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Elevated [CO<sub>2</sub>] compromises maize defences

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**Authors**

VAUGHAN, MARTHA M  
HUFFAKER, ALISA  
SCHMELZ, ERIC A  
et al.

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## Original Article

Effects of elevated [CO<sub>2</sub>] on maize defence against mycotoxigenic *Fusarium verticillioides*

Martha M. Vaughan<sup>1</sup>, Alisa Huffaker<sup>1</sup>, Eric A. Schmelz<sup>1</sup>, Nicole J. Dafoe<sup>1</sup>, Shawn Christensen<sup>1</sup>, James Sims<sup>1</sup>, Vitor F. Martins<sup>2</sup>, Jay Swerbilow<sup>2</sup>, Maritza Romero<sup>1</sup>, Hans T. Alborn<sup>1</sup>, Leon Hartwell Allen<sup>1</sup> & Peter E. A. Teal<sup>1</sup>

<sup>1</sup>Chemistry Research Unit, Center of Medical, Agricultural, and Veterinary Entomology, U.S. Department of Agriculture, Agricultural Research Service, Gainesville, FL 32608, and <sup>2</sup>Department of Biochemistry, University of Florida, Gainesville, FL 32610, USA

## ABSTRACT

Maize is by quantity the most important C<sub>4</sub> cereal crop; however, future climate changes are expected to increase maize susceptibility to mycotoxigenic fungal pathogens and reduce productivity. While rising atmospheric [CO<sub>2</sub>] is a driving force behind the warmer temperatures and drought, which aggravate fungal disease and mycotoxin accumulation, our understanding of how elevated [CO<sub>2</sub>] will effect maize defences against such pathogens is limited. Here we report that elevated [CO<sub>2</sub>] increases maize susceptibility to *Fusarium verticillioides* proliferation, while mycotoxin levels are unaltered. Fumonisin production is not proportional to the increase in *F. verticillioides* biomass, and the amount of fumonisin produced per unit pathogen is reduced at elevated [CO<sub>2</sub>]. Following *F. verticillioides* stalk inoculation, the accumulation of sugars, free fatty acids, lipoxygenase (LOX) transcripts, phytohormones and downstream phytoalexins is dampened in maize grown at elevated [CO<sub>2</sub>]. The attenuation of maize 13-LOXs and jasmonic acid production correlates with reduced terpenoid phytoalexins and increased susceptibility. Furthermore, the attenuated induction of 9-LOXs, which have been suggested to stimulate mycotoxin biosynthesis, is consistent with reduced fumonisin per unit fungal biomass at elevated [CO<sub>2</sub>]. Our findings suggest that elevated [CO<sub>2</sub>] will compromise maize LOX-dependent signalling, which will influence the interactions between maize and mycotoxigenic fungi.

**Key-words:** C<sub>4</sub> crop; climate change; fumonisin; jasmonic acid; lipoxygenase; mycotoxin; phytoalexins.

## INTRODUCTION

Maize (*Zea mays*) is among the most important food, feed and biofuel crops (Young & Long 2000). In order to meet

rising grain demands, the United States has gradually increased the area of land devoted to maize growth (U.S. Department of Agriculture, National Agricultural Statistics Service). However, severe weather conditions, fungal disease and mycotoxin contamination are major limiting factors of maize productivity. In 2012, these factors reduced the yield in bushels per acre to the lowest in this decade (Bienkowski 2012; Schultz 2012; Smith & Mitchell 2012). Rising atmospheric carbon dioxide concentration ([CO<sub>2</sub>]) is largely responsible for the greenhouse effect leading to climatic conditions that reduce maize yield and increase crop susceptibility to mycotoxigenic fungi. Yet despite future food security and food safety concerns accompanying crop productivity and mycotoxin contamination in the context of global climate change, our understanding of how elevated [CO<sub>2</sub>] in and of itself will affect fungal disease progression and mycotoxin contamination in maize is limited.

*Fusarium verticillioides* is the most ubiquitous mycotoxigenic fungus that infects maize (Marasas 2001; Miller 2001; Yates *et al.* 2003; Murillo-Williams & Munkvold 2008). *F. verticillioides* can cause rot in all parts of the plant and produces polyketide-derived mycotoxins termed fumonisins. High levels of these carcinogenic compounds render the grain unfit for consumption. Environmental conditions associated with climate change, such as high temperatures and drought, increase *F. verticillioides* systemic disease development and fumonisin contamination (Shelby *et al.* 1994; Miller 2001; Battilani *et al.* 2008).

In contrast to C<sub>3</sub> plants, rising atmospheric [CO<sub>2</sub>] will not directly stimulate photosynthesis and enhance the growth and yield of C<sub>4</sub> crops, such as maize. Because of their unique foliar Kranz anatomy and cellular physiology, C<sub>4</sub> plants are capable of concentrating [CO<sub>2</sub>] at the site of the major enzyme involved carbon fixation, ribulose biphosphate carboxylase-oxygenase, to levels practically near saturation (Allen *et al.* 2011). Thus, only in combination with drought can the physiological effect of reduced stomatal conductance (which occurs in both C<sub>3</sub> and C<sub>4</sub> plants at elevated [CO<sub>2</sub>]) benefit maize productivity by increasing water-use efficiency (Ghannoum *et al.* 2000; Long *et al.* 2004; Leakey 2009).

Correspondence: M. M. Vaughan, BFP&M, NCAUR, USDA, 1815 N. University Street, Peoria, IL 61604, USA. E-mail: Martha.vaughan@ars.usda.gov

In the absence of major changes in maize photosynthesis and primary metabolism, the potential effects of elevated  $[\text{CO}_2]$  on maize phytohormone signalling networks and secondary metabolites are of particular importance. Elevated  $[\text{CO}_2]$  has been correlated with increases in *Fusarium pseudograminearum* crown rot in wheat, a  $\text{C}_3$  member of the Poaceae grass family (Melloy *et al.* 2010). However, predictions and inferences from this and other studies are difficult as research investigating the impact of elevated  $[\text{CO}_2]$  on plant–pathogen interactions has been variable and very much dependent on the pathosystem; examples of increased, decreased and unaltered susceptibility have been reported (Chakraborty & Datta 2003; Garrett *et al.* 2006; Eastburn *et al.* 2010). More generalities can be made in the context of plant–insect herbivore interactions at elevated  $[\text{CO}_2]$ , but most of these again apply to  $\text{C}_3$  plants, which also exhibit changes in tissue primary metabolism and nutritional quality (Bryant *et al.* 1983; Lincoln *et al.* 1986; Herms & Mattson 1992; Schadler *et al.* 2007). Nevertheless, in pursuit of understanding the inconsistencies in plant secondary metabolism at elevated  $[\text{CO}_2]$ , studies have demonstrated that  $\text{CO}_2$  enrichment can also have a direct effect on plant defence hormones. Based on these studies, elevated  $[\text{CO}_2]$  suppresses the production of jasmonic acid (JA) and lipoxygenase (LOX)-dependent defences while increasing the production of salicylic acid (SA) and corresponding downstream defences (Zavala *et al.* 2009, 2013; Casteel *et al.* 2012a; DeLucia *et al.* 2012). The mechanism by which elevated  $[\text{CO}_2]$  influences the phytohormone defence response is not well characterized. However, the antagonistic effects of SA appear to play an important role in the down-regulation of JA, and it has been hypothesized that changes in the plant redox state at elevated  $[\text{CO}_2]$  activate the regulatory protein non-expressor of pathogenesis-related genes 1 (NPR1), which mediates this antagonism between SA and JA (Spoel *et al.* 2003; Spoel & Loake 2011).

The potential of rising  $[\text{CO}_2]$  to influence plant LOXs is of particular interest in the context of mycotoxigenic fungal pathogens because there is growing evidence that oxylipin signalling is involved in both pathogenesis and stimulation of mycotoxin production (Gao *et al.* 2007; Tsitsigiannis & Keller 2007). Oxylipins are a diverse class of oxygenated fatty acids, derived from the LOX pathway (Feussner & Wasternack 2002; Howe & Schillmiller 2002; Howe 2004; Gao *et al.* 2007; Gao & Kolomiets 2009). Free fatty acids, typically linolenic and linoleic acids, are oxidized by LOXs at either the 9 or 13 carbon position of the chain. Depending on this regio-specificity they are referred to as 9-LOXs or 13-LOXs. The phytohormone JA is derived from linolenic acid via the activity of 13-LOXs (Howe & Schillmiller 2002). Predominant components of maize biochemical defences are controlled by the JA signalling pathway (Oikawa *et al.* 2004; Yan *et al.* 2012). For example, maize zealexin and kauralexin phytoalexins, which are rapidly induced in response to pathogen infection and show biological activity against a variety of pathogens, are partially regulated by JA (Huffaker *et al.* 2011; Schmelz *et al.* 2011). The defence-related functions of 9-LOX metabolites are not as well characterized, but there is evi-

dence that plant 9-LOX derivatives play an essential role in host susceptibility to mycotoxin contamination (Burow *et al.* 1997; Gao *et al.* 2007; Christensen & Kolomiets 2011). Expression of the maize gene *LOX3* is up-regulated by *F. verticillioides* in lines that accumulate higher levels of mycotoxins (Calvo *et al.* 1999; Gao *et al.* 2007). Furthermore, disruption of maize *LOX3*, which resulted in reduced 9-LOX-derived fatty acid hydroperoxides, dramatically reduced fumonisin production on mutant maize kernels.

In this study, we investigated the impact of elevated  $[\text{CO}_2]$  on the interaction between maize and *F. verticillioides*. The challenges associated with controlled environmental growth that are necessary to conduct regulated pathogen research with mature maize at different  $[\text{CO}_2]$ s were overcome by establishing a correlation between the susceptibility and mycotoxin contamination in maize kernels and stalks. Utilizing the stalk infection assay as a model, we tested our hypothesis that  $\text{C}_4$  maize grown at elevated  $[\text{CO}_2]$  would be compromised in LOX-dependent hormone signalling, which would influence both maize phytoalexin accumulation and *F. verticillioides* fumonisin contamination. In addition, we discuss differences in the impact of elevated  $[\text{CO}_2]$  on  $\text{C}_3$  and  $\text{C}_4$  plant hormone signalling, and consider the implications of our findings on the future of maize crop productivity in the context of changing climate.

## MATERIALS AND METHODS

### Plant material and growth conditions

All experiments were conducted using hybrid *Z. mays* var. Golden Queen, which is a sweet corn commonly grown for fresh market throughout Florida (Southern States Cooperative, Inc., Richmond, VA, USA). Four seeds were germinated in each plastic pot (10.5 × 10.5 × 12 cm high for stalk experiments or 25.5 cm diameter × 23 cm depth for cob/kernel experiments) filled with MetroMix 200 (Sun Gro Horticulture Distribution, Inc., Bellevue, WA, USA) supplemented with 14-14-14 Osmocote (Scotts Miracle-Gro, Marysville, OH, USA). Plants were watered daily and received bi-weekly nutrient supplement with soluble Peters 20-20-20 (The Scotts Company, Marysville, OH, USA). Mature maize plants utilized for *F. verticillioides* ear rot experiments were grown in two rooms of a previously described eight-room sunlit greenhouse on the University of Florida campus at Gainesville (Zhang *et al.* 2014). Temperatures were controlled at 28 °C from 0700 to 1900 h Eastern Standard Time (EST) followed by 1 h of smooth transition to 25 °C overnight. Air relative humidity was controlled between 55 and 60%. Maize for stalk rot experiments were grown in two identical environmental Conviron E15 (Pembina, ND, USA) growth chambers controlled at 28 °C day/25 °C night, 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photo flux density 12 h photoperiod and between 50 and 60% relative humidity (Supporting Information Fig. S1). One chamber/greenhouse room was controlled at current ambient 400  $\mu\text{mol CO}_2 \text{ mol}^{-1}$  air ( $1 \times [\text{CO}_2]$ ) and the other at 800  $\mu\text{mol CO}_2 \text{ mol}^{-1}$  air ( $2 \times [\text{CO}_2]$ ), which is the  $[\text{CO}_2]$  the global atmosphere is projected to reach between 2080 and 2100 (Solomon *et al.* 2007).

## Maize inoculation with *F. verticillioides*

*F. verticillioides* (Northern Regional Research Laboratory stock no. 7415) was grown on V<sub>8</sub> agar for 1 week prior to use. Inoculum was prepared by collecting spores with a moist sterile cotton swab which was then stirred into sterile 0.1% Tween 20 (Fisher BioReagents, Fairlawn, NJ, USA). Spores were quantified using a TC10 Automated Cell Counter (Bio-Rad, Hercules, CA, USA).

Maize cobs were inoculated 2 weeks after pollination by pricking 10 kernels (0.5 cm in depth) on each cob with a 25 gauge needle and syringe filled with  $1 \times 10^6$  spores mL<sup>-1</sup> *F. verticillioides* inoculum (or control 0.1% Tween 20 solution). Following inoculation the sheath was pulled back over the cob and covered with a moist paper towel to maintain high humidity. Treated kernels from two cobs were combined for each of four biological replicates. Consistent results were obtained from two independent experiments conducted between March and May and May and July of 2013. [CO<sub>2</sub>]s were switched between rooms for the repeated experiment.

Maize stalks were inoculated 30 d post sowing at the eight-leaf stage of growth by injecting 0.1 mL of  $1 \times 10^6$  mL<sup>-1</sup> *F. verticillioides* spore solution along a 5 cm vertical incision through the apical meristem. Mock-inoculated (control) stems were identically damaged and treated with sterile 0.1 mL of 0.1% Tween 20 solution. Samples were collected by shaving a 2–3 mm width of tissue from both sides of the inoculation site with a scalpel. Metabolite quantities and the amount of pathogen DNA relative to maize DNA was determined from an average of four biological replicates of which each sample was a pool of eight treated stems. Comparable results were obtained from four independent experiments. Chamber [CO<sub>2</sub>]s were switched between repeated experiments.

To validate that pathogen DNA relative to maize DNA was correlated to spread of disease, the lesion area was analysed by inoculating maize stalks as previously described by Gao *et al.* (2007), with some modifications. Stalks of 4-week-old plants grown at  $1 \times$  [CO<sub>2</sub>] or  $2 \times$  [CO<sub>2</sub>] were wounded 1 cm in depth with a sterile 200 μL pipette tip (Rainin, Columbus, OH, USA) at a 45 angle. The tip was filled with 100 μL of  $1 \times 10^6$  mL<sup>-1</sup> *F. verticillioides* conidial suspension and left in place. The conidial suspension was absorbed within 24 h and the tip fell out on its own as the plant continued to grow. Disease symptoms and severity were assessed 7 d post-inoculation. Stalks were split longitudinally and lesion area was calculated by ImageJ software (ImageJ 1.46r; Wayne Rasband, NIH, Bethesda, MD, USA). Results represent the combined average of two independent replicate experiments of six plants at each [CO<sub>2</sub>].

## Quantification of *F. verticillioides* biomass

DNA was extracted from infected maize tissues using a modified CTAB method (Nicolaisen *et al.* 2009). Briefly, 100–200 mg of homogenized tissue was mixed with 300 μL sterile water and 700 μL CTAB buffer (20 g L<sup>-1</sup> CTAB, 1.4 M NaCl, 20 mM Na<sub>2</sub>EDTA, 0.1 M Tris-HCl) and incubated at 65 °C for 30 min. After centrifugation, the supernatant was trans-

ferred to a new tube containing 500 μL of chloroform (Sigma Aldrich, St. Louis, MO, USA), mixed, and separated again by centrifugation. The aqueous phase was then mixed with two volumes CTAB precipitation solution (5 g L<sup>-1</sup> CTAB, 0.04 M NaCl, pH 8) and incubated at 4 °C overnight. DNA was then pelleted and dissolved in 350 μL 1.2 M NaCl and 350 μL chloroform. The aqueous phase was again separated and precipitated in isopropanol followed by 70% ethanol. Fungal DNA was extracted from 1-week-old mycelium of *F. verticillioides* grown on V<sub>8</sub> agar using the same technique.

The amount of pathogen DNA relative to plant DNA was estimated with qRT-PCR. To determine the quantity of *F. verticillioides* DNA the previously described (Visentin *et al.* 2012) gene-specific primers for beta-tubulin (KC964147) were utilized: Fv\_TUB2\_F (5'-TGCTCAT TTC CAAGATCCGCG-3') and Fv\_TUB2\_R (5'-GTAGTTGAG GTCACCGTAGGAGG-3'). Plant DNA quantification was performed using *Eflα* gene-specific (NM\_001112117) primers: Zm\_Eflα\_F (5'-GCTTCACGTCCAGGTC-3') and Zm\_Eflα\_R (5'-ATAGGCTTGTTGGGTATCA-3').

A fivefold dilution series of pure *F. verticillioides* DNA and pure maize DNA was used to generate standard curves by plotting the Ct values obtained by qRT-PCR against the log( [DNA] ) (Nicolaisen *et al.* 2009). In order to ensure that the priming efficiency was not altered by mixtures of plant and pathogen DNA, standard curves for both primer pairs were also generated with mixtures containing DNA of both species; however, no significant difference was detected between the curves with pure or mixtures of DNA. The R<sup>2</sup> values were 0.993 and 0.990 for the *F. verticillioides* and maize DNA curves, respectively.

## Quantification of fumonisin

The Veratox fumonisin kit (Neogen, Lansing, MI, USA) was used to quantify fumonisin contamination caused by *F. verticillioides* in maize tissues grown at  $1 \times$  [CO<sub>2</sub>] or  $2 \times$  [CO<sub>2</sub>]. The kit uses a direct enzyme-linked immunosorbent assay to determine the total fumonisins (B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>) between the quantization range of 1 and 6 μg g<sup>-1</sup> (Bird *et al.* 2002). As described earlier, the maize cobs and stalks were inoculated with *F. verticillioides*, the pathogen was allowed to establish for 7 d, and tissues were collected for analysis. Fumonisin was extracted from 1 g of ground infected tissue with 70% methanol, diluted 10- or 2.5-fold for infected kernel or stalk tissues, respectively, and then quantified following the kit manufacturers protocol. Results were calculated using Neogen's Veratox 3.0 for Windows software. The amount of fumonisin relative to *F. verticillioides* DNA was estimated by dividing the μg g<sup>-1</sup> fumonisin by the pg of pathogen DNA as quantified by qRT-PCR.

## Gas exchange

Intracellular CO<sub>2</sub> content (Ci), photosynthetic rate (Pn) and stomatal conductance (g<sub>s</sub>) measurements were taken with an open-flow portable leaf photosynthesis system (Li-Cor 6400XT, Li-Cor Inc., Lincoln, NE, USA) equipped

with the standard leaf chamber (6 cm<sup>2</sup> of leaf area) and [CO<sub>2</sub>] injection system (model 6400-01, Li-Cor Inc.) adjusted to a constant [CO<sub>2</sub>] concentration of 400 μmol CO<sub>2</sub> mol air<sup>-1</sup> or 800 μmol CO<sub>2</sub> mol air<sup>-1</sup>. The environmental conditions in the Li-Cor 6400 chamber were adjusted to match those of the growth chambers. Measurements were performed 48 h after control or pathogen treatment between 1100–1300 h on the fifth leaf from the bottom for six individual plants at both [CO<sub>2</sub>]s.

### Primary metabolite analysis

Soluble carbohydrate and starch concentrations were estimated using the previously described anthrone reagent colorimetric method (Hansen & Møller 1975). In short, 200 mg of ground tissue was initially clarified to remove interfering pigments by incubating in 100% acetone. Soluble sugars were then extracted twice in 80% ethanol at 65 °C. The ethanol containing the soluble carbohydrates was then separated from the remaining residue, which contained the insoluble starch. The ethanol was lyophilized and the soluble carbohydrates were analysed following the same set of procedures as for starch analysis. Sugar residues were resuspended in 1.1% HCl and incubated in a 95 °C water bath for 30 min. Samples were then diluted to 10 mL with sterile water. Acidic sugar solution (1 mL) was mixed with 5 mL anthrone reagent (2 g anthrone L<sup>-1</sup> of 72% sulphuric acid) and incubated for 11 min at 95 °C. The absorbance at 630 nm was then measured against water. Standard calibration curves were generated for glucose and starch ( $R^2 = 0.995$  and  $R^2 = 0.993$ , respectively). Total soluble carbohydrates were calculated based on the calibration curve of glucose.

Total soluble protein was extracted from 200 mg stem tissue in 1 mL protein extraction buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 15 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM KCl, and 2 mM EDTA). Samples were centrifuged for 15 min at 10 000 g at 4 °C and the quantity of protein in the supernatant was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer's microplate procedure.

### Quantification of maize fatty acids, phytohormones and terpene phytoalexins

The quantification of free fatty acids, phytohormones, kauralexins and zealexins, were performed using a previously developed method (Schmelz *et al.* 2004, 2011; Huffaker *et al.* 2011). Metabolites were extracted with 1-propanol and methylene chloride, and collected by vapour-phase extraction following carboxylic acid methylation. Analysis was performed using GC/CI-MS and quantification was based on internal standards. Quantity estimates of total fatty acids, zealexins and kauralexins were based on <sup>13</sup>C<sub>18</sub>-linolenic acid. The concentration of zealexins (1–14 as numbered in Huffaker *et al.* 2011) and kauralexins (A1–A3 and B1–B3) reported are cumulative total amounts of related metabolites described (Huffaker *et al.* 2011; Schmelz *et al.* 2011). Quantity estimates of total JA (trans + cis) and SA were based on corresponding deuterated internal standards.

### Zealexin and kauralexin activity assays

Compound isolation and antifungal assays were performed as previously described (Huffaker *et al.* 2011; Schmelz *et al.* 2011) with minor modifications for the zealexin mixture. The mixture of oxygenated zealexins was extracted from infected maize stem tissue with ethyl acetate and solid debris were removed by filtration. The organic layer was concentrated under vacuum and then separated by preparative flash chromatography (CombiFlashRf, Teledyne ISCO, Inc.; Lincoln, NE, USA) on a 130 g C18 (RediSepRF Gold, Teledyne ISCO, Inc.) column in a gradient from hexane to ethyl acetate. A fraction consisting almost exclusively of oxygenated zealexins was obtained from this separation. The mixture was composed of approximately, 50% zealexin #14, 40% zealexin #6, 10% zealexin #13 and 5% zealexin #12, following the same compound number system described by Huffaker *et al.* (2011). Without any further purification this mixture of oxygenated zealexins was dried under vacuum, dissolved in dimethyl sulfoxide (DMSO), and used for the activity assay. Kauralexin B3 was isolated as previously described (Schmelz *et al.* 2011), dissolved in DMSO as the carrier solvent, and similarly used for analysis. Using 96-well microtiter plates, fungal inoculum ( $2 \times 10^4$  spores mL<sup>-1</sup>) was incubated with 0.5 μL of either phytoalexin treatment in DMSO or a DMSO control. Fungal growth in broth media was monitored through periodic measurements of changes in 405 nm optical density using a Synergy4 (BioTek Instruments, Inc.; Winooski, VT, USA) reader.

### Phytohormone and gene expression time course

The time course experiment was performed by inoculating maize stems as described earlier. Tissue samples were collected immediately after inoculation (time 0 min), 15 min, 30, 60 and 120 min post-inoculation. All samples were frozen in liquid N<sub>2</sub> and stored at –80 °C till further analysis. Four biological replicates each derived from two pooled individual plants were collected for each time point except 30 min, for which eight biological replicates were collected also as pool of two plants in each. Phytohormone analysis was conducted as described earlier with all biological samples collected. For gene expression analysis, however, in order to reduce the number of 30 min biological replicates, pairs of two RNA sample extractions were combined so that only four (instead of eight) replicates consisting of a pool of four independent plants were analysed. Samples from other time points were utilized as collected.

### Quantitative real-time PCR

Total RNA was isolated from 200 mg ground tissue with NucleoSpin RNA plant kit (Machery-Nagel, Bethlehem, PA, USA) as per manufacturer's protocol. cDNA was synthesized using SuperScript® II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random decamers. qRT-PCR was performed in a total 20 μL volume with 10 μL of 2× SsoAdvanced SYBR Green supermix (Bio-Rad, Hercules, CA, USA), 300 nM of each primer and 1 μL of template. Sample reactions were all performed in triplicate.

Samples were diluted 10-fold before being used as template in PCR. PCR was performed on a Bio-Rad CFX96 Real Time System using the thermocycling programme, which consisted of an initial denaturation at 98 °C for 2 min, 40 cycles each of 98 °C denaturation for 15 s and 60 °C annealing/elongation for 1 min and a final dissociation curve from 65 to 95 °C.

Threshold cycle (Ct) values of *LOX* genes, *AOS*, *AOC* and *NPR1* were normalized to the geometric mean of two endogenous controls, glyceraldehyde-3-phosphate dehydrogenase (*X07156*) and ribosomal protein L17 (*RPL17*; AF034948) (Vandesompele *et al.* 2002). The amount of each gene transcript was calculated relative to its corresponding average for mock-treated controls at time zero. Transcript fold-changes were calculated by the  $2^{-\Delta\Delta Ct}$  method (Schmittgen & Livak 2008) using CFX Manager software, version 3.0 (Bio-Rad). Calculations were adjusted according to primer efficiencies. Gene-specific oligonucleotides and their efficiencies are listed in Supporting Information Table S1.

### Statistical analysis

Student's *t*-tests were performed to compare variable means at  $1 \times [\text{CO}_2]$  with those at  $2 \times [\text{CO}_2]$ . Variables for which comparisons were made included: relative *F. verticillioides* biomass, lesion area and quantity of fumonisin. For the time course experiment, differences between phytohormone concentrations or transcript levels at  $1 \times [\text{CO}_2]$  in comparison with  $2 \times [\text{CO}_2]$  were determined independently for individual time points with Student's *t*-tests.

For experiments with multiple explanatory factors ( $1 \times [\text{CO}_2]$  or  $2 \times [\text{CO}_2]$  and mock-treated control or *F. verticillioides* inoculation), a more complex statistical analysis was required. To initially determine which factors contributed to differences among means, a  $2 \times 2$  ( $[\text{CO}_2] \times F. verticillioides$ ) full factorial analysis of variance (ANOVA) was performed. To improve the power of the test, in the case of no interaction between potential contributing factors, a pair wise Student's *t*-test was performed on the means of the main effect. (For example, if there is no interaction and *F. verticillioides* inoculation was the main effect of differences, the values from control treatment at  $1 \times [\text{CO}_2]$  and  $2 \times [\text{CO}_2]$  were combined and then compared with the average of the values from *F. verticillioides* treatment at  $1 \times [\text{CO}_2]$  and  $2 \times [\text{CO}_2]$ . Thus, only two means were compared and each mean consisted of an average of eight samples instead of four.) If, however, there was a significant interaction between the factors, a Tukey–Kramer honestly significant difference test was performed to determine differences between individual means.

## RESULTS

### Elevated [CO<sub>2</sub>] increased maize susceptibility to *F. verticillioides*, but did not alter fumonisin contamination

The effect of [CO<sub>2</sub>] on maize susceptibility to *F. verticillioides* caused ear rot and stalk rot was evaluated by comparing the

severity of *F. verticillioides* infection on maize grown at  $1 \times [\text{CO}_2]$  ( $400 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$ ) and  $2 \times [\text{CO}_2]$  ( $800 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$ ) (Fig. 1a,b). Assuming that the relative quantity of *F. verticillioides* DNA to maize DNA is associated with the amount of *F. verticillioides* pathogen within the tissue (Nicolaisen *et al.* 2009); *F. verticillioides* biomass was approximately 2.5-fold higher in maize kernels and stalks at  $2 \times [\text{CO}_2]$  as determined by qRT-PCR (Fig. 1c,d;  $P < 0.02$ ). However, fumonisin did not correspondingly increase with fungal biomass, and there was no significant difference in fumonisin contaminants at  $2 \times [\text{CO}_2]$  in either the kernel or the stalk tissues (Fig. 1e,f). Hence, the amount of fumonisin per pg of *F. verticillioides* DNA was almost twofold lower in kernels and stalks grown at  $2 \times [\text{CO}_2]$  as compared with  $1 \times [\text{CO}_2]$  (Fig. 1g,h;  $P < 0.01$ ). Although magnitudes varied, the general trend of maize susceptibility to *F. verticillioides* pathogen proliferation and fumonisin contamination in stalk tissues was analogous to that of kernels on the cob. Given the practical advantages of working with younger vegetative tissues, the stalk infection assay was used to investigate mechanisms behind increased fungal susceptibility.

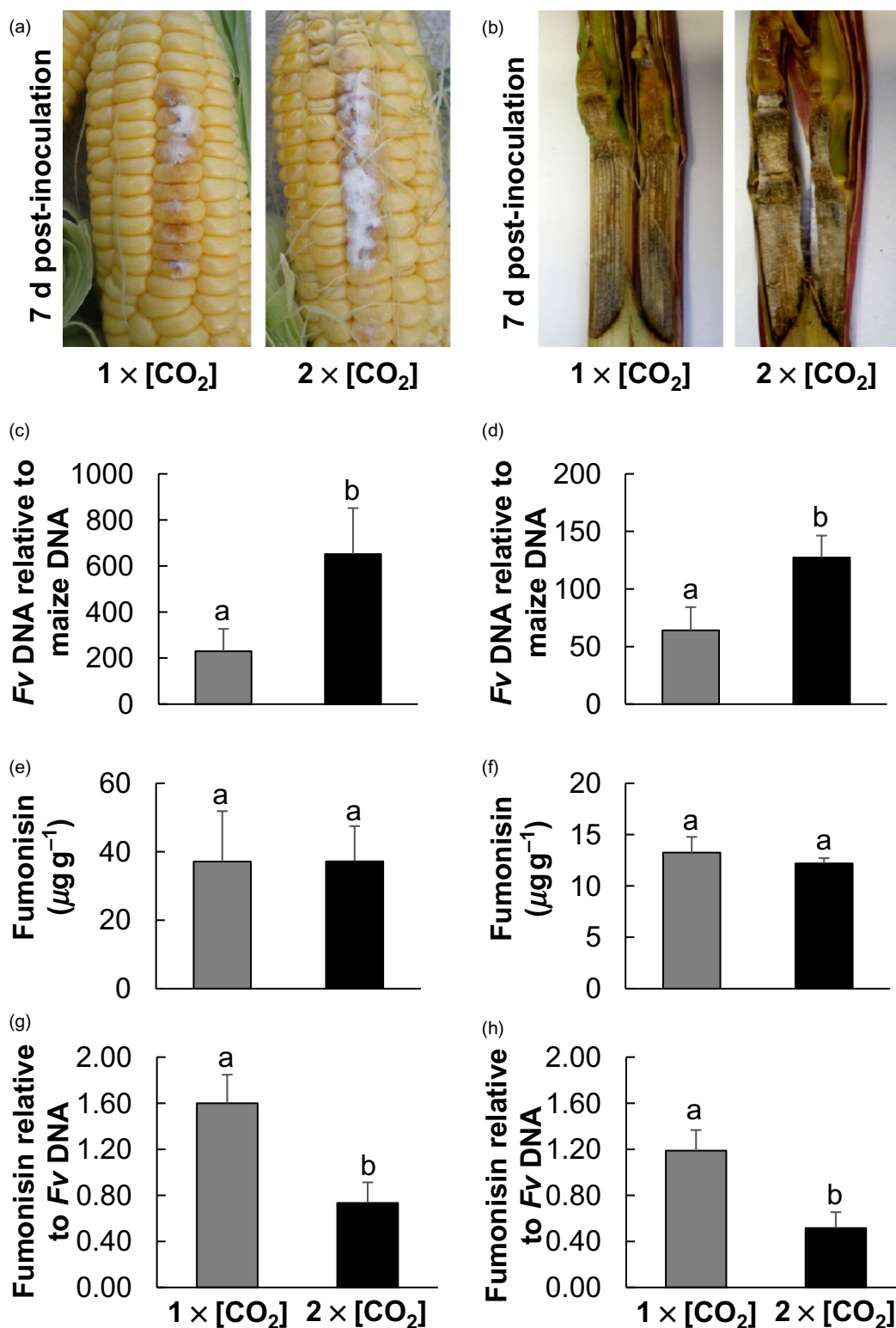
To initially verify that increased pathogen DNA per unit plant DNA translated into increased disease spread, maize stems grown at different [CO<sub>2</sub>]s were point inoculated (as described in the Materials and Methods section) and the lesion area was measured 7 d following. Lesion area in maize grown at  $2 \times [\text{CO}_2]$  was approximately threefold greater than at  $1 \times [\text{CO}_2]$  (Fig. 2;  $P < 0.01$ ), further confirming that maize at  $2 \times [\text{CO}_2]$  was more susceptible to *F. verticillioides* stalk rot.

The occurrence of temporal events is central to ecological performance. Plant resistance or susceptibility to pathogens is largely dependent upon the speed of defence activation (Kim *et al.* 2011). Thus early time points at which initial responses could be detected were considered for metabolite analysis. Significant differences in relative *F. verticillioides* biomass at  $1 \times [\text{CO}_2]$  and  $2 \times [\text{CO}_2]$  could be detected as early as 2 d post-inoculation (Supporting Information Fig. S2;  $P < 0.01$ ).

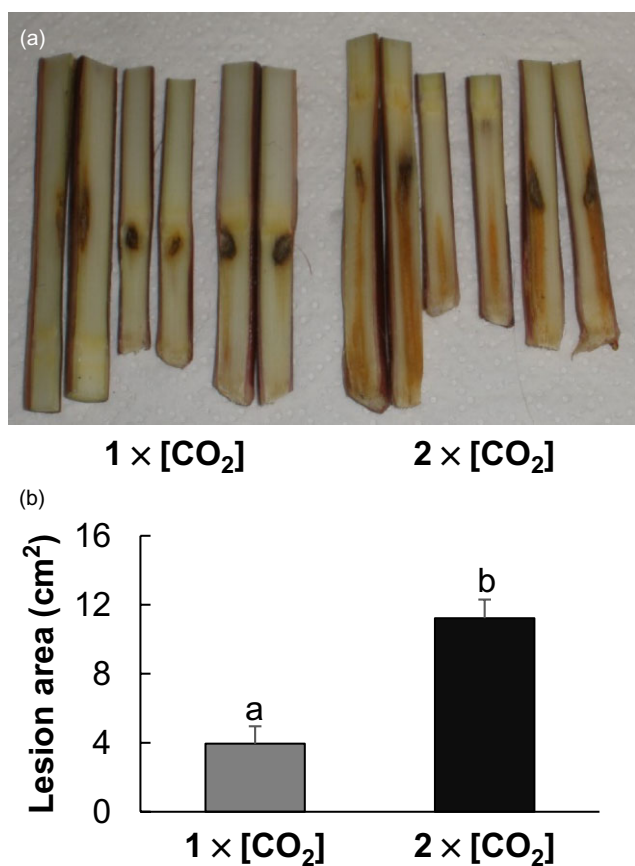
### Primary metabolites were altered in *F. verticillioides*-infected tissues under elevated [CO<sub>2</sub>]

To confirm the occurrence of established patterns of C<sub>4</sub> plant responses to elevated [CO<sub>2</sub>], the intracellular CO<sub>2</sub> content (Ci), photosynthetic rate (Pn) and stomatal conductance (g<sub>s</sub>), were measured for both mock-treated (control) and *F. verticillioides* inoculated maize. As shown by the ANOVA model, there was no significant interaction between [CO<sub>2</sub>] and *F. verticillioides* inoculation. Therefore, since [CO<sub>2</sub>] was the main effect contributing to differences, a pair wise comparison was performed between the mean Ci or g<sub>s</sub> at  $1 \times [\text{CO}_2]$  and  $2 \times [\text{CO}_2]$  (Fig. 3). As expected for C<sub>4</sub> plants,  $2 \times [\text{CO}_2]$  increased Ci and reduced g<sub>s</sub> ( $P < 0.01$ ), but did not affect the Pn ( $P > 0.05$ ).

Consistent with a lack of a difference in Pn, [CO<sub>2</sub>] alone was not a contributing factor to changes in soluble



**Figure 1.** Growth at elevated [CO<sub>2</sub>] increased maize susceptibility to *Fusarium verticillioides* proliferation, but did not effect fumonisin contamination. Representative images of 7 d post *F. verticillioides* inoculated maize kernels (a) and stalks (b) grown at 1 × [CO<sub>2</sub>] (400 µmol CO<sub>2</sub> mol<sup>-1</sup> air) or 2 × [CO<sub>2</sub>] (800 µmol CO<sub>2</sub> mol<sup>-1</sup> air). The average *F. verticillioides* biomass in kernels (c) and stalks (d) grown at different [CO<sub>2</sub>]s was estimated as the amount of pg fungal DNA relative to ng maize DNA via qRT-PCR. The mean amount of fumonisin contamination in 1 × [CO<sub>2</sub>] and 2 × [CO<sub>2</sub>] grown maize kernels (e) and stalks (f) was determined, and the relative amount of fumonisin per pg of *F. verticillioides* DNA in kernel (g) and stalk tissues (h) was estimated. Values represent averages ± SEM. Letters above bars indicate significant differences (Student's *t*-test, *n* = 4, *P* < 0.02).



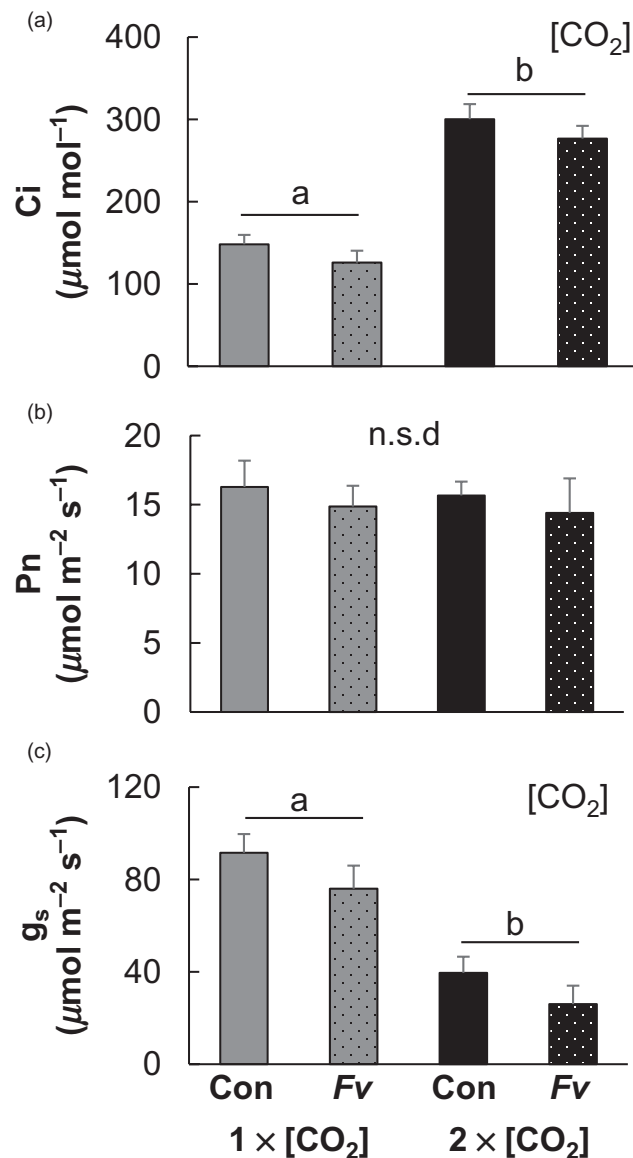
**Figure 2.** Maize grown at elevated [CO<sub>2</sub>] was more susceptible to spread of *Fusarium verticillioides* stalk rot. Picture taken 10 d post point inoculation with *F. verticillioides* in maize stems grown at 1 × [CO<sub>2</sub>] or 2 × [CO<sub>2</sub>] (a). Lesion areas were estimated with ImageJ software (b). Values represent averages ± SEM. (Student's *t*-test, *n* = 12, *P* < 0.001).

carbohydrate, starch or protein concentrations of control maize stem tissues (Fig. 4). However, in combination with *F. verticillioides* infection, the interaction of both factors resulted in significant differences in soluble carbohydrate and starch contents (*P* = 0.004 and *P* = 0.02, respectively). The soluble carbohydrate and starch content increased with *F. verticillioides* infection at 1 × [CO<sub>2</sub>], but not at 2 × [CO<sub>2</sub>]. Total protein content also increased with *F. verticillioides* infection but [CO<sub>2</sub>] was a significant contributing factor (ANOVA *P* = 0.06).

Since free fatty acids are key precursors in the biosynthesis of both 13-LOX and 9-LOX oxylipins (Calvo *et al.* 1999; Gao & Kolomiets 2009; Christensen *et al.* 2013), the concentrations of stearic, oleic, linoleic and linolenic acid were also measured (Fig. 5). The concentration of fatty acids increased with *F. verticillioides* inoculation at 1 × [CO<sub>2</sub>], but not at 2 × [CO<sub>2</sub>]. Hence, the concentration of linolenic acid was twofold less in *F. verticillioides* inoculated maize at 2 × [CO<sub>2</sub>] in comparison with corresponding tissues 1 × [CO<sub>2</sub>]. (Fig. 5c,d; *P* < 0.01).

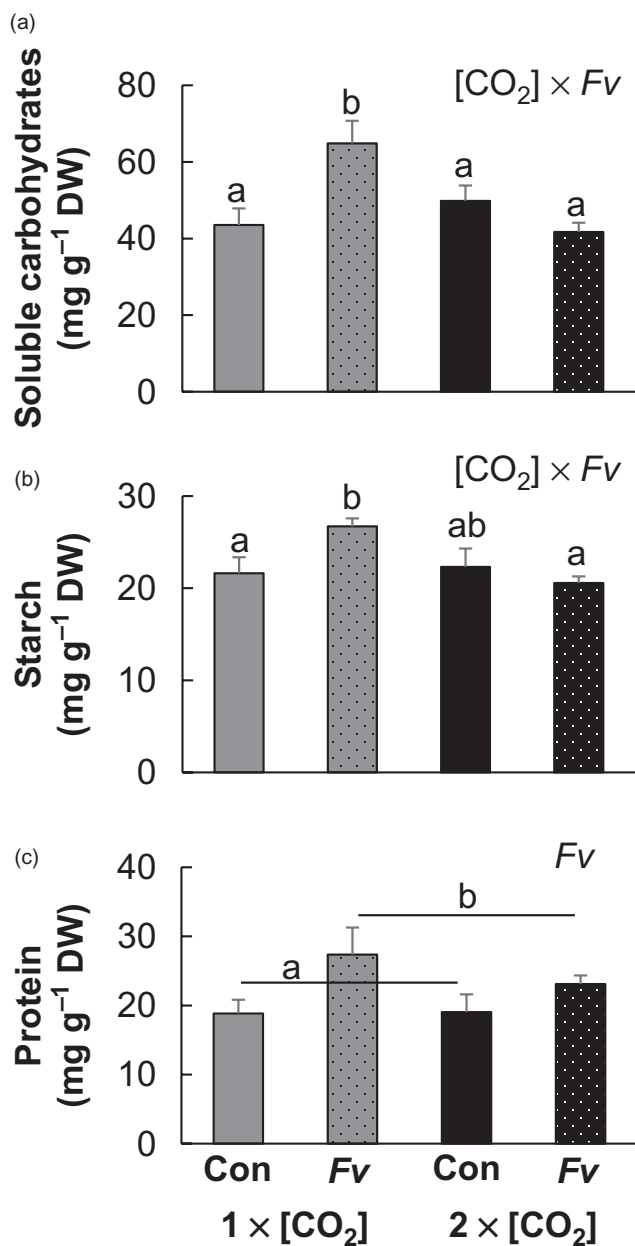
### Elevated [CO<sub>2</sub>] dampened the accumulation of JA and SA following *F. verticillioides* inoculation

To assess differences in defence hormone signalling at 1 × [CO<sub>2</sub>] and 2 × [CO<sub>2</sub>] the amount of JA and SA was

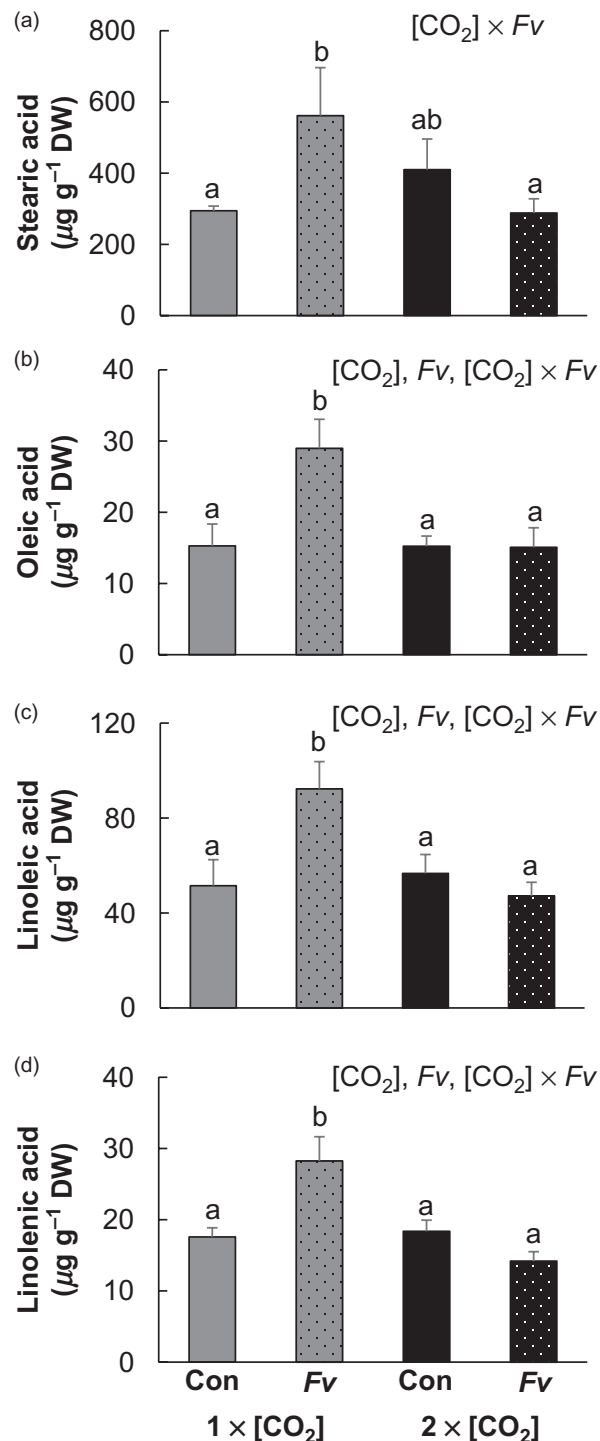


**Figure 3.** Physiological response of maize grown at 2 × [CO<sub>2</sub>] was typical of C<sub>4</sub> species. Gas exchange measurements were taken from the fifth leaf of 1-month-old 1 × [CO<sub>2</sub>] or 2 × [CO<sub>2</sub>] grown maize, which were mock-inoculated (Con) or *Fusarium verticillioides* inoculated (Fv) for 2 d. The average (a) intracellular CO<sub>2</sub> content (Ci), (b) photosynthetic rate (Pn) and (c) stomatal conductance (g<sub>s</sub>) was estimated for each set of plants. The statistically significant main effects of differences determined by a 2 × 2 ([CO<sub>2</sub>] × Fv) full factorial analysis of variance (ANOVA) are indicated in the top right hand corner of each graph. No significant difference (n.s.d.) indicates ANOVA (*P* > 0.05). Because the interaction between the contributing factors was not significant, Student's *t*-tests were performed to independently compare Ci and g<sub>s</sub> means at 1 × [CO<sub>2</sub>] or 2 × [CO<sub>2</sub>] (*n* = 12, *P* < 0.01).

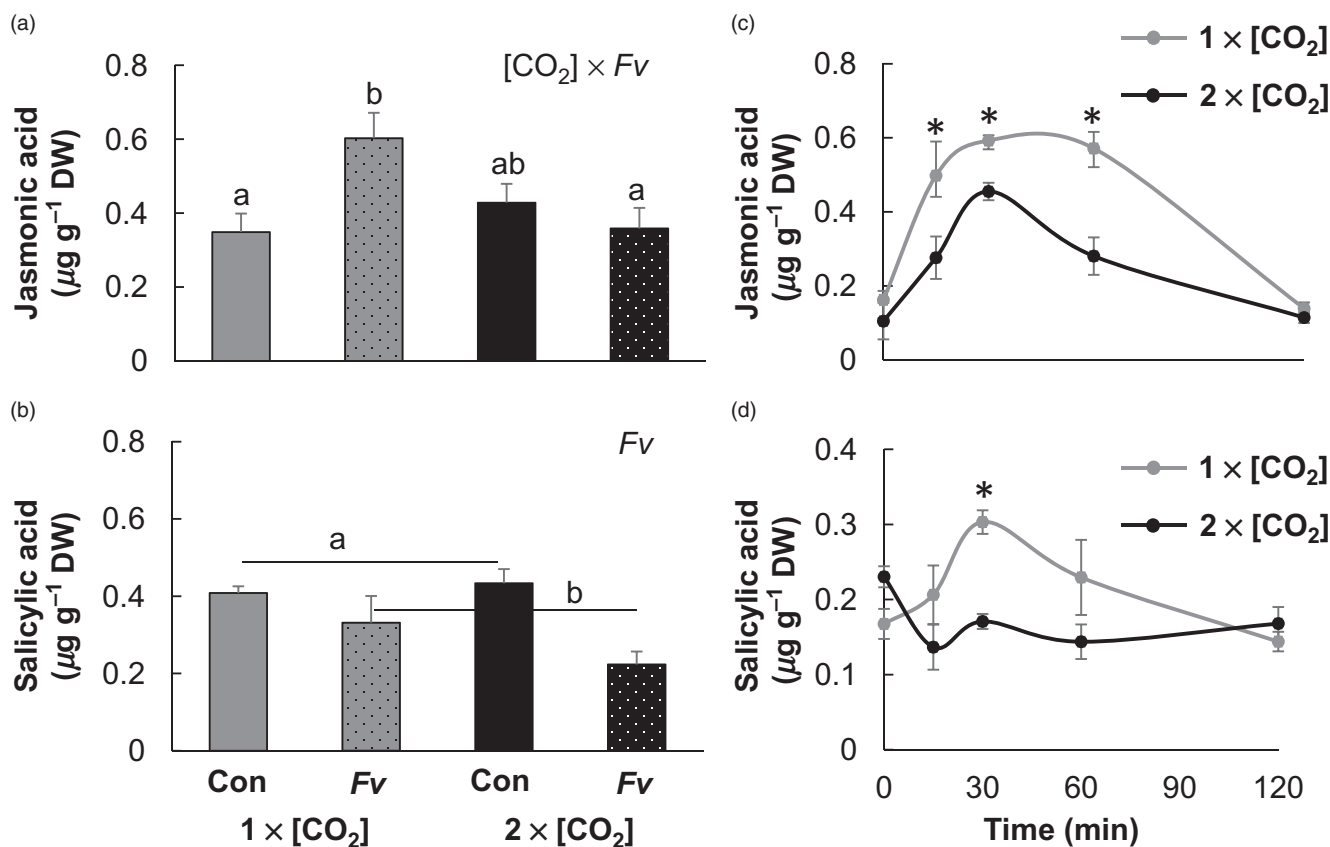




**Figure 4.** Elevated [CO<sub>2</sub>] alone did not alter the nutritional quality of maize stems, but affected the response of primary metabolites to *Fusarium verticillioides* (*Fv*) inoculation. The concentration of (a) total soluble carbohydrates, (b) starch and (c) protein was determined from maize stems 2 d post mock-inoculation (control, Con) or *Fv* inoculation at 1 × [CO<sub>2</sub>] or 2 × [CO<sub>2</sub>]. Statistically significant individual and interacting effects of [CO<sub>2</sub>] exposure and/or *Fv* inoculation are indicated at the top right corner of each graph. For carbohydrate and starch contents, where the 2 × 2 ([CO<sub>2</sub>] × *Fv*) full factorial analysis of variance (ANOVA) revealed a significant interaction between [CO<sub>2</sub>] and *Fv*, differences between individual means were determined by Tukey–Kramer’s honestly significant difference (HSD) ( $n = 4$ ,  $P < 0.05$ ). Conversely, a Student’s *t*-test was performed to independently compare the means of control and *F. verticillioides* inoculated protein concentrations ( $n = 8$ ,  $P < 0.05$ ), since no significant interaction was detected.



**Figure 5.** Free fatty acid precursors of oxylipin biosynthesis were not induced with *Fusarium verticillioides* inoculation at elevated [CO<sub>2</sub>]. The concentration of (a) stearic acid, (b) oleic acid, (c) linoleic acid and (d) linolenic acid in maize stems 2 d post control (Con, mock-treated) and *F. verticillioides* (*Fv*) inoculation at 1 × [CO<sub>2</sub>] and 2 × [CO<sub>2</sub>]. Individual and interacting factors contributing to differences are indicated at the top right corner of each graph. Letters above SEM bars indicate significant differences (2 × 2 full factorial analysis of variance (ANOVA) followed by Tukey–Kramer’s honestly significant difference (HSD),  $n = 4$ ,  $P < 0.05$ ).



**Figure 6.** Elevated [CO<sub>2</sub>] reduced the maize phytohormone response to *Fusarium verticillioides* (*Fv*) inoculation. Mean concentration ± SEM of (a) jasmonic acid (JA) and (b) salicylic acid (SA) in maize stem tissues 2 d after mock treatment (Con) or inoculation with *Fv*. Individual and interacting factors significantly contributing to differences are indicated at the top right corner of each graph. Differences between individual means of JA were determined by Tukey–Kramer’s honestly significant difference (HSD) ( $n = 4$ ,  $P < 0.05$ ). In the case of SA, where no significant interaction was detected between factors, a Student’s *t*-test was performed on the means of control and *Fv* inoculation ( $n = 8$ ,  $P < 0.05$ ). The initial responses of JA (c) and SA (d) was tracked by quantifying phytohormone levels throughout a 2 h time course post *Fv* inoculation of stalks at 1 × [CO<sub>2</sub>] and 2 × [CO<sub>2</sub>]. Error bars represent SEM and asterisks indicate significant differences between 1 × [CO<sub>2</sub>] and 2 × [CO<sub>2</sub>] at a particular time point (Student’s *t*-test,  $n = 4$  for all time points except 30 min,  $n = 8$ ,  $P < 0.01$ ).

determined for control and *F. verticillioides* inoculated tissues at various time points. Control tissues did not exhibit any significant differences in basal phytohormone levels (Fig. 6). Following the same pattern as the fatty acid precursors, at 2 d post-inoculation the concentration of JA increased 1.8-fold (Fig. 6a;  $P < 0.01$ ). However, at the same time point there was no significant difference between JA levels in control and *F. verticillioides* inoculated tissues at 2 × [CO<sub>2</sub>]. At 2 d post-inoculation, [CO<sub>2</sub>] was not a contributing factor to differences in SA levels. *F. verticillioides* inoculation was the only effect resulting in the reduction of SA with pathogen infection (Fig. 6b).

In order to further evaluate the observed effects of [CO<sub>2</sub>] on defence response hormone signalling, a 2 h time course was conducted to track the initial hormone burst following *F. verticillioides* inoculation. The amplitude and duration of JA accumulation in inoculated tissues was significantly reduced at 2 × [CO<sub>2</sub>] (Fig. 6c). Within 15 min the concentration of JA in plants at 1 × [CO<sub>2</sub>] was 45% higher than plants at 2 × [CO<sub>2</sub>] ( $P < 0.05$ ). The maximum difference of twofold

was observed at 60 min ( $P < 0.01$ ). The early accumulation of SA was also impacted by 2 × [CO<sub>2</sub>]. At 30 min post-inoculation, the concentration of SA was 43% less in stem tissues grown at 2 × [CO<sub>2</sub>] than at 1 × [CO<sub>2</sub>] (Fig. 6d;  $P < 0.05$ ).

#### Elevated [CO<sub>2</sub>] reduced the induction of both 9-LOX and 13-LOX gene transcription and down-regulated the transcription NPR1 following *F. verticillioides* inoculation

To test the hypothesis that suppression of JA biosynthesis was regulated at least in part at the level of transcription, qRT-PCR was used to compare transcript levels of LOXs and two key JA biosynthetic genes, allene oxide synthase (*AOS*) and allene oxide cyclase (*AOC*), throughout the same time course following pathogen inoculation. At time zero, there was no significant difference between constitutive transcript levels of the JA-related genes analysed in plants at 1 × [CO<sub>2</sub>]

in comparison with those at  $2 \times [\text{CO}_2]$  (Fig. 7). Transcript abundance of *LOX*, *AOS* and *AOC* genes relative to  $1 \times [\text{CO}_2]$  grown tissues at time zero, tended to increase with time following inoculation; however, with only a few exceptions, the up-regulation of *LOXs* at  $1 \times [\text{CO}_2]$  was significantly higher than at  $2 \times [\text{CO}_2]$  (Fig. 7). Transcript abundance of *LOXI*, which both has 9-*LOX* and 13-*LOX* functionality, was 73% less in  $2 \times [\text{CO}_2]$  grown plants 60 min after inoculation (Fig. 7a). With the exception of *LOX2*, which was unaffected by atmospheric  $[\text{CO}_2]$  (Fig. 7b), all other 9-*LOX* genes, *LOX3*, 4, 5, 6 and 12, had at least one time point at which transcript levels of infected  $1 \times [\text{CO}_2]$  grown plants exceeded those of  $2 \times [\text{CO}_2]$  grown plants. *LOX3* transcript levels were approximately 2.5-fold higher 120 min after inoculation at  $1 \times [\text{CO}_2]$  in comparison with corresponding levels at  $2 \times [\text{CO}_2]$  (Fig. 7c). The 13-*LOXs* (*LOX8*, 9, 10, 11, 13), which have putative functionality in JA production (Chauvin *et al.* 2013), similarly showed a dampened transcriptional response at  $2 \times [\text{CO}_2]$ . Consistent with reduced accumulation of JA at  $2 \times [\text{CO}_2]$ , the transcript abundance of *LOX8*, which is directly involved in the production of substrate for JA biosynthesis (Christensen *et al.* 2013), was 15-fold lower in 30 min inoculated tissues at  $2 \times [\text{CO}_2]$  in comparison with  $1 \times [\text{CO}_2]$  plants at the same time point (Fig. 7g). Transcript abundance of *LOX10*, which has been implicated in the production of green leaf volatiles (Bacon *et al.* 2008; Christensen *et al.* 2013), was the only *LOX* whose induction was stronger  $2 \times [\text{CO}_2]$  (Fig. 7i). Even though transcript levels followed the same oscillating pattern at both  $[\text{CO}_2]$ s, at 30 and 60 min after inoculation, the transcript level of *LOX10* was three- and fivefold higher at  $2 \times [\text{CO}_2]$ , respectively. *AOS* and *AOC* transcription was not effected by  $[\text{CO}_2]$ . Transcript abundance of both genes increased with time following inoculation, but there was no significant differences between the levels at  $1 \times [\text{CO}_2]$  in comparison with  $2 \times [\text{CO}_2]$  (Fig. 7m,n).

Because the early induction of SA was affected at  $2 \times [\text{CO}_2]$ , the transcript abundance of *NPRI*, a key regulator of SA-mediated systemic acquired resistance, was also evaluated throughout the time course. Constitutive (time zero) *NPRI* transcripts were 2.5-fold higher at  $2 \times [\text{CO}_2]$ . However, following *F. verticillioides* inoculation, *NPRI* gene transcription was down-regulated at  $2 \times [\text{CO}_2]$  while it was conversely induced at  $1 \times [\text{CO}_2]$ . This inversion ultimately led to a three- and 3.5-fold higher transcript abundance of *NPRI* 60 and 120 min, respectively, after inoculation at  $1 \times [\text{CO}_2]$ .

### Accumulation of phytoalexin defence metabolites was significantly reduced in maize at elevated $[\text{CO}_2]$

In order to further evaluate the possible downstream effects of dampened *LOX* and JA signalling, the concentration of maize phytoalexins in control and *F. verticillioides* inoculated tissues at  $1 \times [\text{CO}_2]$  and  $2 \times [\text{CO}_2]$  were compared. Treatment with *F. verticillioides* strongly induced the production of zealexin and kauralexin metabolites at both  $[\text{CO}_2]$ s, but in comparison with inoculated plants at  $1 \times [\text{CO}_2]$ , inoculated plants at  $2 \times [\text{CO}_2]$  had 41% less zealexins and 29% less

kauralexins (Fig. 8a,b). A mixture of oxygenated zealexins reduced *F. verticillioides* growth by 14% and purified kauralexin B3 alone reduced *F. verticillioides* growth by as much as 30% (Fig. 8c).

## DISCUSSION

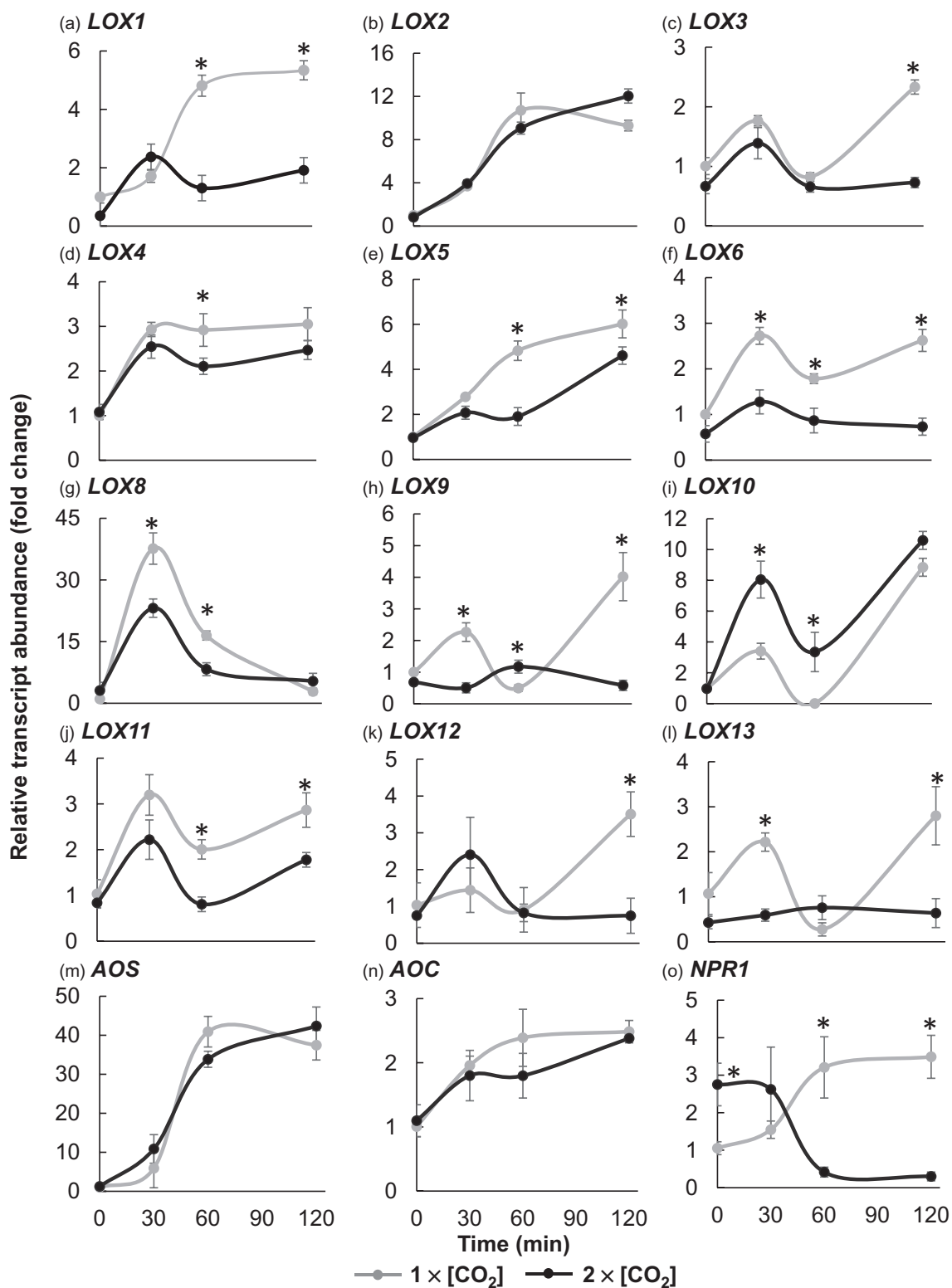
Our research demonstrates that elevated  $[\text{CO}_2]$  enhances maize susceptibility to *F. verticillioides*, but does not alter the severity of fumonisin contamination (Fig. 1). The attenuated induction of maize defences at elevated  $[\text{CO}_2]$  is consistent with increased maize susceptibility to *F. verticillioides* proliferation. In addition, reduced host-derived signals and defence metabolites, which have the potential to aggravate mycotoxin production, may result in the observed decrease in fumonisin per unit of *F. verticillioides* biomass. Nevertheless, when coupled with increased fungal biomass, even reduced host stimulants were sufficient to cause similar quantities of fumonisin in maize at  $2 \times [\text{CO}_2]$  as compared with infected maize at  $1 \times [\text{CO}_2]$ .

Plant–pathogen interaction studies involving other grain crops have likewise shown increased host susceptibility at elevated  $[\text{CO}_2]$  (Chakraborty & Datta 2003; Garrett *et al.* 2006). For example, growth at elevated  $[\text{CO}_2]$  enhances rice susceptibility to *Magnaporthe oryzae* (Kobayashi *et al.* 2006) and wheat susceptibility to *F. pseudograminearum* (Melloy *et al.* 2010). However, our study is unique in that it exemplifies a  $C_4$  grain, and evaluates the effects of elevated  $[\text{CO}_2]$  on maize defences and mycotoxin accumulation. To our knowledge, this represents the first report of the influence elevated  $[\text{CO}_2]$  has on a  $C_4$  crop's defence response in interaction with a mycotoxigenic pathogen.

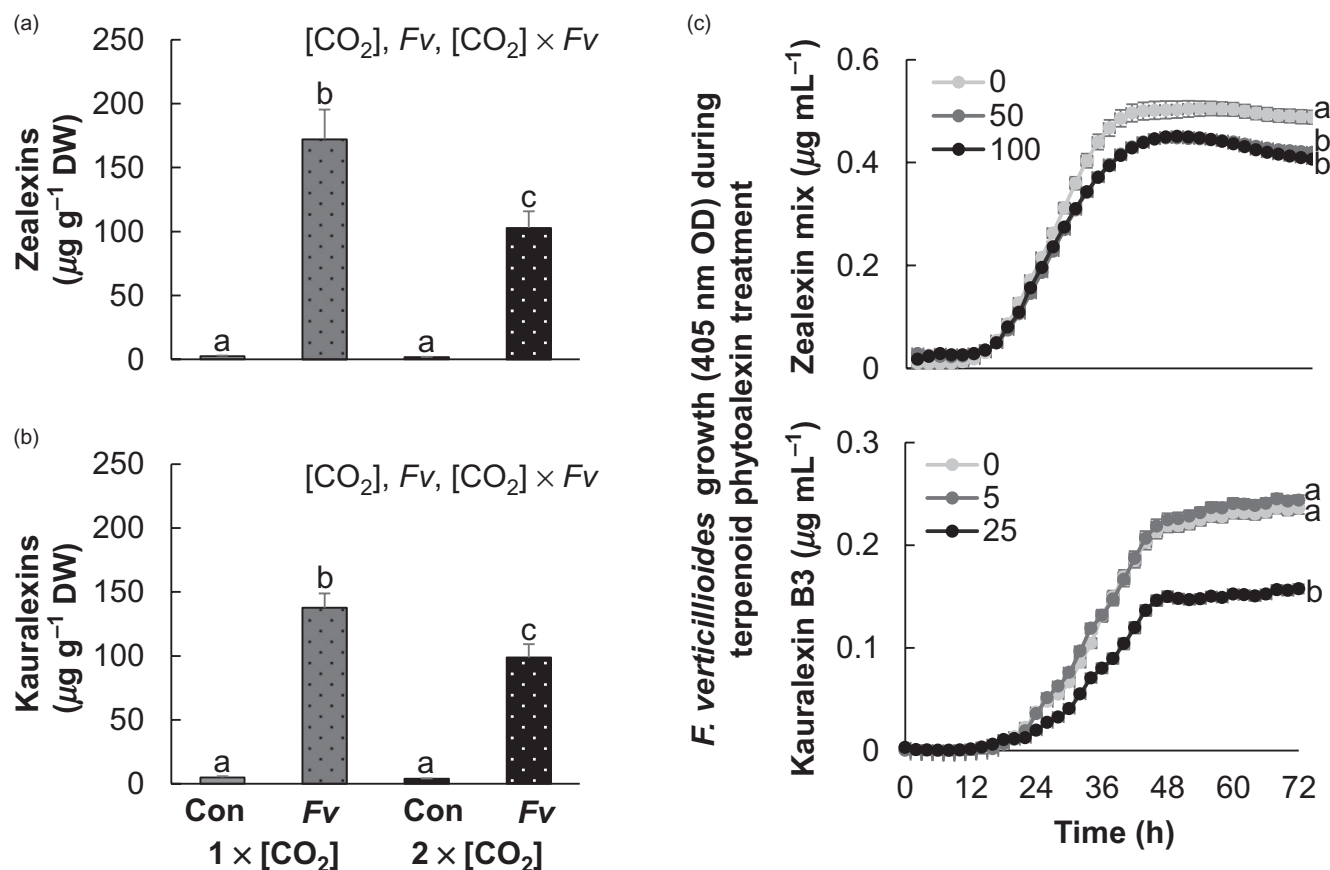
### The lack of an increase in primary metabolites following *F. verticillioides* inoculation correlates with the dampened defence response in maize at elevated $[\text{CO}_2]$

Carbohydrate signalling plays a critical role in the mobilization of plant defences and can even determine the outcome of plant–pathogen interactions (Bolouri Moghaddam & Van den Ende 2012). During a pathogen attack, an increase in endogenous sugar levels can enhance plant resistance (Roitsch *et al.* 2003; Morkunas *et al.* 2005; Gomez-Ariza *et al.* 2007), and many phytohormone-inducible defence-related genes are co-regulated by carbohydrates (Reinbothe *et al.* 1994; Rolland *et al.* 2002; Thibaud *et al.* 2004; Rolland & Sheen 2005). A considerable amount of energy is required for the cascade of defence reactions to be activated, thus a localized increase of carbohydrate supply is important in providing for this additional metabolic demand. The lack of an increase in soluble carbohydrates at  $2 \times [\text{CO}_2]$  with *F. verticillioides* inoculation (Fig. 4a) could therefore compromise the defence response and intensify maize susceptibility to fungal proliferation.

The lack of an increase in free fatty acids following pathogen infection at  $2 \times [\text{CO}_2]$  (Fig. 5) may contribute to impaired JA and 9-*LOX* oxylipin biosynthesis at the level of substrate



**Figure 7.** Elevated [CO<sub>2</sub>] altered the transcriptional response of phytohormone related genes to *Fusarium verticillioides* inoculation. Transcript abundance of *LOX* genes, allene oxide synthase (*AOS*), allene oxide cyclase (*AOC*) and non-expressor of pathogenesis-related genes1 (*NPR1*) in stem tissues of maize grown at 1 × [CO<sub>2</sub>] and 2 × [CO<sub>2</sub>] was analysed via qRT-PCR throughout a 2 h time course following *F. verticillioides* inoculation. Data points have been normalized to the geometric mean of two endogenous controls, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; X07156) and ribosomal protein L17 (*RPL17*; AF034948), and are relative to transcript abundance in maize grown under 1 × [CO<sub>2</sub>] at time zero. Error bars represent SEM and asterisks indicate significant differences between 1 × [CO<sub>2</sub>] and 2 × [CO<sub>2</sub>] at the particular time point (Student's *t*-test, *n* = 4, *P* < 0.05).



**Figure 8.** Terpenoid phytoalexin defence metabolites, which inhibit *Fusarium verticillioides* (*Fv*) growth, were reduced in maize at elevated [ $\text{CO}_2$ ]. The average concentration of (a) total zealexins and (b) kauralexins in two day control (Con, mock-inoculated) and *Fv* inoculated stem tissues of maize plants grown under different [ $\text{CO}_2$ ]. Values represent the total of closely related compounds from each family of phytoalexins. Individual and interacting factors contributing to differences are indicated at the top right corner of each graph. Letters above SEM bars indicate significant differences ( $2 \times 2$  ([ $\text{CO}_2$ ]  $\times$  *Fv*) full factorial analysis of variance (ANOVA) followed by Tukey–Kramer’s honestly significant difference (HSD),  $n = 4$ ,  $P < 0.01$ ). Zealexin and kauralexin phytoalexins inhibit *Fv* growth (c). Average  $\pm$  SEM *Fv* growth throughout a time course in nutrient broth containing (top) a mixture of oxygenated zealexins (Huffaker *et al.* 2011) or (bottom) purified kauralexin B3 (Schmelz *et al.* 2011). Differently coloured lines indicate final concentration of phytoalexin compound(s) within the broth. DMSO solvent controls are indicated as  $0 \mu\text{g mL}^{-1}$ . Letters at the end of each growth curve represent significant differences (ANOVA and Tukey–Kramer’s HSD,  $n = 8$ ,  $P < 0.01$ ).

supply. Previous reports have demonstrated that free fatty acid levels increase after wounding, herbivory or elicitor application (Conconi *et al.* 1996; Ryu & Wang 1998), and this increase of substrate (linolenic acid) is essential for the burst of JA which initiates the defence signalling process (Kallenbach *et al.* 2010). Interestingly, NPR1 is required for the activation mechanism that controls the influx supply of linolenic acid (Kallenbach *et al.* 2010); therefore, the down-regulation of *NPR1* following *F. verticillioides* inoculation at elevated [ $\text{CO}_2$ ] (Fig. 7o) may influence this influx of substrate.

#### The attenuation of induced 9-LOX and 13-LOX gene transcription is consistent with increased *F. verticillioides* growth and reduced fumonisin per unit fungal biomass

Although functional elucidation of individual maize LOXs is still in progress, it is evident that they play an important role

in resistance to fungal pathogens and mycotoxin accumulation (Gao & Kolomiets 2009; Christensen *et al.* 2013; De La Fuente *et al.* 2013). Elevated [ $\text{CO}_2$ ] reduces the induction of most LOX genes including *LOX8* (Fig. 7g), which directly mediates JA production (Acosta *et al.* 2009). The burst of JA following pathogen perception is part of the phytohormone signalling process that regulates the accumulation of zealexin and kauralexin phytoalexins, which have the potential to reduce fungal growth (Fig. 8c) (Huffaker *et al.* 2011; Schmelz *et al.* 2011). Other LOXs not directly involved in providing substrate for JA biosynthesis have also been implicated in regulating maize resistance to pathogens. For example, disruption of *LOX5* or *LOX12* activity has been shown to increase maize susceptibility to *F. verticillioides* (Park *et al.* 2011; De La Fuente *et al.* 2013). Thus the depression of induced LOX gene transcription at  $2 \times [\text{CO}_2]$  is consistent with enhanced maize susceptibility to *F. verticillioides* growth. Furthermore, in support of the hypothesis that

*F. verticillioides* fumonisin production can be stimulated by host 9-LOX oxylipins (Gao *et al.* 2007; Tsitsigiannis & Keller 2007; Christensen & Kolomiets 2011), the dampened induction of 9-LOX gene transcription following infection at 2 × [CO<sub>2</sub>] (Fig. 7c,d,e,f) coincides with less fumonisin production per unit *F. verticillioides* biomass (Fig. 1h). On *lox3* mutant kernels, *F. verticillioides* vegetative growth was not impaired, but fumonisin production was reduced (Gao *et al.* 2007, 2009). The abated induction of *LOX3* at 2 × [CO<sub>2</sub>] may similarly reduce fumonisin production with respect to fungal biomass.

Differences in transcriptional regulation are common in defence-related gene families and reflect their non-redundant/diverse functionality in plants. Maize LOXs are affected by and potentially function in response to wounding [LOX5 (Park *et al.* 2010); LOX8 (Christensen *et al.* 2013); LOX10 (Nemchenko *et al.* 2006)], fungal infection [LOX3 (Gao *et al.* 2008); LOX4 and 5 (Park *et al.* 2010); LOX6 (Gao *et al.* 2007); LOX10 (Nemchenko *et al.* 2006)], herbivory [LOX5 (Park *et al.* 2010)], temperature stress [LOX10 (Nemchenko *et al.* 2006)], drought stress [LOX11 (De La Fuente *et al.* 2013)], circadian rhythm [LOX10 (Nemchenko *et al.* 2006)], and elevated [CO<sub>2</sub>] (based on the present study). Casteel *et al.* (2008) observed analogous regulatory differences in the soybean *LOX* gene family. While the expression of most soybean *LOX*s were affected by [CO<sub>2</sub>] and herbivory, others were not (Casteel *et al.* 2008). In maize the induction of *LOX2* (presently unknown function) does not appear to be influenced by elevated [CO<sub>2</sub>] (Fig. 7b).

### Elevated [CO<sub>2</sub>] influences phytohormone signalling pathways: a comparison between C<sub>3</sub> soybean and C<sub>4</sub> maize

Elevated [CO<sub>2</sub>] similarly increases the susceptibility of C<sub>3</sub> soybean plants to herbivory by compromising *LOX* gene transcription, JA biosynthesis and JA-dependent anti-herbivore defences; however, this is typically accompanied by an increase in SA production (Casteel *et al.* 2008; Zavala *et al.* 2008, 2009; Casteel 2010; Casteel *et al.* 2012a), which does not occur in maize (Fig. 6; Casteel 2010). Opposing the hypothesis of a potential antagonistic effect of SA on JA signalling, our findings suggest that elevated [CO<sub>2</sub>] also compromises the induction of SA (Fig. 6d). Furthermore, following *F. verticillioides* inoculation, the transcription of *NPR1*, which plays a central role in the activations of SA-dependent defence genes, is down-regulated instead of induced (Fig. 7o). Surprisingly, constitutive *NPR1* transcripts are significantly higher in maize grown at 2 × [CO<sub>2</sub>], but it is possible that *NPR1* is kept in an inactivated form under non-induced conditions (Mou *et al.* 2003). *NPR1* transcription is not differentially regulated by [CO<sub>2</sub>] in soybean (Casteel *et al.* 2008), but because *NPR1* mediates the antagonistic relationship between JA and SA signalling pathways it is hypothesized that *NPR1* is post-transcriptionally activated through changes in the cellular redox state at elevated [CO<sub>2</sub>] leading to the down-regulation of JA-dependent defence gene expression (Casteel *et al.* 2012b). There does not appear

to be a down-regulation of constitutive transcripts and/or phytohormone levels in maize that would suggest such a reconfiguration of phytohormone based defences as shown in soybean. The phytohormones are merely inadequately induced following *F. verticillioides* inoculation suggesting more of a desensitized or compromised state of maize grown at elevated [CO<sub>2</sub>] rather than a reconfiguration of defence signalling processes.

### Potential implications of attenuated maize defences at elevated [CO<sub>2</sub>]

It is of increasing concern that predicted global environmental changes, including drought, warmer temperatures and increased insect herbivory, coincide with the major factors affecting maize productivity, predisposition to *F. verticillioides* infection, and fumonisin contamination (Dowd 2003; Chakraborty & Newton 2011; Wu *et al.* 2011; de Sassi & Tylianakis 2012). Our results support these concerns and indicate that increasing atmospheric [CO<sub>2</sub>] will enhance maize susceptibility to *F. verticillioides* by compromising the phyto-immune response. Fortunately, increased fungal biomass at 2 × [CO<sub>2</sub>] does not correspond with an increase in fumonisin thus resulting in an overall reduction in fumonisin per unit pathogen (Fig. 1). However, despite this reduction, our results do not suggest that elevated [CO<sub>2</sub>] will ameliorate the issues of fumonisin contamination in the future. With the augmentation of fungal biomass, the amount of fumonisin in infected tissues at 2 × [CO<sub>2</sub>] is not different from quantities in tissues at 1 × [CO<sub>2</sub>] (Fig. 1e,f) indicating that similar food safety issues will exist in the future at elevated [CO<sub>2</sub>].

The lack of an increase in mycotoxin contaminants and the reduced fumonisin per unit fungal biomass should not lessen the concerns of increased maize susceptibility to *F. verticillioides* disease at elevated [CO<sub>2</sub>]. Particularly in the context of a C<sub>4</sub> crop, which will not benefit from a substantial stimulation in photoassimilation at elevated [CO<sub>2</sub>], increased productivity to meet growing crop demands will rely heavily on disease control. At the current [CO<sub>2</sub>], *Fusarium* rots are responsible for pre-harvest losses of approximately 8.6% of the global maize production (Nayaka *et al.* 2010). Crop losses could substantially escalate if the plants defence response is weakened at elevated [CO<sub>2</sub>] emphasizing the issues of food security. Furthermore, an increase in pathogen biomass per unit plant tissue could further increase transfer of inoculum during successive growing seasons, which would in turn aggravate disease development in future crops (Melloy *et al.* 2010).

While the main focus of our research has been centred on the maize-*F. verticillioides* interaction, a weakened defence response will likely also increase maize susceptibility to other biotic and abiotic stressors. LOXs function in the response to a variety of biotic and abiotic factors as described earlier. Similarly, JA is critical in the mediation of wound and anti-herbivore defences (Kim *et al.* 2003; Oikawa *et al.* 2004; Schmelz *et al.* 2009, 2011; Dafoe *et al.* 2011; Yan *et al.* 2012; Huffaker *et al.* 2013), but also plays a role in responses to drought, salinity and thermotolerance (Xin *et al.* 1997; Clarke

et al. 2009; Yoon et al. 2009). Furthermore, maize zealexin and kauralexin phytoalexins are involved in resistance against multiple pathogens, including *Fusarium graminearum*, *Aspergillus flavus* and *Rhizopus microsporus* (Huffaker et al. 2011; Schmelz et al. 2011). Whether SA-dependent resistance against pathogens, such as *Ustilago maydis*, will be similarly effected in maize at elevated [CO<sub>2</sub>] requires confirmation, but our results suggest that they will be influenced.

The combined effects of elevated [CO<sub>2</sub>] and other abiotic and biotic stress factors on maize susceptibility to infection and mycotoxin contamination warrant further experimentation. If drought, heat and insect damage enhance fumonisin production during increased susceptibility to *F. verticillioides* proliferation at elevated [CO<sub>2</sub>], the current predictions, which already foresee increased fumonisin contaminants in the future maize crop (Miraglia et al. 2009; Chakraborty & Newton 2011; Wu et al. 2011; Magan et al. 2012), could be underestimated. However, it is also possible that the combination of elevated [CO<sub>2</sub>] and drought will negate the impact of elevated [CO<sub>2</sub>] alone, leaving maize susceptibility unchanged as demonstrated in soybean (Casteel et al. 2012a). Given the heightened climate change concerns and the paucity of knowledge regarding the issue, we hope that this study will instigate additional research regarding maize productivity, disease development, and mycotoxin contamination. Ideally in a more organic setting, which combines projected climatic scenarios with multiple abiotic and biotic stress factors in a field-based setting, such as those provided by free air gas concentration enrichment (FACE) experiments. Only through a better understanding of crop responses to individual and combined climatic conditions can effective agricultural management decisions and agronomic practices to be made for adaption to climate changes with minimal agro-economic loss and associated consequences.

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## SUPPORTING INFORMATION

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**Figure S1.** Conviron E15 growth chamber fitted with additional sensors for continuous monitoring of environmental data and [CO<sub>2</sub>] regulation. A temperature and humidity transmitter (Vaisala HMD70Y; Louisville, CO, USA) (A), a carbon dioxide transmitter [Vaisala GMT222 (0–2000 μmol mol<sup>-1</sup>)] (B) and a Quantum sensor (Apogee SQ100; Logan, UT, USA) (C) were installed in each Conviron E15. The sensors from both chambers were connected to a Campbell Scientific Inc (CSI; Logan, UT, USA) datalogger (CR10X) through a Multiplexer (CSI AM 16/32). The data was collected every 10 s and 5 min averages was saved in the datalogger memory. The chamber's air inlet opening includes a pipe that discharges at the circulation fan located in the centre of the chamber below the perforated floor. The air outlet was simply a passive opening through which the 2 × [CO<sub>2</sub>] (800 μmol mol<sup>-1</sup>) chamber air was vented to the outside of the room to keep the air inlet levels at 1 × [CO<sub>2</sub>]. Black polypropylene tube was fed through the inlet pipe to inject the CO<sub>2</sub> directly at the chamber circulation fan for better mixing. Injection of CO<sub>2</sub> from a gas cylinder into the chamber was regulated by a mass flow controller [Brooks 5850i (300 sccm)]. Every 10 s, the CO<sub>2</sub> transmitter output was measured by the datalogger. A programme stored in the datalogger compared the [CO<sub>2</sub>] to the user-selected value to maintain either 400 or 800 μmol mol<sup>-1</sup>. The programme calculated how far the reading deviated from the desired setpoint value and sent a proportional voltage to the Brooks mass flow controller through the Analog Output Module (CSI SMD AO4). The farther the system was from the desired setpoint, the larger the voltage sent with more flow allowed through the mass flow controller and vice versa. The system maintained the [CO<sub>2</sub>] level in the chamber within ±10 μmol mol<sup>-1</sup> of the setpoint. During dark periods when the plant respiration produced CO<sub>2</sub>, the transmitter measures the increase in concentration above set point and the programme shut down the injection until the lights came back on in the chamber and the plants start assimilating the CO<sub>2</sub>. To prevent data loss, the datalogger was powered by a 12 V deep discharge marine battery that was continually recharged with a trickle-charge battery recharger. Therefore, in the event of a power failure, the monitoring sensors will continue to work and record the environmental conditions in the chambers with the exception of the CO<sub>2</sub> transmitter that requires 24 VDC. As a safety precaution, the Brooks mass flow controller has a normally closed valve so the flow would stop in the event of a power failure.

**Figure S2.** Significant differences in *F. verticillioides* biomass in maize stems at 1 × [CO<sub>2</sub>] or 2 × [CO<sub>2</sub>] could be detected as early as 2 d post-inoculation. Representative image of infected maize stalks 2 d post-inoculation (A). Average ± SEM *F. verticillioides* biomass in stalks at 1 × [CO<sub>2</sub>] or 2 × [CO<sub>2</sub>] 2 d post-inoculation estimated as the amount of pg fungal DNA relative to ng maize DNA via qRT-PCR. (Student's *t*-test, *n* = 4, *P* < 0.01).

**Table S1.** Primer pairs used for qRT-PCR expression analysis.