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RESEARCH PAPER

Root and shoot gas exchange respond additively to moderate ozone and methyl jasmonate without induction of ethylene: ethylene is induced at higher O_3 concentrations

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Abstract

The available literature is conflicting on the potential protection of plants against ozone (O_3) injury by exogenous jasmonates, including methyl jasmonate (MeJA). Protective antagonistic interactions of O_3 and MeJA have been observed in some systems and purely additive effects in others. Here it is shown that chronic exposure to low to moderate O_3 concentrations (4–114 ppb; 12 h mean) and to MeJA induced additive reductions in carbon assimilation (A_n) and root respiration (R_r), and in calculated whole plant carbon balance. Neither this chronic O_3 regime nor MeJA induced emission of ethylene (ET) from the youngest fully expanded leaves. ET emission was induced by acute 3 h pulse exposure to much higher O_3 concentrations (685 ppb). ET emission was further enhanced in plants treated with MeJA. Responses of growth, allocation, photosynthesis, and respiration to moderate O_3 concentrations and to MeJA appear to be independent and additive, and not associated with emission of ET. These results suggest that responses of Pima cotton to environmentally relevant O_3 are not mediated by signalling pathways associated with ET and MeJA, though these pathways are inducible in this species and exhibit a synergistic O_3×MeJA interaction at very high O_3 concentrations.

Key words: Air pollution, cotton, ethylene, jasmonate, O_3, ozone, plant hormones, signalling.

Introduction

Ambient ozone (O_3) is the most damaging air pollutant to vegetation (Collins *et al.*, 2000; Fuhrer and Booker, 2003), reducing global crop yield in 2000 by up to 121×10^6 ton (Avnery *et al.*, 2011) through a variety of physiological mechanisms (Wilkinson *et al.*, 2012). O_3 generates reactive oxygen species (ROS) in the apoplast, thereby sharing signalling pathways with ROS-mediated responses to biotic challenges (Langebartels *et al.*, 2002; Moeder *et al.*, 2002). These pathways are non-specific (Moons *et al.*, 1997; Baier *et al.*, 2005; Kangasjarvi *et al.*, 2005) and are induced by multiple abiotic stresses including wounding, drought, and salinity, as well as by O_3 (Conconi *et al.*, 1996; Wang *et al.*, 2001; Glazebrook, 2005; Browse, 2009).

Methyl jasmonate (MeJA) is a naturally occurring, volatile signal metabolite that functions within and between plants (Farmer and Ryan, 1990; Sembdner and Parthier, 1993; Seo *et al.*, 2001). Of the numerous jasmonate (JA) derivatives found in plants, the isoleucine conjugate jasmonoyl-isoleucine (JA-Ile) is the active form to which others are converted (Browse, 2009), although some jasmonate precursors appear to have distinct biological activity (Dave and Graham, 2012). MeJA has been investigated as a crop growth regulator, reducing fruit retention force in grape and citrus (Hartmond *et al.*, 2000; Burns *et al.*, 2003; Fidelibus *et al.*, 2007), and protecting salinity-treated barley against oxidant stress (Tsonev *et al.*, 1998; Walia *et al.*, 2007). JA inhibits O_3-induced lesion proliferation, visible leaf injury, and programmed cell death (PCD) in O_3-sensitive *Arabidopsis thaliana* by suppression of ethylene (ET)-associated signalling pathways (Overmyer *et al.*, 2000, 2003; Rao *et al.*,

2000*a*, *b*, 2002; Shoji *et al.*, 2000; Kanna *et al.*, 2003; Tuominen *et al.*, 2004). Sensitivity to O_3 increased in hybrid *Populus* when JA signalling was compromised (Koch *et al.*, 1998, 2000) and decreased in tobacco and *Arabidopsis* when MeJA was applied exogenously (Orvar *et al.*, 1997; Overmyer *et al.*, 2000; Rao *et al.*, 2000*b*; Kanna *et al.*, 2003). These studies suggest that JA and ET are involved in plant response to acute O_3. At moderate O_3, growth and biomass allocation in Pima cotton were inhibited by both O_3 and foliar MeJA, but effects were additive with no protective, anatagonistic interaction (Grantz *et al.*, 2010*b*).

The role of ET in plant response to O_3 is complex. The magnitude of ET emissions is well correlated with the severity of O_3-induced injury (Tingey *et al.*, 1976; Tuomainen *et al.*, 1997; Overmyer *et al.*, 2000; Nakajima *et al.*, 2002; Rao *et al.*, 2002; Tamaoki *et al.*, 2003). ET activates defence pathways (Lamb and Dixon, 1997; Leubner-Metzger *et al.*, 1998), yet overexpression of ET enhanced O_3 sensitivity in *Arabidopsis* (Overmyer *et al.*, 2000). This may be mediated by ET suppression of JA-induced genes (Shoji *et al.*, 2000). Wounding or exposure to O_3 in tomato induces the classic biphasic emission of ET by activating two sets of 1-aminocyclopropane-1-carboxylate synthase (ACS) genes (Tatsuki and Mori, 1999; Moeder *et al.*, 2002). This leads to tissue generation of H_2O_2 and ultimately PCD (Moeder *et al.*, 2002; Browse, 2009) through the hypersensitive response (HR), in response to both pathogens (Pennel and Lamb, 1997; Ciardi *et al.*, 2001) and O_3 (Schraudner *et al.*, 1998; Overmyer *et al.*, 2000; Rao *et al.*, 2000*b*).

Jasmonates play a prominent role in the O_3–ET signalling pathway as downstream mediators (Tamaoki *et al.*, 2003). MeJA and ET exhibit both antagonistic and synergistic interactions in different systems (Shoji *et al.*, 2000; Schmelz *et al.*, 2003). A fundamental interaction of JA and ET is mediated by JAZ proteins which repress transcription of JA-responsive genes and interact with downstream transcription factors that mediate responses to ET (Guo and Ecker, 2004; Zhu *et al.*, 2011; Wager and Browse, 2012). High levels of JA (specifically JA-Ile) target JAZ proteins for degradation, thereby providing positive feedback for JA activity. Many defensive and pathogenesis-related transcription factors respond to either ET or JA (Xu *et al.*, 1994; Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003), while necrotrophic pathogen-associated transcription factors respond only to the combination of ET plus JA (Xu *et al.*, 1994; Penninckx *et al.*, 1998; Alonso *et al.*, 1999; Glazebrook *et al.*, 2003; Glazebrook, 2005; Broekaert *et al.*, 2006; Browse, 2009). JA is an essential component in induction of phytoalexin synthesis in *Cupressus* cultures by yeast elicitor (Zhao *et al.*, 2004).

There is substantial cross-talk among the many components of intraplant signalling networks (Conconi *et al.*, 1996; Baldwin, 1998; Rojo *et al.*, 1999; Kunkel and Brooks, 2002; Glazebrook, 2005; Fujita *et al.*, 2006; Chassot *et al.*, 2008; Spoel and Dong, 2008). For example, responses to JA and abscisic acid are mediated by components of the ET pathway, even in the absence of ET itself (Alonso *et al.*, 1999; Ghassemian *et al.*, 2000). In many species, MeJA induces synthesis of ET. In contrast, induction of jasmonates by ET has been observed only in a few cases (ODonnell *et al.*, 1996; Watanabe *et al.*, 2001).

The role of JA and ET in plant response to environmentally relevant O_3 exposures remains unclear. Here the impacts and potential interactions of O_3 or MeJA on root and shoot metabolism in Pima cotton, and the role of ET synthesis in mediating these impacts at moderate and very high levels of O_3 exposure are evaluated.

Materials and methods

Hypotheses

The impacts of O_3 and MeJA on gas exchange of shoots and roots of Pima cotton, and the role of ET synthesis in these impacts, are evaluated. Three null hypotheses are tested using analysis of variance (ANOVA): (i) H1—root and shoot carbon metabolism do not respond to O_3 or to MeJA; (ii) H2—ET emission from leaves does not respond to O_3 or to MeJA; (iii) H3—there is no antagonistic $O_3 \times$MeJA interaction.

Three experiments were performed, each with replication in time and space. Two were chronic, long-term exposures to a range of low to moderate O_3 concentrations. The first of these provided measurements of leaf area and root and shoot gas exchange (CHRONIC/GASEX). The second used the same O_3 exposure protocol to provide measurements of ET emission from leaves (CHRONIC/ET). The third applied a single acute, pulse exposure to a wide range of low to very high O_3 concentrations, also to provide measurements of ET emission from leaves (ACUTE/ET).

Plant growth

Initially, two cultivars of Pima cotton (*Gossypium barbadense* L.) were used. Seed of cv. Phytogen 800 (P8; Phytogen Seed Company, Indianopolis, IN, USA) was obtained from a commercial source. Seed of cv. S-6 (S6; J.G. Boswell Company, Corcoran, CA, USA) was obtained from foundation seed stock.

Seeds were planted in moist commercial potting mix (Earthgro Potting Soil; Scotts Company, Marysville, OH, USA) in plastic pots (3.8 cm depth×21 cm height) in a research greenhouse (Kearney Research and Extension Center; 103 msl; 36.598'N 119.503'W). Automated drip emitters irrigated all pots to run-through daily and provided a complete fertilizer solution (1.3 g k^{-1} Miracle Gro, Scotts Miracle-Gro Products Inc., Port Washington, NY, USA) to run-through twice weekly (Grantz *et al.*, 2003, 2008, 2010*b*). Plants were grown on an open greenhouse bench until the onset of O_3 exposure.

Methyl jasmonate application

MeJA (Sigma-Aldrich catalogue no. 392707; 95% purity) was brought to 4.36×10^{-3} M by dilution of 1 ml to 1000 ml in deionized water. Plants were treated with either 160 µl (160 µg of MeJA) of the MeJA solution (+MeJA) or with 160 µl of H_2O (–MeJA). The solution of MeJA was vigorously shaken prior to application and appeared as a single phase. The smallest achievable droplets were applied, using a 0.5–250 µl plastic micropipette tip (Finntip 250; Thermo Electron Corp., Vantaa, Finland). Droplets were distributed uniformly over the adaxial surface of the two youngest fully expanded leaves, twice-weekly, near solar noon.

For CHRONIC/GASEX, application of MeJA began at 24 days after planting (DAP), and for CHRONIC/ET at 21 DAP, in both cases in the O_3 exposure chambers. For ACUTE/ET, application of MeJA began at 22 DAP, on the greenhouse bench.

Variations of this method of application have been used previously without inducing local effects at the site of application (Arnold and Schultz, 2002; Henkes *et al.*, 2008; Grantz *et al.*, 2010*b*).

There were no local effects of volatilized MeJA on non-target (–MeJA) control plants located in the same chambers.

Ozone exposure

Exposures to ozone (O_3) were performed in cylindrical O_3 exposure chambers (continuously stirred tank reactors, CSTRs; Heck *et al.*, 1978) situated in a separate bay of the same greenhouse. Nine CSTRs were arrayed in three blocks, parallel to windows and cooling fans.

O_3 was generated by corona discharge (Model SGC-11, Pacific Ozone Technology, Brentwood, CA, USA) from purified oxygen (Series ATF-15, Model 1242, SeQual Technologies Inc., San Diego, CA, USA). Air containing the desired O_3 concentration was introduced into each CSTR at one complete air exchange per minute into the orbit of a 120 rpm circulating fan for uniform distribution. O_3 was regulated in a single CSTR by a dedicated O_3 monitor (Model 49C, Thermo Environmental Instruments, Franklin, MA, USA), with computerized feedback control (Grantz *et al.*, 2003). O_3 concentration in the chambers was a linear function of control voltage (r^2=0.997). O_3 concentration was maintained proportional to the concentration in a single regulated CSTR, and determined in each CSTR four times per hour with a separate O_3 monitor (Model 49C), through continuously purged Teflon dust filters and tubing using a multiport solenoid valve.

CHRONIC/GASEX and CHRONIC/ET exposures were dispensed as daily half-sine waves, with the same nominal exposure each day (7 d week^{-1}). Daily 12 h mean daylight O_3 exposures (07:00–19:00 h) were 4, 59, and 114 ppb, with daily maxima near solar noon of 4, 89, and 163 ppb. These are low to moderate O_3 concentrations.

ACUTE/ET exposures were dispensed as a single square wave pulse (3.0 h duration). Concentrations of 16±2, 270±4, and 685±13 ppb were imposed in a single block of CSTRs. The O_3 control and monitoring system was the same as used for the chronic exposures with the O_3 generator operating at a higher voltage. These are low to very high O_3 concentrations.

For CHRONIC/GASEX, eight plants were transferred to each CSTR (four per cultivar, two +MeJA, two –MeJA). For CHRONIC/ET, two plants were transferred to each CSTR (one +MeJA, one –MeJA; S6 only). Plants were transferred when cotyledons were fully expanded and the first true leaf was emerging.

For ACUTE/ET, uniform plants of each MeJA treatment (S6 only) were transferred to the CSTRs at ~09:00 h, at 55 DAP. The O_3 pulse was imposed between 10:45–13:45 h Pacific daylight time and the plants returned to the greenhouse bench on the same day.

Leaf and root gas exchange

Leaf area per plant (LA) was determined at 51 DAP with a leaf area meter (LI-3000, LI-COR, Lincoln, NE, USA), following excision between the petiole and stem. Root biomass was obtained from Grantz *et al.* (2010*b*).

Gas exchange measurements of net carbon assimilation (A_n) and stomatal conductance (g_s) were obtained near solar noon at 49 DAP, at steady state on the youngest fully expanded leaves (YFLs). Measurements were obtained with a commercially available gas exchange system (LI-6400; LI-COR Inc.), *in situ* in the growth CSTR. Photosynthetic photon flux density (PPFD) was controlled at 1000 μmol photons m^{-2} s^{-1}, provided by 80% red and 20% blue light-emitting diodes (LI-6400-02B). Ambient (reference) CO_2 concentration in the cuvette was controlled at 400 μmol mol^{-1} using complete scrubbing of CO_2 in ambient air and an integrated CO_2 mixing system (LI-6400-01). Leaf temperature and leaf to air vapour pressure deficit were not controlled and were generally 25–30 °C and 2–3 kPa. A_n and g_s were expressed relative to projected leaf area.

Root respiration (R_r) was measured on freshly sampled fine root tips. Electrodes were calibrated using air-saturated H_2O, and oxygen-free H_2O obtained by adding a small amount of sodium dithioinite to each chamber. A 2 ml aliquot of H_2O was placed in each chamber after several rinsings. When output had become stable (~10 min), the terminal 3–4 cm of fine root was excised and immediately transferred to a respirometer chamber.

Measurements were conducted in liquid phase, with a Clark-type oxygen electrode (Delieu and Walker, 1972). Four respirometer chambers (Oxygraph Oxygen Electrode System; PP Systems, Haverhill, MA, USA) were run in parallel, interfaced with a computer for data acquisition and analysis (Grantz *et al.*, 2003). A magnetic stir bar was placed in each chamber, separated from the root material by a laboratory-designed porous metal screen. Temperature control (25 °C) was maintained by circulation of water through a precision water bath (Model 9100, Isotemp, Pittsburgh, PA, USA) and through the plastic housing of each respirometer chamber. Specific root respiration (R_r) was expressed relative to the oven-dry mass of root material in each respirometer chamber, obtained following the measurement.

Ethylene emission

CHRONIC/ET was assayed for ET emission at 54 DAP. ACUTE/ET was assayed at 55 DAP. Incubation for ET emission began immediately following removal from the CSTR in early afternoon. The YFLs were sampled by excising leaf disks with a cork borer (2.3 cm diamter; 4.15 cm^2), avoiding the midrib and large veins. Cut edges of the leaf disks were sealed with petroleum jelly held at the melting point. One leaf disk [~20 mg dry weight (dwt)] was suspended on edge in each glass vial (64.7 ml). Vials were sealed with screw-top, gas-tight serum caps and incubated in darkness for 24 h at 23–24 °C.

At the conclusion of the incubation period, the head space of each vessel was sampled through an air-tight septum with a 22 gauge (0.7 mm) hypodermic needle and a gas-tight syringe. An aliquot of 12 ml was extracted from each vial.

The 12 ml aliquot was injected into a sample collection column of a gas chromatograph (Carle AGC-400, EG and G, Chandler Engineering, Tulsa, OK, USA). A 2.0 ml sample was automatically introduced from the sample collection column into a 30 ml min^{-1} helium carrier gas and passed through an 8% NaCl/alumina column (F-1, 80/100 mesh), held at 70 °C, followed by a flame ionization detector.

The concentration of ET in the sample gas was determined against an authentic standard also injected as 12 ml into the collection column. Peaks were identified by elution time and quantified relative to local baseline. Emission of ET was expressed as ng g dwt^{-1} h^{-1}, using an ET concentration derived from peak height, volume of the incubation vessel, biomass of the leaf sample, and incubation time in the vial. The detection threshold was ~3 ppb, equivalent to < 1 ng g dwt^{-1} h^{-1} in the final units.

Experimental design

CHRONIC/GASEX was performed on four plants per CSTR of each of the two related cultivars (two +MeJA and two –MeJA), and repeated once (n=6). The cultivars did not differ in their responses to O_3 or to MeJA and were pooled as subsamples. The two plants of each cultivar in each O_3×MeJA treatment were also pooled as subsamples. Runs did not differ and were pooled prior to analysis by ANOVA, as a split-plot, randomized complete block design (SAS v. 9.2; PROC GLM), with a non-default error term [O_3×block].

CHRONIC/ET was performed on two plants per CSTR (one +MeJA and one –MeJA) of the single cultivar, Pima S-6. The experiment was repeated twice (n=9). Runs were pooled and analysed as a split-plot, randomized complete block design with no subsamples.

ACUTE/ET was performed on six plants per CSTR (three +MeJA and three –MeJA) of the single cultivar, Pima S-6, and was repeated once (n=6). One sample was discarded as an outlier

(n=5 for the highest O_3). Runs were pooled and analysed by ANOVA as a completely randomized design.

ET emission data were log transformed to approximate more closely a normal distribution prior to ANOVA. In all cases, mean separation ($P < 0.05$) was performed with Duncan's multiple range test.

Results

Leaf responses

The CHRONIC/GASEX protocol led to a systemic response to MeJA, observed as darkly pigmented circular areas (~500 μm diameter) on the adaxial surface of leaves (Fig. 1). This pigmentation was apparent on all leaves of all +MeJA plants, independent of O_3 exposure level, including those leaves younger and older than the two leaves that received direct application (e.g. four leaf insertion levels are shown in Fig. 1). This response was not observed on the cotyledons nor on leaves of control (–MeJA) plants in the same CSTR. MeJA did not induce any localized injury at the sites of foliar microapplication nor additional symptoms on the leaves receiving direct application.

O_3 accelerated leaf senescence, so that O_3-induced visible symptoms of bronzing and purple discoloration were observed on older leaves (not shown). No O_3-induced pigmentation was observed on the two YFLs.

LA was reduced by ~15% by MeJA (Fig. 2; compare the dotted and solid lines) and by ~55% by moderate O_3 in –MeJA plants (Fig. 2; open symbols). Responses of LA to increasing O_3 were parallel in +MeJA and –MeJA plants, so that the highly significant impacts of both factors were strictly additive (i.e. with no significant O_3×MeJA interaction) (Table 1).

Fig. 1. Response of leaf surface appearance of a low O_3-treated plant to application of MeJA (+MeJA) to young leaves of Pima cotton, cv. S-6 in the CHRONIC/GASEX experiment. The discrete dark-pigmented, areas were observed on all leaves of +MeJA plants, and independently of O_3 exposure.

Photosynthetic activity of the YFLs, observed as midday net carbon assimilation (A_n), was reduced by 13% by MeJA at low O_3 (Fig. 3; compare open and filled symbols). A_n was also reduced by O_3 (Fig. 3; open symbols; Table 1). These impacts were both highly significant but these responses to O_3 in the +MeJA and –MeJA plants were also parallel, and effects were strictly additive with no significant O_3×MeJA interaction (Table 1). The effect on A_n of O_3 was much larger than that of MeJA (Fig. 3).

Root responses

In contrast to effects on A_n, the CHRONIC/GASEX protocol stimulated root respiration (R_r) substantially (Fig. 4). The response of R_r to MeJA was greatest (35%) at low O_3, though enhancement was significant at all O_3, and there was no significant O_3×MeJA interaction (Table 1). In contrast to MeJA, O_3 had little effect on R_r, inducing an upward trend of 7–10% (non-significant) in –MeJA plants (Fig. 4; open symbols). O_3 had little effect and MeJA a large effect on R_r, in contrast to effects on A_n (Fig. 4).

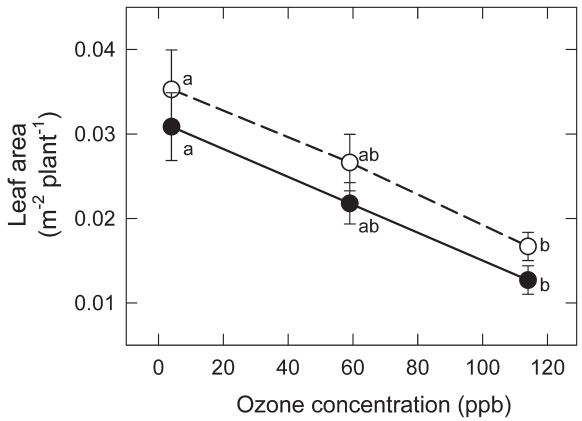

Fig. 2. Response of whole plant leaf area, LA, of Pima cotton to chronic exposure to a range of low to moderate O_3 in the CHRONIC/GASEX experiment, in leaves treated with MeJA (+MeJA; 160 μg plant^{-1}; filled symbols, solid lines) or untreated (–MeJA; open symbols, dashed lines). Points associated with the same letter within a line do not differ ($P < 0.05$). The effects of O_3 and MeJA were highly significant ($P < 0.01$; Table 1).

Table 1. *Analysis of variance (P-values) of midday photosynthetic gas exchange, root respiration, and calculated whole plant carbon balance, following long-term exposure to moderate O_3 in Pima cotton (CHRONIC/GASEX experiment)*

	Pima cotton		
	O_3	MeJA	O_3×MeJA
Leaf area (m^2 plant^{-1})	0.008	0.000	0.945
Assimilation (μmol m^{-2} s^{-1})	0.001	0.000	0.671
Stomatal conductance (μmol m^{-2} s^{-1})	0.000	0.007	0.742
Root respiration (μmol g dwt^{-1} s^{-1})	0.941	0.001	0.623
Carbon balance (μmol plant^{-1})	0.001	0.000	0.469

Shoot productivity and whole root system respiration declined proportionally, for example by ~70% each with increasing O_3 in – MeJA plants. Loss of shoot productivity was driven by similar inhibition of LA and A_n, while declines in root system respiration were driven by O_3 impacts on allocation of biomass to roots. The effects of O_3 and MeJA on A_n and R_r both negatively impacted plant carbon balance (CB), suggesting that impacts on productivity might be more closely associated with CB than with the individual gas exchange components. CB was estimated as the difference between A_n scaled for leaf area, photoperiod, and contribution to canopy carbon acquisition (shoot productivity), and R_r scaled for total root biomass (Grantz *et al.*, 2010*b*) (root system respiration). CB declined by approximately a third in response to MeJA at all O_3 and by ~75% at the highest chronic O_3 in both +MeJA and –MeJA (not shown). Despite substantial additive impacts of MeJA and O_3 on CB, there was no O_3×MeJA interaction (not shown).

The responses of LA, A_n, R_r, and CB allow rejection of hypothesis H1, that growth and metabolism of root and shoot do not respond to O_3 or to MeJA. In contrast, hypothesis H3 cannot be rejected under these conditions of low to moderate chronic O_3 exposure. There was no evidence of O_3×MeJA interaction that could provide the basis for protection by MeJA against ambient O_3.

Ethylene is not induced at moderate O_3 concentrations

The unexpected absence of an antagonistic and potentially protective O_3×MeJA interaction suggested that the required signalling components may by absent in this system. Tests were carried out to determine whether CHRONIC/ET exposure to the range of low to moderate O_3 concentrations affected the JA–ET signalling pathways.

Very low emissions of ET from the YFL (<5 ng g dwt^{-1} h^{-1}) were observed under all combinations of O_3 and MeJA exposure conditions (Fig. 5). There was a slight downward trend in ET emission (non-significant; Table 2) with increasing O_3. Exogenous MeJA also had no significant effect on emission of ET (Fig. 5; Table 2), and the MeJA×O_3 interaction was non-significant (Table 2).

ET was not induced despite the substantial O_3 concentrations imposed (up to 163 ppb, midday hourly average) and clear impacts of both O_3 and MeJA on multiple endpoints. Hypotheses H2 and H3, that ET is not induced by MeJA or moderate O_3, and that there is no antagonistic O_3×MeJA interaction under these conditions, cannot be rejected. This result provides a potential rationale for the lack

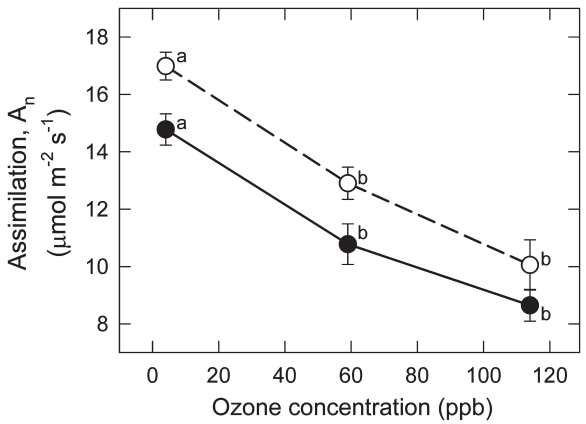

Fig. 3. Response of net carbon assimilation, A_n, of Pima cotton to chronic exposure to a range of low to moderate O_3 in the CHRONIC/GASEX experiment, in leaves treated with MeJA (+MeJA; 160 μg plant^{-1}; filled symbols, solid lines) or untreated (–MeJA; open symbols, dashed lines). Points associated with the same letter within a line do not differ ($P < 0.05$). The effects of O_3 and MeJA were highly significant ($P < 0.01$; Table 1).

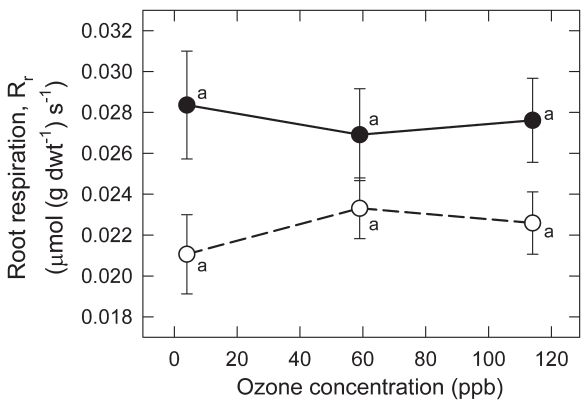

Fig. 4. Response of root respiration, R_r, of Pima cotton to chronic exposure to a range of low to moderate O_3 in the CHRONIC/GASEX experiment, in leaves treated with MeJA (+MeJA; 160 μg plant^{-1}; filled symbols, solid lines) or untreated (–MeJA; open symbols, dashed lines). Points associated with the same letter within a line do not differ ($P < .05$). The effect of MeJA was highly significant ($P < 0.01$; Table 1).

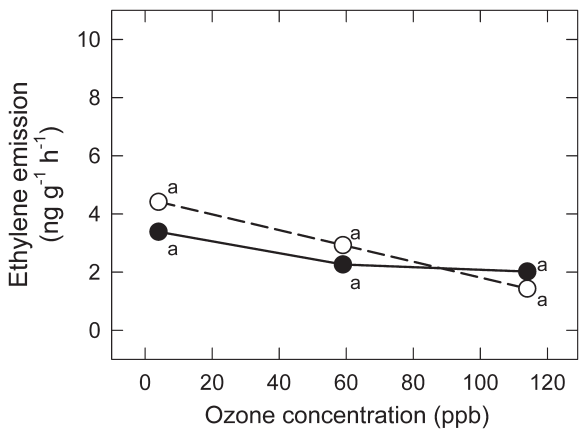

Fig. 5. Response of emission rate of ET (dry weight basis) of Pima cotton to (a) chronic exposure to a range of low to moderate O_3 in the CHRONIC/ET experiment, in leaves treated with MeJA (+MeJA; 160 μg plant^{-1}; filled symbols, solid lines) or untreated (–MeJA; open symbols, dashed lines). Points associated with the same letter within a line do not differ ($P < 0.05$). Neither the effect of O_3 nor the effect of MeJA, nor the O_3×MeJA interaction, was significant (Table 2).

of protective O_3×MeJA interaction under the CHRONIC/GASEX conditions.

Ethylene is induced at very high O_3 concentrations

The absence of ET emission in CHRONIC/ET raised the question of whether the leaves of Pima cotton are competent in O_3-induced ET emission and the HR and PCD signalling pathways that MeJA could influence. This was tested in plants exposed to a 3 h square wave pulse to a broad range of low to very high O_3 concentrations (16±2, 270±4, and 685±13 ppb).

At low O_3 (14 ppb and 16 ppb), the results of CHRONIC/ET and ACUTE/ET were consistent, with similar ET emissions (2.74 ng g dwt^{-1} h^{-1} and 2.66 ng g dwt^{-1} h^{-1}; compare Figs 5 and 6). The intermediate O_3 concentration in the pulse protocol (270 ppb) was greater than twice the concentration at the high end of the moderate exposure regime. These exposures did not induce a significant increase in ET emission, in either –MeJA or +MeJA leaves, though both exposures are well above typical ambient levels.

At the highest acute O_3 exposure (685 ppb) in the ACUTE/ET protocol, ET emission was induced by O_3, increasing significantly by 324% (Fig. 6; open symbols) in –MeJA leaves, and by 1604% in +MeJA leaves (Fig. 6; filled symbols). The O_3 effect on ET emission was highly significant (Table 2), regardless of whether ET emissions were expressed relative to leaf mass (Fig. 6) or leaf area (not shown).

The MeJA stimulation of ET emission was also significant (Table 2). MeJA enhanced ET emissions by 13% at low O_3, but this increased to 45% and 354% in +MeJA leaves (relative to –MeJA leaves) at successively higher O_3 exposures (Fig. 6). This O_3×MeJA interaction ($P = 0.094$; Table 2) was not observed under more moderate O_3 exposure regimes.

At very high O_3, both hypotheses H2 and H3 may be rejected. Pima cotton exhibited significant induction of ET signaling pathways under appropriate conditions of O_3 exposure.

Table 2. *Analysis of variance (P-values) of ethylene emission following long-term exposure to moderate O_3 or pulse exposure to high O_3 (CHRONIC/ET and ACUTE/ET experiments)*

		O_3	MeJA	O_3×MeJA
Long-term moderate O_3 (n=9)	Log ethylene emission [log (ng g^{-1} h^{-1})]	0.304	0.581	0.402
Pulse high O_3 (n=6)	Log ethylene emission [log (ng g^{-1} h^{-1})]	0.000	0.017	0.094

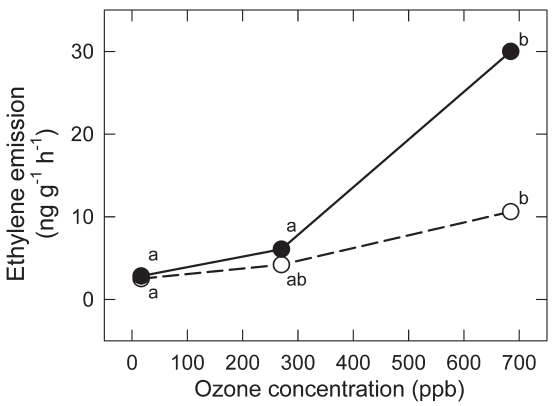

Fig. 6. Response of emission rate of ET (dry weight basis) of Pima cotton to an acute pulse exposure to a range of low to very high O_3 in the ACUTE/ET experiment, in leaves treated with MeJA (+MeJA; 160 μg $plant^{-1}$; filled symbols, solid lines) or untreated (–MeJA; open symbols, dashed lines). Points associated with the same letter within a line do not differ ($P < 0.05$). Both the effect of O_3 and the effect of MeJA were significant ($P < 0.01$; Table 2). The O_3×MeJA interaction was significant at $P < 0.10$ ($P = 0.094$; Table 2).

Discussion

Acute and chronic exposures to O_3 have been shown to reduce productivity, photosynthetic gas exchange, and allocation of biomass below-ground (Cooley and Manning, 1987; Reiling and Davison, 1992; Grantz *et al.*, 2006; Booker *et al.*, 2009; Chen *et al.*, 2009). While the spatial heterogeneity of foliar symptoms may be more pronounced under shorter regimes of higher O_3 concentration than under chronic exposure protocols (Chen *et al.*, 2009), differences in the induction of signalling pathways are not well defined. This may lead to potential confusion in development of protective strategies based on emerging knowledge of signalling metabolites.

Growth and gas exchange

A range of MeJA application rates produced concentration-specific effects on growth and allocation in Pima cotton (Grantz *et al.*, 2010*b*). These paralleled those induced by moderate O_3. These ranges of O_3 and MeJA did not induce necrotic lesions indicative of HR but were sufficient to induce systemic impacts. Similar responses to O_3 and to MeJA of leaf and root tip gas exchange were observed in the present study. For all endpoints examined to date, including growth, gas exchange, and ET emission, there was no O_3×MeJA interaction at moderate O_3.

Leaf tissues in the YFLs have direct access to both O_3 and MeJA through stomatal uptake and cuticular diffusion. Above-ground processes are more accessible and thus typically better characterized than their below-ground counterparts. In the present study, O_3 reduced A_n of the YFLs and LA of the whole plant substantially, consistent with previous observations of gas exchange, leaf chlorophyll, and shoot growth, both in this species (Grantz and

Yang, 1996; Grantz and Shrestha, 2006; Grantz *et al.*, 2006, 2010*b*) and in many others (Cooley and Manning, 1987; Reiling and Davison, 1992; Morgan *et al.*, 2003; Ashmore, 2005; Booker *et al.*, 2009). O_3 impacts on A_n are associated with reduced transcript, protein, and activity of Rubisco (Dann and Pell, 1989).

A_n was also substantially reduced by MeJA, consistent with previous observations in other species (Wiedhase *et al.*, 1987; Staswick *et al.*, 1992; Creelman and Mullet, 1995; Tung *et al.*, 1996; Arnold and Schultz, 2002; Henkes *et al.*, 2008).

Below-ground structures have no direct access to O_3 or O_3 breakdown products (Turner *et al.*, 1973), nor to MeJA. Root impacts are also more difficult to characterize than those in the shoot. In the present study, O_3 had only a modest stimulatory effect on R_r, but a large inhibitory impact on root biomass (Grantz *et al.*, 2010*b*), while MeJA had a large stimulatory impact on R_r and a modest inhibition of root biomass. In previous studies, R_r increased significantly with increasing O_3 exposure in Pima cotton and muskmelon (*Cucumis melo* L.; Grantz *et al.*, 2003), but little impact on R_r was observed in yellow nutsedge (*Cyperus esculentus*; Grantz *et al.*, 2010*a*). The responses to O_3 by the root system were similar to earlier observations (Cooley and Manning, 1987; Reiling and Davison, 1992; Grantz *et al.*, 2006). Variability in physiological processes below-ground is typically large (Bryla *et al.*, 1997; Lambers *et al.*, 2002), and often contributes the largest errors in determination of whole plant CB (Ryan, 1991).

Jasmonates down-regulate core metabolism and photosynthesis, reduce allocation below-ground, and accelerate senescence, while up-regulating production of feeding deterrents and toxins (Herms and Mattson, 1992; Feys *et al.*, 1994; Berger *et al.*, 1996; Henkes *et al.*, 2008; Browse, 2009). The below-ground responses to MeJA observed in the present study were consistent with earlier observations (Staswick *et al.*, 1992; Creelman and Mullet, 1995; Tung *et al.*, 1996; Arnold and Schultz, 2002; Henkes *et al.*, 2008; Grantz *et al.*, 2010*b*). In *Arabidopsis*, these responses are regulated by both ET and MeJA (Schmidt *et al.*, 2010).

Reductions in the estimates of whole plant CB induced by both O_3 and MeJA were substantial. Nevertheless, at moderate O_3 there was no O_3×MeJA interaction. Root system respiration was highest and shoot productivity was lowest in the +MeJA plants subjected to the highest chronic O_3 exposure. These whole plant impacts were driven nearly equally by effects on total shoot productivity and total root system respiration. However, the responses of these components were driven differently by changes in carbon allocation (dominant for roots) and in physiological activity (co-dominant with allocation in shoots). In C_3 and C_4 grasses, a similar estimate of CB declined in response to simulated herbivory (Thorne and Frank, 2009). In yellow nutsedge, CB was positively correlated with reproductive output (Grantz *et al.*, 2010*a*). Further evaluation of the CB parameter, with appropriate measurements at whole shoot and root system scales, may provide considerable insight into the impacts of O_3 on vegetation.

Ethylene

ET emission is highly correlated with O_3 injury (Tingey *et al.*, 1976; Tamaoki *et al.*, 2003), and clearly linked to induction of HR and PCD (Overmyer *et al.*, 2000; Rao *et al.*, 2000*b*). Lesion proliferation is limited by JA (Kanna *et al.*, 2003; Overmyer *et al.*, 2003; Tuominen *et al.*, 2004), apparently serving to deny palatable necrotic tissue to necrotrophs.

A potential explanation for the consistent lack of protection by MeJA against moderate O_3 exposure in Pima cotton is that the JA–ET signalling network is not induced. This is supported by the present data. The exposures to moderate O_3 or MeJA did not induce ET signalling. The alternative explanation, excessive cuticular or stomatal resistance that prevented penetration of either material, can be dismissed based on the systemic responses to both O_3 and MeJA that were observed. Another alternative explanation can be dismissed based on the ACUTE/ET protocol. ET was assayed after 4 weeks of exposure in the chronic experiment, and after 3 h of exposure in the pulse protocol, suggesting that a burst of ET at the onset of moderate O_3 exposure could have been overlooked. However, the intermediate pulse exposure (270 ppb) did not elicit emission of ET, though the O_3 concentration was substantially higher than the highest O_3 in the chronic exposure.

Despite the lack of ET signalling at moderate O_3, the presence of these pathways and the sensivity of ET emission in Pima cotton to O_3 were confirmed under very high O_3 exposure conditions, using the acute 3 h square wave pulse exposure. In both the CHRONIC/ET and ACUTE/ET experiments, the low and intermediate O_3 exposures were consistent in not inducing significant emission of ET. Only at much higher O_3 (685 ppb) was ET induced and a synergistic O_3×MeJA interaction observed. This interaction does not imply protection by MeJA, since ET emission is associated with O_3 injury (Tingey *et al.*, 1976; Tuomainen *et al.*, 1997; Overmyer *et al.*, 2000; Nakajima *et al.*, 2002; Rao *et al.*, 2002; Tamaoki *et al.*, 2003).

In Pima cotton, exposure to O_3 at ambient levels or slightly above induces substantial developmental and physiological responses without inducing emission of ET. These responses were consistently additive and independent of ET induction. At higher O_3, ET is induced and an O_3×MeJA interaction is observed. Superambient O_3 may be required to elicit the ET and JA coordination of responses to O_3 that are well known in responses to other biotic and abiotic stresses, mediated through PCD and HR (Kangasjarvi *et al.*, 1994, 2005; Schlagnhaufer *et al.*, 1995; Overmyer *et al.*, 2005). The present data appear to rule out application of MeJA as an anti-ozonant in Pima cotton under current global conditions.

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