al.* 2007). While the background temperature during the experiments was low, it is possible that the combination of eC and eT increased leaf temperatures more than just the eT temperature alone, thus triggering some stress-related transcriptional responses. The GO-term enrichment analysis of the transcripts associated with module 5 were also in agreement with a trend towards up-regulation of secondary metabolites involved in defense response.

Many amino acids are precursors to secondary metabolites that change in response to various environmental perturbations. In this study, the eT and eCeT treatments resulted in significantly greater levels of amino acids derived from oxaloacetate and pyruvate. Accumulation of such amino acids is well known in abiotic stresses and heat (Joshi *et al.* 2010; Kaplan *et al.* 2004; Rizhsky *et al.* 2004; Guy *et al.* 2008; Obata & Fernie 2012; Obata *et al.* 2015). The role of branched chain amino acids and other amino acids in improving heat tolerance is complex and not fully understood, but likely provides support for an increase in production of secondary metabolites to influence defense response during temperature stress (Kaplan *et al.* 2004; Du *et al.* 2011). Additionally, increases in amino acid pools have the potential to serve as osmolytes under heat stress (Joshi *et al.* 2010) or an alternative electron donor for the mitochondrial electron transport chain (Araujo *et al.* 2013). High levels of Ile, Val, and Phe were observed in the leaves of Bermuda grass and mature kernels of rice exposed to elevated temperature and were thought to improve heat tolerance (Du *et al.* 2011; Yamakawa & Hakata 2010; Kaplan *et al.* 2004). The buildup of transcripts in the brown and green modules involved in defense and secondary metabolite biosynthesis in combination with the increase in amino acids suggest up-regulation of defense metabolism in the eCeT treatment at 30 days post-treatment exposure.

Plants grown under abiotic stress conditions use compatible osmolytes, low molecular mass compounds, to provide an adaptive mechanism. Accumulation of these soluble sugars such as fructose, sucrose, and glucose are documented to signal stress conditions and improve tolerance to cold, heat, and drought stress ((Du *et al.* 2011; Wahid *et al.* 2007; Kaplan *et al.*

2004; Rizhsky *et al.* 2004). The eT plots, regardless of [CO₂], showed increased soluble sugar content; including melibiose. Melibiose is a common osmolyte and a byproduct of the hydrolysis of raffinose, via invertase (ElSayed *et al.* 2014), and has been identified to accumulate under various environmental conditions including drought stress and elevated temperature in both C₃ and C₄ species (Rizhsky *et al.* 2004; Kaplan *et al.* 2004; Du *et al.* 2011). Figure 2.2 shows the relationship of four transcripts negatively associated with melibiose, the strongest of which was MAP3K. MAPKs are highly conserved signaling modules in eukaryotes involved in cellular responses, including response to stimuli. These cascades trigger activation of transcription factors, via phosphorylation, and other cellular responses, to signal a stimulus response (Xu and Zhang 2015). This transcript was annotated as ANP2/NPK1; these enzymes activate stress-related MAPKs activated by ROS (Kovtun *et al.* 2000). In this study, there was an inverse relationship between MAP3K expression and melibiose content, suggesting that there was less stress-related signaling in the eT plots.

The second transcript highly associated with melibiose was a caffeic acid/ 5-hydroxyferulic acid O-methyltransferase 1 (COMT). Switchgrass and maize mutants have shown that downregulation of COMT impacts lignin content of biofuel crops specifically decreasing the S-lignin units and improving ethanol yields (Liu *et al.* 2017; Guillaumie *et al.* 2008). Downregulation of this transcript could signal a shift from lignin biosynthesis to secondary defense metabolism.

The final two transcripts with a strong negative relationship to melibiose were CRK6 and CYP71A23, annotated as CYP72A26 in the maize genome (Jameson *et al.* 2008). Both of these transcripts are expressed in the guard cells. CRKs are induced by pathogen infection, and transgenic *Arabidopsis* plants with significantly elevated levels of CRK5 had enhanced leaf growth and increased pathogen resistance (Chen *et al.* 2003). CRK6 was found to be involved in ROS mediated signal processes with increased transcript abundance after exposure to pathogens, SA, and ROS (Chen *et al.* 2003; 2004). The decrease in abundance of transcripts involved in guard cell metabolism is interesting and could potentially indicate a stomatal response within the eT and eTeC plots. The correlation between transcripts that are tightly regulated with complex feedback interactions between environment and cellular metabolism and melibiose lead us to suggest that melibiose functions in osmotic adjustment under eT and eTeC to protect cellular components and enhance acclimation to eT.

Integration of growth, metabolomic, and transcript data – Timepoint B

Time Point B (50 d of treatment) allowed us to test the correlation of leaf biomass and area with metabolite and transcript levels; however, no significant correlations were observed. The low temperatures reported on the day of sampling may have greatly affected the metabolite and transcript levels, hindering the correlation of these datasets with leaf measurements taken at the end of the season. Field conditions are also inherently more variable than controlled environment conditions, but field experiments better represent the variation expected in the future. After 50 d of treatment, we did observe significant correlations between transcripts and metabolites, with the most notable being xylose (Table 2.2). Xylose is a monosaccharide and a precursor to pyruvate and acetyl-CoA.

The use of linear correlation analysis allowed for the identification of associations that might not have been predicted with traditional methods. Three transcripts, shown in Figure 2.3, had a strong positive correlation with xylose content. The strongest correlation was with *Z. mays* cinnamoyl-CoA reductase (CCR1). CCR1 is the entry point into the lignin branch of the phenylpropanoid pathway (Piquemal *et al.* 1998). The position of CCR in the phenylpropanoid pathway gives this enzyme direct control over the direction of the metabolic flux toward flavonoids or monolignols (Sattler *et al.* 2017). *ZmCCR1* mutants with a moderate down-regulation of CCR1 activity increased the digestibility of cell walls without severely modifying lignin content (Tamasloukht *et al.* 2011). The overexpression of *SbMyb60* transcription factor in *Sorghum bicolor* was found to directly impact the monolignol biosynthesis with higher abundance of syringyl lignin, cell wall composition, with increases in soluble phenolic and aromatic amino acids (Scully *et al.* 2016; 2018). The observed increase in abundance of sinapic acid, phenylalanine, and α -Tocopherol under the eT treatment, regardless of [CO₂], point to an impact of eT on lignin composition, possibly increased S-type concentrations, although that would need to be tested. In addition to components of cell wall structure, monolignol polymers have been induced under various biotic and abiotic stress conditions (Tronchet *et al.* 2010; Vincent *et al.* 2005). A similar heating experiment with both C₄ and C₃ grasses was performed at the prairie heating and CO₂ enrichment site located in Wyoming, USA. A metabolomic profiling of *Bouteloua gracilis*, a C₄ grass, identified similar responses including an increase in phenolic and sugar content within the senesced leaves of plants grown under elevated temperature and the

combination treatment, along with an increase in amino acid content under the elevated temperature with ambient [CO₂] (Suseela *et al.* 2014).

Sugars are continuously moving thru the phloem from source to sink and convey signaling information along the way, including regulation of flowering time (Gibson 2005). In several species adding exogenous sucrose and possibly other sugars promotes flowering (Cho 2018). Interestingly, xylose was identified to have two significant positive correlations with flowering time-related genes, FT and ZCN12 (AtTFL1, Fig. 2.3 c, d). The *Arabidopsis* orthologs are two closely related genes that are key integrators of flowering transition pathways. These two genes have antagonistic functions in the flower transition pathway; FT promotes flowering and TFL1 suppresses the transition (Shannon *et al.* 1991). Unlike the ortholog gene, ZCN12 belongs to the FT-like II group, primarily expressed in the leaf blades, and is activated after floral transition and is continually express through late stages of vegetative development (Danilevskaya *et al.* 2010). FT-like proteins mediate numerous processes including growth, plant architecture, fruiting and tuber formation (Pin & Nilsson 2012). Recent evidence has shown FT can act as a cell autonomous regulator of stomatal guard cell opening and closure (Kinoshita *et al.* 2011). Kinoshita and colleagues showed overexpression of FT in *Arabidopsis* increased H⁺-ATPases activity within the guard cells, suggesting that FT likely influences the stomatal opening/ closure via regulation of H⁺-ATPase. The accumulation of FT related transcripts within eT and eCeT could offer an additional regulation of stomatal function correlating with the osmotic adjustment provided from the accumulation of soluble sugars, such as melibiose and xylose.

Conclusion

This study investigated tropical Guinea grass response to global atmospheric change and rising temperature in the field. Although warming treatments in temperate regions often decreased productivity, heating the canopy 2 °C above ambient in this experiment resulted in increased leaf area and biomass. Field transcriptomics and metabolomics identified metabolic pathways that were altered by growth at elevated [CO₂] and temperature. Melibiose and xylose content were greater in plants exposed to elevated temperature, and content of these sugars was significantly correlated to the expression of genes involved in secondary metabolism, defense responses, and stomatal function. We hypothesize that the metabolite and transcript responses were associated with alleviation of stress and greater growth at elevated temperature.

TABLES AND FIGURES

Table 2.1: List of metabolites with significant changes in the experimental treatments ($p \leq 0.01$). The $\log_2$ normalized median values $\pm$ standard error and $\log_2$ fold change ($\log_2(\text{FC})$) for each treatment are shown. Metabolites significantly changed are denoted by bold value ($p \leq 0.01$) or * ($p \leq 0.05$). Orange cells show increased metabolite content and blue represents decreased metabolite content in the treatments.

Compound	Ambient	Elevated [CO ₂]		Elevated Temperature		Combination	
	$\log_2(\tilde{x}) \pm \text{SE}$	$\log_2(\tilde{x}) \pm \text{SE}$	$\log_2(\text{FC})$	$\log_2(\tilde{x}) \pm \text{SE}$	$\log_2(\text{FC})$	$\log_2(\tilde{x}) \pm \text{SE}$	$\log_2(\text{FC})$
1,3-Diaminopropane	9.21 $\pm$ 0.27	9.81 $\pm$ 0.23	0.60	10.91 $\pm$ 0.13	1.70	9.95 $\pm$ 0.24	0.75
1-Benzylglucopyranoside	9.61 $\pm$ 0.21	10.56 $\pm$ 0.17	0.95	10.89 $\pm$ 0.26	1.28	10.76 $\pm$ 0.15	1.15
2-Methylmalic acid	11.27 $\pm$ 0.48	8.25 $\pm$ 0.17	-3.02	9.60 $\pm$ 0.17	-1.67	10.30 $\pm$ 0.38	-0.97
α -ketoglutaric acid	9.93 $\pm$ 0.33	7.78 $\pm$ 0.21	-2.15	10.57 $\pm$ 0.25	0.63	9.21 $\pm$ 0.25	-0.72
γ -tocopherol	8.48 $\pm$ 0.17	8.90 $\pm$ 0.14	0.42	9.12 $\pm$ 0.18	0.64	8.26 $\pm$ 0.12	-0.22
Arabinose	12.68 $\pm$ 0.12	13.21 $\pm$ 0.17	0.52	13.92 $\pm$ 0.25	1.24	13.34 $\pm$ 0.25	0.65
Arabitol	10.90 $\pm$ 0.22	11.61 $\pm$ 0.26*	0.71	11.67 $\pm$ 0.14	0.77	11.02 $\pm$ 0.16	0.12
Dehydroascorbic acid	11.54 $\pm$ 0.21	11.81 $\pm$ 0.14	0.27	12.57 $\pm$ 0.15	1.02	11.71 $\pm$ 0.19	0.17
Ethanolamine	14.58 $\pm$ 0.15	15.07 $\pm$ 0.13*	0.49	15.23 $\pm$ 0.11	0.65	14.70 $\pm$ 0.16	0.12
Fructose	20.19 $\pm$ 0.19	20.57 $\pm$ 0.21	0.38	21.27 $\pm$ 0.11	1.08	20.38 $\pm$ 0.23	0.19
Galactose	15.85 $\pm$ 0.18	16.71 $\pm$ 0.21	0.87	16.88 $\pm$ 0.18	1.03	16.22 $\pm$ 0.20	0.37
Gluconic acid	10.03 $\pm$ 0.05	10.68 $\pm$ 0.23	0.65	10.98 $\pm$ 0.17	0.95	10.77 $\pm$ 0.20	0.74
Glucose	20.55 $\pm$ 0.08	21.10 $\pm$ 0.21*	0.55	21.47 $\pm$ 0.09	0.92	20.54 $\pm$ 0.23	-0.01
Glyceric acid	16.47 $\pm$ 0.12	17.20 $\pm$ 0.09	0.72	17.25 $\pm$ 0.15	0.78	16.54 $\pm$ 0.20	0.07
Inositol	14.78 $\pm$ 0.18	15.50 $\pm$ 0.19	0.71	15.83 $\pm$ 0.15	1.05	15.47 $\pm$ 0.16	0.68
Isoleucine	9.59 $\pm$ 0.17	10.40 $\pm$ 0.25	0.81	10.75 $\pm$ 0.22	1.16	10.58 $\pm$ 0.25	0.98
Leucine	9.45 $\pm$ 0.33	10.11 $\pm$ 0.11	0.66	10.95 $\pm$ 0.39	1.50	10.44 $\pm$ 0.35	0.98
Maltose	15.06 $\pm$ 0.15	15.50 $\pm$ 0.14	0.44	15.74 $\pm$ 0.07	0.68	14.98 $\pm$ 0.26	-0.08
Melibiose	11.08 $\pm$ 0.23	11.91 $\pm$ 0.19	0.84	13.02 $\pm$ 0.27	1.94	12.24 $\pm$ 0.37	1.17
Neochlorogenic acid	16.72 $\pm$ 0.21	16.96 $\pm$ 0.11	0.24	17.32 $\pm$ 0.14	0.60	17.17 $\pm$ 0.13	0.45
O-acetylsalicylic acid	10.73 $\pm$ 0.11	11.00 $\pm$ 0.12	0.27	11.79 $\pm$ 0.19	1.06	11.09 $\pm$ 0.22	0.36

Table 2.1 (continued).

Oxalic acid	13.46 ± 0.16	13.54 ± 0.15	0.08	11.92 ± 0.31	-1.54	12.04 ± 0.31	-1.42
Phenylalanine	9.49 ± 0.13	9.83 ± 0.21	0.34	10.76 ± 0.36	1.27	10.81 ± 0.40	1.31
Quinic acid	17.30 ± 0.22	18.22 ± 0.12	0.92	18.14 ± 0.12	0.84	17.68 ± 0.13	0.39
Ribitol	10.25 ± 0.25	10.81 ± 0.17	0.55	11.57 ± 0.21	1.32	10.90 ± 0.22*	0.64
Ribose	14.92 ± 0.17	15.25 ± 0.10	0.33	15.56 ± 0.12	0.64	15.13 ± 0.18	0.21
Serine	14.04 ± 0.11	13.92 ± 0.11	-0.12	15.15 ± 0.16	1.11	14.36 ± 0.26	0.32
Sinapic acid	7.30 ± 0.19	7.74 ± 0.15	0.45	7.99 ± 0.20	0.69	7.98 ± 0.18	0.69
Threitol	8.97 ± 0.12	9.28 ± 0.09	0.31	10.23 ± 0.15	1.26	9.78 ± 0.16	0.81
Threonine	11.90 ± 0.29	12.38 ± 0.11	0.46	13.30 ± 0.12	1.38	12.77 ± 0.26	0.85
Valine	10.57 ± 0.40	11.19 ± 0.15*	0.61	12.22 ± 0.18	1.64	11.91 ± 0.26	1.34
Xylose	8.80 ± 0.20	9.71 ± 0.15*	0.91	10.26 ± 0.26	1.46	10.08 ± 0.26	1.27

Table 2.2: Number of significant linear correlations between metabolite content and gene expression measured at time point B (50 d of treatment). Correlations have $|r| \geq 0.75$ and $p < 0.01$.

Metabolite	Positive	Negative
1-Benzylglucopyranoside	1	0
α -Tocopherol	2	0
β -Sitosterol	1	0
O-acetylsalicylic acid	2	0
Sinapic acid	4	1
Stigmasterol	1	0
Valine	2	0
Xylose	10	1

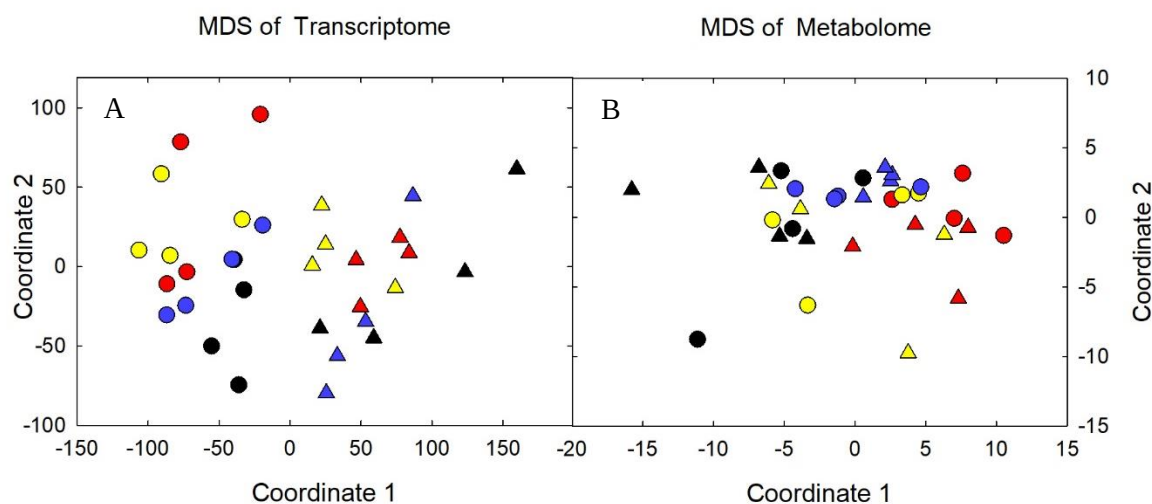


Figure 2.1: Multidimensional Scaling (MDS) plots for the transcript and metabolite experiments. (A) MDS plot using transcript data, showing a clear time point separation (circles vs triangles) as well as separation of elevated temperature and ambient temperature in Timepoint A (circles). (B) MDS plot using metabolite data, showing no clear separation of time points, but a clear separation of ambient treatment (black) from elevated temperature treatment (red). *Time Point A (Circles), Time Point B (Triangles), Ambient (black), Elevated [CO₂] (blue), Elevated Temperature (red), Combination (yellow)*

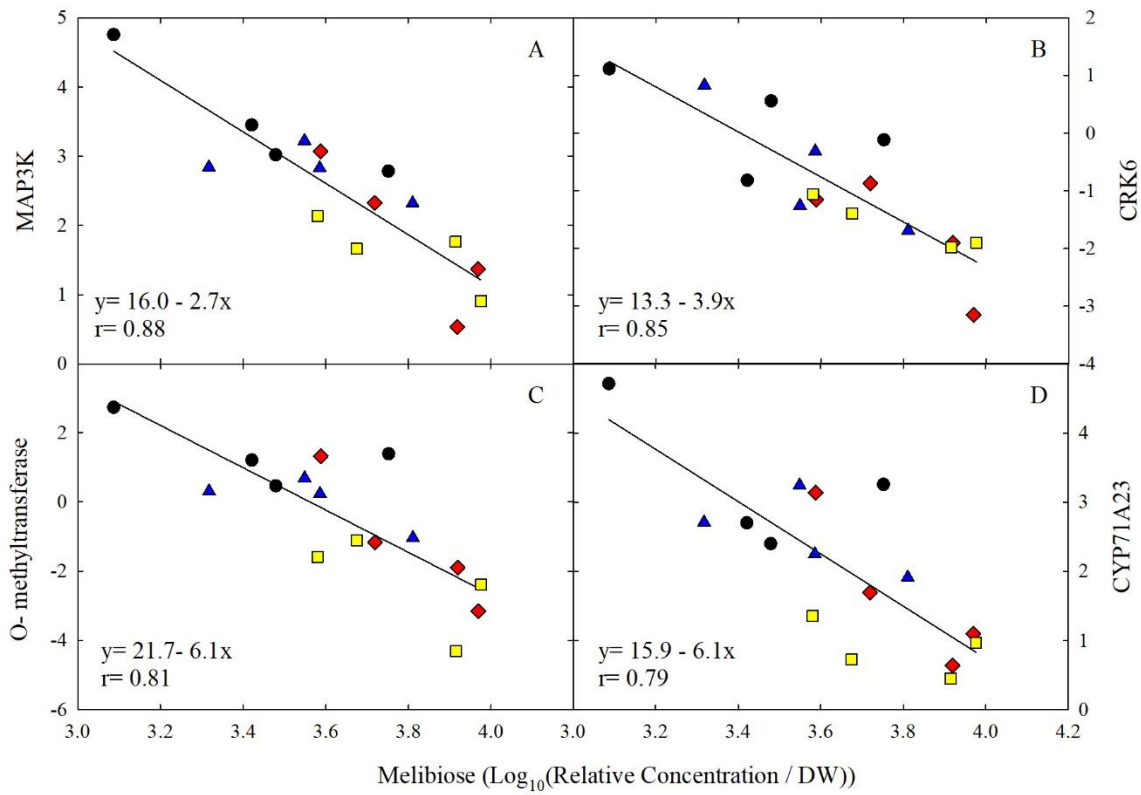


Figure 2.2: Linear correlation between gene expression and melibiose content. Significant linear relationships between melibiose and transcripts present within the weighted gene correlation network analysis module 3, for time point A (30 d of treatment). Correlations listed have $|r| \geq 0.70$ and $p < 0.01$. Normalized expression values for each transcript are plotted on the y axis: A) At1g54960 - mitogen-activated protein kinase kinase kinase 2 (MAP3K); B) At4g23160 - cysteine-rich receptor-like protein kinase 8 (CRK8); C) At5g54160- O-methyltransferase; D.) At3g48300 - cytochrome P450 (CYP71A23). *Ambient (black, circle), elevated $[\text{CO}_2]$ (blue, triangle), elevated temperature (red, diamond), combination (yellow, square).*

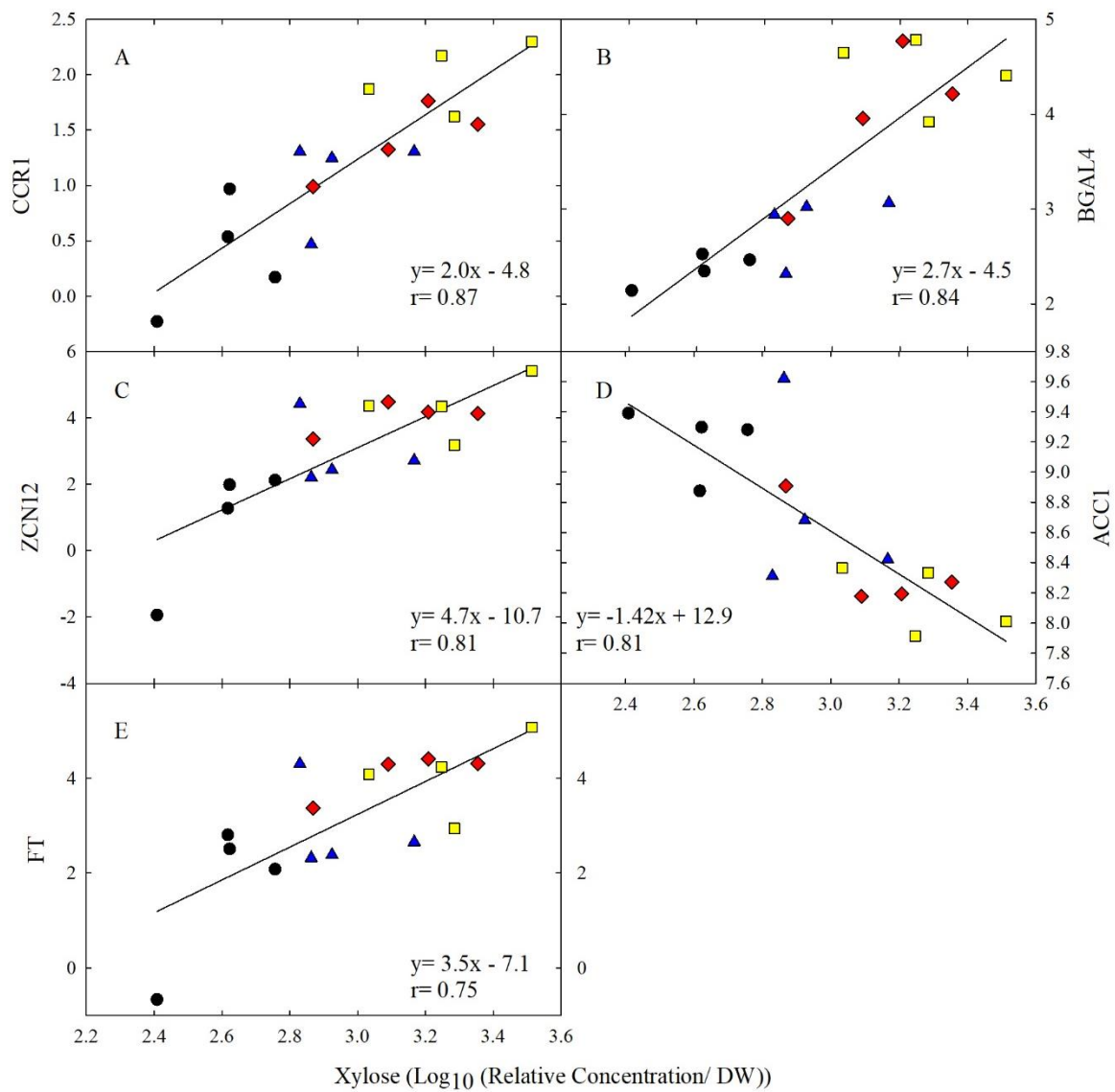


Figure 2.3: Linear correlation between gene expression and xylose content. Significant linear relationships between xylose content and transcript expression measured at time point B (50 d of treatment). Correlations listed have $|r| \geq 0.70$ and $p < 0.01$. Normalized expression values for each transcript are plotted on the y axis: A) At5g19440 – NAD(P)-binding protein (CCR1); B) At5g56870 – β -galactosidase 4 (BGAL4); C) At5g03840 – terminal flower 1/Zea centroradialis (ZCN12); D) At1g36160-acetyl-CoA carboxylase 1(ACC1); E) At1g65480- flowering locus T (FT). Ambient (black, circle), elevated $[\text{CO}_2]$ (blue, triangle), elevated temperature (red, diamond), combination (yellow, square).

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CHAPTER III: AGE-DEPENDENT INCREASE IN α -TOCOPHEROL AND PHYTOSTEROLS IN MAIZE LEAVES EXPOSED TO ELEVATED OZONE POLLUTION

Abstract

Tropospheric ozone is a major air pollutant that significantly damages crop production around the world. Crop metabolic responses to rising chronic ozone stress have not been well-studied in the field, especially in C₄ crops. In this study, we investigated the metabolomic profile of leaves from two diverse maize (*Zea mays*) inbred lines and the hybrid cross during exposure to season-long elevated ozone (~100 nL L⁻¹) in the field using free air concentration enrichment (FACE). The hybrid line, B73 x Mo17, showed an acceleration of chlorophyll loss under elevated ozone accompanied by a significant change in the metabolite profile. In contrast, the metabolite profile, although significantly different between the two inbred lines (B73 and Mo17), was not different in ambient and elevated ozone treatments. Phytosterols and α -tocopherol levels increased in B73 x Mo17 leaves as they aged, and to a significantly greater degree in elevated ozone stress. Untargeted metabolomic profiling revealed that inbred and hybrid lines of maize differ in ozone response and metabolic responses provide stabilization of membranes and chloroplast reactive oxygen species quenching in the responsive hybrid line.

Introduction

Tropospheric ozone (O₃) is a secondary air pollutant formed from the reaction of nitrogen oxides and volatile organic compounds in the presence of UV radiation. From 1980 to 2011, O₃ concentrations ([O₃]) in the United States are estimated to have caused a yield loss of approximately 10% for rain-fed maize, with an estimated economic cost of \$7.2 billion per year (McGrath *et al.* 2015). Ozone diffuses through the stomata into the intercellular airspace, where it rapidly reacts to form other reactive oxygen species (ROS). Antioxidants within the apoplast quickly detoxify the ROS, but if the capacity is overwhelmed, the redox state of the cell is altered, initiating molecular damage and signaling cascades both in the apoplast and symplast (Vainonen & Kangasjarvi 2015). Ozone-induced oxidative stress can accelerate senescence, impair photosynthesis, and alter metabolism, ultimately resulting in a reduction of plant productivity and crop yield (Choquette *et al.* 2019; Kangasjarvi *et al.* 2005).

While much is known about the biochemistry of metabolic and signaling responses within plant cells to O₃, major knowledge gaps remain with regard to: (1) the mechanisms underlying genetic variation in O₃ response, and (2) the nature of biochemical O₃ responses in the production environment of a farm field (Ainsworth 2017). Much of our understanding of plant metabolic responses to O₃ stress comes from acute experiments in controlled environments (Vainonen & Kangasjarvi 2015), yet it is also well known that the mechanisms of response to chronic O₃ exposure, generally defined as long-term exposure to concentrations around 100 nL L⁻¹ or lower, differ from acute responses (Grantz & Vu 2012; Vahala *et al.* 2003). Increased emissions of precursor pollutants have primarily increased crop exposure to chronic O₃ stress, with tropospheric [O₃] increasing from ~10 nL L⁻¹ in the late 1800's to ~40-50 nL L⁻¹ today (Brauer *et al.* 2016; Monks *et al.* 2015). In many countries, O₃ concentrations continue to increase (Brauer *et al.* 2016), further intensifying chronic O₃ exposure. Free Air gas Concentration Enrichment (FACE) technology enables crops to be grown under standard agronomic conditions with elevated, chronic O₃ exposure (Choquette *et al.* 2019; Morgan *et al.* 2006; Tang *et al.* 2011).

Upon entering the leaf, O₃ forms ROS in the apoplast, reacts with the plasma membrane and a number of proteins within it (e.g., NADPH oxidases, aquaporins, receptor-like kinases, and G-proteins), and quickly elicits an increase in cytosolic calcium (Short *et al.* 2012). Peroxidation and denaturation of membrane lipids can occur with O₃ exposure (Loreto & Velikova 2001; Pell *et al.* 1997). The intracellular response to the influx of ROS depends upon the duration and intensity of O₃ exposure, with calcium, hormone signaling, and MAP kinase cascades all playing a role in regulation of transcriptional and biochemical changes (Vainonen and Kangasjarvi 2015). Under chronic O₃ stress, transcriptional changes have been associated with decreased photosynthesis (Leitao *et al.* 2007; Li *et al.* 2019; Pell *et al.* 1997), increased rates of mitochondrial respiration and antioxidant production (Gillespie *et al.* 2012; Yendrek *et al.* 2015), increased hormone biosynthesis (Kangasjarvi *et al.* 2005; Vainonen & Kangasjarvi 2015)), and early activation of several *senescence associated genes* (SAGs) (Betzelberger *et al.* 2012; Fiscus *et al.* 2005; Gillespie *et al.* 2012; Kangasjarvi *et al.* 2005; Miller *et al.* 1999). Shifts in metabolism from carbon assimilation to defense and detoxification in combination with early senescence, are thought to be major drivers of reduced plant productivity under elevated [O₃] (Dizengremel 2001; Feng *et al.* 2011; Morgan *et al.* 2006; Yendrek *et al.* 2017a).

To limit abiotic stresses that cause production of ROS in various cellular compartments of plants, a diverse signaling network integrates antioxidant metabolism, hormones, sugars, and plant secondary metabolites to regulate plant defense responses and growth (Munne-Bosch, 2005). In addition to antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) that are often upregulated in response to O₃ stress (Moura *et al.* 2018; Singh *et al.* 2014), a wide range of metabolites act as non-enzymatic ROS scavengers. These include carotenoids, α -tocopherol, ascorbic acid, glutathione, and phenolic compounds (Foyer & Noctor 2005). Plant steroids, such as phytosterols and brassinosteroids (BRs), influence many aspects of plant growth, including senescence and tolerance to abiotic stress (Vriet *et al.* 2012). Phytosterols help maintain plasma membrane fluidity and permeability during stress conditions (Griebel & Zeier 2010). In the chloroplasts, α -tocopherol is an important scavenger that protects photosynthetic machinery by quenching singlet oxygen or inhibiting the progression of lipid peroxidation (Havaux *et al.* 2005), preserving the rate of photosynthesis. But it is unknown if these metabolites play a role in O₃ response under chronic exposure and field conditions. If so, breeding or biotechnology might be used to leverage the protective roles of phytosterols and non-enzymatic antioxidants to improve tolerance to O₃-induced oxidative stress.

More broadly, metabolomics provides a tool to explore biochemical signatures that may be predictive of environmental stress effects on primary productivity, even in field conditions. Studies have examined the relationship between leaf metabolites and physiological traits in the field under various abiotic stress conditions in maize (Obata *et al.* 2015; Riedelsheimer *et al.* 2012), rice (Melandri *et al.* 2020), and Guinea grass (Wedow *et al.* 2019). But, metabolomic responses of maize to elevated [O₃] have not yet been widely characterized. Maize is one of the world's most widely-grown crops, and roughly 32% of the global production occurs in the U.S. (USDA FAS 2020). It is also a model species for the C₄ plant functional group that includes many other important crops used to produce food, fuel, forage and fiber, including sorghum and sugarcane. To address knowledge gaps regarding the response of maize to elevated [O₃], we examined the metabolomic profile of maize inbred (B73 and Mo17) and hybrid (B73 x Mo17) lines grown at the FACE facility at the University of Illinois, Urbana-Champaign. The response of B73, Mo17 and the hybrid B73 x Mo17 to elevated [O₃] was compared in replicated plots exposed to either ambient [O₃] (~40 nL L⁻¹) or elevated [O₃] (100 nL L⁻¹). This elevated [O₃] treatment was roughly 2.5 times the current average summer concentration in central Illinois, but

consistent with current [O₃] in polluted regions of Asia (Hong *et al.* 2019) where climate change is expected to increase O₃ pollution further. Additionally, many regions with high O₃ events overlap with the most productive croplands across the world (Ainsworth 2017; Ramankutty *et al.* 2008). In this study, leaf metabolomic profiles were investigated at three time points from early to middle stages of leaf senescence as characterized by chlorophyll content. Metabolite content was then linked to leaf mass per unit area and grain yield to identify potential biochemical markers for O₃ response in maize. B73 and Mo17 are two classic elite inbred maize lines (Stuber *et al.* 1992), which serve as the parents for widely used mapping populations to study the genetic architecture of numerous traits (Balint-Kurti *et al.* 2007; Mickelson *et al.* 2002; Pressoir *et al.* 2009; Sorgini *et al.* 2019; Wassom *et al.* 2008). We aimed to test the predictions that (1) maize metabolomic signatures would be altered under elevated [O₃], with a more pronounced effect as the leaves aged; (2) an increase in metabolites associated with oxidative stress and/or membrane stabilization would occur in sensitive maize lines; (3) the contents of key metabolites would be correlated with variation in yield loss under elevated [O₃].

Materials and methods

Field site and experimental conditions

In 2015, maize (*Zea mays*) inbred (B73 and Mo17) and hybrid (B73 x Mo17) lines were studied at the FACE research facility in Savoy, IL (<https://soyface.illinois.edu/>). The facility is a 32-ha farm where maize and soybean (*Glycine max*) are grown in annual rotation as commonly practiced in the region. Maize inbred and hybrid lines were planted with a precision planter in rows spaced 0.76 m apart and 3.35 m in length at a density of eight plants m⁻¹ in four pairs of ambient [O₃] and elevated [O₃] plots ($n = 4$) (Appendix C Supplemental Fig. C.1a). Hybrid and inbred lines were grown in parallel experiments to avoid the larger hybrids altering the light environment or interfering with fumigation of smaller inbred lines, resulting in the use of 16 plots in total. Each plot was octagonal in shape and 20 m in diameter. One replicate pair of plots of hybrid lines was dropped from the analysis due to water logging where it was located in the field. Each FACE plot and control plot were divided into five sub-blocks with genotypes planted in one row per sub-block (Appendix C Supplemental Fig. C.1 b,c). Additional site information and field conditions were described in Yendrek *et al.* (2017a).

Ozone enriched air was delivered to the experimental rings with FACE technology as described in Yendrek et al. (2017b). The target fumigation for the elevated [O₃] rings was 100 nL L⁻¹ from 10:00 to 18:00 throughout the growing season when it was not raining, and the wind speed was greater than 0.5 m s⁻¹. Based on 1 min average [O₃] collected in each ring, the fumigation was within 10% of the 100 nL L⁻¹ target for 56% of the time and within 20% of the target for 79% of the time in 2015. Weather conditions were measured with an on-site weather station as reported previously in Yendrek *et al.* (2017a).

Sampling protocol and tissue handling

Leaf material for metabolomic profiling of B73 x Mo17, B73, and Mo17 lines were taken on three dates corresponding to leaf physiological maturity, early- and mid-senescence (Fig. 3.1 a-c). Samples were taken in the hybrid experiment on: 27 July 2015, 7 August 2015, and 17 August 2015 (day of year (DOY): 208, 219, and 229), and in the inbred experiment on: 4 August 2015, 13 August 2015, and 24 August 2015 (DOY: 216, 225, and 236). Different plants within the replicate rows were sampled on each date. All material was collected from the plots between 12:00 and 14:00. Approximately 100 cm² of leaf material was cut from the middle of the leaf subtending the ear with scissors, wrapped in tinfoil and immediately placed in liquid nitrogen, before being stored at -80°C. One row per sub-block, five total, was sampled per genotype per ambient and elevated [O₃] plot.

Leaf chlorophyll content, LMA and seed yield measurements⁵

Chlorophyll content was estimated from measurements collected with a SPAD meter (Konica-Minolta SPAD-502 Chlorophyll meter, Japan). Measurements were collected from the leaf subtending the ear of three plants per genotype at five locations per ring. Six readings were taken from the middle third of each leaf and the average value was recorded. Measurements were taken approximately every three days starting at anthesis, continuing through leaf senescence (DOY 204-255). The equation: chlorophyll content (µg cm⁻²) = (99*SPAD)/(144-SPAD) was used to convert SPAD values to chlorophyll content (Cervic *et al.* 2012). The analysis of chlorophyll loss over time was tested by fitting a quadratic equation to the data where: Chl = y₀ +

⁵ Leaf chlorophyll measurements were taken by Charles Burroughs, seed yield was measured by Lorena Rios Acosta

$\alpha \cdot x + \beta \cdot x^2$, where x equals day of year. To test for differences in chlorophyll loss over time in ambient and elevated $[O_3]$, a single quadratic model was first fit to the data for each genotype (PROC NLIN, SAS 9.4, SAS Institute, Cary, NC), then models were fit to each genotype and treatment combination. An F statistic was used to test if the model with genotype and treatment produced a significantly better fit to the data, that is, if there were significant differences in the response of chlorophyll over time in ambient and elevated $[O_3]$, following the approach of Yendrek *et al.* (2017a).

Leaf mass per unit area (LMA) was measured from leaf disks taken at the same time as samples for metabolomic profiling. Three leaf disks (2.0 cm dia) per row were cut with cork borers, placed into coin envelopes and dried in an oven at $\sim 60^\circ\text{C}$ for one week. Samples were then weighed and data from the five rows within each plot were averaged for a plot-level estimation of LMA (g m^{-2}). The effect of O_3 in the hybrid experiment was tested using analysis of variance and the mixed model procedure (Proc Mixed, SAS) with treatment as a fixed effect and block as a random term in the model. For the inbred experiment, the model included fixed effect terms for inbred line and treatment, and a random term for block. Significant differences between treatments for each time point were determined by Tukey tests with a threshold of $p < 0.05$.

At maturity, ears were harvested from eight plants in each of the five rows per genotype per plot, dried for ~ 1 week in an oven at $\sim 60^\circ\text{C}$, then shelled and weighed to estimate yield (g plant^{-1}). The five yield estimates genotype per plot were averaged to obtain plot-level means, and analysis of variance was used to test for O_3 effects as described above for LMA.

Metabolomic profiling by GC-MS

Untargeted metabolomic profiling was performed with 15 mg of lyophilized leaf tissue according to the protocol described in Ulanov and Widholm (2010). A total of 30 samples per time point for B73 x Mo17, and 40 samples per time point for B73 and Mo17 were processed for metabolomic profiling. Samples were extracted with methanol: acetone: H_2O (1:2:1, v/v/v) at ambient temperature. For quality control (QC), 10 μl of leaf extract was taken from each sample and pooled, then run and analyzed after every nine biological samples. Samples and QC were dried under vacuum and derivatized with 75 μl methoxyamine hydrochloride (Sigma-Aldrich, MO, USA) (40 mg ml^{-1} in pyridine) for 90 min at 50°C , then with 125 μl MSTFA + 1%TMCS

(Thermo, MA, USA) at 50°C for 120 min followed by an additional 2-h incubation at room temperature. An internal standard (30 μ L hentriacontanoic acid) was added to each sample prior to derivatization. Samples were analyzed on a gas chromatography/mass spectroscopy (GC/MS) system (Agilent Inc, Palo Alto, CA, USA) consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 mass selective detector, and a HP 7683B autosampler. Gas chromatography was performed on a ZB-5MS capillary column (Phenomenex, Torrance, CA, USA). The inlet and MS interface temperatures were 250°C, and the ion source temperature was adjusted to 230°C. An aliquot of 1 μ L was injected with the split ratio of 10:1. The helium carrier gas constant flow rate was 2.4 ml min⁻¹. The temperature program was 5 min isothermal heating at 70°C, followed by an oven temperature increase of 50 C min⁻¹ to 310°C, and a final 10 min at 310°C. The mass spectrometer was operated in a positive electron impact mode at 69.9 eV ionization energy in m/z 50-800 scan range.

Raw data files were processed with the metaMS.GC workflow hosted on the workflow4metabolomics (W4M) server (Giacomoni *et al.* 2015). Default settings were used except for minimum class fraction, specified at 0.6. Spectra were normalized to internal standards and leaf dry weights to account for tissue and water content differences over time. Batch correction was done with the all loess sample regression model, W4M tool. Peak annotation used a custom-built database and AMDIS 2.71 (NIST, Gaithersburg, MD, USA) program. All known artificial peaks were identified and removed. After annotation and filtering 51, 48, and 50 annotated metabolites were identified in hybrid line at time points A, B, and C (Appendix A Supplemental Table 3.1), and 45, 41, and 46 annotated metabolites were identified in the inbred lines at time points A, B, and C (Appendix A Supplemental Table 2). The instrument variability was within the standard acceptance limit of 5%.

Statistical analysis of metabolite data

The hybrid and inbred metabolite data were analyzed separately because they were grown in different field plots and harvested on different dates (Appendix C Supplemental Fig. C.1). For each dataset, metabolite data were log₁₀ transformed and processed with univariate analysis to identify and remove outliers (studentized residual ≥ 4). The five observations for a genotype within each FACE or control plot were averaged for analysis and time points were analyzed independently. Additionally, each metabolite was tested independently using a one-way analysis

of variance model for the hybrid experiment and a two-way ANOVA for the inbred experiment with treatment and genotype as fixed effects. Statistical differences in least squared mean estimates between ambient and elevated [O₃] for each time point were determined by Tukey tests with a threshold of $p < 0.05$ (Kuehl 2000). The above statistical analysis was done using SAS software (SAS, Version 9.4, Cary, NC).

Multivariate statistics were performed using R (version 3.5.1; The R Foundation for Statistical Computing). Multivariate clustering analysis was done with the log₁₀- transformed and Pareto scaling normalized data, with identified outlier observations removed. Missing values were estimated prior to multivariate analysis using k-nearest neighbor (KNN) in the MetaboAnalystR package (Chong & Xia 2018). The total number of missing values within the hybrid datasets was between 4.8 – 6.4% and for the inbred datasets was between 4.2 – 6.4% of all observations. Principal component analysis (PCA) was performed using the prcomp function (stat package) for the hybrid and inbred experiments independently for each time point. A singular value decomposition data matrix was applied per normalized dataset. When the unsupervised PCA identified a clear treatment separation, a supervised partial least square – discriminant analysis (PLS-DA) was performed, with the mixOmics package (Rohart *et al.* 2017). The number of latent variables included in the model was selected by testing the predictability value (Q^2) using an increasing number of latent variables from one to ten. The relative importance of the metabolites in the models was summarized using PLS-DA loadings, with significant loadings considered when the contribution was greater than 0.1.

Pearson linear correlation analysis was performed with physiological traits (chlorophyll and LMA) based on the class separation observed in the multivariate clustering. Correlations with an adjusted p value (False Discovery rate, (Benjamini & Hochberg 1995) of 0.05 or less, and a correlation coefficient of absolute value |0.55| or greater were considered significant and biologically meaningful in this analysis.

Statistical analyses for the linear regressions of sterol:chlorophyll (relative concentration/ 100 mg DW: $\mu\text{g}/\text{cm}^2$) and day of year (DOY) were performed using the plot means of all variables and linear regression (Proc Reg; SAS). An F statistic was used to test if treatments had significantly different slopes, with each metabolite analyzed independently. Differences in slopes between ambient and elevated [O₃] were considered significant when $p < 0.05$.

Results

Leaf metabolomic profiles of inbred and hybrid lines exposed to ambient and elevated [O₃]

Leaf metabolomic profiles of the leaf subtending the ear from two inbred lines (B73 and Mo17) and the hybrid cross (B73 x Mo17) were measured in ambient and elevated [O₃] on three dates in 2015. These three time points captured the initial and middle stages of leaf senescence, characterized by leaf chlorophyll content (Fig. 3.1 a-c). Between 48 and 51 annotated metabolites were detected in more than three-fourths of the samples in B73 x Mo17 (Appendix A Supplemental Table 1). Between 41 and 46 annotated metabolites were detected in the inbred lines (Appendix A Supplemental Table 2).

Multivariate clustering of the leaf metabolite profile of B73 x Mo17 across time points showed a clear shift in leaf metabolism under elevated [O₃] as the leaves aged (Fig. 3.2). When the PCA plots revealed strong discrimination between ambient and elevated [O₃], partial least square-discriminant analysis (PLS-DA) was performed to identify maximum separation among treatments and to rank individual metabolite contributions to the class separation. On the first date of sampling, when the flag leaf was relatively young (time point A, DOY 208), there was no clear O₃ treatment separation for metabolite pools in B73 x Mo17 (Fig. 3.1a). Both PCA and PLS-DA showed a clear separation of the metabolite profiles in ambient and elevated [O₃] (along component 1) from samples taken at time point B, DOY 219 (Fig. 3.2b) and time point C, DOY 229 (Fig. 3.2c). Statistical tests (1-way ANOVA) identified 20 metabolites with significant changes in elevated [O₃] in time point B (Appendix A Supplemental Table 3), and 10 metabolites with significant changes in time point C (Appendix A Supplemental Table 3).

In contrast to the hybrid response where elevated [O₃] altered the metabolite signature in aging leaves, the inbred leaf metabolite profile did not show a significant response to elevated [O₃] in any of the time points (Fig. 3.3 a-c). This was true whether both inbred lines were analyzed together (Fig. 3.3 a-c) or independently (data not shown). All time points showed a strong genotype separation between B73 and Mo17 along the first principle component. Similarly, the two-way ANOVA confirmed significant differences in metabolite content between inbred lines, but no strong treatment effect. Statistical tests (ANOVA) identified 22, 37, and 24 metabolites with significant changes between genotypes in time point A, B, and C respectively (Appendix A Supplemental Table 4). In contrast, each of the time points had only 3 metabolites with significant changes in elevated [O₃] (Appendix A Supplemental Table 4).

Trends in hybrid metabolite signature under elevated [O₃]

PLS-DA ranks the variables contributing to each class loading, identifying metabolites that differentiate ambient from elevated [O₃] (Appendix C Supplemental Fig. C.2). Secondary metabolites quinic acid, stigmasterol, and α -tocopherol were associated with elevated [O₃] in both time points (Appendix C Supplemental Fig. C.2, Fig. 3.4 b,c). Sitosterol, which is converted to stigmasterol under many abiotic stress conditions, contributed to the ambient [O₃] signal in both time points (Appendix C Supplemental Fig. C.2, Fig. 3.4 b,c). Palmitate and linoleate are poly-unsaturated fatty acids, functioning as membrane lipids, and contributed to the signal of ambient [O₃] (Appendix C Supplemental Fig. C.2, Fig. 3.4 e,f). The sugar, benzyl glucopyranoside, and two essential compounds of the TCA cycle, citric acid and malic acid, were associated with elevated [O₃] in both time points. Pyruvate, which is converted to acetyl-CoA in the initial step of the citric acid cycle, contributed to the ambient [O₃] signal (Appendix C Supplemental Fig 2, Fig. 3.4 e,f). Similarly, threonine, glycerol-3-P, and glycerophosphoglycerol were associated with the ambient [O₃] loading in the PLS-DA analysis in time points B and C.

Phytosterol response to elevated [O₃] differs between hybrid and inbreds

Chlorophyll content was measured throughout the growing season to track leaf senescence (Fig. 3.1 a-c). The hybrid showed a clear acceleration of leaf senescence under elevated [O₃], losing 25% more chlorophyll over time compared to ambient [O₃] (Fig. 3.1a). The acceleration of leaf senescence was much less pronounced in the inbred lines (Fig. 3.1 b, c). This result agreed with the general trend observed in the leaf metabolome, with a lack of treatment response in the inbred lines. The ratio of phytosterol content to chlorophyll content has been used as an index of leaf senescence (Li *et al.* 2017). The ratios of α -tocopherol, campesterol, and stigmasterol to chlorophyll increased over time in B73 x Mo17, especially in elevated [O₃] (Fig. 3.5 a-c). These trends were due to both the decline in chlorophyll (Fig. 3.1) and an increase in α -tocopherol, campesterol, and stigmasterol content in aging leaves, especially in elevated [O₃] (Appendix A Supplemental Table 1, 2). A significant difference between ambient and elevated [O₃] in the accumulation of α -tocopherol relative to chlorophyll (Fig. 3.5a) was observed, along with trends towards greater accumulation of stigmasterol and campesterol relative to chlorophyll (Fig. 3.5 b, c). The reverse trend was seen in the sitosterol:chlorophyll ratio with decreasing ratios in the

elevated O₃ condition (Fig. 3.5d). The inbred lines showed an age-dependent increase in α -tocopherol:chlorophyll ratio, but no effect on elevated [O₃] (Fig. 3.6).

LMA and yield correlations with leaf metabolite content

Both LMA and yield were significantly reduced in elevated [O₃] in the hybrid B73 x Mo17, but were not significantly affected by elevated [O₃] in the inbred lines (Table 3.1). Leaf metabolite concentrations were correlated with LMA and yield using Pearson linear correlation analysis. For time point A in the hybrid, there was no treatment separation between ambient and elevated [O₃], so data were pooled across treatments and then correlated with LMA and yield (Table 3.2). Stigmasterol, campesterol, and ethanolamine contents measured in recently mature leaves were negatively correlated with yield, while alanine was positively correlated with yield.

As multivariate clustering for time points B and C showed significant differences in ambient and elevated [O₃] in B73 x Mo17, correlations were done separately for each O₃ treatment (Table 3.3). The majority of the significant correlations observed in time point B were between yield and metabolite content in elevated [O₃]. Benzyl glucopyranoside, campesterol, glycerohexose, and malic acid content were negatively correlated with yield in elevated [O₃], but not in ambient [O₃] (Table 3.3). Scyllo inositol had a strong positive correlation with yield in ambient [O₃], and the reverse trend in elevated [O₃] (Table 3.3). Ferulic acid, itaconic acid, and citric acid measured at time point C (when leaves were senescing) were positively correlated with yield in ambient [O₃], but not in elevated [O₃] (Table 3.4).

LMA was not correlated with many individual metabolites. Sitosterol and LMA showed opposite correlations in ambient and elevated [O₃] when measured at time point B (Table 3.3). Benzyl glucopyranoside and LMA were negatively correlated in time point B in ambient [O₃], the reverse of the correlation identified with yield (Table 3.3). In time point C, LMA and α -tocopherol were positively correlated in elevated [O₃], but not in ambient [O₃] (Table 3.4). Two significant linear correlations were identified in the ambient [O₃] treatment with LMA, o-coumaric acid and malonic acid (Table 3.4). Itaconic acid, pyruvic acid, ribose, alanine, and pyroglutamic acid were all negatively correlated with LMA in time point C in elevated [O₃] (Table 3.4).

The inbred leaf metabolite profile separated by genotype rather than treatment, as seen in the PCA plots (Fig. 3.3 a-c). Due to the class separation based on genotype, Pearson linear

correlations were analyzed independently for each genotype, with ambient and elevated [O₃] treatments pooled (Appendix A Supplemental Table 6). Itaconic acid measured at leaf maturity (time point A) was positively correlated with yield (Appendix A Supplemental Table 6). Stigmasterol and LMA were negatively correlated in B73 at time point B. The final time point showed a moderate positive relationship between raffinose and LMA. The strongest relationships within the Mo17 inbred line were between malic acid and LMA in time point A, and sucrose and LMA in time point C (Appendix A Supplemental Table 6).

Discussion

Field metabolomics can be a powerful approach for profiling the metabolite changes of plants in response to environmental stress (Melandri *et al.* 2020; Wedow *et al.* 2019). The physiological response of crops to O₃ pollution has been well-studied, with elevated [O₃] decreasing carbon assimilation, accelerating senescence and cell death, and reducing economic yield (Ainsworth 2017). However, the metabolite profile underpinning the response to O₃ in maize has not been investigated and could provide information for targets to improve O₃ tolerance in maize and C₄ crops. We found that maize metabolomics signatures were altered by elevated [O₃] in a hybrid line (Fig. 3.2), but not in two inbred lines (Fig. 3.3). As predicted, there was a more pronounced difference in metabolite signatures in the hybrid line as leaves aged (Fig. 3.2). In the hybrid, we discovered a significant increase in α -tocopherol and phytosterols in elevated [O₃], supporting the prediction that metabolites associated with oxidative stress and membrane stabilization would be key responses to O₃ stress.

Accelerated senescence, as indicated by a reduction in chlorophyll content was observed under elevated [O₃] in B73 x Mo17 (Fig. 3.1a), but was less pronounced in the inbred lines B73 and Mo17 (Fig. 3.1 b,c). The metabolomic profile reflected the observed acceleration of senescence in the hybrid as the leaves aged. Initially, when chlorophyll content was similar in ambient and elevated [O₃], the B73 x Mo17 metabolomic profile lacked a clear treatment separation (Fig. 3.2a). However, as chlorophyll was lost more rapidly at elevated [O₃] during senescence, a clear separation in metabolite profiles between ambient and elevated [O₃] was apparent (Fig 3.2b,c). The inbred genotypes had no discernible difference in their metabolomic profiles in ambient and elevated [O₃] at any of the time points, although B73 was very different from Mo17 (Fig. 3.3). These results agree with our prediction that a metabolomic signature

develops with the accumulation of O₃ damage in aging leaves and reflects altered biochemistry. Ozone-induced acceleration of senescence has been previously reported to impact grain yield losses due to a decrease in photosynthetic capacity and shortened leaf lifespan (Emberson *et al.* 2018). A reduction in photosynthetic carbon assimilation in maize ear leaves during grain filling was previously reported and was greater in hybrid maize compared to inbred lines (Yendrek *et al.* 2017a). Additionally, the average yield reduction under elevated [O₃] was 30.4% for B73 x Mo17, and only 5.0% for Mo17, and 2.1% for B73. This variation in yield reduction under elevated [O₃] is also consistent with the chlorophyll, senescence and metabolite responses of the inbred and hybrid lines. Greater flux of O₃ into more vigorous hybrids may be one explanation for the observed differences.

The metabolites contributing to the O₃ treatment separation during leaf senescence in B73 x Mo17 included TCA cycle intermediates, and specialized metabolites such as phytosterols and fatty acids (Fig. 3.4). In the latter two time points, citric acid and malic acid had a significant influence on the elevated O₃ loadings. This trend is consistent with increased flux through the TCA cycle and greater rates of mitochondrial respiration in plants exposed to O₃ stress (Betzberger *et al.* 2012; Dizengremel *et al.* 2012; Yendrek *et al.* 2015). The shift in metabolism from photosynthetic carbon assimilation to the TCA cycle and mitochondrial respiration supplies energy for repair and detoxification of the plant cells against oxidative damage (Dizengremel 2001). The additional influence of specialized secondary metabolites including α -tocopherol, benzyl glucopyranoside, stigmasterol, and quinic acid is consistent with a shift to defense and repair under elevated O₃ stress (Fig. 3.4). Steryl esters (SEs), palmitate and linoleate, were associated with ambient O₃, possibly inferring differences in membrane fluidity or properties between ambient and elevated [O₃]. Phosphoenolpyruvate (PEP) and pyruvate are both required for the synthesis of three plastid-localized biochemical pathways: shikimate pathway, isoprenoid synthesis, and fatty acid synthesis (Mene-Saffrane 2018). The maize hybrid B73 x Mo17 appeared to shift the balance and competition for precursors between α -tocopherol, SEs, and additional metabolites downstream of these pathways, including aromatic amino acids in elevated [O₃].

We found that α -tocopherol increased to a greater extent as leaves aged in elevated [O₃] in the hybrid B73 x Mo17 (Fig. 3.5 a-c), but not in either inbred (Fig. 3.6). The protective function of tocopherols to preserve cell membrane integrity during the final stages of leaf

development has well documented (Falk & Munne-Bosch 2010; Fryer 1992; Lira *et al.* 2017). α -tocopherol levels have been shown to increase under high light (Krieger-Liszkay & Trebst 2006), drought (Ahkami *et al.* 2019; Munne-Bosch & Alegre 2000), and high temperature stress (Spicher *et al.* 2017). Within the chloroplast, where highly reactive singlet oxygen ($^1\text{O}_2$) is formed, α -tocopherol is an essential scavenger protecting the thylakoid membranes against lipid peroxidation (Piller *et al.* 2014). In high light conditions, the rapid turnover of α -tocopherol and plastoquinone have been correlated with the increased turnover rate of the D1 protein within PSII, thereby protecting the photosynthetic process (Krieger-Liszkay & Trebst 2006). Experimental studies exposing plants to elevated $[\text{O}_3]$ have reported conflicting results regarding α -tocopherol levels in leaf extracts; beech leaves in full sun showed a significant increase (Garcia-Plazaola & Becerril 2001), and a decrease or no change was observed in spinach leaves (Calatayud *et al.* 2003; 2004). We found that the response of α -tocopherol to elevated $[\text{O}_3]$ varied with maize genotype and leaf age (Fig. 3.5a; Fig. 3.6). Previous experimental variation may also be attributed to the timing of sampling and the progression of leaf senescence as we would not have identified a relationship between α -tocopherol and O_3 in B73 x Mo17 had samples only been taken at the initial time point (Appendix A Supplemental Table 3). Elevated α -tocopherol concentrations in plants exposed to elevated $[\text{O}_3]$ are potentially quenching reactive oxygen species that form during the disassembly of the photosynthetic membranes during senescence and/or inhibiting lipid peroxidation (Rogers & Munne-Bosch 2016). It would be interesting to experimentally test if further increasing α -tocopherol in aging leaves would improve O_3 tolerance.

Senescing hybrid leaves exposed to elevated $[\text{O}_3]$ showed a trend towards accumulating campesterol and stigmasterol (Fig. 3.5b, c) at the expense of sitosterol (Fig. 3.5d). Phytosterols are proposed to modulate membrane integrity to improve abiotic stress tolerance (Dufourc 2008; Kuczynska *et al.* 2019). In stress conditions stigmasterol and sitosterol interact with phospholipids to maintain the permeability and fluidity of the plasma membrane (Dalal *et al.* 2016; Griebel & Zeier 2010). Heat stressed hard fescues up-regulated ethyl sterol content, including stigmasterol and sitosterol, and the more heat tolerant variety showed a significantly greater increase in stigmasterol compared to a heat sensitive variety (Wang *et al.* 2017). In the current study, we found greater concentrations of stigmasterol and campesterol in hybrid leaves exposed to elevated $[\text{O}_3]$, but not in inbred lines (Fig. 3.5; Appendix A Supplemental Table 7, 8).

Genotypic variation in phytosterol concentrations has been documented in wheat (Nurmi *et al.* 2010), rice (Kumar *et al.* 2018), and potato (Hancock *et al.* 2014), and genetic variation in the concentrations of phytosterols during drought was associated with differences in stress tolerance (Kumar *et al.* 2015). It is interesting that in elevated [O₃], increased phytosterol content only occurred in the hybrid genotype that also showed accelerated senescence and significant yield loss, not in the inbred lines. This suggests that increased phytosterol content was not associated with O₃ tolerance, instead with changes to membranes in aging leaves. Furthermore, campesterol is a precursor of brassinosteroids (Fujioka & Yokota 2003), and brassinosteroid signaling mutants display a delayed senescence phenotype (Clouse & Sasse 1998). Thus, the accumulation of campesterol in aging leaves exposed to elevated [O₃] in B73 x Mo17 could further promote leaf senescence. Such fluctuations in the levels of one phytosterol at the expense of another are proposed to alter plant responses to environmental stimuli (Aboobucker & Suza 2019). Our work also supports the hypothesis that the balance of various phytosterols may be a key signaling response for additional cellular defenses (Griebel & Zeier 2010; Schaller 2003).

Previous experiments have identified metabolites associated with maize grain yield under drought and heat stress (Obata *et al.* 2015), and here we investigated potential metabolite markers associated with yield under elevated [O₃] stress. In the B73 x Mo17 genotype, stigmasterol and campesterol showed negative correlations to yield at the first time point, before any treatment effect could be identified (Table 3.2). In the second time point, campesterol content was negatively correlated with yield in the elevated [O₃] treatment, but not in ambient [O₃] (Table 3.3). There were no metabolites associated with yield in both ambient and elevated [O₃] in either time point B or C (Table 3.3; Table 3.4), when PCA and PLS-DA analysis revealed strong treatment effects on the leaf metabolite profile. This supports previous work that identified different metabolite correlations with yield under drought stress compared to heat stress (Obata *et al.* 2015). LMA and α -tocopherol were positively correlated under elevated [O₃] (Table 3.4). A reduction in LMA is generally linked to decreasing leaf nitrogen content and leaf area under elevated O₃ conditions (Oikawa & Ainsworth 2016). The positive relationship identified suggests a potential protective role of α -tocopherol in stabilizing thylakoid membranes and preventing excessive degradation of chlorophyll under elevated [O₃]. The strong relationships identified in this study provide potential metabolic signatures for improving O₃

tolerance and provide metabolite markers that can now be screened across a diverse genetic background.

Conclusion

Field experiments can be more variable than controlled environment experiments (Lovell *et al.* 2016), and metabolites respond rapidly to environmental changes (Caldana *et al.* 2011), both of which present challenges for field metabolomic studies. Recent criticism of metabolomics data includes the low repeatability and strong effects of environmental perturbations on plant metabolism (Tucker *et al.* 2020). We found that environmental conditions influenced the metabolomic profiles; yet there were clear patterns of response in hybrid maize. Moreover, we discovered that inbred lines have significantly different metabolite profiles from one another but lacked a response to elevated [O₃]. Our study emphasized that metabolomic profiling is a vital tool that can be used alongside additional ‘omic’ and standard physiological measurements to improve understanding of plant metabolic responses to stress. Despite the dynamic and variable nature of field-based metabolomics, we identified novel markers of O₃ response in hybrid maize, which can be broadly tested across diverse germplasm.

TABLES AND FIGURES

Table 3.1. Leaf mass per unit area (LMA) measured at three time points on the leaf subtending the ear and yield (kernel mass per plant) measured at maturity in ambient and elevated [O₃]. Bold font indicates significant differences in ambient and elevated [O₃] ($p < 0.05$).

	LMA Time Point A		LMA Time Point B		LMA Time Point C		Yield	
Genotype	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated
B73 x Mo17	3.0±0.08	3.0±0.08	3.3±0.08	3.3±0.05	3.4±0.08	3.2±0.07	175.9±4.9	126.8±6.0
B73	3.9±0.1	3.9±0.08	4.4±0.1	4.2±0.1	4.4±0.1	4.3±0.1	100.2±3.4	96.9±4.2
Mo17	3.6±0.09	3.5±0.06	3.5±0.08	3.5±0.08	3.7±0.08	3.7±0.1	82.2±4.1	78.4±2.8

Table 3.2. Significant Pearson linear correlations ($p < 0.05$) between yield and metabolite content for hybrid line, B73 x Mo17. Linear correlation statistics for time point A (DOY: 208) contain all samples from both ambient and elevated [O₃].

Time Point	Trait	Metabolite	All (r)
A	Yield	Stigmasterol	-0.72
	Yield	Campesterol	-0.68
	Yield	Alanine	0.57
	Yield	Ethanolamine	-0.55

Table 3.3. Significant Pearson linear correlations ($p < 0.05$) of yield and leaf mass per unit area (LMA) with metabolite content sampled at time point B (DOY: 219) for hybrid line, B73 x Mo17 in ambient and elevated [O₃].

Time Point	Trait	Metabolite	Ambient (r)	Elevated (r)
B	Yield	Benzyl glucopyranoside	0.15	-0.60
	Yield	Campesterol	0.01	-0.58
	Yield	Glycerohexose	-0.26	-0.56
	Yield	Malic acid	0.14	-0.55
	Yield	Inositol, scyllo	0.56	-0.54
B	LMA	Sitosterol	-0.58	0.55
	LMA	Threonic acid	-0.50	-0.57
	LMA	Benzyl glucopyranoside	-0.57	-0.32

Table 3.4. Significant Pearson linear correlations ($p < 0.05$) of yield and leaf mass per unit area (LMA) with metabolite content sampled at time point C (DOY: 229) for hybrid line, B73 x Mo17 in ambient and elevated $[O_3]$.

Time Point	Trait	Metabolite	Ambient (r)	Elevated (r)
C	Yield	Ferulic acid	0.81	0.02
	Yield	Itaconic acid	0.68	-0.04
	Yield	Citric acid	0.57	-0.29
C	LMA	α -tocopherol	0.14	0.60
	LMA	Maltose	-0.25	0.60
	LMA	Malonic acid	-0.59	0.05
	LMA	o-coumaric acid	-0.67	0.03
	LMA	Itaconic acid	-0.05	-0.64
	LMA	Pyruvic acid	0.21	-0.63
	LMA	Ribose	0.11	-0.60
	LMA	Alanine	-0.12	-0.58
	LMA	Pyroglutamic acid	-0.40	-0.57

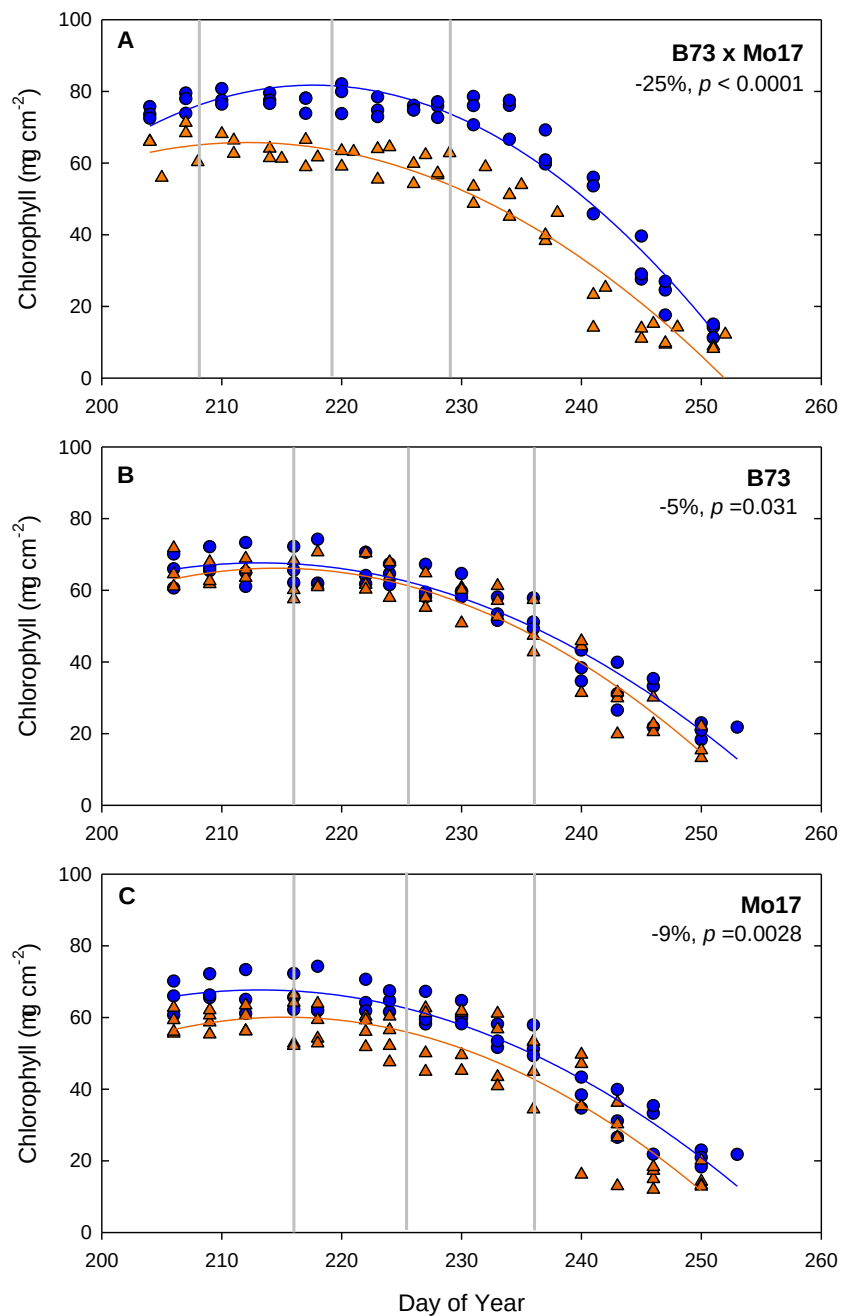


Figure 3.1. Chlorophyll content estimated from SPAD measurements of the leaf subtending the ear from leaf maturity through senescence for hybrid line B73 x Mo17 (A), and inbred lines B73 (B) and Mo17 (C) measured at ambient (blue symbols) and elevated [O₃] (orange symbols). The area under the curve was integrated to calculate the percentage change in chlorophyll content over the lifetime of the leaf, and is shown in the top right of each plot, along with significance values. Vertical lines indicate the measurement dates for leaf metabolite content.

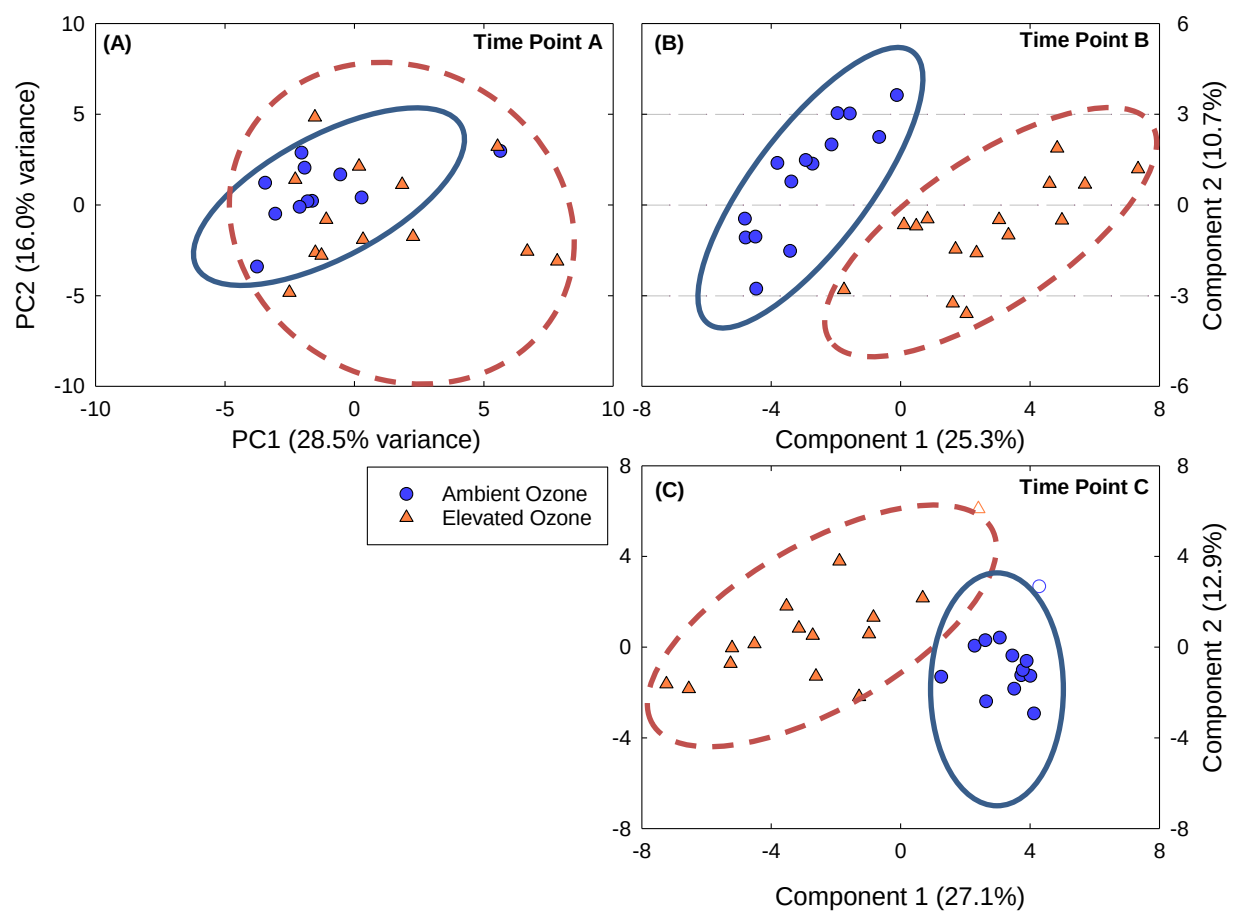


Figure 3.2. Multivariate clustering of the metabolomic profile of B73 x Mo17 hybrid at different time points. (A) Principle component analysis (PCA) of time point A (DOY: 208) showing no strong treatment separation; (B) Partial least square – discriminant analysis (PLS-DA) of time point B (DOY: 219), and (C) PLS-DA of time point C (DOY: 229). Ellipses show 95% confidence intervals and shapes without fill are outside the confidence ellipse for PLS-DA.

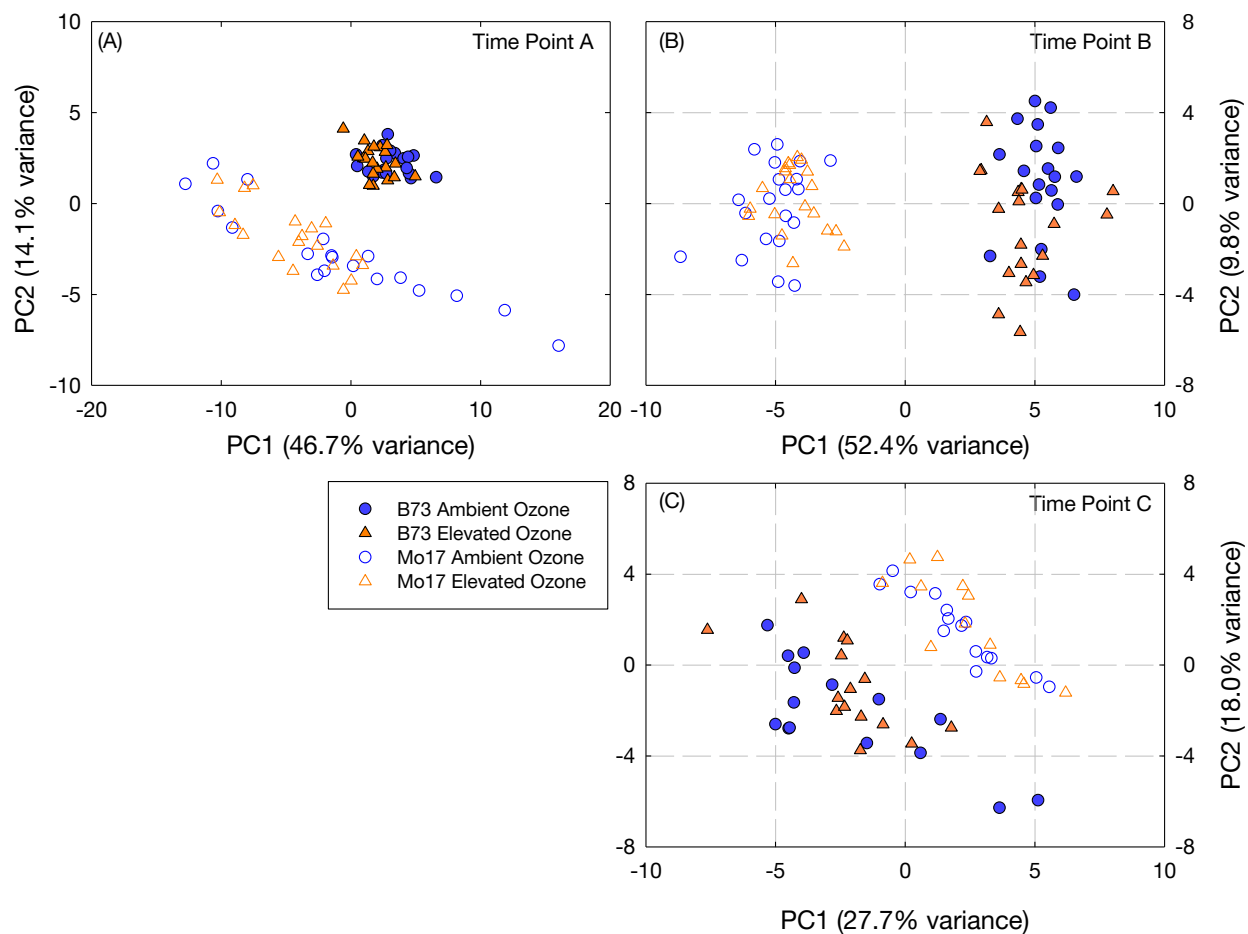


Figure 3.3. Clustering from principle component analysis of the metabolomic profile of inbred lines B73 (filled symbols) and Mo17 (open symbols) grown at ambient (blue symbols) and elevated [O₃] (orange symbols) sampled at time points (A) DOY: 216, (B) DOY: 225, and (C) DOY: 236.

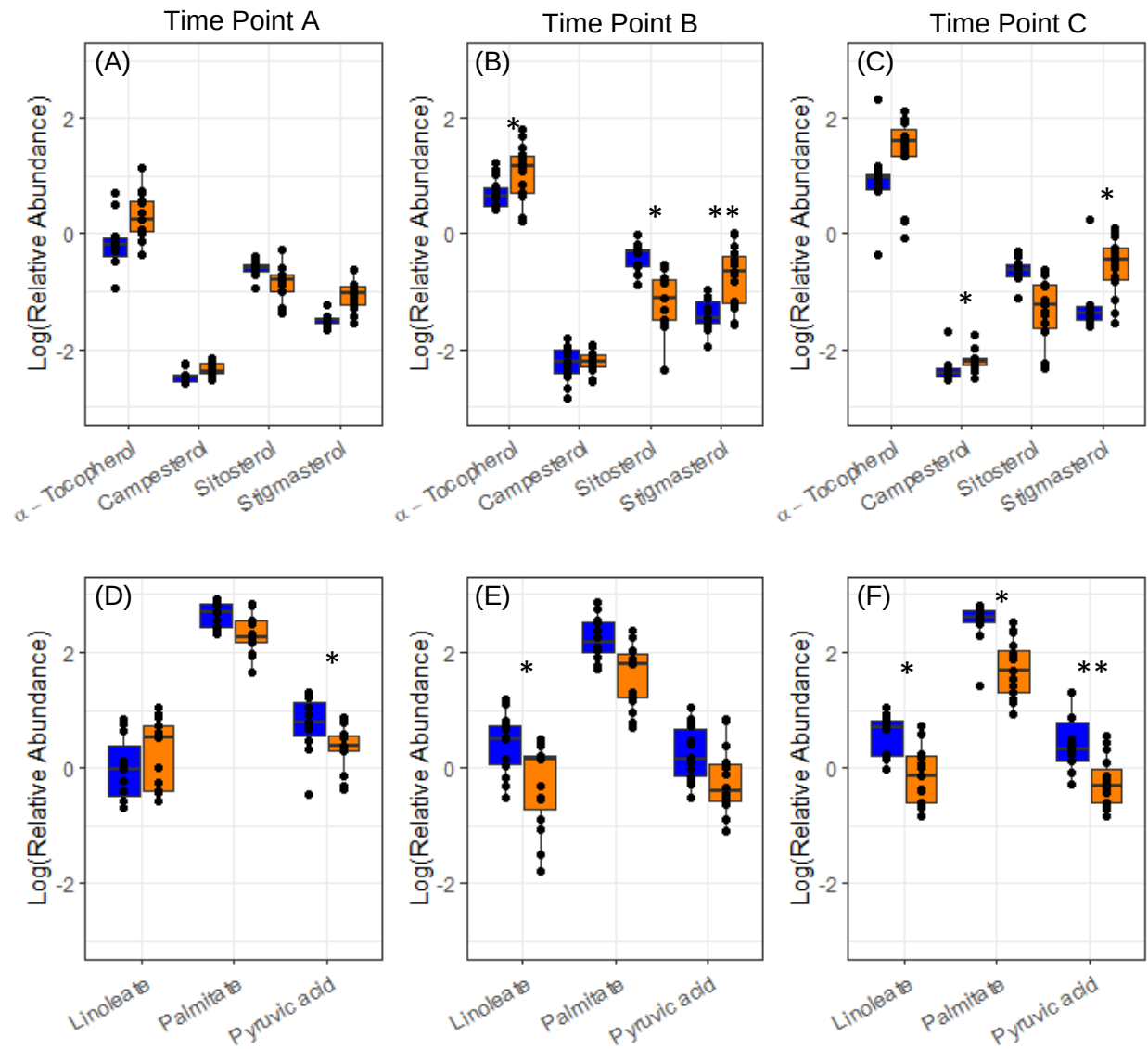


Figure 3.4. Leaf metabolite content for B73 x Mo17. Box plots show the relative abundance of selected metabolites sampled at time point A (A,D), time point B (B,E), and time point C (C,F). Statistical analyses for all metabolites are provided in Appendix A Supplemental Table 3. Blue represents ambient [O₃] and orange represents elevated [O₃]. Black dots show individual values from each sample. (* p < 0.05, ** p < 0.01).

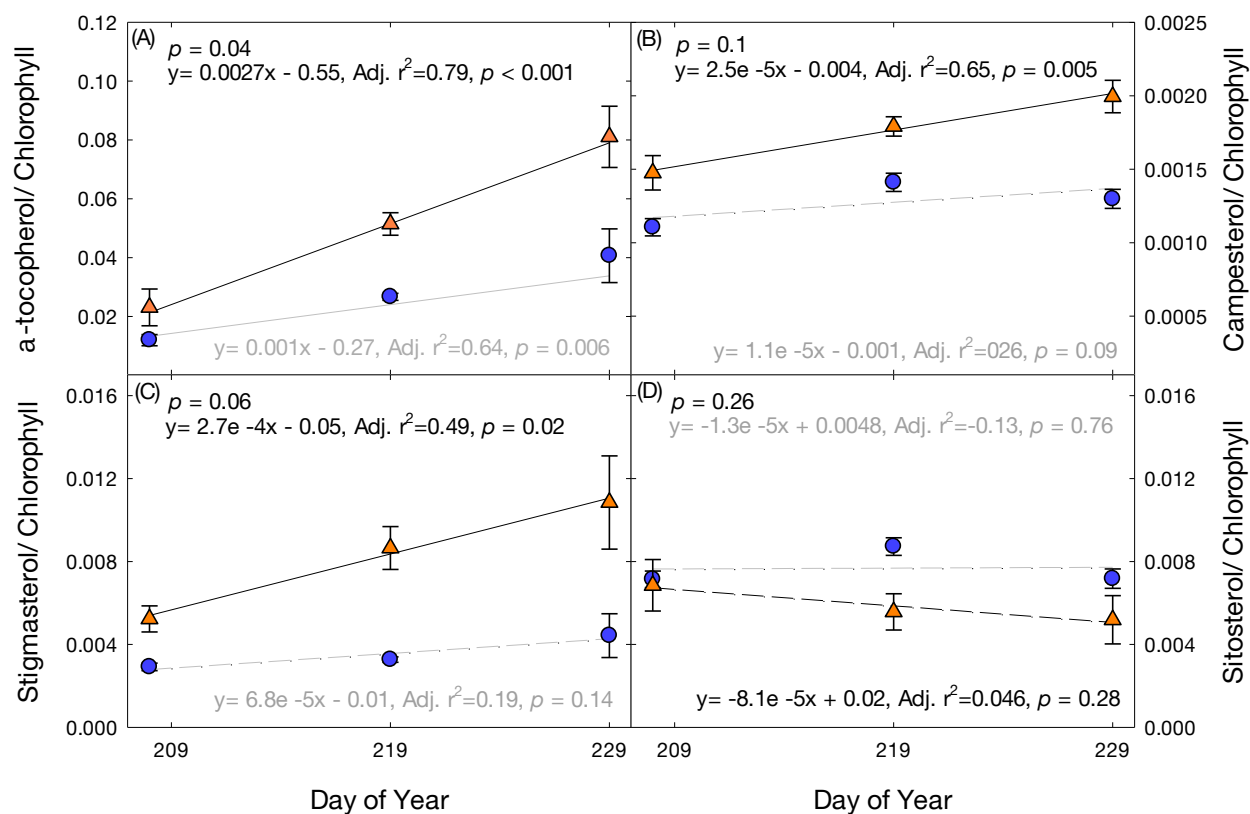


Figure 3.5. Ratio of leaf metabolite (relative concentration / 100 mg DW) content to chlorophyll content ($\mu\text{g}/\text{cm}^2$) over time for hybrid B73 x Mo17. (A) α -tocopherol/chlorophyll ratio; (B) campesterol/chlorophyll ratio; (C) stigmasterol/chlorophyll ratio; (D) sitosterol/ chlorophyll ratio. Solid lines indicate statistically significant relationships, while dashed lines are not significant. p -value in the top left indicates significantly different slopes in the linear regressions in ambient (blue symbols, grey lines) and elevated $[O_3]$ (orange symbols, black lines).

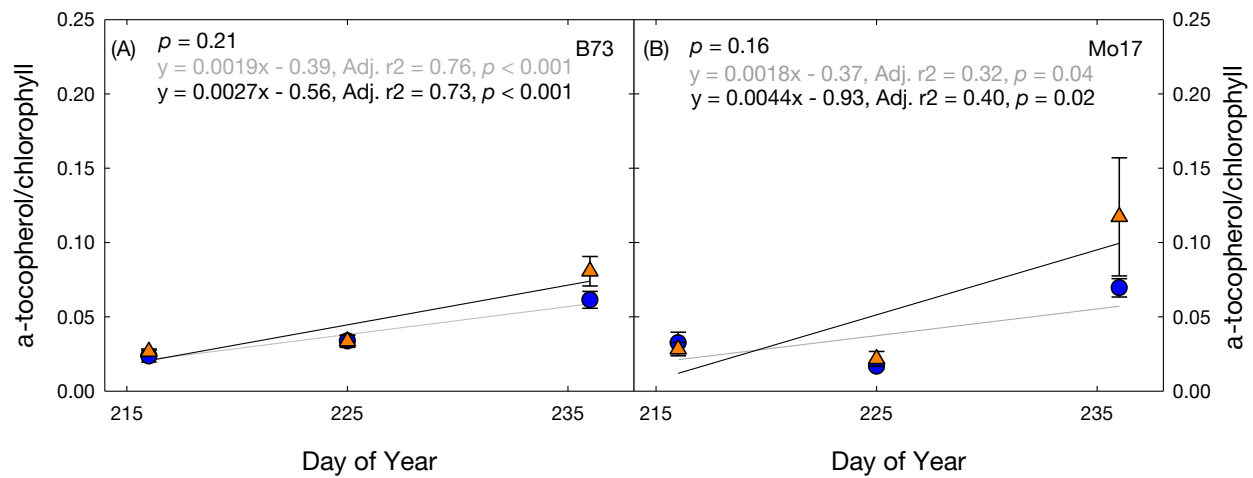


Figure 3.6. Ratio of α -tocopherol (relative concentration/ 100 mg DW) to chlorophyll ($\mu\text{g}/\text{cm}^2$) content measured over time in (A) B73 and (B) Mo17 grown at ambient (blue symbols) and elevated $[O_3]$ (orange symbols). p -value in the top left indicates the test of differences in slope of the regression line between ambient and elevated $[O_3]$.

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CHAPTER IV: USE OF METABOLOMIC TECHNIQUES TO TRACE THE FATE OF OZONE IN LEAVES

Abstract

Ozone (O₃) is a greenhouse gas and considered the most damaging air pollutant to plants. Ozone enters leaves through the stomata, and once in the apoplast, it reacts to produce other reactive oxygen species (ROS) initiating a cellular response. The specific ROS initially formed after O₃ exposure and the antioxidants involved in apoplastic detoxification vary among plant species and even genotypes within a species. Very little is known about how C₄ crops respond to acute O₃ exposure. To address this gap, maize (*Zea mays*) and foxtail millet (*Setaria viridis*) were grown in growth chambers under ambient air (<10 ppb) and then exposed to 200 ppb [O₃]. Leaf material was taken for nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) from the youngest fully expanded leaf after 0, 1, 5, and 24 hours of O₃ exposure. EPR results showed higher concentrations of hydroxyl radicals in the maize leaves grown under elevated O₃, following 24 hours of exposure. Attached leaf EPR measurements done with millet identified an increase in superoxide radical at very high concentrations of O₃. Untargeted metabolomics performed with ¹H-NMR revealed no change in either ascorbate or glutathione concentrations in either maize or millet exposed to O₃. While there were a variety of altered metabolites following O₃ stress in the two species and experiments, changes in isoleucine and pyruvate were common among all experiments. Understanding of the nature and lifetime of O₃ induced ROS and antioxidant responses in C₄ grasses will assist in modeling the fate of O₃ and can help identify strategies for improving tolerance in C₄ crops.

Introduction

Upon entry of O₃ into the leaf via the stomata, a variety of reactive oxygen species (ROS) are formed, including hydrogen peroxide (H₂O₂), singlet oxygen (¹O₂), superoxide radicals (O₂^{•−}), hydroxyl radicals (•OH), and nitric oxide (Ahlfors *et al.* 2009; Grimes *et al.* 1983; Heath 1987). Ozone immediately reacts with molecules present within the intercellular air space resulting in an intercellular O₃ concentration ([O₃]) close to zero (Hill & Littlefield 1969). While a small amount of ROS can activate beneficial protective systems by initiating changes in gene expression (Foyer 2018; Martin & Sies 2017), excess ROS damages biological membranes, including photosynthetic machinery (Foyer & Shigeoka 2011; Heath 1987; Lyons *et al.* 1999;

Rebouças *et al.* 2017). To minimize the production and damage of ROS, a diverse signaling network integrates antioxidants, hormones, sugars, and plant secondary metabolites to respond to this environmental stress (Munne-Bosch 2005). The antioxidant system consists of both enzymatic and non-enzymatic antioxidants, carotenoids, α -tocopherol, and phenolic compounds (Foyer & Noctor 2005). The specific nature of the ROS and antioxidants involved in plant responses to O_3 vary among species, with most of the experiments performed in C_3 species, creating a knowledge gap for C_4 species. This gap ultimately hinders the ability to improve O_3 tolerance in food and bioenergy crops.

Cellular responses to O_3 are dependent on treatment concentrations and exposure length. Acute O_3 exposure is typically defined as exposure to short-term, large dosage events (>100 ppb); in contrast, chronic O_3 exposure results from exposure to a relatively low concentration extended over a long period of time. Following acute O_3 exposure, a burst of ROS within the guard cells and rapid closure of stomata has been measured and hypothesized to prevent additional O_3 flux into the leaf (Vahisalu *et al.* 2010). ROS are also measured in the apoplastic space and can be amplified by generation of $O_2^{\cdot-}$ and H_2O_2 from extracellular peroxidases (PRX) and plasma membrane-bound NADPH oxidase (RBOH) (Vainonen & Kangasjarvi 2015). Antioxidants present in the apoplast detoxify ROS, but if the capacity is overwhelmed, ROS are likely to interact with membrane proteins and enter cells, initiating signaling cascades altering gene expression and a cellular biphasic ROS burst (Vainonen & Kangasjarvi 2015). The combination of ROS, oxidized ascorbate, and the influx of Ca^{2+} signals to mitogen-activated protein kinases (MAPK), plastids and the nucleus, resulting in altered gene expression, increased hormone biosynthesis and antioxidant metabolism, increased respiration, and decreased photosynthesis (Ainsworth 2017).

A biphasic burst of ROS has been measured following acute O_3 exposure (Kangasjarvi *et al.* 2005); however, few studies have identified the specific ROS species and have only inferred the presence of ROS in relation to the increase in antioxidant enzymes, such as superoxide dismutase, or antioxidant metabolites (Singh *et al.* 2014; Vainonen & Kangasjarvi 2015). Electron paramagnetic resonance (EPR) spectroscopy has been used to characterize and quantify the generation of paramagnetic molecules and free radicals following exposure to oxidative stress (Mehlhorn *et al.* 1990; Miller *et al.* 1999). Radicals can be measured with either indirect or direct methods and both have been successfully performed in oxidative stress experiments

(Balasaraswathi *et al.* 2017; Filek *et al.* 2016; Nowicka *et al.* 2017). To allow for quantification of transient radicals ($\cdot\text{OH}$, $\text{O}_2^{\cdot-}$, $^1\text{O}_2$) and EPR silent radicals (NO , H_2O_2), spin trap reagents can be used to stabilize the radicals for detection (Villamena & Zweier, 2004). The spin trap is perfused into the plant leaves, using vacuum infiltration, and either the leaf or perfusion are loaded into the EPR for analysis. Hydroxyl radicals were first identified in wild carrot (*Daucus carota*) exposed to elevated $[\text{O}_3]$ using a spin trap (Grimes *et al.* 1983). The presence of $\text{O}_2^{\cdot-}$ within leaves exposed to O_3 was first identified with EPR attached leaf measurements in bluegrass (*Poa pratensis* L.) and ryegrass (*Lolium perenne* L.) (Runeckles & Vaartnou 1997). Very few studies have implemented EPR to directly measure ROS in any plant species, with no current literature in C_4 species. Quantification and classification of ROS formed following the first 24 hours of exposure to O_3 will be critical for modeling the biochemical response in some of the most productive food and energy crops.

There are variations in the roles that ascorbate and glutathione play in detoxification of ROS in the apoplast and cell. For example, glutathione content was 80% higher in soybean exposed to elevated $[\text{O}_3]$, but was not affected in garden pea or common bean (Yendrek *et al.* 2015). In other species, increased ascorbate concentrations within the apoplast have been correlated with plant tolerance to elevated $[\text{O}_3]$ (Menser 1964; Moldau *et al.* 1997). Yet, in a study of tropical maize responses to O_3 stress, no change in ascorbate content was observed, while thiol content increased (Singh *et al.* 2014). Additionally, non-antioxidant metabolites detoxify the cell during O_3 stress. These secondary metabolites include α -tocopherol and phytosterols identified in chapter III of this thesis. Metabolomics offers the ability to screen a large number of metabolites to develop additional hypotheses and identify interesting relationships that may not have been otherwise predicted (Langridge & Fleury 2011). Nuclear magnetic resonance (NMR) detects and unambiguously identifies metabolites that are present in high abundance. The use of NMR will help unravel the response of antioxidants and secondary metabolites to acute O_3 exposure.

It is important to accurately estimate how environmental stress, including elevated $[\text{O}_3]$, will impact crop yields to enable proper mitigation strategies. C_4 species are some of the most productive bioenergy, food, and feed crops worldwide, including maize, sorghum, and sugarcane. These crops supply a critical amount of food for a significant proportion of the world; however, they are also essential in feedstock and biofuel production. Seven grasses have been

identified as potential targets for feedstock and biofuel development, five of which are C₄ grasses (Perlack *et al.* 2005). Maize is the world's most widely grown crop, with about 32% of the global production occurring in the U.S. (USDA FAS 2020). Foxtail millet is a close relative to maize, sorghum, and sugarcane, and with its small size and rapid life cycle is a model C₄ species (Brutnell *et al.* 2010). This research aimed to improve molecular understanding of acute O₃ response in these two important model C₄ species by employing both EPR and NMR to identify free radicals and specific metabolites formed following exposure to acute O₃ stress.

Material and Methods

Maize Experiment

Plant Material and Growth

Maize (*Zea mays*) inbred line, B73, was planted directly into 1 L pots filled with soil (LC1 Sunshine Mix, SunGro Horticulture, Agawam, MA, USA) on June 9th, 2019, and grown in environmental growth chambers (Chagrin Falls, Ohio) set to a 9h/15h day/night schedule at 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, 60% RH, and 25°C day / 21°C night conditions. Osmocote 19-5-8 slow-release fertilizer (The Scotts Company, Marysville, OH) was incorporated into the top inch of the soil, and plants were watered as needed. Maize plants were grown under low [O₃] (<10 nL L⁻¹) until the day of sampling, July 2nd, 2018 (maize experiment #1). On the day of sampling, O₃ was produced by a variable output UV-C light bulb ballast (HVAC 560 O₃ generator, Crystal Air, Langley, Canada), added to the airstream and monitored with an O₃ analyzer (Thermo Electron 49i, Thermo Scientific, Waltham, MA, USA). Plants were exposed to 200 nL L⁻¹ [O₃] for 24 hours, with samples taken after 0, 1, 5, and 24 h of exposure.

¹H-NMR

Leaf material from the youngest fully expanded leaf was cut and immediately flash frozen in liquid N₂. After complete lyophilization, 100 mg dried and ground material was weighed, placed in a 2 mL tube and sent to the Pacific Northwest National Laboratory (PNNL) Environmental Molecular Sciences Laboratory (EMSL) for untargeted metabolite identification with ¹H-NMR. Metabolites were extracted with chloroform: methanol (3:7 v/v) (Walker *et al.* 2016). The extracted pellet was re-suspended in 180 μL DSS-d₆ (internal standard). Samples were run on 600 MHz ¹H frequency NMR spectrometer and classified with 1D ¹H NMR metabolomics

software, Chenomx NMR Suite. Final concentrations were adjusted based on initial leaf weight. The leaf metabolite extraction and analysis were the same across experiments unless otherwise noted.

EPR

A second leaf section (20-30 mg) was cut and placed in a 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) spin trapping solution (Khramtsov *et al.* 1999). The perfusion solution contained 10 mM DEPMPO, 0.5 M HEPES buffer, with 0.01 μ L Tween 20. Stock spin trap solution was purified with activated charcoal. The leaves in solution were vacuum infiltrated repeatedly to ensure the spin trap was incorporated into the leaves. Perfusion was quenched in liquid N₂. At the time of measurements, each sample was thawed on ice and an aliquot was placed in a quartz capillary tube. Spectra were taken with the Bruker Elexsys 580 electron paramagnetic resonance (EPR) spectrometer. Signal intensity was evaluated as a height of the central peak of EPR spectrum. EPR conditions were as follows: 10 mW, microwave power; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 s⁻¹.

Statistics

Metabolite data were log₁₀ transformed and processed with univariate analysis to identify and remove outliers (studentized residual ≥ 4), and metabolites with less than 75% coverage across samples were also removed. Less than 5% of the final data was removed due to outliers. Each metabolite was tested independently using a one-way analysis of variance model with time point as a fixed effect. Statistical differences in least squared mean estimates between time points were determined by pdiff with an additional Bonferroni correction, and a significance threshold of $p < 0.05$ (Kuehl 2000). Statistical analysis was done using SAS software (SAS, Version 9.4, Cary, NC).

Foxtail millet Experiments

Plant Material and Growth

Green foxtail millet (*Setaria viridis*) accession A10 was planted directly into soil (Sungrow MetroMix) on March 8th, 2019 (foxtail millet experiment #1) and on July 15th, 2019 (foxtail

millet experiment #2). Plants were grown in growth chambers set to a 9h/15h day/night schedule with 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, 60% RH, and 30°C day / 23°C night conditions. Osmocote 19-5-8 slow-release fertilizer (The Scotts Company, Marysville, OH) was incorporated into the top inch of the soil, and plants were watered as needed.

¹H-NMR

Millet plants were kept at low $[\text{O}_3]$ ($<10 \text{ nL L}^{-1}$) until the days of sampling, March 24th, 2019 and July 15th, 2019. On the day of sampling, O_3 was added to the airstream and monitored with an O_3 analyzer (Thermo Electron 49i, Thermo Scientific, Waltham, MA, USA). Ozone was produced and monitored as described above for the maize experiment. For foxtail millet experiment #1, plants were exposed to 200 $\text{nL L}^{-1} \text{O}_3$ for 24 hours, with samples taken after 0, 1, 5, and 24 h following exposure. For foxtail millet experiment #2, an additional set of plants were kept at low $[\text{O}_3]$ ($<10 \text{ nL L}^{-1}$) and sampled at the corresponding time points, to serve as a control for diurnal variation in metabolite content and a direct treatment comparison. The youngest fully expanded leaf was cut, immediately quenched in liquid N_2 , and lyophilized. Leaf material was pooled from two plants, ground, and weighed (10-15 mg) for untargeted metabolomic extraction. Samples were re-suspended with 200 μL DSS-ds (internal standard) for each sample. The final concentration was adjusted based on leaf weight. Extraction and analysis were the same as stated above.

Statistics

Initial quality control was performed as described above for the maize experiment. For foxtail millet experiment #1, each metabolite was tested independently using a one-way analysis of variance model with time point as a fixed effect. One sample was removed from the millet experiment #1 as it was an extreme outlier. In the foxtail millet experiments, less than 5% of the total data were removed. Statistical differences in least squared mean estimates between time points were determined by paired differences of means (pdiff statement, SAS) with an additional Bonferroni correction and statistical threshold of $p < 0.05$ (Kuehl 2000). For foxtail millet experiment #2, each metabolite was tested independently using a two-way analysis of variance model with treatment and time point as fixed effects. One sample was removed from millet experiment #2 as it was an extreme outlier.

Attached leaf EPR

The youngest fully expanded millet leaf was inserted into the EPR sampling cavity, using a custom silicon cavity (Fig. 4.1). These plants were grown under low $[O_3]$ ($<10 \text{ nL L}^{-1}$) as described above until the time of sampling. Multiple genotypes of millet were tested (A10, 0529, and B100). A10 was the only suitable genotype for attached leaf analysis based on leaf shape and ease of transfer to the EPR cuvette (Fig. 4.1). Control measurements were made with a blank cavity exposed to ambient air or elevated $[O_3]$ and did not differ from one another (data not shown). Ozone was generated with a UV-C light bulb ballast, pumped into a box to allow the concentration to stabilize and monitored as described above. Ozone-enriched air was then pumped from the mixing box to the EPR spectrometer. The $[O_3]$ at the sampling cavity was tested and not found to be significantly different from the measurement in the mixing box. The leaf was measured in the cavity for 5 scans (40 sec each) in ambient air to create a baseline spectrum. After the baseline spectrum was obtained under ambient air, O_3 was introduced into the airstream ($300\text{-}400 \text{ nL L}^{-1}$) and leaves were exposed for 30 min. Scans were taken throughout O_3 exposure. After 30 minutes, O_3 was turned off, and the leaf was measured for an additional 20 minutes.

Results

Leaf metabolite profiles of maize and foxtail millet after initial $[O_3]$ exposure

Leaf metabolite profiles of the youngest fully expanded leaf from maize and foxtail millet were measured after 0, 1, 5, and 24 hours following the initial exposure to an acute dose of $[O_3]$. These time points were selected to track the initial antioxidant, metabolite, and reactive ROS response of O_3 exposure (Kangasjarvi *et al.* 2005). ^1H -NMR experiments identified 40, 31, and 17 compounds per experiment (Appendix A, Supplemental File 1). Statistical tests for the maize experiment #1 and foxtail millet experiment #1 (one-way ANOVA) identified 6 (Appendix A, Supplemental File 1) and 18 (Fig. 4.3) metabolites significantly changing after exposure to O_3 . The two-way ANOVA did not identify any metabolites with significant differences in ambient and elevated $[O_3]$ in the millet experiment #2 (Appendix A, Supplemental File 1).

Amino acids were most commonly increased after five hours of O_3 exposure and were identified in the list of metabolites that substantially changed over the course of 24 h of O_3 exposure in both maize and millet. Isoleucine and pyruvate were both significantly altered in the

maize and millet experiments (Fig. 4.2, Fig. 4.3). Isoleucine was significantly increased following five hours of O₃ exposure, and this trend was also seen in the foxtail millet experiment #2, although not significant ($p = 0.1$, Fig. 4.4). In comparison, pyruvate decreased following five hours of O₃ exposure in maize and millet. We also specifically tested the response of two of the most abundant and stable antioxidants, glutathione and ascorbate (Foyer and Noctor, 2011). In contrast to many O₃ publications, no significant response of ascorbate or glutathione to O₃ exposure was observed in any of the experiments (Fig. 4.5). There was a considerable difference in the leaf concentration ($\mu\text{mol/g}$) between the two antioxidants, with glutathione present in low concentrations in both maize and millet.

Quantification of the ROS with EPR

Spin trapping allows for the quantification and identification of ROS formed during oxidative stress. Figure 4.6 shows an example EPR spectrum of spin trap (DEPMPO) labeled maize leaf perfusions prior to and following 24 h of O₃ exposure. The spectrum is characteristic of a hydroxyl adduct, with a 2x increase in hydroxyl between O₃ treatments. This experimental result was unable to be replicated with millet leaves due to difficulties with spin trap infiltration into the leaves. Additional spin traps were tested in millet leaves, including *N-tert-Butyl- α -phenylnitrone* (PBN) and 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), but due to the low permeability of each of these spin traps through the leaf, neither was suitable for measurements.

Attached leaf *in situ* EPR measurements allow for the identification of ROS as they are formed. Figure 4.7 shows the spectrum of an intact millet plant measured on two consecutive days. Leaves were exposed to 300-400 nL L⁻¹ [O₃]. When O₃ was introduced into the air system, the superoxide radical immediately increased in concentration and slowly decreased back to ambient concentrations ($\sim 2000\text{s}$, Fig. 4.7 b,d). The radical intensity was much lower on the following day (Fig. 4.7d) but took approximately the same amount of time ($\sim 2500\text{s}$) to decrease back to ambient radical levels. This preliminary result was difficult to replicate at lower O₃ concentrations ($< 300\text{ nL L}^{-1}$).

Discussion

The mechanisms of O₃ response in C₃ plants have been well studied, including specific ROS formed following O₃ exposure, antioxidant systems up-regulated, signaling pathways affected,

and subsequent physiological responses (Vainonen & Kangasjarvi 2015). The physiological impacts of C₄ plants have also been studied under elevated O₃ including decreased photosynthesis and accelerated leaf senescence (Moura *et al.* 2018; Yendrek *et al.* 2017). Differences in sensitivity between genotypes is also established in C₄ plants (Choquette *et al.* 2019; Moura *et al.* 2018). However, studies have not measured the metabolomic response of C₄ species to acute O₃ stress. To address this gap, different metabolomic platforms were used to study the initial response of C₄ plants to elevated [O₃]. ¹H-NMR identified some metabolites potentially involved in the initial O₃ response of maize and millet, and EPR measurements identified a significant increase in hydroxyl radical after 24 hours of acute O₃ stress in maize. These preliminary studies have developed methods that can be used to further quantify metabolites and ROS formed following exposure.

Isoleucine was on the only metabolite altered consistently after acute O₃ exposure in both maize and millet (Fig. 4.2d, 4.3e, 4.4a). An upregulation of amino acids was observed in maize experiment #1 and millet experiment #1, along with a significant change in pyruvate concentrations. Increased amino acid concentrations have been measured in previous chronic O₃ studies, and were interpreted as evidence of increased flux through the TCA cycle (Betzberger *et al.* 2012; Dizengremel *et al.* 2012; Yendrek *et al.* 2015). Increases in mitochondrial respiration and TCA cycle biproducts are thought to occur shortly after exposure and may provide additional energy and reductant for detoxification (Dizengremel *et al.* 2012; Gillespie *et al.* 2012). The significant changes in amino acids concentration for maize experiment #1 and millet experiment #1 did not have a control group to allow for comparison of concentrations. The second millet experiment was specifically designed to separate changes in amino acid concentrations caused by O₃ flux from changes in concentrations due to diurnal variations.

Although none of the metabolites identified in millet experiment #2 were significant, some treatment trends could be inferred and inform future experiments. The increase in isoleucine concentrations 5 hours after exposure was observed in the elevated O₃ treatment but not the ambient O₃ treatment (Fig. 4.4a), supporting the findings of the maize and the first millet experiments. Additionally, alanine followed the treatment trend observed in the initial experiments, with a decrease immediately after O₃ exposure. Alanine is synthesized from pyruvate and is the primary C₃ acid shuttled from the bundle sheath to mesophyll cells in C₄ photosynthesis (Furbank 2011). The reduction in both alanine and pyruvate might indicate a

decline in photosynthesis and an increase respiration under elevated O₃ stress (Dutilleul *et al.* 2003). Additional experiments to quantify TCA intermediates, amino acids, and C₄ photosynthesis metabolites would be needed to test this idea.

Ascorbate and glutathione are two of the most abundant antioxidant molecules in leaves and their abundance and redox states are important for detoxification of O₃-induced ROS (Foyer & Noctor 2005; Kangasjarvi *et al.* 2005). Therefore, we examined those molecules in maize and millet exposed to acute O₃ exposure. Ascorbate content was much greater than glutathione content in both maize and millet, although there was no effect of O₃ identified in the ¹H-NMR experiments for either C₄ species (Fig. 4.5), contrary to expectation. Ascorbate and glutathione are both difficult to measure with ¹H-NMR because they are often buried under peaks of more saturating metabolites and are of low abundance. Therefore, it is possible that either antioxidant was altered to a small degree, and these changes were below the detectable limit of ¹H-NMR. Additional experiments need to be performed to resolve these questions.

This chapter was reliant on NMR based metabolomics. NMR has the ability to quantify metabolites of high abundance in an inherently quantitative method. Structural information can be provided for each compound, which is essential in plant metabolomics, where many compounds have the same chemical composition (Alseekh & Fernie 2018). However, NMR has disadvantages, especially in complex plant samples, where many metabolites are present in low concentrations that are difficult to detect. Manual fitting is a common protocol for identification and quantification of individual metabolites in NMR experiments. This method is time consuming, prone to bias, slow, and dependent on operator experience (Ravanbakhsh *et al.* 2015). The large amount of sample variation could be due to manual fitting making it difficult to achieve significant results without a large number of replications. For instance, the sugar region of a ¹H-NMR spectrum is difficult to accurately quantify, due to saturation and overlapping peaks within the spectrum. One solution to reduce overall sample error would be to incorporate binning methods that employ machine learning techniques rather than the subjective opinion of a user (Cassiede *et al.* 2018; Padayachee *et al.* 2019).

To achieve greater coverage of the metabolome, mass spectrum (MS)-based approaches are often coupled with NMR. MS-based approaches are highly sensitive, robust, and can identify hundreds of metabolites (Fang *et al.* 2019). LC/GC-MS would offer a more sensitive detection and quantification of sugars, organic acids, and amino acids that cannot be provided with NMR

(Barding *et al.* 2012). The troubled sugar region in NMR spectrum can be accurately resolved using MS-based methods. Additionally, in each of the three experiments, phenolic compounds were present within the elevated O₃ samples, and not in the initial time point or control samples. These phenolics were present in low concentrations, making them difficult to quantify with ¹H-NMR. Additionally, the facility did not have many phenolics present in the library, hindering the ability to positively identify the compounds. Many of these phenolics could be identified with MS-based methods, further supporting the need for a combined approach.

ROS can be difficult to measure due to their short lifetime (μs-ms) and non-paramagnetic nature, resulting in EPR silence. Few studies have directly measured the ROS formed after O₃ exposure; more recent literature has used the application to measure additional oxidative stresses. Maskimovic and colleagues extracted apoplastic fluid from maize root to quantify hydroxyl radical adduct with DEPMPO spin trap (Maksimovic *et al.* 2014). The difference in the accumulation of ROS has also been reported between tolerant and sensitive genotype. Oksanen *et al.* (2004) associated a difference in hydrogen peroxide accumulation due to the sensitivity of an Aspen clone to O₃ damage. In spin trapped maize samples, a 2-fold increase in hydroxyl radical was observed after 24 hours of O₃ exposure (Fig. 4.6). Although multiple spin traps were used to attempt to obtain sufficient perfusion with the millet leaves, none were successful possibly due to differences in the thickness of the epidermal wax layer between species. Due to the hydrophilic nature of many spin traps, detection of radicals formed in the lipid phase is not possible (Villamena & Zweier 2004). Radicals formed following lipid peroxidation are a consequence of O₃ stress, often damaging photosystem II complex and generating additional ROS, mainly superoxide (Farmer & Mueller 2013). Runeckles and Varrtnou (1997) were unable to detect singlet oxygen using a spin trap, due to the lack of infiltration to the areas of radical formation. The use of new spin traps or experimental methods could enable the detection of lipid peroxidation radicals, but this remains a significant unknown in the field. Additionally, many of the spin traps are costly, making it impractical to perfuse whole leaves or many samples for measurements and to troubleshoot protocols. One solution is to combine this approach with *in vivo* attached leaf studies. The ability to measure ROS as they are formed *in vivo* would provide vital evidence for the formation, length, and concentrations that are largely unknown in the plant response to O₃ stress and oxidative stress in general.

Ozone-induced ROS can be monitored *in vivo* with the EPR spectrometer enabling non-destructive measurements. Many of the initial studies to identify the ROS involved in the plant response to O₃ were completed with attached leaf EPR measurements. EPR measurements identified variation in the level of O₃ tolerance between wheat genotypes, with a greater than 2-fold increase in O₂^{•-} between tolerant and sensitive species (Reichenauer *et al.* 1998). Valavanidis *et al.* (2004) identified a 20-25% increase in Mn²⁺ signal in diseased pine needles using detached leaf EPR. An increase in Mn²⁺ signal under O₃ stress is characteristic of damage to the D1-protein of photosystem II (Sersen & Kralova 2013). Figure 4.1 shows an attached leaf measurement of a millet plant. The leaf is placed in an EPR cuvette and O₃ induced superoxide radicals were identified at very high concentrations of O₃ (300-400 nL L⁻¹, Fig. 4.7) but were difficult to measure at more moderate O₃ levels (100 nL L⁻¹). Interestingly, when the same plant was measured two days in a row, the intensity of the radical peak was diminished on the second day (Fig. 4.7 c, d), suggesting priming from the original stress and an attenuated response. This is consistent with plant responses to pathogens (Conrath *et al.* 2006). Although we were able to show the feasibility of measuring ROS *in vivo* with a non-destructive technique, we consider these to be preliminary experiments and the techniques needs to be developed further to identify factors that hinder the ability to measure radicals.

Conclusion

Metabolomics can be a powerful approach when multiple platforms are used in combination to achieve a holistic view of the metabolome (Stitt & Fernie, 2003). Implementing H-NMR and EPR studies this project identified a significant increase in amino acid content, hydroxyl radical, and superoxide radical formation following initial O₃ exposure in two C₄ species. Additional metabolomic profiling methods, such as ¹H-NMR and GC/LC-MS, in combination with the proposed EPR analysis, would confirm information regarding the antioxidants and secondary metabolites expressed under O₃ stress.

FIGURES

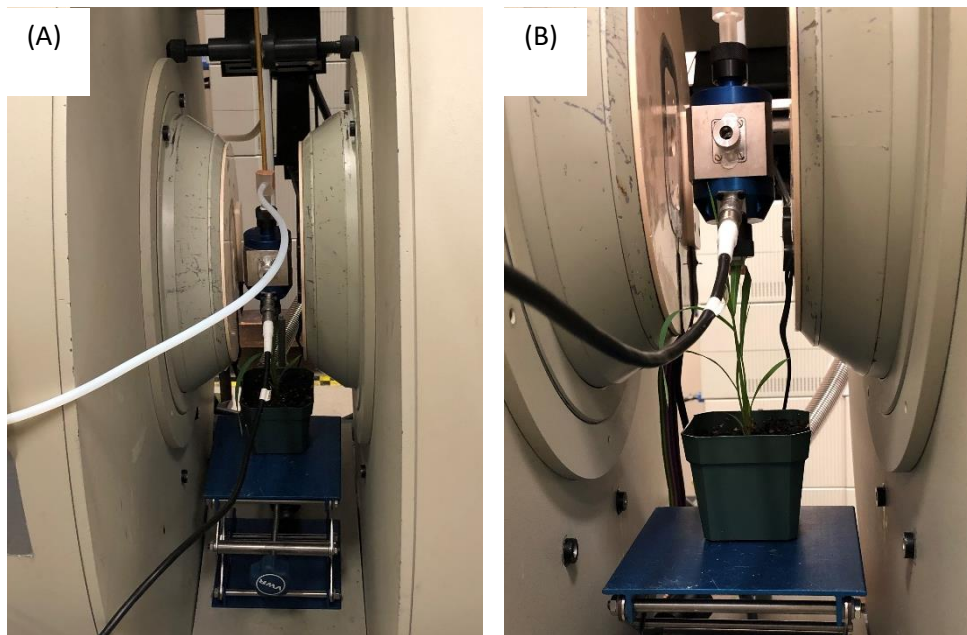


Figure 4.1. Attached leaf EPR measurements. (A) Picture shows white tube that carries O₃ from the mixing box, to the surface of the leaf. (B) Image of millet leaf inserted in the EPR cavity.

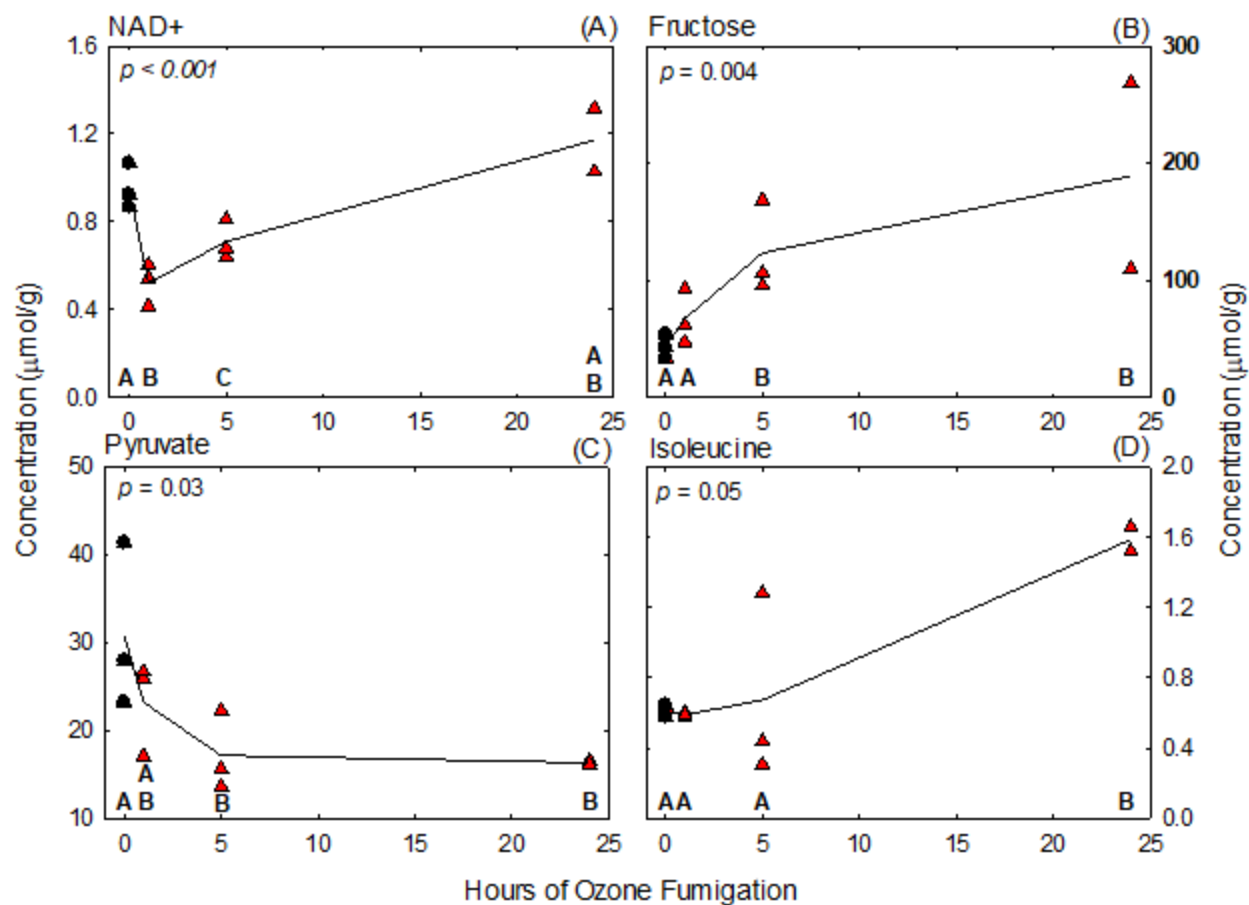


Figure 4.2. Maize experiment #1. Maize leaf metabolites concentrations (μmol/g) significantly altered following initial exposure to elevated [O₃] (200 ppb). Black points indicate measurements before O₃ was introduced into the airstream, triangle points indicate samples taken at 1, 5, and 24 hours after initial exposure. Individual sub-replicates (n=3) are shown, with mean values represented in the trend line. p-value in the top left indicates time point effect and letters denote the significant differences between groups.

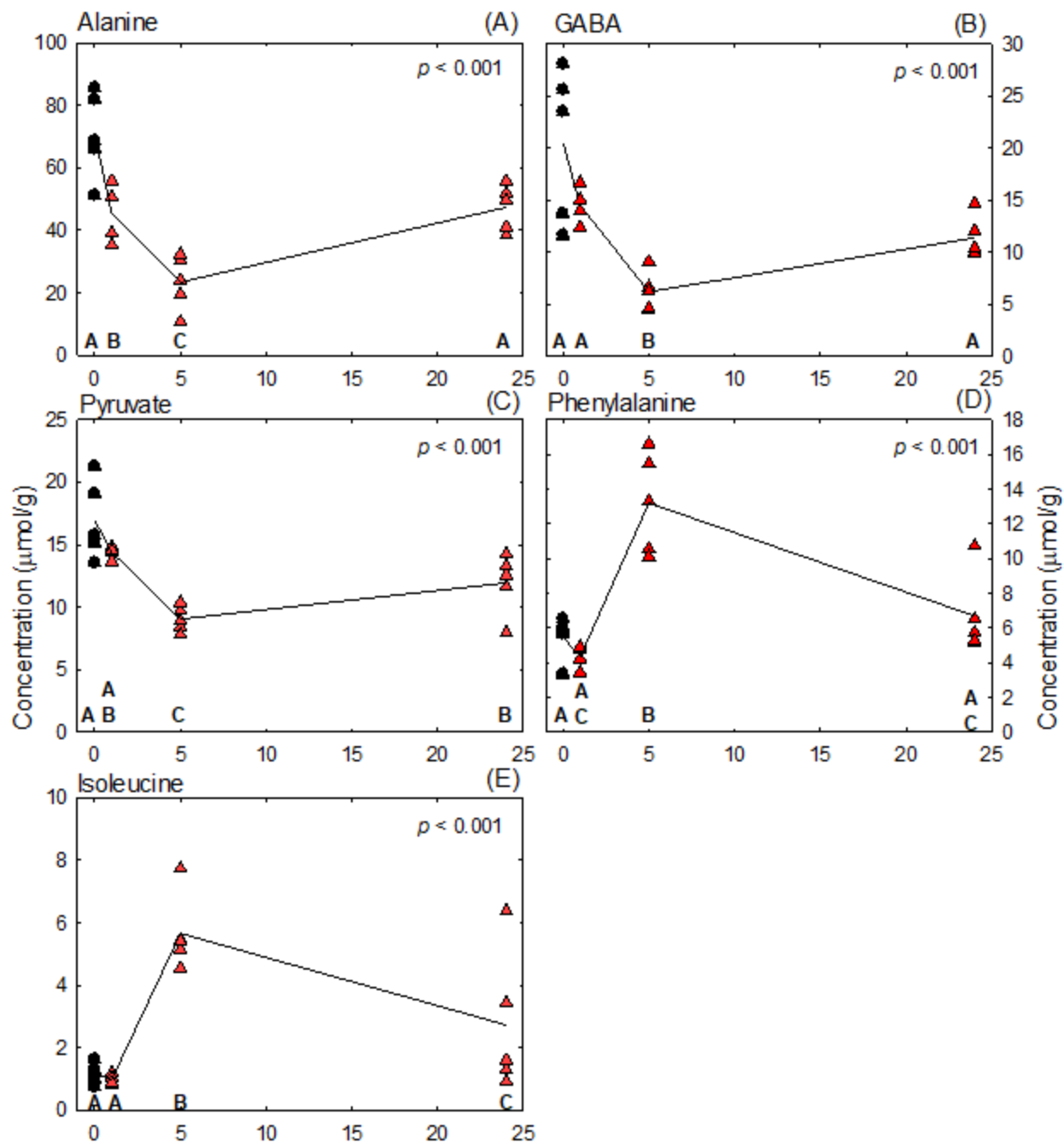


Figure 4.3. Millet experiment #1. Millet leaf metabolites concentrations ($\mu\text{mol/g}$) significantly altered following initial exposure to elevated $[O_3]$ (200 ppb). Black points indicate measurements before O_3 was introduced into the airstream, triangle points indicate samples taken at 1, 5, and 24 hours after initial exposure. Individual sub-replicates (n=4-5) are shown, with mean values represented in the trend line. p-value in the top left indicates time point effect and letters denote the significant differences between groups.

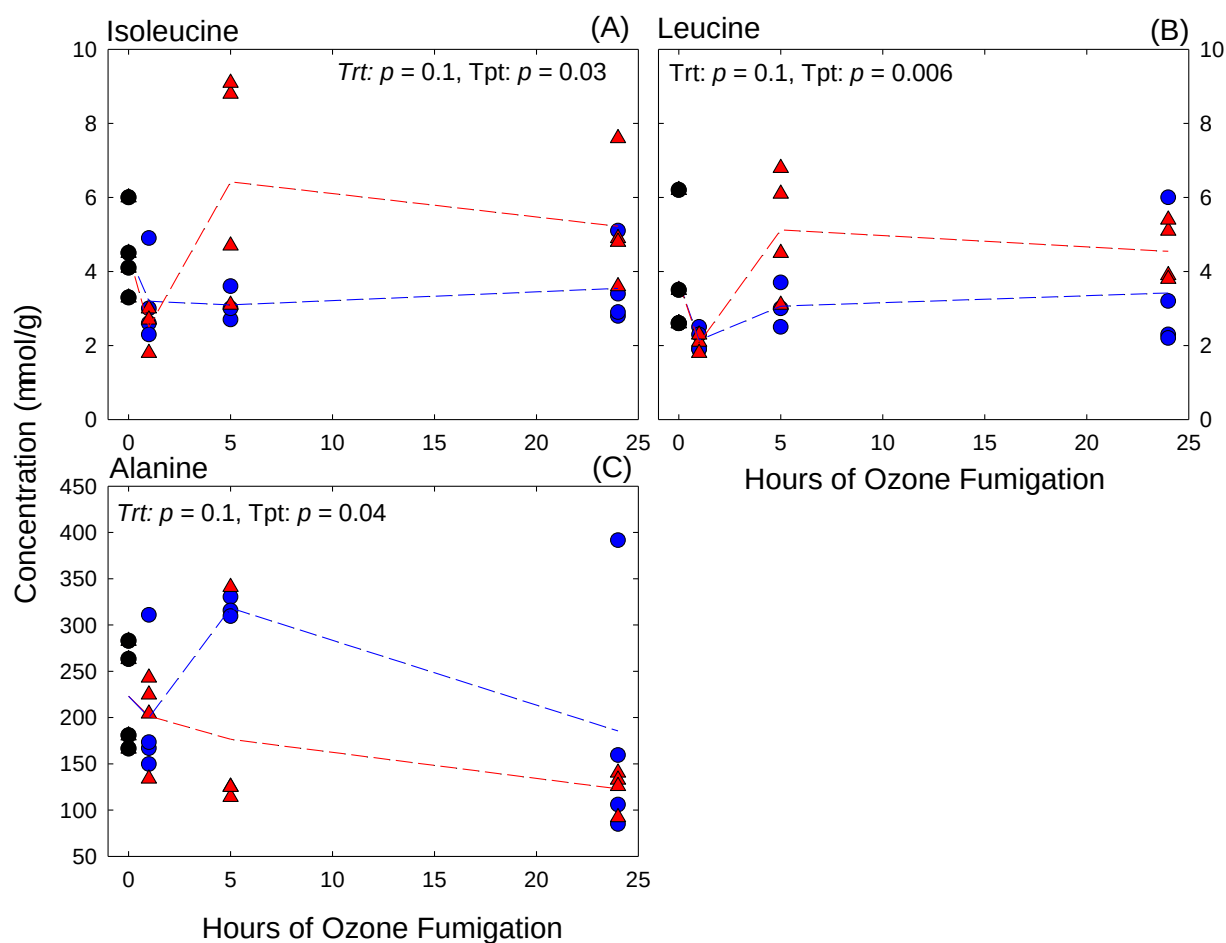


Figure 4.4. Millet experiment #2. Millet leaf metabolites concentrations ($\mu\text{mol/g}$) following initial exposure to elevated $[\text{O}_3]$ (200 ppb). Black points indicate measurements before O_3 was introduced into the airstream, triangle points indicate samples taken in O_3 treatment (1, 5, and 24 hours after initial exposure), and circle points indicate ambient O_3 treatment. Individual sub-replicates ($n=4$) are shown, with mean values represented in the trend line (blue-ambient O_3 , red- elevated O_3). p-value in the top left indicates time point (Tpt) and treatment (Trt) effect while letters denote the significant differences between groups.

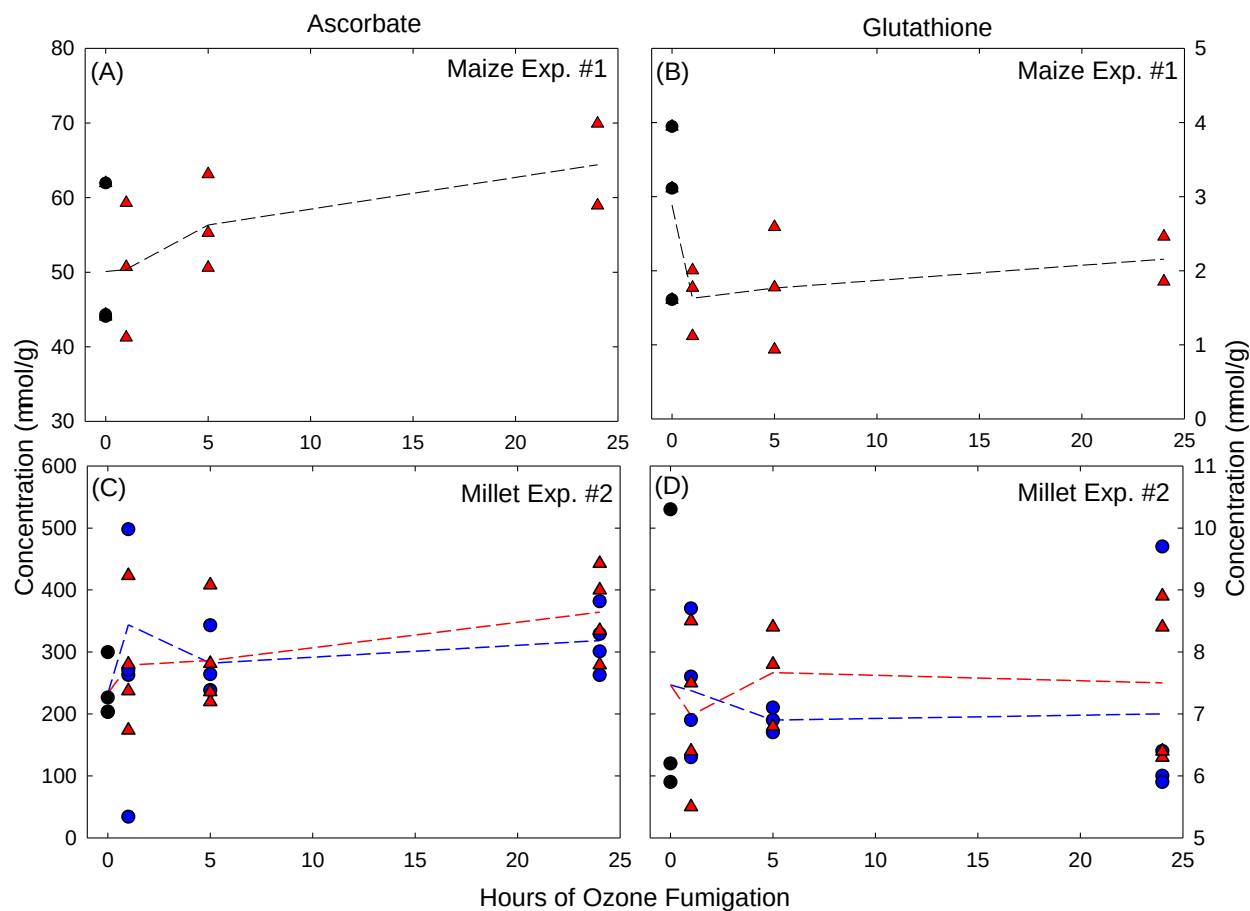


Figure 4.5. Maize and millet leaf ascorbate (A,C) and glutathione (B,D) concentrations ($\mu\text{mol/g}$) following initial exposure to elevated $[\text{O}_3]$ (200 ppb). Black points indicate measurements before O_3 was introduced into the airstream, circle points (blue) indicate ambient O_3 treatment, and triangle points (red) indicate samples taken in elevated O_3 treatment (1, 5, and 24 hours after initial exposure). Individual sub-replicates ($n=4$) are shown, with mean values represented in the trend line (blue-ambient O_3 , red- elevated O_3).

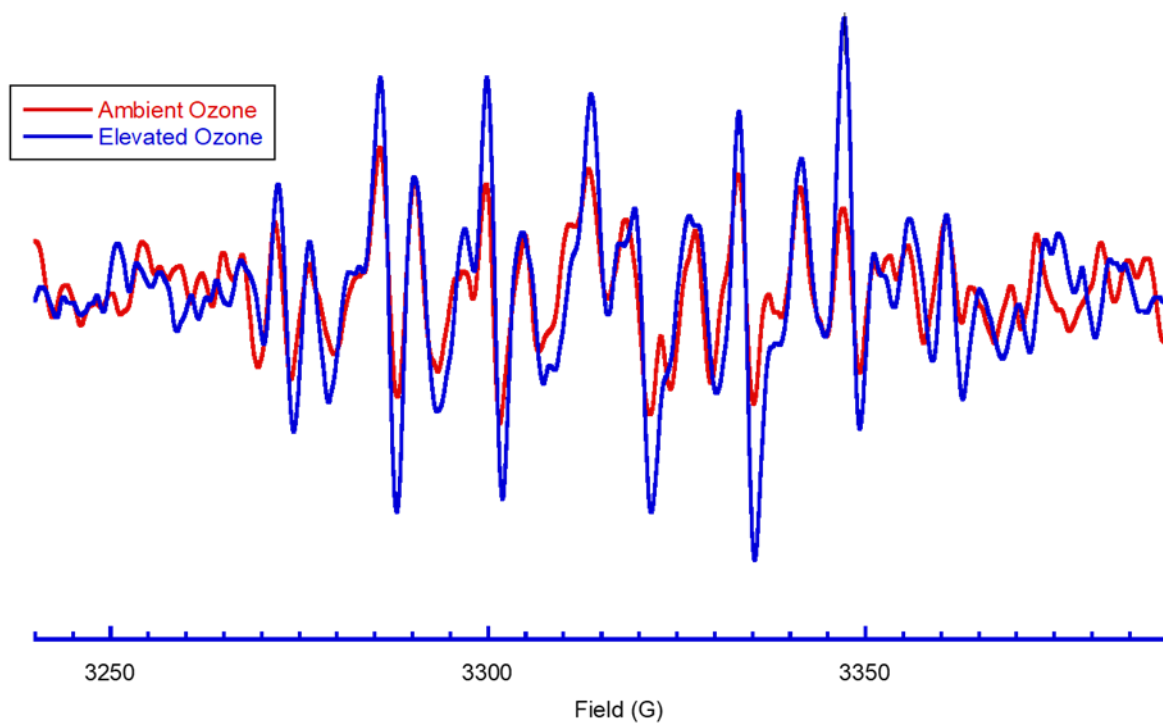


Figure 4.6. EPR hydroxyl adduct spectrum labeled with spin trap (10 mM DEPMPO), from maize experiment #1. Red line shows spectrum from ambient O₃ sample, blue line indicates elevated O₃ sample (24 hr of exposure, 200 [ppb]).

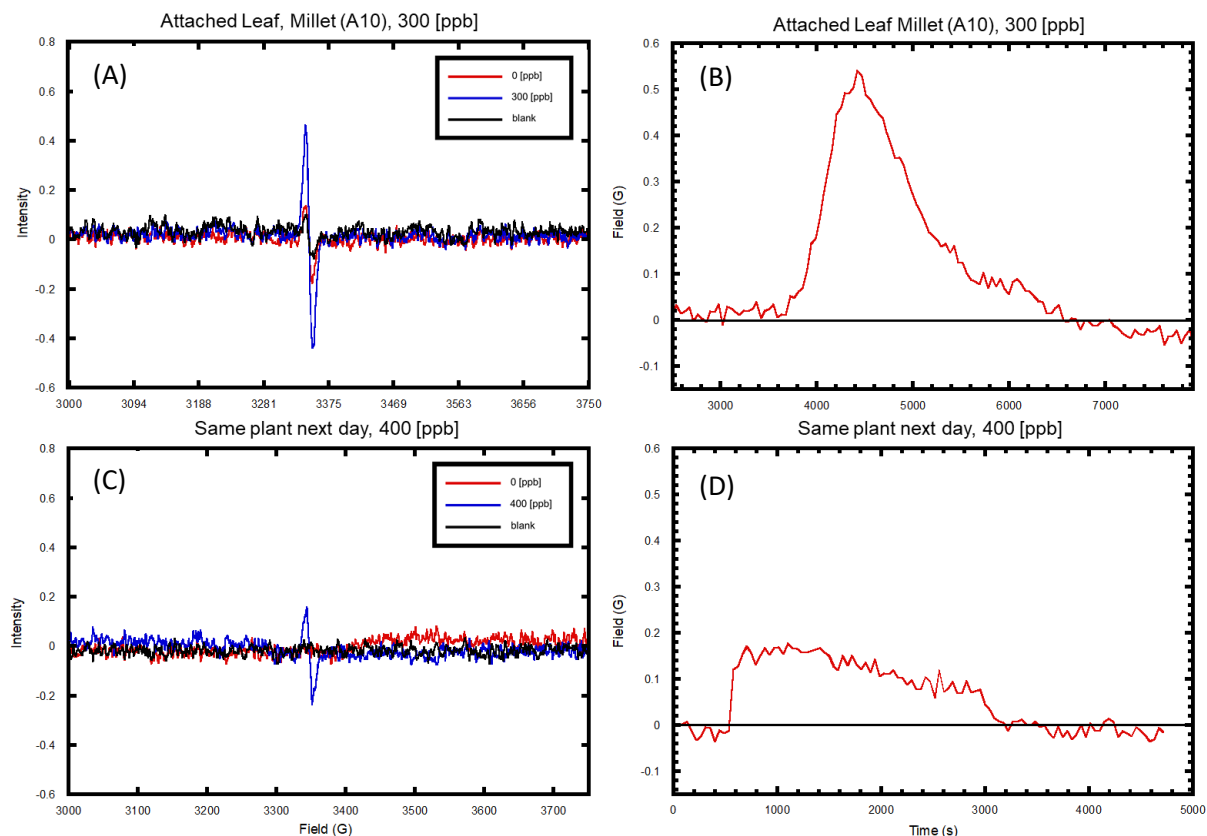


Figure 4.7. Attached millet leaf EPR measurements. (A) Single spectrum scan of; black line- base line spectrum, red- ambient O₃ spectrum prior to exposure, blue- elevated 300 [ppb] O₃ exposure. (B) Sum of all spectrum combined, with central peak indicating the formation and decay of radical. (C) Single spectrum scan of the same plant measured the following day under 400 [ppb] O₃, lines indicate the same conditions as in panel A. (D) Sum of all spectrum combined for the same plant on the following day, with central peak indicating the formation and decay of radical.

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CHAPTER V: CONCLUDING REMARKS

Global climate change disproportionately affects countries and regions of the world with some of the highest rates of food insecurity (FAO 2016). Carbon dioxide concentrations ($[\text{CO}_2]$) are continuing to increase yielding a warmer global surface temperature, and tropospheric ozone concentrations ($[\text{O}_3]$) are only expected to decrease under the most optimistic IPCC scenario (RCP 2.6) (Eyring *et al.* 2013). The combination of environmental stresses will impose food supply challenges across the world with an expected demand increase of approximately 65% by 2100 (FAO 2016). Understanding the response of crops to abiotic stress is the first step towards adapting agriculture to a changing environment. Metabolomics profiling offers an ideal platform to measure the biochemical response of plants. This thesis aimed to quantify changes in metabolite profiles of agronomically important C_4 grasses in response to abiotic stress.

Chapter II aimed to understand the effects of elevated $[\text{CO}_2]$ and warming singly and in combination, on the metabolomic and transcriptomic profiles of guinea grass. Elevated $[\text{CO}_2]$ had virtually no impact on the transcriptome and very little impact on the metabolome. These results are consistent with the lack of response of maize to elevated $[\text{CO}_2]$ in the absence of other stresses (Leakey *et al.* 2006). In elevated temperature conditions, regardless of $[\text{CO}_2]$, content of amino acids and secondary metabolites were altered, along with gene expression. The response seen in both the transcriptome and metabolome were hypothesized to increase growth and alleviate stress associated with elevated temperature. The chapter showed that a tropical C_4 grassland species had an unpredicted response to global climate change.

Chapter III provided evidence for differences in the metabolomic response between inbred and hybrid maize lines to season long elevated $[\text{O}_3]$. The metabolome of two maize inbred lines and the hybrid were analyzed following the course of leaf senescence. No treatment effect was observed in the inbred lines, although the two inbred lines had different metabolomics signatures. In contrast the metabolite profile of the hybrid line responded to elevated $[\text{O}_3]$ with the response becoming more pronounced as leaves aged. Phytosterols and α -tocopherol were significantly correlated with yield and leaf mass area reductions observed under elevated $[\text{O}_3]$ in the hybrid genotype. The positive relationship identified suggests a potential protective role these metabolites to stabilize thylakoid membranes and prevent excessive degradation of chlorophyll under elevated $[\text{O}_3]$. The historical and field-based literature regarding plant response to elevated O_3 almost exclusively focus on the role of ascorbate and glutathione as the main antioxidants

protecting plants from ROS (Ainsworth 2017). Although the role of phytosterols and α -tocopherol has been identified in other abiotic stresses (Foyer 2018) it was not expected in an elevated O₃ stress further supporting the importance of metabolomic profiling to identify unexpected relationships. The metabolomic signature identified in hybrid has the potential to be used as a marker to improve O₃ tolerance. This chapter showed the feasibility of using field-based metabolomic profiling to gain insight into physiological responses.

Chapter IV investigated the initial reactive oxygen species (ROS) and antioxidants following exposure to acute [O₃]. No significant differences between antioxidants was observed in either maize or millet after initial O₃ exposure. However, a number of amino acids were following acute O₃ exposure, potentially upregulating flow through the TCA cycle. Increased hydroxyl radical and superoxide were observed in maize and millet leaves following O₃ exposure, using two separate electron paramagnetic resonance (EPR) techniques. The ability to measure ROS *in vivo* with EPR has only been proposed in C₄ species; this chapter demonstrated that it could be successfully applied to studies of acute O₃ stress. However, additional metabolomic and EPR studies are needed to fully understand the response of C₄ crops to acute O₃ exposure. This thesis emphasized that metabolomic profiling is a vital tool that can be used alongside additional ‘omic’ and standard physiological measurements to improve understanding of plant metabolic responses to environmental stress.

The whole of this thesis emphasizes the power of using metabolite profiling to improve the understanding of the biochemical response to abiotic stresses. The ability to identify clear treatment differences in multiple species, using this approach is powerful and somewhat surprising. Metabolomic data is notorious for the significant amount of noise within a dataset. Even without considering the field variation that comes with sampling from rings acres apart, each step of the process introduces another set of variables to be accounted for in the repeatability of the data. To avoid degregation of unstable metabolites, leaves need to be quenched in liquid nitrogen as quickly as possible. In order to sample eight rings in the span of two hours multiple sampling teams are required, introducing variation in the speed of quenching, and again contributing to noise. Everything from the sample preparation to peak quantification introduces another set of errors.

Plants contain hundreds of thousands of individual metabolites with current metabolomic tools unable to identify the majority of these chemical compounds. In both of the GC-MS based

untargeted profiling experiments within this thesis, about 100 metabolites were positively identified with at least another 50 to 100 more unknown and unable to be classified. The significant amount of information lost in these unknowns is a current bottleneck within the field. It is common practice to take a quick survey of these unknowns, look for extreme differences, then throw them out of the experiment because they cannot be identified. Until methods address this issue, valuable information is being lost in the metabolomic profiling studies that could be vital to the conclusions being made. For example in Chapter 3, the fatty acid pathway was altered in the hybrid genotype under elevated O_3 . However, many of the compounds important in this pathway were not identified and no clear conclusion could be drawn. The lack of identification of compounds critical to that pathway left a gap in knowledge of the response of maize exposed to O_3 stress. Improved identification of metabolites within any one platform would greatly improve the aims of profiling studies to identify biochemical signatures in response to environmental stresses.

Even with all of the variables and unknowns present in any metabolomic based experiment, treatment separations were identified reflecting phenotypic responses. This was seen in both of the studies presented in Chapter 2 and Chapter 3. In Chapter 3, a significant response of α -tocopherol to elevated $[O_3]$ was identified in aging leaves of hybrid maize reflecting the acceleration of senescence observed under elevated $[O_3]$. The metabolomic profile clearly reflected the increased senescence phenotype seen in the hybrid genotype, and the lack of a phenotype in the inbred. An interesting next experiment would be to identify if accumulation of α -tocopherol in maize, or other crops, could improve crop tolerance to elevated $[O_3]$. These results provide evidence for the power of metabolomics profiling experiments to understand the plant response to an abiotic stress. The ability to generate novel and unexpected hypothesis, such as the link between O_3 tolerance and α -tocopherol, from these experiments is vital to advancing the goals to improve crops in an increasingly harsh world.

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APPENDIX A: SUPPLEMENTAL INFORMATION

I. Supplemental Files for Chapter II

The supplemental files references in Chapter II may be found in a folder named

ChapterII_supplemental. Description of each file is below.

Supplemental File 1: Differentially expressed genes (DEG) within each weighted gene correlations network analysis (WGCNA) module for time point A. Values show the elevated CO₂ and elevated temperature (eCeT) treatment compared to ambient control (C) based on LIMMA analysis.

Supplemental File 2: Differentially expressed genes (DEGs) in combined elevated CO₂ and elevated temperature (eCeT) treatments from time points A and B. The log₂ fold change (log₂FC) is shown along with statistical analysis of differential gene expression.

Supplemental File 3: Linear correlation coefficient (r) and significance (Adj.P) of linear relationships between metabolites and transcripts sampled at Time Point B.

II. Supplemental Files for Chapter III

Supplemental files mentioned in Chapter III may be found in supplemental folder named

Chapter III_supplemental. Descriptions of each file is below.

Supplemental Table 1: Mean, standard error, minimum and maximum metabolite content measured in ambient and elevated [O₃] in the hybrid B73 x Mo17 at timepoint A (DOY 208), timepoint B (DOY 219) and timepoint C (DOY 229).

Supplemental Table 2: Mean, standard error, minimum and maximum metabolite content measured in inbred lines B73 and Mo17 at timepoint A (DOY 216), timepoint B (DOY 225) and timepoint C (DOY 236).

Supplemental Table 3: Statistical analysis (ANOVA) for individual metabolites, LMA and yield from the hybrid line B73 x Mo17.

Supplemental Table 4: Statistical analysis (ANOVA) for individual metabolites, LMA and yield for the inbred lines B73 and Mo17.

Supplemental Table 5: Linear correlations between grain yield or LMA and metabolite content in hybrid line B73 x Mo17.

Supplemental Table 6: Linear correlations between grain yield or LMA and metabolite content in inbred lines B73 and Mo17 (grey).

III. Supplemental File for Chapter IV

The supplemental file mentioned in Chapter IV may be found in supplemental folder named **Chapter IV_supplemental**. Description of the file is below.

Supplemental File 1: Normalized ^1H -NMR leaf metabolite values for each experiment and statistical analysis (ANOVA) for individual metabolites.

APPENDIX B: SUPPLEMENTAL FIGURES AND TABLE FOR CHAPTER II

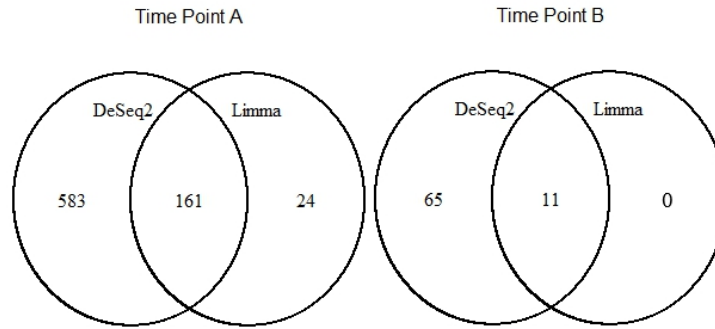
I. Tables and Figures

Supplemental Table B.1: Weighted gene correlation network analysis (WGCNA) signed network statistics for time point A with GO-term enrichment analysis. Minimum module size was 75 genes. The genes contained within a module were used to identify significantly increased GO-Enrichment biological process terms ($P < 0.01$). Metabolites listed were significantly correlated with the corresponding module ($P < 0.05$, $|r| \geq 0.75$). *Module*: WGCNA gene grouping, *Number of Genes*: total number of genes within each module, *Number of DEG*: total number of differentially expressed genes within each module, *Metabolite (P,r)*: Linear correlation between module eigengene based connectivity and gene significance for metabolite (p value and correlation coefficient), *GO-term enrichment*: gene ontology enrichment of total number of genes within the module.

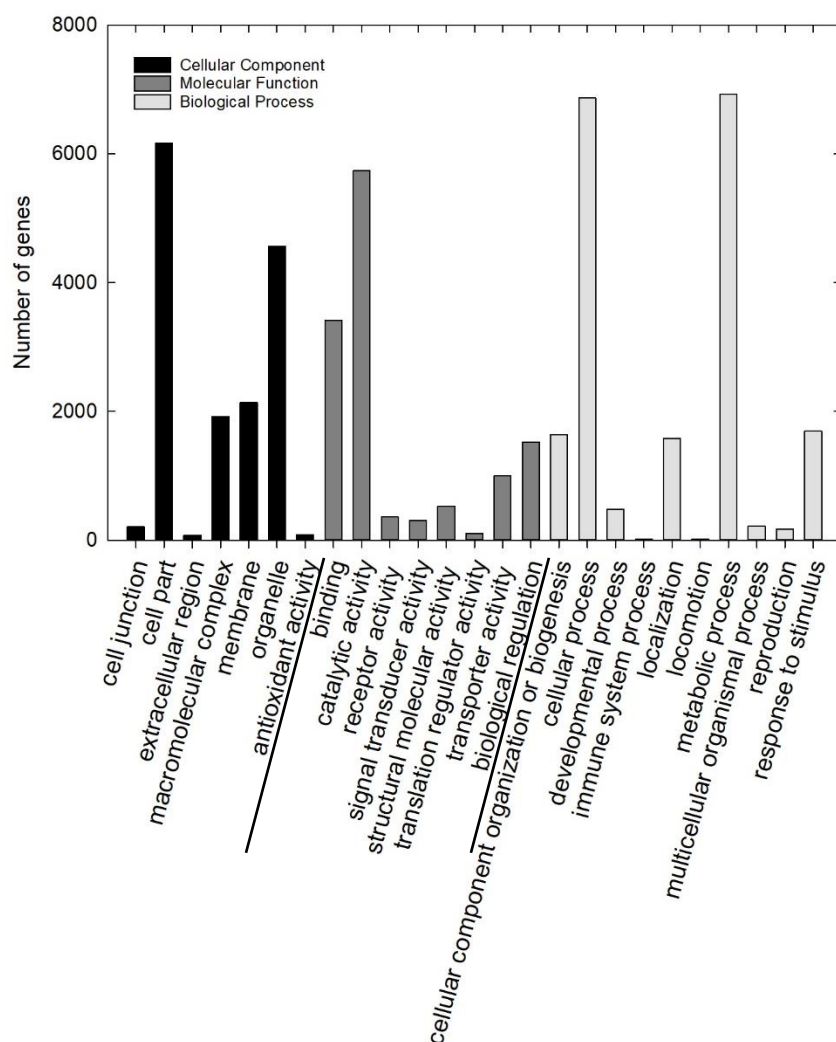
Module	Number of Genes	Number of DEG	Metabolite (P, r)	GO-term Enrichment (GO-term ID; Fold Enrichment)
1	1719	2	-	Phosphate-containing compound metabolic process (GO:0006796; 1.67)
2	1260	9	-	Gibberellin biosynthetic process (GO:0009686; 12.82) Cell surface receptor signaling (GO:0007166; 3.81) Protein phosphorylation (GO:0006468; 2.74)
3	948	84	Melibiose (5.8e-04, -0.76)	Defense response to oomycetes (GO:0002229; 12.7) Transmembrane receptor protein tyrosine kinase signaling (GO:0007169; 6.94) Response to bacterium (GO:0009617; 6.94)
4	536	2	-	Protein phosphorylation (GO:0006468; 2.71) Defense response (GO:0006952; 2.19)
5	484	35	-	Oxylipin biosynthesis (GO:0031408; 17.06) Terpenoid biosynthesis (GO:0046246; 9.22) Response to wounding (GO:0009611; 5.03) Hormone biosynthesis (GO:0042446; 4.24) Secondary metabolite biosynthesis (GO:0044550; 3.85) Defense response to other organisms (GO:0098542; 2.28)

Supplemental Table B.1 cont.

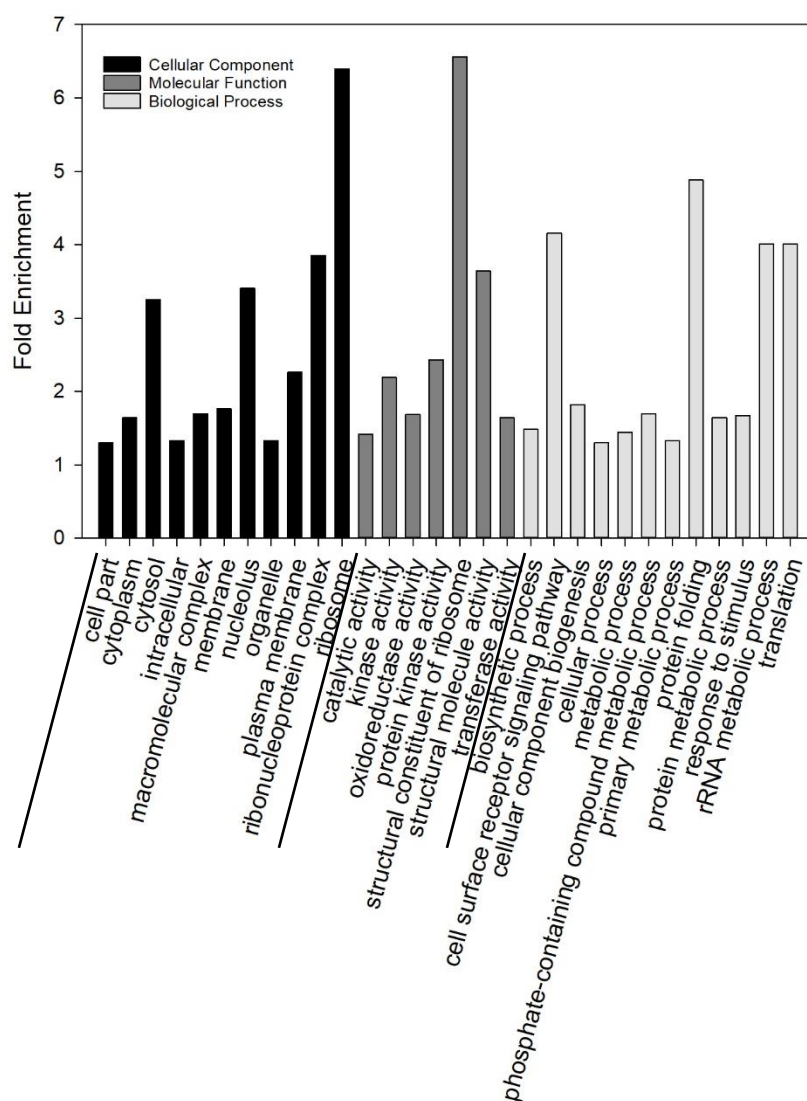
				Oxidation-reduction process (GO: 0055114; 2.23)
6	444	2	-	Response to wounding (GO: 0009611; 6.20)



Supplemental Figure B.1. Comparison of statistical methods for RNAseq. Venn diagram of differentially expressed genes ($p < 0.05$) in the combined elevated $[\text{CO}_2]$, elevated temperature treatment comparing LIMMA and DESeq2 analyses for time points A and time point B.



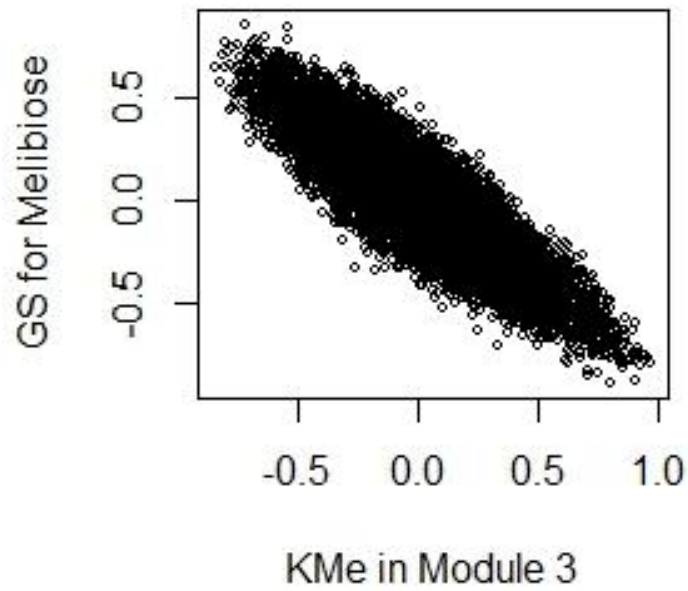
Supplemental Figure B.2: Gene ontology ‘slim’ classification of the *Panicum maximum* transcriptome. The number of unigenes assigned to each GO slim terms in the *P. maximum* transcriptome is shown in the bar chart. Transcripts were classified into three categories: cellular components, molecular functions and biological processes. 11,538 total classified unigenes. False discovery rate of 0.05 was applied and categories with less than 10 genes were removed.



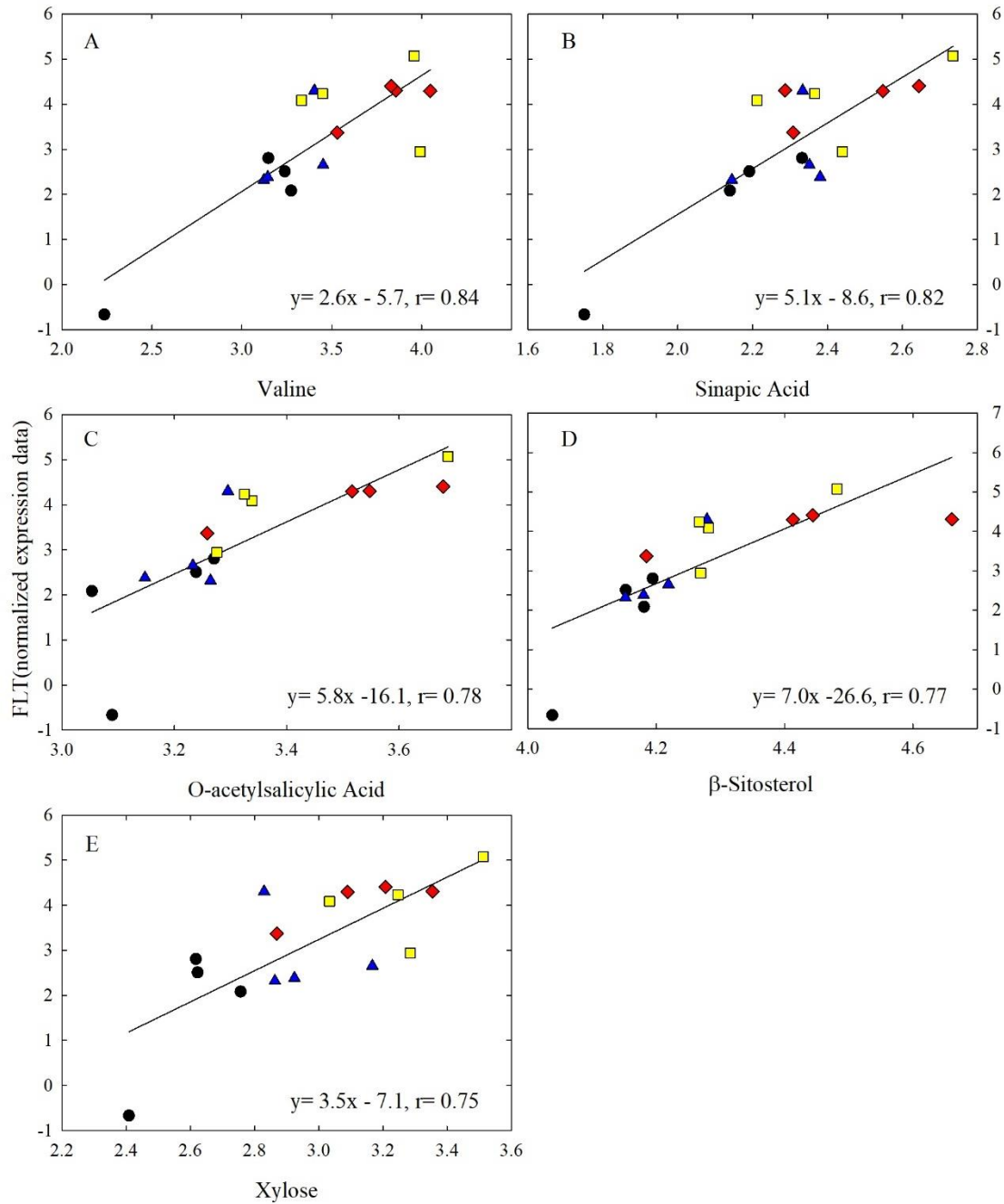
Supplemental Figure B.3: Gene ontology ‘slim’ classification of differentially expressed genes (DEG) in plants grown in the combined elevated [CO₂], elevated temperature treatment in time point A.

The average fold enrichment for groups of transcripts assigned to each GO slim term is shown.

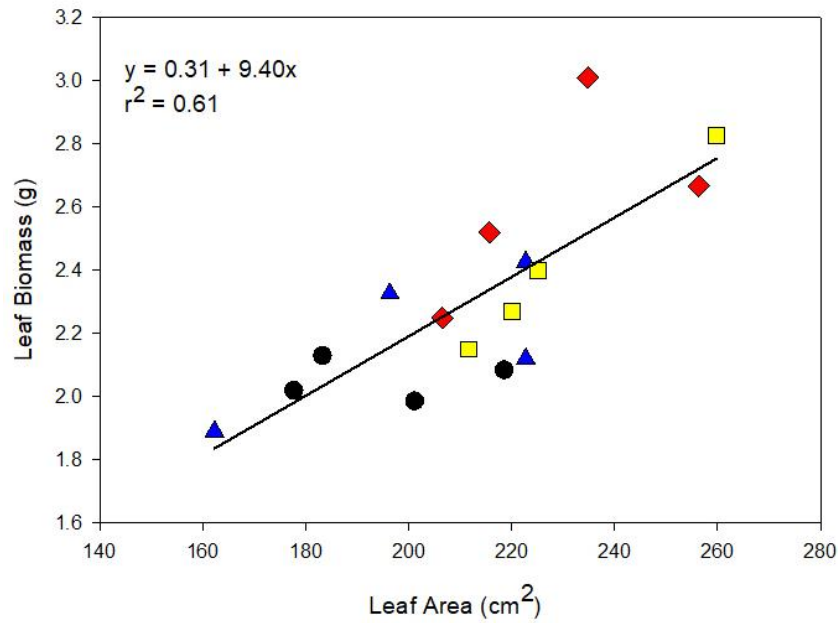
Transcripts were classified into three categories: cellular components, molecular functions and biological processes. 666 total genes were classified. A false discovery rate of 0.05 was applied.



Supplemental Figure B.4: Correlation between module membership in Module 3 and gene significance (GS) for melibiose. Eigengen based connectivity of module membership measure is plotted on the x axis (KMe). Correlation coefficient -0.87 and $p < 0.0001$.



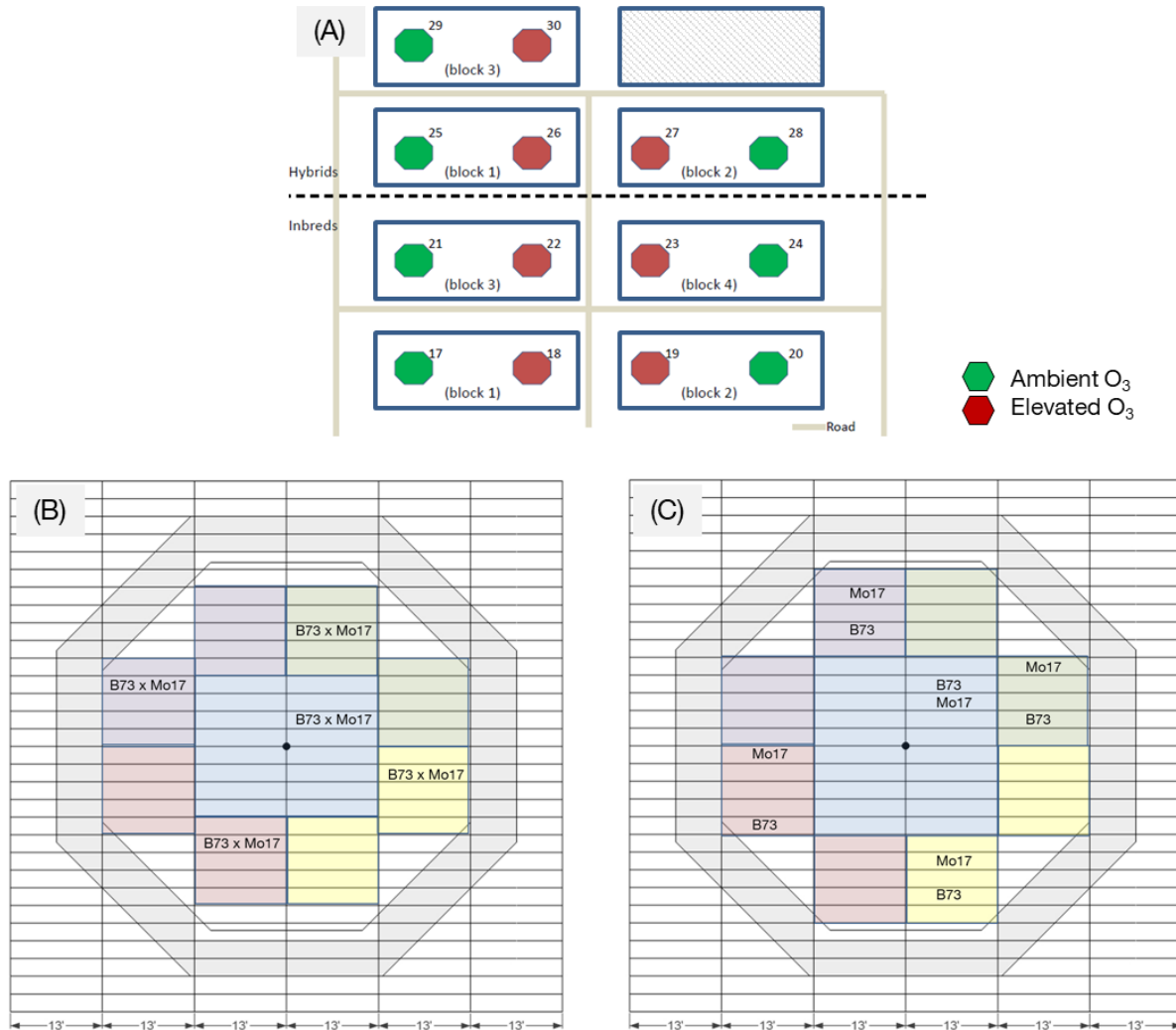
Supplemental Figure B.5: Linear correlations, from Time Point B, with Flowering Locus T and metabolite content. 'Atlg65480' FLT (normalized expression level) correlation with metabolite [Log₁₀ (relative concentration/ 100mg DW)] (A) Valine, (B) Sinapic acid, (C) O-acetylsalicylic acid, (D) β-Sitosterol, (E) Xylose. Ambient (black, circle), elevated [CO₂] (blue, triangle), elevated temperature (red, diamond), combination (yellow, square). Linear regression equation and correlation coefficient are denoted on each panel.



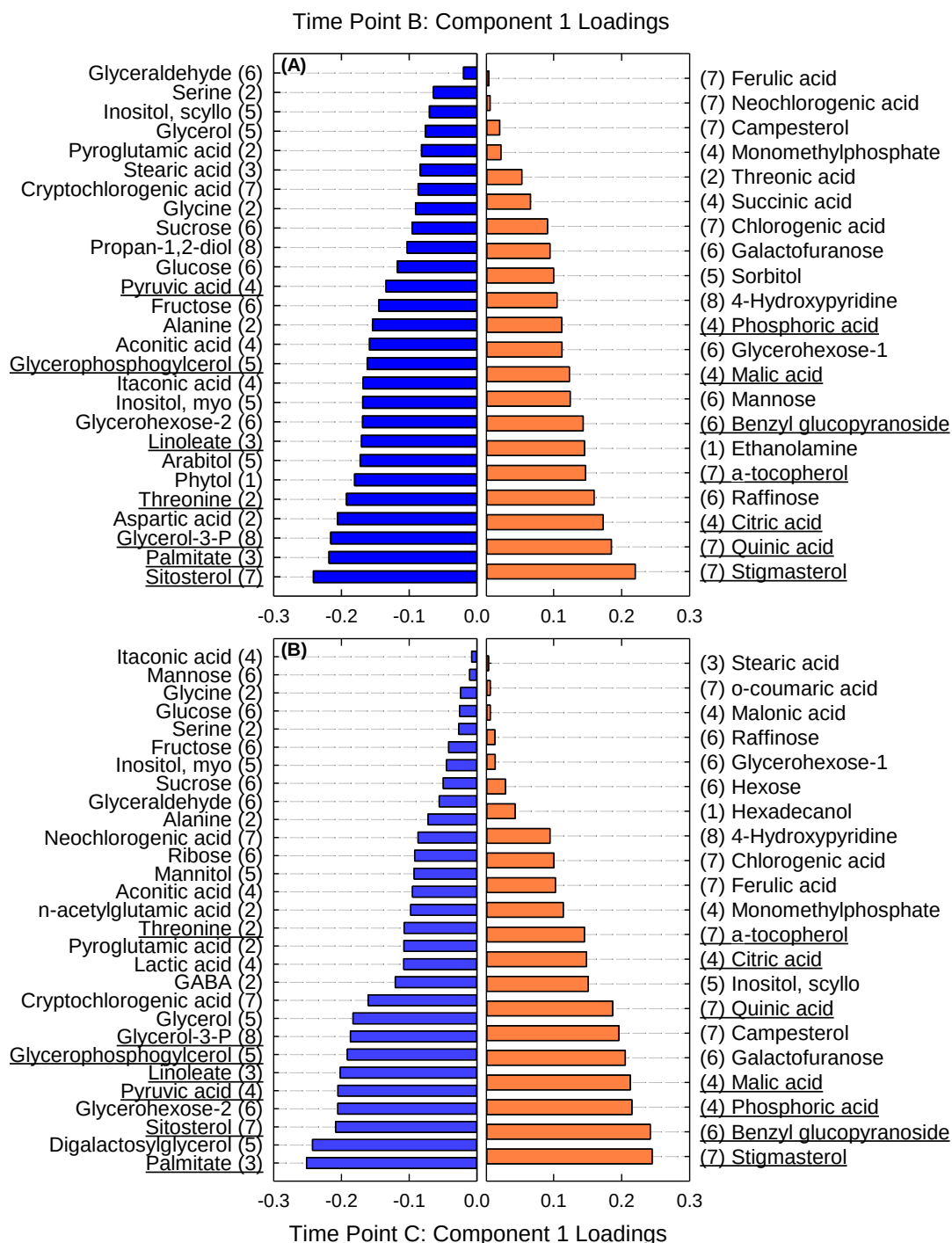
Supplemental Figure B.6: Relationship between individual leaf biomass and leaf area. The linear correlation of Guinea grass individual leaf area and leaf biomass measured on Aug 22-29, 2014. *Ambient [CO₂] and temperature (black, circle), Elevated [CO₂], ambient temperature (blue, triangle), Ambient [CO₂], Elevated temperature (red, diamond), Elevated [CO₂] and temperature (yellow, square).*

APPENDIX C: SUPPLEMENTAL FIGURES FOR CHAPTER III

I. Figures



Supplemental Figure C.1: (A) The experimental field layout with four replicate ambient (green) and elevated (red) O₃ rings, one replicate was dropped from the hybrid experiment (grey box). The spatial layout of a (B) hybrid and (C) inbred ring with different color shading indicating the five sub-blocks within the ring. Nine genotypes were assigned to a 'set' and were randomly assigned to different sub-blocks in each of the four replicate ambient and elevated O₃ rings.



Supplemental Figure C.2. PLS-DA loading plot of each metabolite feature selected for the first component for (A) time point B (DOY: 219) and (B) time point C (DOY: 229). Bars indicate the expression value for each metabolite in blue (ambient ozone) and orange (elevated ozone). Underlined metabolites are commonly expressed within an ozone environment across the two sampling dates. The numbers indicate a general metabolite classification: (1) alcohol, (2) amino acid, (3) fatty acid, (4) organic acid (5) polyol, (6) sugar, (7) specialized metabolite (8) other.