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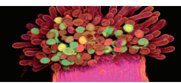
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RESEARCH PAPER

Lower incidence and severity of tomato virus in elevated CO_2 is accompanied by modulated plant induced defence in tomato

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ABSTRACT

Elevation in atmospheric CO_2 concentration broadly affects plant phenology and physiology, and these effects may alter the performance of plant viruses. The effects of elevated CO_2 on the susceptibility of tomato plants to *Tomato yellow leaf curl virus* (TYLCV) were examined for two successive years in open top chambers (OTC) in the field. We experimentally tested the hypothesis that elevated CO_2 would reduce the incidence and severity of TYLCV on tomato by altering plant defence strategies. Our results showed that elevated CO_2 decreased TYLCV disease incidence (by 14.6% in 2009 and 11.8% in 2010) and decreased disease severity (by 20.0% in 2009 and 10.4% in 2010). Elevated CO_2 also decreased the level of TYLCV coat protein in tomato leaves. Regardless of virus infection, elevated CO_2 increased plant height and aboveground biomass. Additionally, elevated CO_2 increased the leaf C:N ratio of tomato, but decreased soluble protein content in leaves. Notably, elevated CO_2 increased the salicylic acid (SA) level in uninfected and infected plants. In contrast, elevated CO_2 reduced jasmonic acid (JA) in uninfected plants while it increased JA and abscisic acid (ABA) in virus-infected plants. Furthermore, combined exogenous SA and JA application enhanced resistance to TYLCV more than application of either SA or JA alone. Our results suggest that the modulated antagonistic relationship between SA and JA under elevated CO_2 makes a great contribution to increased tomato resistance to TYLCV, and the predicted increases in tomato productivity may be enhanced by reduced plant virus susceptibility under projected rising CO_2 conditions.

INTRODUCTION

The atmospheric CO_2 concentration has risen from 280 ppm to 379 ppm since the Industrial Revolution; it now exceeds any level in the past 65,000 years and is predicted to double by 2100 (Anderson 2006; IPCC 2007; Fu *et al.* 2010). The rise in CO_2 is projected to increase the productivity of agroecosystems by enhancing photosynthesis, C:N ratio and water-use efficiency, particularly in C_3 crops (Jablonski *et al.* 2002; Long *et al.* 2006). Although the growth and physiological responses of numerous plant species to elevated CO_2 are well documented (Drake *et al.* 1997; Saxe *et al.* 1998), few studies have assessed how plant interactions with virus will change under future climatic conditions (Aranda *et al.* 2009; Fu *et al.* 2010).

Plant disease expression is determined by three key parameters: 'the inherent susceptibility of the host, the inoculum potential of the pathogen, and the impact of the environment on pathogenesis' (McNew 1960; Mcelrone *et al.* 2010). Interaction of the three parameters can be conceptualised by the disease triangle model to illustrate how shifts in any one of them can essentially impact the disease expression in a given pathosystem (Scholthof 2007; Mcelrone *et al.* 2010). Thus, the altered environmental conditions associated with climate change will potentially modify plant disease expression through changes in host plants, as well as to reshape the co-evolutionary relationships between plants and pathogens (Garrett *et al.* 2006; Eastburn *et al.* 2011). Indeed, Matros *et al.* (2006) and Ye *et al.* (2010) demonstrated that elevated CO_2 concentration leads to a shift in the composition of secondary metabolites in tobacco and to increased resistance against infection by *Potato virus Y*, and DeLucia *et al.* (2008) reported that elevated O_3 increase salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signalling gene transcriptions of plants and thereby enhanced resistance to plant viruses. Similarly, our previous research showed that increased resistance to *Cucumber mosaic virus* under elevated CO_2 was associated with altered secondary metabolites and leaf chemistry (Fu *et al.* 2010). However, whether elevated CO_2 alters the induced defence response of the host plants against viruses is still unclear.

Plants have evolved many defence responses to pathogens, herbivorous insects and others biotic stressors (Schenk *et al.* 2000; Felton *et al.* 2005; Staskawicz *et al.* 2005). Extensive reports indicate that the induction of SA, JA and abscisic acid (ABA) plays crucial roles in plant disease resistance, leading to direct defence-related gene expression (Delaney *et al.* 1994; Thaler *et al.* 2004; Adie *et al.* 2007). Salicylate-mediated

responses have been established to have a role in resistance to pathogens in plants (Malamy *et al.* 1990; Brading *et al.* 2000) and jasmonate-mediated responses in resistance to herbivores (Farmer *et al.* 1992; Thaler *et al.* 2004). Several lines of evidence suggest, however, that this dichotomy is too simplistic. First, each of these responses has 'cross-talk' or reciprocal down-regulation by the other (Kunkel & Brooks 2002; Jones *et al.* 2007). Second, salicylate-dependent responses are induced by some herbivores (Felton *et al.* 2005; Kaloshian & Walling 2005) while jasmonate-mediated responses are induced by some microbial pathogens (Thaler *et al.* 2004; Dong *et al.* 2007). Finally, jasmonate-mediated responses have been reported to be essential for resistance to several microbial pathogens in plants (O'Donnell *et al.* 2003; Stout *et al.* 2006), and salicylate is necessary in resistance to some herbivores (Walling *et al.* 2007). Thus, the perceived dichotomy that salicylate is induced by and impacts pathogens while jasmonate is induced by and impacts insect herbivores has been replaced by a more nuanced understanding of the complexity in such interactions (Thaler *et al.* 2010).

Elevated CO_2 re-allocates carbon and nitrogen resources among tissues and alters the synthesis of nutrients and secondary metabolites in plants (Dyckmans *et al.* 2000; Matros *et al.* 2006; Sun *et al.* 2010). Previous studies indicate that elevated CO_2 affects induced defence signalling pathways of plants. For example, elevated CO_2 increased SA and ABA levels in tomato *(Lycopersicon esculentum)* infected by the oomycete *Phytophthora parasitica* but suppressed the JA-induced pathway and related gene expression against Japanese beetle in soybean (Jwa & Walling 2001; Zavala *et al.* 2008). To our knowledge, limited experimental studies have documented the interactions between phytohormones and the spread of virus disease under elevated CO_2.

Tomato yellow leaf curl virus (TYLCV) causes severe damage to tomato crops in many tropical and subtropical regions worldwide (Czosnek & Laterrot 1997; Zhou *et al.* 2009). In China, TYLCV is the most important viral pathogen of tomato in the major tomato-producing areas (Zhu *et al.* 2008). In this study, we hypothesised that elevated CO_2 would reduce the incidence and damage caused by TYLCV on tomato (*L. esculentum*) by altering the plant defence strategies. Our aims were to determine whether elevated CO_2 altered (i) the susceptibility of tomato plants to TYLCV, and (ii) the level and the relationship between phytohormones (SA, JA and ABA) involved in plant induced defence in tomato plants infected by TYLCV.

MATERIAL AND METHODS

Plant growth and harvest

Seeds of tomato (*L. esculentum* cv. YF 8, a host of TYLCV) were purchased from the Institute of Vegetables and Flowers, Beijing Academy of Agricultural and Forestry Sciences, Beijing, China, and were sown about 0.5-cm deep in sterilised soil. One week after germination, when the cotyledons were beginning to expand, the seedlings were transplanted singly into plastic pots (15-cm diameter and 13-cm height) containing sterilised loamy field soil. The pots were placed in ventilated insect proof cages (1.5-m long, 1.0-m wide, 1.5-m high, 80 mesh) in octagonal open top chambers (OTC, 1.6-m wide, 4.2-m diameter and 2.4-m high) until they reached the three to four leaf stage. The experiment was carried out in eight OTCs located at the Observation Station on Global Change Biology of the Institute of Zoology, Chinese Academy of Sciences, Xiaotangshan County, Beijing, China (40°11′ N, 116°24′ E). Four of the OTCs were continuously maintained at the current ambient CO_2 level (about 375 ppm) and four at an elevated level (about 750 ppm, the predicted level in about 100 years; IPCC 2007). During the experiment, which was performed in 2009 and then repeated in 2010, CO_2 concentrations were monitored and controlled by an infrared CO_2 transmitter (Ventostat 8102; Telaire Co., Goleta, CA, USA) and were maintained throughout the experiment. CO_2 concentrations were measured hourly. In ambient CO_2 chambers, the CO_2 concentrations (mean ± SD per day) were 363 ± 35 in 2009 and 372 ± 43 ppm in 2010. In elevated CO_2 chambers, the CO_2 concentrations were 758 ± 56 in 2009 and 746 ± 39 ppm in 2010. Details of the automatic control system for CO_2 concentrations and OTCs are provided in Chen *et al.* (2005). Air temperature was measured three times daily (08:00, 14:00 and 18:00 h) throughout the experiment and did not differ significantly between the two sets of OTCs during the 2 years (24.8 ± 3.40 and 25.7 ± 4.5 °C in ambient CO_2 chambers versus 25.5 ± 4.55 and 26.2 ± 3.9 °C in elevated CO_2 chambers in 2009 and 2010, respectively).

Each ventilated cage contained 25 seedlings, and each of the eight OTCs contained two ventilated cages. Within each OTC, the 25 plants in one cage were inoculated with TYLCV, and the 25 plants in the other cage were inoculated with LB culture medium as a control. Thus, this experiment had two levels of CO_2 and two levels of virus infection. Plants were maintained in the OTCs for about 2 months (27 July to 29 September in 2009 and 15 July to 20 September in 2010). Pot placement was re-randomised within each OTC once every 2 weeks. No insecticides were used. All plants were watered every 1 to 2 days (about 200 ml plant^{-1}) as necessary.

Tomato yellow leaf curl virus clone and agro-inoculation

The TYLCV infection of tomato plants was achieved using *Agrobacterium tumefaciens*-mediated infectious inoculation (Zhou *et al.* 2009; Al Abdallat *et al.* 2010), and the infectious clone (pBINPLUS-SH2-1.4A) of TYLCV- Israel [China: Shangai2] was constructed into *A. tumefaciens* strain EHA105 as described previously (Zhou *et al.* 2009). The infectious clone of TYLCV was provided by Professor Xueping Zhou (Institute of Biotechnology, Zhejiang University, Hangzhou, China). The culture of TYLCV clone was grown in LB culture medium with kanamycin (50 μg ml^{-1}) and rifampicin (50 μg ml^{-1}) at 28 °C (250 rpm) for 24 h (OD_{600} = 1.5), after which 0.2 ml of the culture was injected three times into the phloem (about 1 mm in depth) of the tomato stem at the three to four leaf stage to achieve inoculation; a sterile syringe (1 ml) with a bevelled needle was used for injection. This TYLCV inoculation is mainly according to the injection method for *Tomato yellow leaf curl Yunnan virus* (Zhou *et al.* 2004). Inoculated plants were grown in ventilated cages in OTCs (27 July to 10 August in 2009 and 15 July to 28 July in 2010).

Infection incidence and leaf sampling

Virus infection of the tomato plants in the OTCs was determined 6 weeks after agro-inoculation in the two successive years (29 September 2009, 20 September 2010). We surveyed all TYLCV-inoculated tomato plants in each OTC (n > 95 for both ambient and elevated CO_2 in each year of the study) for disease incidence (percentage of plants showed disease symptoms). A disease index was used to quantify disease severity (Curvers *et al.* 2010), and index values were determined as follows:

$$(\mathrm{DI}) = \Sigma \mathrm{N}_i \times R_i/(N \times R_h) \times 100$$

where N_i represents the number of plants in disease symptom ranking *i*, R*i* represents the disease symptom rank (*i* = 0–4), N represents the total number of plants investigated, and R_h represents the highest disease symptom rank. Disease symptoms were ranked mainly according to Friedmann *et al.* (1998): 0 = no visible symptoms: inoculated plants show the same growth and development as non-inoculated plants; 1 = very slight yellowing of leaflet margins on the apical leaf; 2 = some yellowing and minor curling of leaflet ends; 3 = widespread leaf yellowing, curling, and cupping, with some reduction in size, but plants continue to develop; 4 = severe plant stunting and yellowing, and pronounced cupping and curling; plants cease to grow. Plant height, biomass, and stem diameter were determined for uninfected and infected plants grown in 375 ppm and 750 ppm CO_2 in the year of 2010. In each experiment, 20 plants in each OTC were randomly investigated, and 80 (20 × 4 OTCs) plants per treatment were measured. Stem diameters were measured with calipers at the middle of the stem between the fifth and sixth leaf from top; each point was measured twice. Plant height (from base to terminal) and fresh aboveground mass were determined at the same time.

For analysis of biochemical and physiological parameters, recently developed and mature leaves were harvested, immediately frozen in liquid nitrogen, and transferred to −80 °C until they were assayed (assay details are described below). Two replicates per treatment from each chamber were analysed (n = 8).

Coat protein determination

Plants with obvious disease symptoms were used for immunological detection of virus coat protein. Newly expanded leaves (fourth leaf from apex) were harvested for testing. An total of 0.5 g frozen leaf tissue was homogenised in 5 ml TYLCV extraction buffer (100 mg ml^{-1}) containing 0.05 M Tris-HCL and 0.05 M Na_2SO_3 (pH 8.5). An aliquot of 100 µl cleared extract was analysed using a triple antibody sandwich enzyme-linked immunoabsorbent assay (TAS-ELISA) with polyclonal antibodies (IgG) conjugated to the surface of the microtitre plate to capture the antigen of interest. A monoclonal antibody (Mab) raised in mice against TYLCV was used to detect the antigen. An anti-species antibody–enzyme conjugate was used to detect the Mab (Adgen, UK), according to the manufacturer's instructions. Plant extracts of 100 µl were submitted to determine the coat protein level using a microplate spectrophotometer at 405 nm wavelength (Spektra max® Plus; Molecular Devices, Sunnyvale, CA, USA).

Foliar chemical determination

About 20 g of frozen tomato leaves were dried at 80 °C for 72 h for C and N determination. Dried leaves from each treatment were ground in a mill. Total non-structural carbohydrates (TNCs), mainly starch and sugars, in leaves were assayed by acid hydrolysis following the method of Tissue & Wright (1995). Nitrogen content in leaves was determined using Kjeltec nitrogen analysis (Foss automated Kjeltec™ Instruments, Model 2100; Foss, Hoganas, Sweden). A 0.5-g sample of frozen tomato leaves was used for soluble protein determination. Samples were homogenised on ice in 1:10 (fresh weight/buffer volume ratio) 100 mM phosphate buffer containing 100 mM KCl and 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 10,000 *g* for 10 min, and the supernatant was subjected to chemical component analysis. Protein concentration was determined using the Bradford (1976) assay with bovine serum albumin (BSA) as standard. Samples from the year 2010 were determined.

Endogenous JA and ABA quantification

Endogenous JA and ABA were mainly quantified according to Mauch-Mani *et al.* (2008). Frozen foliar tissue (0.4 g) was ground with 4 ml 80% methanol (80 methanol: 20 water, V/V) on ice and kept at −20 °C for 12 h, then a mixture of internal standards containing 6 µl [9,10]-dihydro-JA (50 ng μl^{-1}) and 6 µl of D3-ABA (50 ng μl^{-1}) was added. After centrifugation (7500 *g*, 20 min), the supernatant was condensed to aqueous and then frozen at −20 °C and thawed three times. After centrifugation at 3000 *g* for 20 min, the supernatant was adjusted to pH 2.5–3.0 with 3 M HCl and subsequently extracted with an equal volume of ethyl acetate; the organic fraction was evaporated under vacuum. The solid residue was re-suspended in 0.1 M acetic acid and loaded on a C18 column (Waters, Milford, MA, USA). The C18 column was eluted sequentially with a series of solvent mixtures (0.1 M acetic acid/methanol (83:17, 60:40 and 60:40, v/v)), taking 5.0 ml of the solvent mixture each time. The eluents of the last 4 ml in 40% methanol and of the first 3 ml in 60% methanol were collected together. After evaporation of the solvent and esterification of the residue with excess diazomethane, the sample volume was adjusted to 50 µl with ethyl acetate. A 1-µl volume of this sample was directly injected into the GC/MS system (6890N/5973N; Agilent Technologies, Santa Clara, CA, USA) with a DB-5-MS column (30 m × 0.32 mm × 0.25 µm, J&W Scientific, Agilent Technologies). Endogenous JA and ABA and their internal standards were analysed using full scan mode. The GC-MSD Chemistration G1701EA and NIST 2003 mass library were used to determine the retention time of the samples.

Endogenous SA quantification

The SA was extracted and quantified essentially as described in Malamy *et al.* (1992) and Matros *et al.* (2006). The free forms of SA were determined. Frozen foliar tissue (0.4 g) was ground and extracted with 3 ml 90% methanol (90 methanol:10 water, v/v). The extract was vortexed and centrifuged at 7500 *g* for 20 min. The residue was re-suspended in 2 ml

methanol, and the supernatant was separated twice and then mixed. The sample was dried under vacuum, and the pellets were dissolved in 1.5 ml 5% trichloroacetic acid, vortexed, and centrifuged at 7500 *g* for 15 min. The supernatant was extracted three times with a mixture comprising equal volumes of ethyl acetate and cyclohexane. The organic phase was dried, re-suspended in 3 ml 70% methanol, loaded onto a C_{18} column (Waters) and then collected. After evaporation of the solvent in the collected sample, 500 µl of acetonitrile were added to the residue. The solution was passed through a 0.45-µm filter.

All samples were analysed with HPLC (1100; Agilent Technologies), and the fractions were collected by injecting 10 µl of the sample onto a 5-µm C18 reverse phase column (250 mm × 4.6 mm; Agilent). SA was detected by excitation at 295 nm and emission at 405 nm and identified by the retention time of the parallel standard SA samples. Quantitative analysis of SA was completed by plotting the results against the standard curve.

Effects of exogenous SA, JA and SA plus JA on TYLCV incidence

The SA and JA (Sigma, St Louis, MO, USA) were dissolved in ethanol at 50 mg ml^{-1} and dispersed in water to achieve a 1 mM SA and 1 mM JA solutions (Thaler *et al.* 1999). A carrier solution, which consisted of ethanol dispersed in water (5 µl ml^{-1}), was used as a control treatment. Tomato plants were treated immediately after mechanical TYLCV inoculation, when the plants had three to four fully expanded true leaves. There were four chemical treatments in the experiment: SA treatment, JA treatment, SA treatment followed by JA treatment 30 min later, and control solution. About 1 ml SA solution, JA solution or control solution was applied per plant, on average. Each treatment had 30 plants, which were caged in a ventilated insect-proof mesh (1.5-m long, 1.0-m wide, 1.5-m high, 80 mesh) and were maintained about 50 m apart to avoid cross-contamination. Six weeks after treatment, the infection incidence was quantified as described earlier. The experiment was replicated three times.

Statistical analyses

The experimental design consisted of four blocks, and each block was split into paired OTCs, one with elevated CO_2 and one with ambient CO_2. We used a split–split plot analysis of data values within an OTC to avoid pseudoreplication. Treatment effects on infection incidence, disease index, coat protein level, plant TNC content, N content, soluble protein content, C:N ratio, SA, JA and ABA content, height, aboveground biomass and stem diameter were pooled within chambers and evaluated using ANOVA (SPSS 13.0, SPSS lnc., Chicago, IL, USA). Effects were considered significant if $P < 0.05$. The effect of block and the interaction of block and other factors was not significant ($P > 0.29$), and are not presented in order to facilitate data presentation in tables and text. A least significant difference (LSD) test was used to analyse the TYLCV infection rate after chemical treatment (SPSS 13.0). One-Way ANOVA were used to separate the levels within the same variable. Proportional data (infection rate) were arcsine transformed to ensure that data were normally distributed before analysis.

RESULTS

Effect of elevated CO_2 on TYLCV incidence and severity

The infection incidence and disease index of TYLCV were significantly reduced under elevated CO_2 in the OTCs (Fig. 1). Infection incidence was 14.6% lower in 2009 and 11.8% lower in 2010 under elevated CO_2 than under ambient CO_2 (Fig. 1A). The disease index was 20.0% lower in 2009 and 10.4% lower in 2010 under elevated CO_2 than under ambient CO_2 (Fig. 1B).

Effect of elevated CO_2 on TYLCV coat protein

All tested uninfected tomato leaves (controls) were negative 6 weeks after inoculation (OD 405 the same as the negative control). Among virus-inoculated plants, levels of TYLCV coat protein in leaves were 37.3% lower in 2009 and 30.2% lower in 2010 under elevated CO_2 than under ambient CO_2, respectively (Fig. 2A and B).

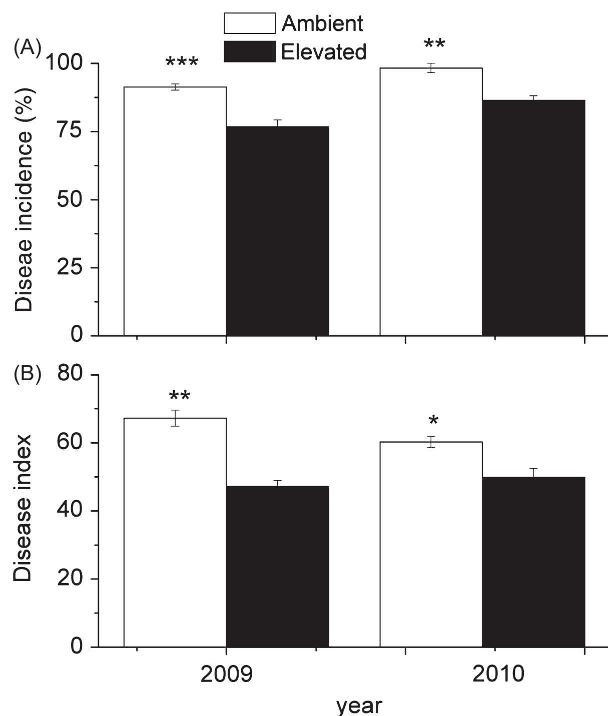

Fig. 1. Tomato yellow leaf curl virus (TYLCV) incidence (A) and severity (B) measured on TYLCVinoculated tomato plants (*L. esculentum*) grown under ambient or elevated CO_2 in 2009 and 2010. Disease incidence refers to the percentage of plants infected, and disease severity refers to the disease index (see Methods for explanation of the disease index). We applied 25 subsamples per OTCs and four OTCs per treatment, values represent means (±SE) of four replicates. Paired treatment bars within a year are significantly different at $P < 0.01$ (**) or $P < 0.05$ (*).

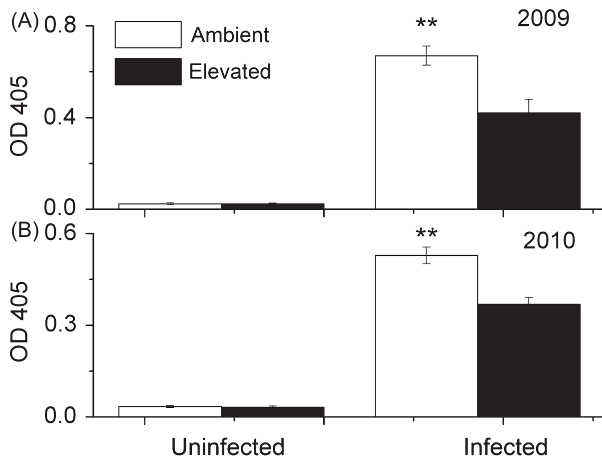

Fig. 2. TYLCV coat-protein levels in inoculated tomato plants grown under ambient or elevated CO_2 in 2009 (A) and 2010 (B). Coat-protein levels were monitored by ELISA 6 weeks after inoculation. The titre of TYLCV coat protein at 405 nm was measured. Values represent means (±SE) of 8 replicates. Paired treatment bars within inoculated plants are significantly different at P < 0.01 (**) or P < 0.05 (*).

Plant aboveground height, fresh weight and stem diameter

Elevated CO_2 increased tomato plant height by 40.8% and 36.5%, and increased the aboveground biomass by 23.3% and 14.3%, in uninfected plants and TYLCV-infected plants in 2009, respectively (Table 2, Fig. 3A and C). Elevated CO_2 increased plant height by 36.9% and 26.0% and increased the aboveground biomass by 53.9% and 28.7%, in uninfected plants and TYLCV-infected plants in 2010, respectively (Table 2, Fig. 3B and D). Under elevated CO_2, the stem diameter of TYLCV-infected plants increased by 6.4% and 7.1% in 2009 and 2010, respectively (Table 2, Fig. 3E and F). TYLCV infection decreased plant height and aboveground biomass under ambient and elevated CO_2 but increased stem diameter under elevated CO_2 (Table 2, Fig. 3).

Table 1. ANOVA results (P-values) for the effects of CO_2 level, *Tomato yellow leaf curl virus* and their interactions on foliar chemical constituents of tomato.

independent variable	dependent variable: nitrogen	TNCs	protein	C:N
CO_2[a]	<0.05*	<0.001***	<0.001***	<0.001***
Virus (V) [b]	0.057	<0.01***	0.237	<0.01**
CO_2 × V	0.148	0.730	<0.01**	0.342

TNCs: total non-structural carbohydrates.
[a]CO_2 levels (ambient and elevated).
[b]virus (uninfected and infected).
Significance levels are indicated by * P < 0.05, ** P < 0.01, and *** P < 0.001.

Foliar chemicals in tomato were significantly altered in elevated CO_2

Elevated CO_2 increased the foliar TNCs by 53.6% and 96.8, and the C/N ratio by 86.9% and 107.7%, in uninfected and TYLCV-infected plants, respectively (Table 1, Fig. 4A and C), whereas virus infection decreased foliar TNC content by 39.0% and 21.8%, and decreased C:N by 40.6% and 34.0% under ambient and elevated CO_2, respectively (Table 1, Fig. 4

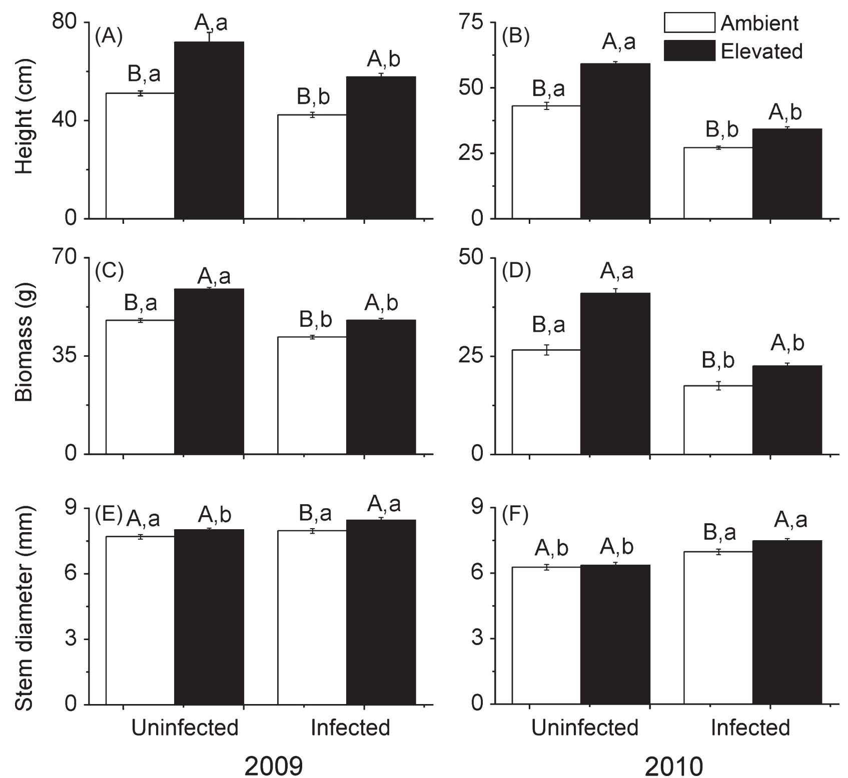

Fig. 3. Plant height (A, B), aboveground biomass (C, D), and stem diameter (E, F) in 2009 and 2010 of tomato plants grown under ambient (375 ppm) or elevated CO_2 (750 ppm) as affected by TYLCV infection. Uninfected = uninfected with virus, infected = TYLCV infected. Values are the means (±SE) of 80 replicates. For each pair of bars, different uppercase letters indicate significant differences between CO_2 levels (oneway ANOVA: df = 1.158; P < 0.05). Different lowercase letters indicate significant differences between virus treatments (plus or minus virus) (one-way ANOVA: df = 1.158; P < 0.05).

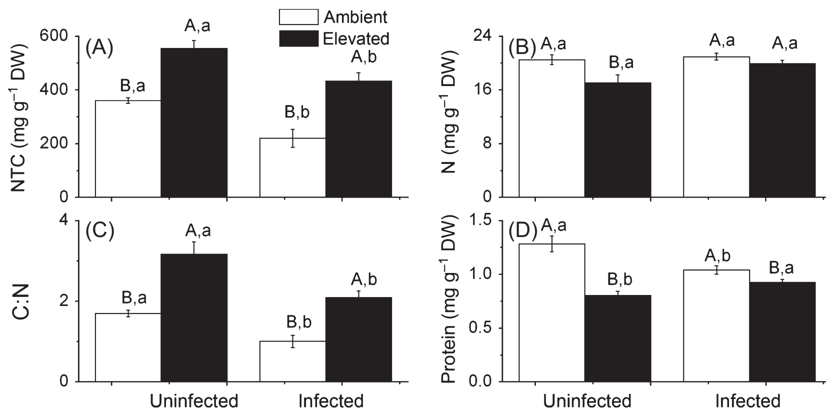

Fig. 4. Chemical content of tomato leaves grown under ambient or elevated CO_2 and as affected by TYLCV infection in 2010. (A) total nonstructural sugar (TNC) content, (B) nitrogen content, (C) C/N and, (D) soluble protein content. Uninfected = uninfected with virus, infected = TYLCV infected. Values are the means (±SE) of 8 replicates. For each pair of bars, different uppercase letters indicate significant differences between CO_2 levels (one-way ANOVA: df = 1.14; P < 0.05). Different lowercase letters indicate significant differences between virus treatments (plus or minus infection) (one-way ANOVA: df = 1.14; P < 0.05).

Table 2. P-values from ANOVA for the effect of CO_2 level, *Tomato yellow leaf curl virus*, time course and their interactions on plant height, aboveground biomass, stem diameter, CPs and phytohormones of tomato.

independent variable	dependent variable						
	height	biomass	stem diameter	CPs	SA	JA	ABA
CO_2[a]	<0.001***	<0.001***	<0.001***	<0.05*	<0.001***	<0.01**	>0.05
Virus (V)[b]	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	>0.05	>0.05
Year (Y)[c]	<0.01**	<0.001***	<0.05*	>0.05	>0.05	<0.01*	<0.05*
CO_2 × V	<0.01**	<0.05*	>0.05	<0.05*	<0.001***	<0.001***	<0.05*
CO_2 × Y	<0.01**	<0.05*	<0.05*	>0.05	>0.05	<0.05*	>0.05
V×Y	<0.001***	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
CO_2 × V × Y	<0.05*	>0.05	<0.05*	>0.05	>0.05	<0.05*	<0.01**

CPs: coat proteins.
Plant phytohormones were free salicylic acid (free SA), conjugate salicylic acid (conjugate SA), jasmonic acid (JA) and abscisic acid (ABA).
[a]CO_2 levels (ambient and elevated).
[b]virus (uninfected and infected).
[c]Year (2009 and2010).
Significance levels are indicated by * P < 0.05, ** P < 0.01, and *** P < 0.001.

A and C). Elevated CO_2 decreased the N content in uninfected plants by 16.8% (Table 1, Fig. 4B). Under elevated CO_2, foliar soluble protein content decreased in uninfected plants and in TYLCV-infected plants (Table 1, Fig. 4D).

Induction of SA, JA and ABA

Elevated CO_2 increased the SA level by 105.6% and 81.7% in uninfected plants, and by 122.7% and 119.1% in TYLCV-infected plants in 2009 and 2010, respectively (Table 2, Fig. 5 A,B,C and D). Virus infection significantly increased SA level by 244.4% and 257.0% under ambient CO_2, and by 264.9% and 331.4% under elevated CO_2 in 2009 and 2010, respectively (Table 2, Fig. 5A and B). Elevated CO_2 reduced JA level by 53.8% and 61.2% in uninfected plants in 2009 and 2010, but increased JA level in virus-infected plants by 166.7% in 2009 (Table 2, Fig. 5C and D). Under ambient CO_2, virus infection decreased the accumulation of JA by 46.2% and 66.2% in 2009 and 2010, respectively; under elevated CO_2, however, the response was positive, with an increase of 266.7% and 196.7% in 2009 and 2010, respectively (Table 2, Fig. 5C and D). Virus infection induced higher ABA levels under elevated CO_2 than under ambient CO_2, and the induction pattern of ABA was similar to that of JA (Table 2, Fig. 5 E and F).

Effects of exogenous SA, JA and SA plus JA on TYLCV incidence

Treatment of TYLCV-inoculated tomato plants with SA plus JA decreased the incidence of virus infection (Fig. 6). Treatment with SA alone also reduced the incidence of infection, but treatment with JA alone did not affect the incidence of infection.

DISCUSSION

Although the direct effects of elevated CO_2 on plant physiology and growth have been well documented (Drake *et al.* 1997; Saxe *et al.* 1998), limited experimental data are available on the effects of increased atmospheric CO_2 on the susceptibility of individual plant species to viruses (Aranda *et al.* 2009). Here, we document a reduction in TYLCV incidence and severity under elevated CO_2 and provide evidence that the reduction in disease is potentially related to changes in leaf chemistry and phytohormones.

In the few studies that have examined pathogen-induced disease under elevated CO_2, the main disease parameters measured have been disease incidence and severity (Thompson & Drake 1994; Mcelrone *et al.* 2005). Our 2-year study (2009, 2010) showed that elevated CO_2 reduced the disease

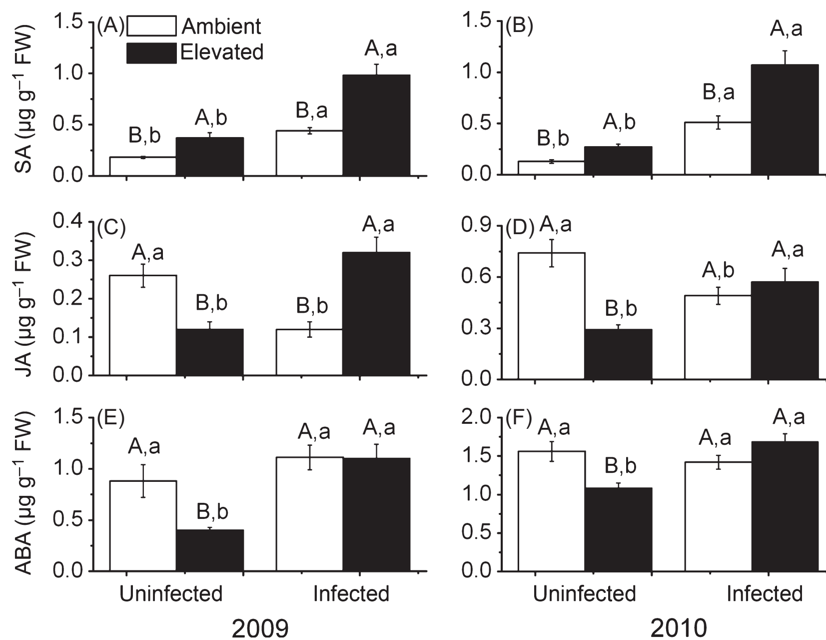

Fig. 5. Levels of endogenous phytohormones in uninfected and TYLCV-infected tomato plants grow under ambient and elevated CO_2 in 2009 and 2010. (A) SA (free salicylic acid) in 2009, (B) SA in 2010, (C) JA (jasmonic acid) in 2009, (D) JA in 2010, (E) ABA (abscisic acid) in 2009 and (F) ABA in 2010. Uninfected = uninfected with virus, infected = TYLCV infected. Values are the means (±SE) of 8 replicates. For each pair of bars, different uppercase letters indicate significant differences between CO_2 levels (one-way ANOVA: df = 1.14; $P < 0.05$). Different lowercase letters indicate significant differences between virus treatments (plus or minus infection) (one-way ANOVA: df = 1.14; $P < 0.05$).

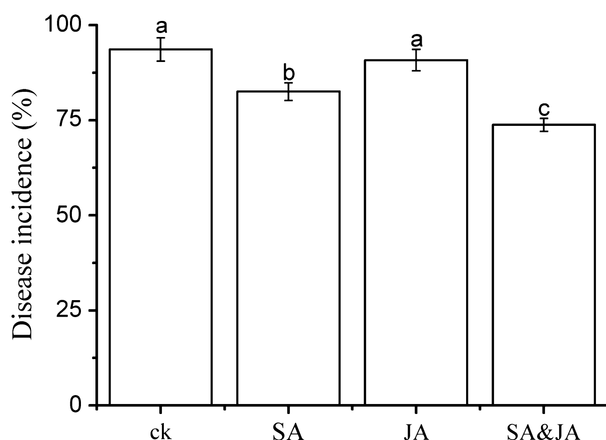

Fig. 6. Virus infection incidence of TYLCV-inoculated plants as affected by exogenous applications of SA, JA, SA followed by JA 30 min later (JA&SA), and control solution (ck). Each treatment was applied to 30 plants and was repeated three times. Values are the means (±SE). Different lowercase letters indicate significant differences between treatments (LSD tests: df = 3.8; $P < 0.05$). Statistical analysis results: F = 6.629, P = 0.015.

incidence and disease severity in TYLCV-inoculated tomato plants and simultaneously decreased the level of viral coat protein in infected plant leaves. Similar results were found by Matros *et al.* (2006), who reported that elevated CO_2 decreased PVY coat protein, which in turn decreased PVY infection in tobacco.

In this study, virus infection markedly decreased plant aboveground biomass in ambient and elevated CO_2, while elevated CO_2 increased the aboveground biomass and stem diameter in infected tomato plants in the two successive years. Additionally, elevated CO_2 also increased plant height in TYLCV-infected plants, which is considered one of the most important parameters in ranking the symptoms of plants infected by viruses (Friedmann *et al.* 1998; Lapidot *et al.* 2001). TYLCV is one of the most economically damaging plant pathogens in tomato worldwide, with yield losses of up to 100% (Zhou *et al.* 2009). The results suggest that yield losses caused by virus in tomato could be alleviated in the future in elevated CO_2 conditions.

Resource availability is the primary factor determining whether there is a surplus of photosynthate available for allocation to plant defence (Bryant *et al.* 1988), and it is clear that C- or N-based nutrient availability regulates the C/N ratio and therefore alters the secondary metabolites in a plant (Dyckmans *et al.* 2000; Matros *et al.* 2006; Fu *et al.* 2010). Plant secondary metabolites, such as plant hormones, play a crucial role in plant resistance to pathogens (Adie *et al.* 2007; Mauch-Mani *et al.* 2008). In the current study, elevated CO_2 increased the foliar TNCs and the C/N ratio in both uninfected and infected plants. Thus, more carbon resources could be utilised for virus defence under elevated CO_2.

Plant hormones (*i.e.* ABA, JA and SA) involved in resistance induced by biotic stress are also likely to be affected by elevated CO_2 (Matros *et al.* 2006; Mauch-Mani *et al.* 2008; Zavala *et al.* 2008). Previous studies reported that elevated CO_2 increased SA/ABA levels, resulting in higher resistance to pathogen stress in some crop species (Jwa & Walling 2001; Matros *et al.* 2006); however, the JA-induced pathway was suppressed by elevated CO_2 in soybean, which compromised plant defence against Japanese beetle (Zavala *et al.* 2008). Moreover, cross-talk among these phytohormone-mediated resistance pathways has also been documented in many studies. ABA was proven to be required for JA biosynthesis, while it was reported to antagonise SA-mediated defences in the process of pathogen infection (Adie *et al.* 2007; Grant *et al.* 2009). The JA and SA defence pathways have been mainly considered mutually antagonistic (Schenk *et al.* 2000; Kunkel & Brooks 2002), but evidence of synergistic interactions has also been reported (Mur *et al.* 2006). In this study, TYLCV infection increased the SA level and suppressed the JA level under ambient CO_2 but increased the SA, JA and ABA levels simultaneously under elevated CO_2. Likewise, SA and JA

levels in plant tissues seem to be antagonistic (when one rises, the other falls) under ambient CO_2 in response to virus infection, but tend to be synergistic under elevated CO_2. Pathogen infection was reported to induce both salicylate-dependent and jasmonate-dependent responses in plants (Kaloshian & Walling 2005; Dong *et al.* 2007), and simultaneously increased SA-mediated and JA-mediated defence responses of plants under elevated O_3 have been demonstrated to enhance resistance to plant viruses (DeLucia *et al.* 2008). Moreover, combined exogenous SA and JA application induced stronger resistance against TYLCV than application of either SA or JA alone. Therefore, the modulated interaction between SA and JA under elevated CO_2 makes a large contribution to lowering TYLCV incidence and severity.

After perceiving a biotic threat, plants fine-tune the balance of defence pathways to orchestrate the 'best' defence response against the intruder (Reymond & Farmer 1998; Kunkel & Brooks 2002). The cross-talk between the SA and JA/ABA pathways is thought to minimise expression of costly and ineffective defences that divert C and N resources from plant vegetative growth (Walling *et al.* 2007). The current research suggests that the induced defence pattern is more effective under elevated CO_2 than under ambient CO_2. To the best of our knowledge, our report is the first to explore the potential mechanism related to modulated plant-induced defence for reduced virus incidence and severity under elevated CO_2. These findings imply that the predicted increases in tomato productivity may be enhanced by reduced susceptibility to plant viruses under projected rising CO_2 conditions.

Studies conducted at OTC facilities allow for realistic disease assessment because plants are exposed to field conditions (Fu *et al.* 2010). Such studies conducted for 2 years also allow us to assess the concurrent effects of variations in temperature and precipitation. The lower plant height, biomass and stem diameter and higher levels of JA and ABA in tomato tissues in 2010 were probably associated with varied microclimatic conditions. High temperature (>35 °C) was reported as a heat stress for tomato growth (Chen *et al.* 1981). Days of high temperature (maximum temperature >35 °C) that occurred in ambient and elevated CO_2 chambers in 2010 were more than in 2009 during the period of the experiment (data not shown), and high temperature might have had adverse effects on plant growth and positive effects on plant hormone induction. Future research with multiple-year field studies will need to consider both the direct impacts of atmospheric change and the resulting alterations in regional climate.

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