Plant, Cell and Environment (2007) **30**, 654–661 doi: 10.1111/j.1365-3040.2007.01646.x

Isoprene synthase expression and protein levels are reduced under elevated O_3 but not under elevated CO_2 (FACE) in field-grown aspen trees

CARLO CALFAPIETRA[1,2], AMY E. WIBERLEY[2], TANYA G. FALBEL[2], AUTUMN R. LINSKEY[2], GIUSEPPE SCARASCIA MUGNOZZA[1,3], DAVID F. KARNOSKY[4], FRANCESCO LORETO[3] & THOMAS D. SHARKEY[2]

[1]University of Tuscia, Department of Forest Environment and Resources (DISAFRI), Via S.Camillo de Lellis snc, 01100 Viterbo, Italy, [2]University of Wisconsin, Department of Botany, 430 Lincoln Dr, Madison, WI 53706, USA, [3]CNR – Istituto di Biologia Agroambientale e Forestale, Via Salaria Km. 29.300 00016 Monterotondo Scalo (Roma), Italy and [4]Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931, USA

ABSTRACT

Emission of hydrocarbons by trees has a crucial role in the oxidizing potential of the atmosphere. In particular, isoprene oxidation leads to the formation of tropospheric ozone and other secondary pollutants. It is expected that changes in the composition of the atmosphere will influence the emission rate of isoprene, which may in turn feedback on the accumulation of pollutants and greenhouse gases. We investigated the isoprene synthase (*ISPS*) gene expression and the ISPS protein levels in aspen trees exposed to elevated ozone (O_3) and/or elevated carbon dioxide (CO_2) in field-grown trees at the Aspen Free-Air Carbon Dioxide Enrichment (FACE) experimental site. Elevated O_3 reduced *ISPS* mRNA and the amount of ISPS protein in aspen leaves, whereas elevated CO_2 had no significant effect. Aspen clones with different O_3 sensitivity showed different levels of inhibition under elevated O_3 conditions. The drop in ISPS protein levels induced a drop in the isoprene emission rate under elevated O_3. However, the data indicated that other mechanisms also contributed to the observed strong inhibition of isoprene emission under elevated O_3.

Key-words: *Populus*; forestry plantations; global change; VOC.

INTRODUCTION

Isoprene is the main non-methane volatile organic compound (VOC) emitted by plants (Guenther *et al.* 2000). It is estimated that VOC emission from plants accounts for 503 Tg C $year^{-1}$, almost double the anthropogenic emission (Guenther *et al.* 1995). Most of this carbon is emitted as isoprene, especially by poplars, aspen, oaks and eucalyptus trees, with additional emissions of monoterpenes from conifers and a limited number of broadleaved species.

Isoprene is rapidly oxidized in the atmosphere leading to the formation of ozone and other secondary pollutants when anthropogenic nitrogen oxides and sunlight are present, thus affecting the atmospheric composition (Chameides *et al.* 1988; Monson 2002). Changes in the atmospheric composition (IPCC 2001) are expected to affect the capacity of plants to emit such compounds. Constable *et al.* (1999) estimated that isoprene emission will increase by 80% by simulating an increase in temperature coupled with a doubling in atmospheric CO_2 concentration. However, the estimations at large scale may differ from the effects at the leaf level because of the indirect effects of global change on leaf area index (LAI), canopy temperature, water and nutrient availability, and length of growing season.

At the leaf level, the increase in CO_2 concentration generally induces a decrease in isoprene emission both in closed environments (Rosenstiel *et al.* 2003; Pegoraro *et al.* 2004; Possell, Hewitt & Beerling 2005) and in open ecosystems (Centritto *et al.* 2004; Scholefield *et al.* 2004). This has been attributed to a decrease in the availability of one of the precursors in the isoprene synthesis pathway (pyruvate) occurring under elevated CO_2 (Rosenstiel *et al.* 2003). However, Scholefield *et al.* (2004) demonstrated a strong inhibitory effect of elevated CO_2 on isoprene synthase (ISPS) activity, the enzyme responsible for isoprene formation. Whether this resulted from low substrate availability or was the cause for the general reduction of isoprene biosynthesis is still unknown. The data on monoterpene synthases suggest that these enzymes might have an important role in explaining the drop of emission under elevated CO_2 of VOCs formed by the same biosynthetic pathway as isoprene (Loreto *et al.* 2001a).

Even less is known about the effect of ozone (O_3) on the ISPS and on the rate of isoprene emission by plants. In laboratory experiments, ozone fumigation was found to stimulate emission of monoterpenes in *Quercus ilex* (Loreto *et al.* 2004) and to decrease emission of isoprene in *Quercus pubescens* (Velikova *et al.* 2005). In addition, leaves emitting isoprene showed less damage by high O_3 concentrations than non-emitting leaves (Loreto & Velikova 2001). A species-specific effect was found in monoterpene emission rates from Mediterranean species

Correspondence: C. Calfapietra. Fax: +39 0761 357389; e-mail: carlocalf@unitus.it

exposed to elevated O_3 concentrations in open top chambers (Llusià, Peñuelas & Gimeno 2002). The interaction between elevated CO_2 and O_3 concentrations on VOCs was studied in an open top chamber experiment on birch (Vuorinen *et al.* 2005), although no clear effect on the emission of the different compounds was found.

The aim of the present study was to analyse how ISPS in aspen is affected by increases in tropospheric CO_2 and O_3 concentration and how this could in turn affect the rate of isoprene emission. We carried out our study in a free-air CO_2 and O_3 enrichment (FACE) site where global change is simulated in a natural environment conserving the interactions between plant and the surrounding environment. Firstly, we measured mRNA levels to gain insight into a possible transcriptional regulation. Secondly, we determined the amount of ISPS protein present in the leaves. Thirdly, we related ISPS amount with isoprene emission rates measured from a subsample of the leaves. We sampled from clones with different ozone sensitivity available at the Aspen FACE site, previously selected from wild populations in the Great Lakes Region (Isebrands *et al.* 2001).

MATERIALS AND METHODS

Experimental treatments and leaf samplings

The study was carried out at the Aspen FACE site located near Rhinelander in northern Wisconsin, where elevated atmospheric CO_2 and/or elevated O_3 concentrations have been maintained in an aggrading aspen ecosystem since 1998, using the FACE technique (Karnosky *et al.* 1999; Dickson *et al.* 2000). The experimental plots included elevated CO_2 and elevated O_3 treatments in the following combinations: control (ambient CO_2 and ambient O_3 concentrations), CO_2 (elevated CO_2 and ambient O_3 concentrations), O_3 (ambient CO_2 and elevated O_3 concentrations) and $CO_2 + O_3$ (elevated CO_2 and elevated O_3 concentrations). The ambient CO_2 concentration is about 370 μL L^{-1} and the target is 560 μL L^{-1} in the elevated CO_2 plots, whereas the O_3 concentration in the elevated O_3 treatment is kept at about 1.5× ambient O_3 concentration. The mean ambient O_3 concentration during the growing season before leaf gas exchange measurements was 45.65 ppbv (hourly average for the period 0900–1700 h). Accumulated ozone exposure over a threshold of 40 ppb (AOT 40) was 3.88 μL L^{-1} h in the ambient O_3 plots and 9.54 μL L^{-1} h as average in the elevated O_3 plots for the 30 d period preceding the leaf gas exchange measurements. AOT 40 is an exposure-plant response index function set by the United Nations Economic Commission for Europe (UNECE) and US-Environmental Protection Agency. It is calculated as the sum of differences between the hourly averaged O_3 concentration and the threshold value of 40 ppb for each hour that the averaged O_3 concentration exceeds 40 ppb.

Different species are present in each experimental plot, but this study focused on the pure aspen stand (*Populus tremuloides* Michx.).

Thirty mature leaves exposed to full sun were collected in each of the four treatment combinations described above from different aspen clones in order to take into account a broad range of genotypic variability, especially with regard to ozone sensitivity. Leaves were collected in equal number from the following clones: 8L, 271, 216 and 42E, where the first two are considered tolerant to ozone, 216 moderately tolerant and 42E sensitive (Coleman *et al.* 1995a,b; Isebrands *et al.* 2001). The leaves were collected on 15 June, 13–15 July and 15 August 2005, and values were pooled to represent mean summer conditions. The leaves were collected in the central hours of the day (1000–1600 h) to avoid a timing effect observed in a similar unpublished experiment at the *ISPS* transcript levels. Furthermore, leaves at similar developmental stages were sampled in this study as a clear pattern in the *ISPS* transcript levels has been observed moving from the leaves at the top to the ones at the bottom of the stem (Wiberley *et al.* 2005).

Leaf punches (Ø 12 mm) were obtained avoiding the main veins and were immediately frozen in dry ice before being stored at –80 °C.

ISPS gene expression

Total RNA was extracted for each leaf by grinding two or three leaf punches in liquid nitrogen and subsequently following the protocol by Chang, Puryear & Cairney (1993) and its modifications by Haruta, Pedersen & Constabel (2001).

RNA concentration was determined using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA), and 0.625 μg RNA from each sample was reverse transcribed using oligo(dT)15 primer and M-MLV reverse transcriptase (RT) according to the manufacturer's instructions (Promega Corp., Madison, WI, USA).

Before the RT, total RNA was treated with RQ1 RNase-Free DNase according to the manufacturer's instructions (Promega Corp.), in order to remove genomic DNA previously detected in some samples. Quantitative polymerase chain reaction (QPCR) was carried out on 1 μL of the RT product, using an Mx3000P real-time (RT)-PCR system with Brilliant SYBR green QPCR master mix (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions.

The primers used for the analysis of the *ISPS* gene were: forward 5′ – CAGAAACAGAAACAGAAACCAGAC – 3′; reverse 5′ – GGACATTATCTATCAGTTCAAGCAGA – 3′. The resulting PCR segment length was 201 bp. The thermal profile was set with a hot start (10 min, 95 °C); 40 PCR cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s; 95 °C for 1 min; 55 °C for 30 s.

The target sequence had previously been amplified from a poplar RNA preparation and reverse transcription, with concentration determined as described for RNA extracts.

Dilutions of this, each containing a known number of copies of the target amplicon, were used to create a standard curve to quantify the QPCR product in the leaf samples as described in Wiberley *et al.* (2005).

A number of samples (including the ones showing the highest and the lowest numbers of copies of the target amplicon) have been tested with regular PCR using RT products with the 18 S-pair primers to detect possible bias in total RNA amount sent to the RT.

ISPS protein amount

Total soluble protein was extracted grinding one leaf punch per sample in 200 μL of sample loading buffer (1 M Tris-HCl, pH 6.8; 10% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue). Before loading the protein gels, each sample was vortexed and heated at 70 °C for 10 min. The protocol described by Wiberley *et al.* (2005) was followed to load the protein gels and to blot proteins onto membranes. A polyclonal primary antibody for ISPS was generated using ISPS of *Populus trichocarpa*. *P. trichocarpa ISPS* was PCR amplified from *P. trichocarpa* total RNA sample previously shown to have many copies of *ISPS* message. Pfu polymerase and the following primer pairs were used: the forward primer introduced an NcoI cloning site just upstream of the chloroP prediction for the chloroplast transit peptide cleavage site 5′ TACCAT GGCATGTTCTGTAAGCACAGAAAACGT-3′ and the reverse primer put a NotI site just after the C-terminus of the protein, with no stop codon 5′ CAGCGGCCGC TCTCTCAAAGGGTAGAATAGGCTC-3′. This PCR product was cloned into pET28a expression vector (Novagen, Madison, WI, USA) resulting in a hexahistidine tag fused to the C-terminus of the bacterially expressed protein. The construct was transformed into *E. coli* strain BL21(DE3) and was induced for 6 to 8 hours at room temperature (25 °C) with 0.75 mM IPTG. The protein was purified from bacterial pellets using standard procedures on a Ni-NTA resin, with the addition of 20% glycerol to the lysis and wash buffers, and 50% glycerol to the 250 mM imidazole elution buffer. This resulted in an enzymatically active preparation of ISPS that was used to generate rabbit polyclonal antisera by Covance, Inc. (Denver, PA, USA). The antibodies were highly specific for ISPS.

The primary antibody was diluted 1:5000, and the secondary donkey anti-rabbit antibody coupled to horseradish peroxidase was diluted 1:2500. Blots were exposed to Kodak (Rochester, NY, USA) Biomax MS X-ray film for 30 s–3 min. Membranes were stained with Coomassie blue to visualize all of the protein and check for equal loading. The concentration of *Populus* ISPS protein used as a standard was determined by Bradford (1976) assay. Western blot films were scanned and bands were quantitated using Adobe Photoshop version 6.0 (Adobe Systems Incorporated, San Jose, CA, USA). Relative band weight was defined as the ratio between the weight (number of pixels divided by the relative luminosity) of ISPS protein band of each sample and the weight of the ISPS protein band of the standard.

A calibration curve was obtained by loading the ISPS protein standard described earlier at a concentration of one-fourth-, one-half-, one-, two-, four-, eightfold (relative protein concentrations) the one used in all membranes for reference (3.7 ng μL^{-1}). The relationship between relative band weight and relative protein concentration was linear until a relative band weight of about 4 ($y = 0.956x$, $R^2 = 0.988$, $P = 0.0005$). Thus, the samples with a relative band weight above 4 (about 5% of the total) were reloaded on gel with lower concentrations in order to obtain bands falling in the linear phase.

Leaf gas exchange and isoprene emission

Leaf gas exchange measurements were conducted in the period 13–15 July 2005 on the leaves collected for ISPS analyses. Before leaf collection, leaf gas exchange was measured in the field as described by Wolfertz *et al.* (2003). A Li-Cor 6400 (Li-Cor, Inc., Lincoln, NE, USA) was used with the leaf temperature set at 30 °C and the photosynthetic photon flux density (PPFD) at 1000 μmol m^{-2} s^{-1}. Ambient air was used with O_3 concentration as high as the growth conditions. CO_2, provided by a cartridge, was set at 370 μL L^{-1} for all measurements in order to focus on the long-term adaptation of the leaf to elevated CO_2 rather than on a direct effect of CO_2 concentration on the isoprene synthesis, which has been more investigated so far (Loreto & Sharkey 1993; Scholefield *et al.* 2004). This could hide a possible effect of CO_2 concentration on g_s, which, however, is not likely to affect isoprene emission (Monson & Fall 1989). Furthermore, the possible effect of that on leaf temperature is offset, maintaining the leaf at constant temperature (30 °C). Air flow was set at 300 μmol s^{-1}, allowing a frequent replacement of the air in the cuvette. Ten millilitres of the air exiting the leaf cuvette were collected by syringe and analysed for isoprene content by gas chromatography as described by Loreto & Sharkey (1993) with the following modifications: the column was maintained at 52 °C, and the liquid isoprene standard was serially diluted to 128 nmol mol^{-1} in N_2. Leaf isoprene emission rates were calculated as described by Singsaas *et al.* (1997).

Statistics

A two-way analysis of variance (ANOVA) was carried out to determine the effects of elevated CO_2, elevated O_3 and their interaction on *ISPS* mRNA copy numbers, ISPS protein levels, leaf gas exchange and isoprene emission. Statistical analyses were performed in SYSTAT 11 (Systat Software, Inc., Richmond, CA, USA) using general linear models procedure. Thereafter, the significance within each combination of CO_2 and O_3 treatments was calculated using Bonferroni's multiple comparison procedure. Differences between means were considered significant at $P < 0.05$.

Percentage effects of O_3 treatment on *ISPS* mRNA copy numbers and protein amounts were calculated for the four clones separately to provide additional information, and differences between elevated and ambient O_3 were tested with a *t*-test and considered significant at $P < 0.05$. The elevated O_3 effect was presented in percentage as the ratio of the mean values in (elevated O_3 – ambient O_3)/ambient O_3.

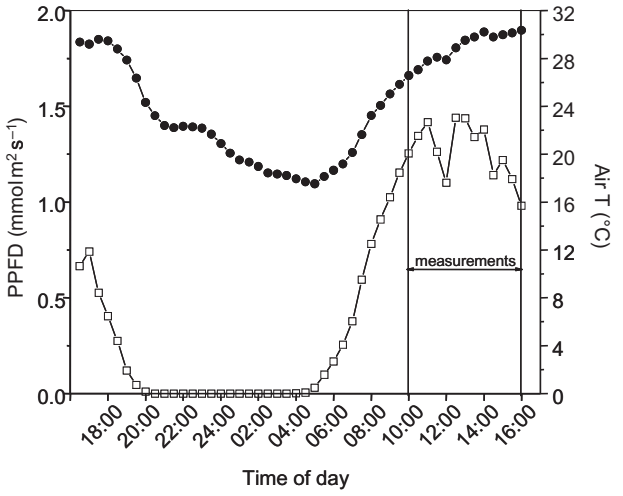


Figure 1. Average daily meteorological conditions measured at the Aspen Free-Air Carbon Dioxide Enrichment (FACE) site from 13 to 15 July 2005 when leaf gas exchange measurements were carried out (the values averaged over the 3 d of measurements). Photosynthetic photon flux density (PPFD, open squares) and air temperature (T, filled circles) measured during sampling and in the 24 h before are shown.

Regression lines and relative statistics were realized with SigmaPlot version 10.0 (Systat Software, Inc.).

RESULTS

Meteorological data measured at the site showed that the measurements were carried out in typical summer conditions although with a maximum temperature of 30 °C, slightly above normal for Northern Wisconsin (Fig. 1). Meteorological conditions were stable during the field campaign (Fig. 1), and temperature and light were artificially controlled inside the cuvette during the leaf gas exchange measurements.

The number of copies of *ISPS* mRNA (Fig. 2) dropped significantly under elevated O_3 ($P = 0.02$), whereas elevated CO_2 did not show any effect ($P = 0.68$). The percentage decrease under elevated O_3 was higher for 42E and 216 than 8L and 271 (Fig. 2).

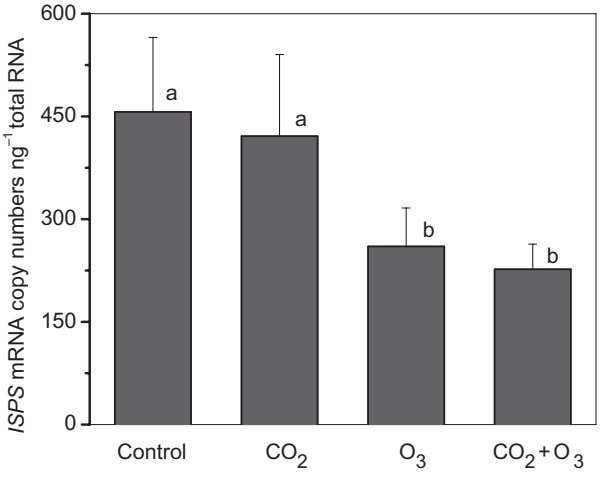
Similar to *ISPS* mRNA number of copies, the levels of ISPS protein (Fig. 3) in the leaves were significantly reduced under elevated O_3 ($P = 0.03$). However, the multiple comparison revealed that the effect was significant only under ambient CO_2. No effect of elevated CO_2 was observed ($P = 0.31$). The percentage decrease under elevated O_3 varied among clones, with 271 and 216 showing lower effect compared with 42E and 8L (Fig. 3). Values ranged from 258 μg m^{-2} under elevated O_3 to 411 μg m^{-2} under control conditions. The ratio between *ISPS* copy numbers over ISPS protein amount showed that the effect of O_3 was slightly higher on the first than the latter as the regression line is located below the 1:1 line (Fig. 4). However, the intercept was not significantly different from 0 and the slope was not significantly different from 1, suggesting that the effect of O_3 on the *ISPS* mRNA copy numbers is likely to remain similar to the effect on the amount of ISPS protein across a relatively wide range of values.

Isoprene emission rates (Table 1) were considerably decreased by elevated O_3 exposure ($P < 0.001$), whereas elevated CO_2 was not significant ($P = 0.50$). The multiple comparison showed that the effect of O_3 was strong under ambient CO_2 but not significant under elevated CO_2. Overall, the decrease under elevated O_3 was 58%, slightly higher than for assimilation rate (A) (37%, $P < 0.001$). An increase of the assimilation rates by 24% ($P = 0.003$) was observed under elevated CO_2, although only the overall effect resulted significant. The multiple comparison showed that the CO_2 effect was not statistically significant in either ambient or elevated O_3, tested separately (Table 1).

Stomatal conductance (g_s) was strongly decreased ($P < 0.001$) by O_3 treatment (Table 1), but the effect was significant only under ambient CO_2. Elevated CO_2 did not show a significant effect ($P = 0.42$) on g_s.

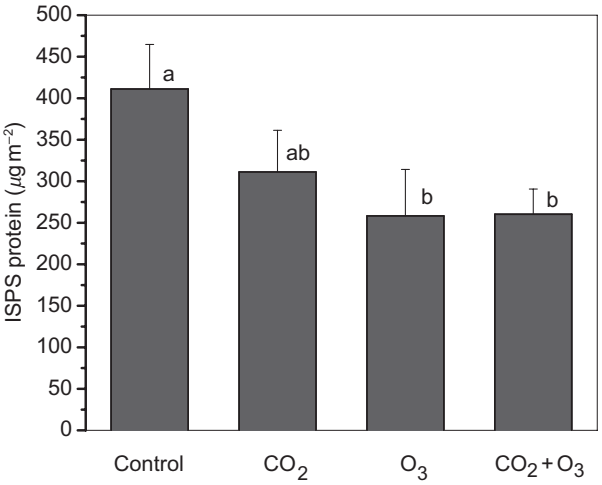
No effect of elevated O_3 ($P = 0.56$) or elevated CO_2 ($P = 0.22$) were observed on the intercellular CO_2 concentration (C_i). However, multiple comparison revealed a significant CO_2 effect at ambient O_3 and a significant O_3 effect at elevated CO_2.

Amounts of ISPS protein in the leaves versus their isoprene emission rates showed a positive relationship



Variation under O_3	8L	271	216	42E
%	–33.9	–28.2	–53.1*	–59.8*

Figure 2. Isoprene synthase (*ISPS*) mRNA copy numbers under different combinations of ambient or elevated CO_2 and O_3 per nanogram of total RNA. Each bar is the average (with SE as error bar) of $n = 30$ leaves. Means followed by different letters are significantly different, $P < 0.05$. The table below shows the percentage variation under elevated O_3 compared with ambient O_3 on the number of *ISPS* mRNA copy numbers for the different aspen clones. The asterisk shows significant differences between elevated and ambient O_3, $P < 0.05$.



Variation under O_3	8L	271	216	42E
%	–38.6*	–4.1	–21.8	–39.4*

Figure 3. Isoprene synthase (ISPS) protein amount in leaves under different combinations of ambient or elevated CO_2 and O_3. Each bar is the average (with SE as error bar) of $n = 30$ leaves. Means followed by different letters are significantly different, $P < 0.05$. The table below shows the percentage variation under elevated O_3 compared with ambient O_3 on the amount of ISPS protein for the different aspen clones. The asterisk shows significant differences between elevated and ambient O_3, $P < 0.05$.

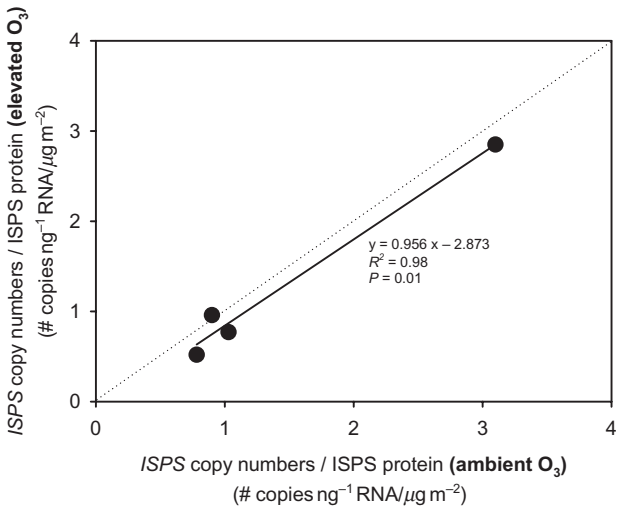


Figure 4. Relationship between the ratio of isoprene synthase (*ISPS*) mRNA copy numbers over the ISPS protein amount under ambient O_3 and elevated O_3. Each point represents the ratio of the mean values of *ISPS* mRNA copy numbers and of the ISPS protein amount for each clone. The equation, R^2 and P of the fit (solid line) are shown; the intercept of the fit is not significantly different from 0 and the slope is not significantly different from 1. The dotted line represents the 1:1 line.

according to a fit with equation $y = a\ (1 - e^{-bx})$ (Fig. 5). Elevated O_3 induced a shift of this relationship: isoprene emission rates were lower under elevated O_3 compared with the ambient O_3 at similar amounts of ISPS protein.

DISCUSSION

ISPS mRNA copy numbers and amounts of protein were considerably reduced by elevated O_3 concentrations in the leaves of field-grown aspen trees. The data on mRNA are in agreement with Fares *et al.* (2006) who found a decrease under O_3 exposure in potted seedlings of *Populus alba*.

Mean values of mRNA and of amount of ISPS protein show a similar pattern among the different treatments (Figs 2 & 3). This is quite interesting if we consider that in another experiment, Mayrhofer *et al.* (2005) did not find a correlation plotting the *ISPS* mRNA versus the amount of ISPS protein of each sample. This is probably due to the strong diurnal variability of the transcript levels and to the strong seasonal pattern of the ISPS protein (Mayrhofer *et al.* 2005; Wiberley *et al.* 2005). Regardless of the ratio between the two, which may vary in different clones or temporally within the same leaves, the O_3 effect is likely to remain similar across the transcriptional and the translational level (Fig. 4). The effect was slightly higher for mRNA with a mean decrease of 45% rather than on the amount of ISPS protein (26%), but our data suggest that this difference in the O_3 effect was not significant.

Although clonal variability was not an objective of the present work, we observed an interesting trend of increased response to O_3, moving from clones with less sensitivity to O_3 to the most sensitive ones. However, this was only true for the *ISPS* transcript levels, whereas for the *ISPS* protein levels, the tolerant clone 8L showed a strong decrease similar to the one of the sensitive clone 42E. Thus, it is difficult from this study to assess whether more tolerant clones can maintain higher isoprene emission rates under

	Control	CO_2	O_3	$CO_2 + O_3$
Isoprene (nmol m^{-2} s^{-1})	11.2^{a} (1.0)	8.2^{ab} (1.9)	3.5^{b} (1.4)	4.7^{b} (1.0)
A (μmol m^{-2} s^{-1})	11.2^{ac} (0.9)	13.1^{a} (0.8)	6.5^{b} (0.4)	8.9^{bc} (0.7)
g_s (mol m^{-2} s^{-1})	0.24^{a} (0.03)	0.21^{a} (0.02)	0.11^{b} (0.01)	0.17^{ab} (0.01)
C_i (μL L^{-1})	274^{a} (5)	243^{b} (9)	255^{ab} (8)	270^{a} (3)

Mean values ($n = 12$) with SE in brackets are shown. For each parameter, means followed by different letters are significantly different, $P < 0.05$.

Table 1. Isoprene emission rates, assimilation rates (A), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) of aspen leaves under different combinations of ambient or elevated CO_2 and O_3

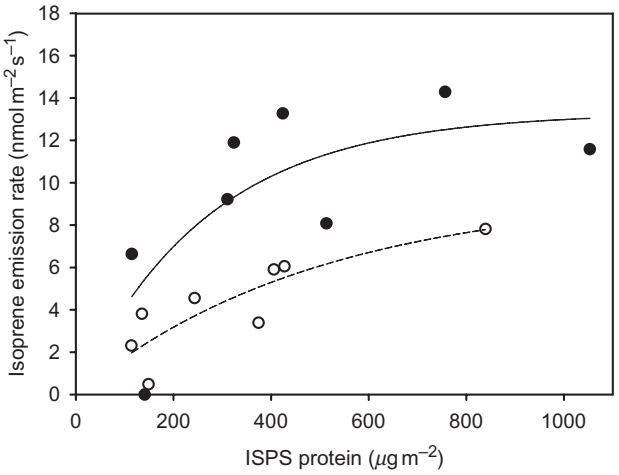


Figure 5. Relationship between isoprene synthase (ISPS) protein amount in the leaves and isoprene emission rate measured under ambient O_3 [black circles, solid fit with equation: $y = 13.30\ (1 - e^{-0.0037x})$, $R^2 = 0.57$, $P = 0.03$] and elevated O_3 [white circles, dashed fit with equation $y = 9.52\ (1 - e^{-0.0020x})$, $R^2 = 0.72$, $P = 0.008$]. Each point represents the value of each clone in the ambient or elevated O_3 plots.

high O_3 and if this can result in a benefit against ozone damage as suggested by Loreto & Velikova (2001). Sensitivity to O_3 is mainly linked with O_3 uptake through stomata (Martin *et al.* 2000; Fowler *et al.* 2001); however, the antioxidant role of certain compounds deserves more detailed investigation (Musselman *et al.* 2006). In particular, the presence of isoprenoids in the leaves has a strong influence on the sensitivity to O_3 (Loreto & Fares, in press).

We observed an inhibitory effect of elevated O_3 on the isoprene emission rates even more pronounced than on *ISPS* transcript levels and protein amounts. The effect of O_3 was also very strong on the assimilation rates suggesting that the pathways of photosynthesis and isoprene were altered to a similar extent by elevated O_3.

We found a good correlation between ISPS protein in the leaves and isoprene emitted by those leaves. Elevated O_3 treatment affected this relationship indicating that the decrease in the rate of isoprene emission under elevated O_3 cannot be explained only by a reduction in the amount of ISPS protein. Even though g_s dropped significantly under elevated O_3, we did not find any correlation with isoprene emission rate ($R^2 = 0.02$, data not shown), further indicating that the stomatal control of isoprene emission is weak (Monson & Fall 1989). We might hypothesize an effect of O_3 on dimethylallyl diphosphate (DMADP), the substrate for the formation of isoprene or an effect on the ISPS protein activity, although both DMADP and the ISPS protein activity are well correlated with the amount of the ISPS protein (Mayrhofer *et al.* 2005).

The drop in isoprene emission rates is in agreement with that found by Velikova *et al.* (2005) with *Q. pubescens* exposed to a localized O_3 fumigation system. Their experiment on O_3 exposure was carried out in laboratory conditions on potted plants using ozone-free air during the leaf gas exchange measurements. This approach rules out the reactions that may naturally occur between isoprene and O_3 at the leaf-atmosphere boundary as we know that O_3 promptly reacts with a series of compounds once it enters the leaf (Mehlhorn & Wellburn 1987; Chameides 1989; Beauchamp *et al.* 2005). Isoprene might be one of such reactants given its high concentration within the leaf (Loreto *et al.* 1998). If this is true, then the ozone effect might reflect both an inhibition of isoprene biosynthesis and the disappearance of a fraction of isoprene which has reacted with ozone, although we hypothesize the latter is much smaller than the former. The lifetime of isoprene in our measurement conditions was estimated to be about 8 h (Ciccioli, personal communication: calculations based on a value of 1.27 E-17 cm^3 molecule^{-1} s^{-1} for $K_{O_3\ isoprene}$ and on an air O_3 concentration of 100 ppbv), indicating that reactions of isoprene with O_3 in the cuvette were likely to be negligible.

The elevated O_3 effect on *ISPS* transcript levels, ISPS protein levels and isoprene emission was not altered by elevated CO_2. Elevated CO_2 did not have any significant effect itself. Some interactions were observed between elevated CO_2 and elevated O_3 on g_s and C_i, which, however, could have a marginal effect on the isoprene emission rates.

A decrease of isoprene emission rates was observed under elevated CO_2 (Rosenstiel *et al.* 2003; Pegoraro *et al.* 2004; Possell *et al.* 2005), but only Centritto *et al.* (2004) found this in open field conditions. Rapparini *et al.* (2004) found that this inhibition was lost in a natural spring over long-term elevated CO_2 exposure. Moreover, no response was observed on field-grown *Populus* trees (Loreto *et al.* 2001b). We observed a slight decrease in isoprene emission rates under elevated CO_2 only under ambient O_3 in line with a slight decrease of the ISPS protein levels, both of them being not statistically significant. We know that foliar respiration rates at this site were not affected by elevated CO_2 (Davey *et al.* 2004), thus, based on the hypothesis of Rosenstiel *et al.* (2003), we might speculate that elevated CO_2 did not induce a decrease of DMADP levels. Our data on the ISPS, combined with the isoprene emission rates measured at the same CO_2 concentration between treatments, suggest that any possible change of isoprene emission under elevated CO_2 is not likely driven by a long-term adaptation of the leaf. These results at leaf level should be combined with other findings to predict canopy level isoprene emission rates under climate change. In particular, an increase in atmospheric CO_2 concentration should be associated with an increase in air temperature (IPCC 2001) and an increase in LAI, although not for all the ecosystems (Ainsworth & Long 2005). These effects would both determine a considerable increase of the isoprene emission at canopy level. On the contrary, the expected increase of UV radiation (IPCC 2001) could lead to an increased O_3 formation and thus to a possible decrease of the isoprene emission as a result of the findings of this study.

Moreover, the statistical analysis used in this study used leaves as pseudo-replicates rather than plots as real replicates, because of the lack of an adequate number of

samples from all the plots. This approach led likely to overestimate the significance of the treatments.

In conclusion, ISPS in field-grown aspen trees adapted to long-term exposure to global change appears to be affected by increasing O_3 rather than CO_2 concentrations. The response is consistent from the transcriptional level to the translational level. Although the change in ISPS protein levels induces a change in isoprene emission rates under elevated O_3, we hypothesize that other mechanisms also occur to explain such a significant drop. A more detailed investigation is needed in order to prove these hypotheses. We found that the response to O_3 of ISPS transcript levels and protein differs among the aspen clones studied. Whether these differences are related to O_3 sensitivity is still unknown. Ozone uptake is mainly due to a stomatal component and is associated to O_3 sensitivity. However, there might be a contribution to stomatal uptake related to detoxification processes, including a possible antioxidant action by isoprenoids.

ACKNOWLEDGMENTS

This research was supported by a *Marie Curie International Fellowship* within the 6th European Community Framework Programme ('GLOBALVOC', contract MOIF-CT-2005-007692) and by the Italy–USA Bilateral Programme on Climate Change of the Italian Ministry of Environment. The Aspen FACE experiment is principally funded by the US Department of Energy. Other Aspen Face support comes from the Office of Science (BER), the National Science Foundation, the USFS Global Change Program, Michigan Technological University and the USFS North Central Research Station. The research in Madison was funded in part by NSF grants IBN-0212204 and IOS-0640853.

The authors acknowledge R. Teclaw and W. Jones for providing meteorological data and values of O_3 concentrations in the experimental plots. The authors thank J.P. Schnitzler for providing ISPS antibodies for preliminary tests and I. Beritognolo for useful suggestions on the procedures at the gene level.

REFERENCES

Ainsworth E.A. & Long S.P. (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytologist* **165,** 351–372.

Beauchamp J., Wisthaler A., Hansel A., Kleist E., Miebach M., Niinemets U., Schurr U. & Wildt J. (2005) Ozone induced emissions of biogenic VOC from tobacco: relationships between ozone uptake and emission of LOX products. *Plant, Cell & Environment* **28,** 1334–1343.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248–254.

Centritto M., Nascetti P., Petrilli L., Raschi A. & Loreto F. (2004) Profiles of isoprene emission and photosynthetic parameters in hybrid poplars exposed to free-air CO_2 enrichment. *Plant, Cell & Environment* **27,** 403–412.

Chameides W.L. (1989) The chemistry of ozone deposition to plant leaves. Role of ascorbic acid. *Environmental Science and Technology* **23,** 595–600.

Chameides W.L., Lindsay R.W., Richardson J. & Kiang C.S. (1988) The role of biogenic hydrocarbons in urban photochemical smog: Atlanta as a case study. *Science* **241,** 1473–1475.

Chang S., Puryear J. & Cairney J. (1993) Simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* **11,** 113–116.

Coleman M.D., Dickson R.E., Isebrands J.G. & Karnosky D.F. (1995a) Carbon allocation and partitioning in aspen clones varying in sensitivity to tropospheric ozone. *Tree Physiology* **15,** 593–604.

Coleman M.D., Isebrands J.G., Dickson R.E. & Karnosky D.F. (1995b) Photosynthetic productivity of aspen clones varying in sensitivity to tropospheric ozone. *Tree Physiology* **15,** 585–592.

Constable J.V.H., Guenther A.B., Schimel D.S. & Monson R.K. (1999) Modelling changes in VOC emission in response to climate change in the continental United States. *Global Change Biology* **5,** 791–806.

Davey P.A., Hunt S., Hymus G.J., DeLucia E.H., Drake B.G., Karnosky D.F. & Long S.P. (2004) Respiratory oxygen uptake is not decreased by an instantaneous elevation of $[CO_2]$, but is increased with long-term growth in the field at elevated $[CO_2]$. *Plant Physiology* **134,** 520–527.

Dickson R.E., Lewin K.F., Isebrands J.G., *et al.* (2000) Forest atmosphere carbon transfer storage-II (FACTS II) – The aspen free-air CO_2 and O_3 enrichment (FACE) project in an overview. USDA Forest Service, North Central Research Station. General Technical Report NC-214. 68 pp.

Fares S., Barta C., Brilli F., Centritto M., Ederli L., Ferranti F., Pasqualini S., Reale L., Tricoli D. & Loreto F. (2006) Impact of high ozone on isoprene emission, photosynthesis and hystology of developing *Populus alba* leaves directly or indirectly exposed to the pollutant. *Physiologia Plantarum* **128,** 456–465.

Fowler D., Flechard C.R., Cape J.N., Storeton-West R.L. & Coyle M. (2001) Measurements of ozone deposition to vegetation quantifying the flux, the stomatal and non-stomatal components. *Water, Air and Pollution* **130,** 63–74.

Guenther A., Hewitt C.N., Eriksson D., *et al.* (1995) A global model of natural volatile organic compound emissions. *Journal of Geophysical Research-Atmospheres* **100,** 8873–8892.

Guenther A., Geron C., Pierce T., Lamb B., Harley P. & Fall R. (2000) Natural emissions of non-methane volatile organic compounds, carbon monoxide, and oxides of nitrogen from North America. *Atmospheric Environment* **34,** 2205–2230.

Haruta M., Pedersen J.A. & Constabel C.P. (2001) Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates. *Physiologia Plantarum* **112,** 552–558.

IPCC (2001) Climate change 2001: The scientific basis. Contribution of working group I to the third assessment report of the Intergovernmental Panel on Climate Change (eds J.T. Houghton, Y. Ding, D.J. Griggs, M. Noguer, P.J. van der Linden, X. Dai, K. Maskell & C.A. Johnson). Cambridge University Press, Cambridge, UK and New York, NY, USA.

Isebrands J.G., McDonald E.P., Kruger E., Hendry G., Pregitzer K., Sober J. & Karnosky D.F. (2001) Growth responses of *Populus tremuloides* clones to interacting carbon dioxide and tropospheric ozone. *Environmental Pollution* **115,** 359–371.

Karnosky D.F., Mankovska B., Percy K., *et al.* (1999) Effects of tropospheric O_3 on trembling aspen and interaction with CO_2: results from an O_3-gradient and a FACE experiment. *Water, Air, and Soil Pollution* **116,** 311–322.

Llusià J., Peñuelas J. & Gimeno B.S. (2002) Seasonal and species-specific response of VOC emissions by Mediterranean woody

plant to elevated ozone concentrations. *Atmospheric Environment* **36,** 3931–3938.

Loreto F. & Sharkey T.D. (1993) On the relationship between isoprene emission and photosynthetic metabolites under different environmental conditions. *Planta* **189,** 420–424.

Loreto F. & Velikova V. (2001) Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiology* **127,** 1781–1787.

Loreto F. & Fares S. (in press) Ozone flux in leaves: a protection rather than a damage index? *Plant Physiology*.

Loreto F., Ciccioli P., Brancaleoni E., Cucinato A. & Frattoni M. (1998) Measurement of isoprenoid content in leaves of Mediterranean *Quercus* spp. by a novel and sensitive method and estimation of the isoprenoid partition between liquid and gas phase inside the leaves. *Plant Science* **136,** 25–30.

Loreto F., Fischbach R.J., Schnitzler J.-P., Ciccioli P., Brancaleoni E., Calfapietra C. & Seufert G. (2001a) Monoterpene emission and monoterpene synthase activities in the Mediterranean evergreen oak *Quercus ilex* L. grown at elevated CO2 concentrations. *Global Change Biology* **7,** 709–717.

Loreto F., Fischbach R.J., Schnitzler J.-P., Ciccioli P., Calfapietra C., Baraldi R. & Rapparini F. (2001b) Isoprenoid emission by forest trees grown at elevated CO_2 concentrations. In *Forest Research: A Challenge for an Integrated European Approach* (ed. K. Radoglou) pp. 175–178. NAGREF, Thessaloniki, Greece.

Loreto F., Pinelli P., Manes F. & Kollist H. (2004) Impact of ozone on monoterpene emission and evidence for an isoprene-like antioxidant action of monoterpens emitted by *Quercus ilex* leaves. *Tree Physiology* **24,** 361–367.

Martin M.J., Farage P.K., Humphries S.W. & Long S.P. (2000) Can the stomatal changes caused by acute ozone exposure be predicted by changes occurring in the mesophyll? A simplification for models of vegetation response to the global increase in tropospheric elevated ozone episodes. *Australian Journal of Plant Physiology* **27,** 211–219.

Mayrhofer S., Teuber M., Zimmer I., Louis S., Fischbach R.J. & Schnitzler J.P. (2005) Diurnal and seasonal variation of isoprene biosynthesis-related genes in grey poplar leaves. *Plant Physiology* **139,** 474–484.

Mehlhorn H. & Wellburn A.R. (1987) Stress ethylene formation determines plant sensitivity to ozone. *Nature* **327,** 417–418.

Monson R.K. (2002) Volatile organic compound emissions from terrestrial ecosystems: a primary biological control over atmospheric chemistry. *Israel Journal of Chemistry* **42,** 29–42.

Monson R.K. & Fall R. (1989) Isoprene emission from aspen leaves. The influence of environment and relation to photosynthesis and photorespiration. *Plant Physiology* **90,** 267–274.

Musselman R.C., Lefohn A.S., Massman W.J. & Heath R.L. (2006) A critical review and analysis of the use of exposure- and flux-based ozone indices for predicting vegetation effects. *Atmospheric Environment* **40,** 1869–1888.

Pegoraro E., Rey A., Bobich E.G., Barron-Gafford G., Grieve K.A., Malhi Y. & Murthy R. (2004) Effect of elevated CO_2 concentration and vapour pressure deficit on isoprene emission from leaves of *Populus deltoides* during drought. *Functional Plant Biology* **31,** 1137–1147.

Possell M., Hewitt N.C. & Beerling D.J. (2005) The effects of glacial atmospheric CO_2 concentrations and climate on isoprene emissions by vascular plants. *Global Change Biology* **11,** 60–69.

Rapparini F., Baraldi R., Miglietta F. & Loreto F. (2004) Isoprenoid emission in trees of *Quercus pubescens* and *Quercus ilex* with lifetime exposure to naturally high CO_2 environment. *Plant, Cell & Environment* **27,** 381–391.

Rosenstiel T.N., Potosnak M.J., Griffin K.L., Fall R. & Monson R.K. (2003) Increased CO_2 uncouples growth from isoprene emission in an agriforest ecosystem. *Nature* **421,** 256–259.

Scholefield P.A., Doick K.J., Herbert B.M.J., Hewitt C.N.S., Schnitzler J.-P., Pinelli P. & Loreto F. (2004) Impact of rising CO_2 on emission of volatile organic compounds: isoprene emission from *Phragmites australis* growing at elevated CO_2 in a natural carbon dioxide spring. *Plant, Cell & Environment* **27,** 393–401.

Singsaas E.L., Lerdau M., Winter K. & Sharkey T.D. (1997) Isoprene increases thermotolerance of isoprene-emitting species. *Plant Physiology* **115,** 1413–1420.

Velikova V., Tsonev T., Pinelli P., Alessio G.A. & Loreto F. (2005) Localized ozone fumigation system for studying ozone effects on photosynthesis, respiration, electron transport rate and isoprene emission in field-grown Mediterranean oak species. *Tree Physiology* **25,** 1523–1532.

Vuorinen T., Nerg A.N., Vapaavuori E. & Holopainen J.K. (2005) Emission of volatile organic compounds from two silver birch (*Betula pendula* Roth) clones grown under ambient and elevated CO_2 and different O_3 concentrations. *Atmospheric Environment* **39,** 1185–1197.

Wiberley A.E., Linskey A.R., Falbel T.G. & Sharkey T.D. (2005) Development of the capacity for isoprene emission in kudzu. *Plant, Cell & Environment* **28,** 898–905.

Wolfertz M., Sharkey T.D., Boland W., Kühnemann F., Yeh S. & Weise S.E. (2003) Biochemical regulation of isoprene emission. *Plant, Cell & Environment* **26,** 1357–1364.

Received 17 August 2006; received in revised form 28 November 2006; accepted for publication 13 January 2007

