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Impact of rising CO_2 on emissions of volatile organic compounds: isoprene emission from *Phragmites australis* growing at elevated CO_2 in a natural carbon dioxide spring†

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ABSTRACT

Isoprene basal emission (the emission of isoprene from leaves exposed to a light intensity of 1000 μmol m^{-2} s^{-1} and maintained at a temperature of 30 °C) was measured in *Phragmites australis* plants growing under elevated CO_2 in the Bossoleto CO_2 spring at Rapolano Terme, Italy, and under ambient CO_2 at a nearby control site. Gas exchange and biochemical measurements were concurrently taken. Isoprene emission was lower in the plants growing at elevated CO_2 than in those growing at ambient CO_2. Isoprene emission and isoprene synthase activity (IsoS) were very low in plants growing at the bottom of the spring under very rich CO_2 and increased at increasing distance from the spring (and decreasing CO_2 concentration). Distance from the spring did not significantly affect photosynthesis making it therefore unlikely that there is carbon limitation to isoprene formation. The isoprene emission rate was very quickly reduced after rapid switches from elevated to ambient CO_2 in the gas-exchange cuvette, whereas it increased when switching from ambient to elevated CO_2. The rapidity of the response may be consistent with post-translational modifications of enzymes in the biosynthetic pathway of isoprene formation. Reduction of IsoS activity is interpreted as a long-term response. Basal emission of isoprene was not constant over the day but showed a diurnal course opposite to photosynthesis, with a peak during the hottest hours of the day, independent of stomatal conductance and probably dependent on external air temperature or temporary reduction of CO_2 concentration. The present experiments show that basal emission rate of isoprene is likely to be reduced under future elevated CO_2 levels and allow improvement in the modelling of future isoprene emission rates.

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†*This work is dedicated to the memory of Dr Wolfgang Zimmer.*

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INTRODUCTION

Isoprene (C_5H_8) is produced and emitted by many higher plants (e.g. Kesselmeier & Staudt 1999) although its function is not clear. It may provide enhanced leaf thermotolerance (Sharkey & Singsaas 1995), it may scavenge ozone (Sauer *et al*. 1999) and it may act as a signal to promote flowering in neighbouring plants (Terry *et al*. 1995) but none of these hypotheses have been proved beyond doubt.

Isoprene is very reactive in the atmosphere and is believed to play a key role in atmospheric chemistry (e.g. Fehsenfeld *et al*. 1992). It rapidly reacts with the hydroxyl radical ($OH^{\cdot}$) and in fact its emission may control the concentration of $OH^{\cdot}$ in the atmosphere. Since the $OH^{\cdot}$ concentration determines the lifetime of methane in the atmosphere (methane being the third most important 'greenhouse gas' in the atmosphere), it can be hypothesized that feedback loops exist between isoprene emission and global warming.

In the context of global change, there is therefore considerable interest in understanding how isoprene emissions may change in the future, as global CO_2 concentrations increase. Such changes can be studied by fumigating plants with CO_2, either in laboratory facilities on potted plants or in large CO_2 enrichment facilities on soil-rooted plants. Exposure to elevated CO_2 often results in a decrease of isoprene emission of potted plants (e.g. Loreto & Sharkey 1990) but growth at elevated CO_2 may not cause the same effect. For instance, isoprene emission was reduced in potted aspen but was enhanced in potted oaks grown at elevated CO_2 in a growth cabinet (Sharkey, Loreto & Delwiche 1991). The response of isoprene to field experiments of CO_2 enrichment also yielded results that were difficult to interpret. Tognetti *et al*. (1998) reported that isoprene emission is stimulated in *Quercus pubescens* leaves. This experiment was conducted with plants grown at naturally high concentration of CO_2, on the same exper-

imental site as our work, and is therefore particularly important to compare with our results. On the other hand, isoprene emission of leaves grown at elevated CO_2 in a Free-Air-CO_2-Enhancement (FACE) facility was substantially unchanged in *Populus alba, P. nigra, and P.* × *euroamericana* (Loreto *et al*. 2001a), and was depressed in poplars grown at CO_2 levels higher than ambient in Biosphere-2 mesocosms (Rosenstiel *et al*. 2003). Other isoprenoids are formed through the same metabolic pathway as isoprene and probably share the same functions in some Mediterranean plants. The emission of monoterpenes from Q*uercus ilex* plants grown at elevated CO_2 in open top chambers (OTC) was generally lower than the emission from plants grown in OTC with no CO_2 enrichment (Loreto *et al*. 2001b). This experiment indicated that the observed response could be attributed to a biochemical regulation (a reduced activity of monoterpene synthases).

An alternative approach to the field studies with artificial CO_2 enrichment, is to use plants that grow naturally in areas of elevated CO_2, such as those around natural CO_2 springs. The CO_2 spring of 'il Bossoleto' is an excellent site for studies on CO_2 enrichment (Van Gardingen *et al*. 1995) and has been previously chosen to study the effect of elevated CO_2 on isoprene emission (Tognetti *et al*. 1998). Il Bossoleto was one of the two experimental sites of an EC-Environment project dedicated to predictions of isoprenoid emissions under future CO_2 levels and here we present results obtained with one of the most important natural grasses, *Phragmites australis*. *Phragmites* is ubiquitous around the world and is a significant emitter of isoprene (Loreto & Velikova 2001), especially when the emission is integrated over the packed stand.

MATERIALS AND METHODS

Sampling sites

The Bossoleto natural CO_2 spring is located in Central Italy (43°17′ N, 11°35′ E, 272 m a.s.l), near the village of Rapolano Terme, Tuscany, Italy. The virtually pure CO_2 (> 99%) of geological origin is released by a number of vents at the base and the lower flanks of the natural crater, creating a steep vertical gradient, and accumulating in the bowl in the evenings and overnight. Concentrations around the vents approach 100% at these times, but during the day the mean long-term CO_2 concentration is approximately twice the present day global average (Van Gardingen *et al*. 1995). Three veins of freshwater are present in the bottom of the crater and this allows the growth of a dense stand of *Phragmites*. Plants were sampled in several areas of the spring. The main area of sampling was approximately 25 m from the main vents where CO_2 concentrations varied from 400 to 1200 μmol mol^{-1}, with an average of approximately 1000 μmol mol^{-1}. A second sampling area was selected at the bottom of the spring, where CO_2 concentration was >1000 μmol mol^{-1} for most of the day and dropped to around 400–1000 μmol mol^{-1} only during the middle hours of the day, when measurements were therefore taken. Plants were also sampled at three intermediate areas between the main sampling area and the bottom of the crater. The control site was approximately 1500 m from the vents, an open wasteland that is meteorologically and morphologically similar to the Bossoleto spring, with similar floral and faunal associations, but far enough to be free from CO_2-enrichment. The *Phragmites* grows in a depression permanently receiving humidity from a subterraneous vein of freshwater, a condition similar to that found in the CO_2 spring. At both sites, temperature, relative humidity, photosynthetically active radiation (PAR) and CO_2 concentration were measured and controlled using two environmental gas monitoring systems (EGM-1; PP-Systems, Hitchin, Herts., UK and Li-Cor 6400; Li-Cor Lincoln, NE, USA).

Gas exchange measurements and environmental control at leaf level

System I

A CIRAS-1 photosynthesis analysis system (PP Systems) was used to monitor exchanges of CO_2 and H_2O on the last fully expanded leaf of *Phragmites* plants, and to establish controlled environmental conditions before and during measurements. In particular, relative humidity, PAR and leaf temperature were maintained at 50%, 1000 μmol m^{-2} s^{-1}, and 30 °C, respectively. These are the conditions at which isoprene *basal emission* is measured. Basal emission is the emission that is used to model and predict isoprene emission after correction to take into account temperature and light dependency of its biosynthesis (Guenther *et al*. 1993, 1995). The CO_2 concentration in the cuvette was controlled automatically by using a CIRAS CO_2 regulator and cartridge unit. Leaves at the main Bossoleto sampling area were maintained at 1000 μmol mol^{-1} CO_2, and leaves at the control site at 360 μmol mol^{-1} since these concentrations were representative of the average CO_2 concentration experienced by the plants throughout their life cycle. For measurements made at the bottom of the Bossoleto spring, the CO_2 concentration was set at 2000 μmol mol^{-1}. All leaves were allowed 60 min in the cuvette in order to stabilize gas exchange and for the cuvette environment to equilibrate prior to sampling. Eight leaves were sampled in this way from the control site and 10 leaves from the Bossoleto site. These experiments were made during September 2001.

System II

The Li-Cor 6400 portable system was also used to control environmental parameters and monitor gas exchange by *Phragmites* leaves at the control site and at the Bossoleto site. Leaf samples and environmental settings were chosen as for the CIRAS system. The only differences were that: (1) the cuvette allowed measurement of gas exchange on a fixed leaf area of 5 cm^2; and (b) the CO_2 concentration for plants in the Bossoleto spring was set at double that of the control site (700 and 350 μmol mol^{-1}, respectively). One of

the experiments was performed by rapidly switching CO_2 concentration from the growth concentration to the concentration experienced by the other stand, without stabilizing leaves at the new conditions. In another experiment, emission measurements were also made at varying vertical distances from the bottom of the Bossoleto spring and in this case the leaf was maintained at the CO_2 concentration actually monitored in the air at the time of the measurement. Measurements were made during July 2001.

Isoprene sampling and detection

System I: off-site isoprene analysis

When other gas-exchange parameters (i.e. photosynthesis and transpiration rates) were stable, isoprene was collected via a 'T' piece in the return airflow from the cuvette to the CIRAS-1 onto dual-bed adsorption-traps, containing Tenax TA and Carbotrap (100 mg of each; Supelco, Bellefonte, USA) packed into stainless steel tubes. Air was drawn from the cuvette at a rate of 200 mL min^{-1} for a period of 1 min. Airflow was controlled using a mass flow controller (MKS type 1179 A; MKS, Andover, USA). Samples were replicated five times. After a 15-min period, this process was repeated, giving 10 samples per leaf. Sample tubes, precleaned prior to use, were kept at <5 °C until analysis.

Isoprene peak identification was accomplished with gas chromatographic separation and mass selective detection (GC–MS). Desorption and analysis of isoprene was carried out using a Perkin Elmer ATD400 (Perkin-Elmer, Norwalk, CT, USA) connected by a thermal transfer line maintained at 200 °C to a Hewlett-Packard 5890 A gas chromatograph with a Hewlett Packard 5970 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). The preconcentrated samples were thermally desorbed at 280 °C for 6 min at 30 mL min^{-1} onto a Tenax TA cold trap maintained at −30 °C. Secondary desorption was at 250 °C for 5 min. A fused silica capillary (50 m × 0.32 mm inside diameter coated with Al_2O_3/KCl) PLOT column was used to detect and quantify isoprene. An initial oven temperature of 120 °C was maintained for 1 min, then increased to 165 °C at 3 °C min^{-1} followed by an increase at 45 °C min^{-1} to 200 °C for 10 min. The isoprene peak quantification was accomplished using a thermal desorption system (Perkin Elmer ATD400) connected to a Perkin Elmer gas chromatograph (GC) Autosystem configured with a flame ionization detector and PLOT column. Column and tube desorption conditions were as described above for the GC-MS. The limit of detection for isoprene was approximately 0.1 ng on column.

System II: on-site isoprene analysis

Isoprene emission was measured by diverting a small part (40 mL) of the air exiting the cuvette from the cuvette outflow normally used to match the two infrared gas analysers (IRGAs) of the system directly into a portable GC (Syntech GC855 series 600; Syntech, Groningen, The Netherlands) placed near to the gas-exchange system. The air was pumped into the GC via a Graphite-Tenax (60–80 mesh, 8 cm) trap, desorbed at 240 °C, transferred to a 13-m capillary column (indide diameter 0.53 mm, packed with 95% dimethylpolysiloxane, 5% diphenylpolysiloxane) under a flow of pure N_2, and the isoprene present was detected after 6 min by photoionization (PID at 10.6 eV). The GC was calibrated with several concentrations of gaseous isoprene. Additional comparisons were performed with simultaneous measurements of isoprene trapped in carbon cartridges and analysed by GC-MS (see Rapparini *et al*. 2004).

Chlorophyll fluorescence analysis

The ratio between variable and maximal fluorescence (F_v/F_m) was measured in dark-adapted (60 min) leaves with a plant efficiency analyser (PEA, v2.05; Hansatech Instruments Ltd, Kings Lynn, Norfolk, UK). When using the system II, chlorophyll fluorescence was measured with a Mini-PAM (Walz, Effeltrich, Germany) modulated fluorometer, as explained in detail by Loreto & Delfine (2000). Briefly, the fluorescence probe was inserted in the middle of the LED arrays with the tip reaching the upper window of the gas-exchange cuvette. The non-photochemical quenching of fluorescence was calculated from the maximal and minimal fluorescence in dark-adapted and illuminated leaves, according to Van Kooten & Snel 1990).

Macro-elemental analysis

Leaf samples were dried and sealed inside bags and brought back to the Lancaster laboratory for carbon and nitrogen analysis. Leaves were dried for a further 3 d at 40 °C. They were then weighed, and ground in liquid nitrogen in a pestle and mortar. Approximately 400 μg of leaf was then sealed inside a tin capsule for flash combustion on a Carlo Erba EA1108 elemental analyser (Carlo Erba, Milan, Italy). Data were analysed using SPSS V 10.0.4 (SPSS Inc. Chicago, IL, USA).

Biochemical analyses

Destructive samplings were carried out at the control site and the Bossoleto site to analyse biochemical and photochemical properties of the *Phragmites* leaves. Sampling in the Bossoleto was carried out at midday and at the bottom of the spring. Samples for determination of isoprene synthase (IsoS) activity were also collected in the main station on the slope of the spring, about 25 m from the vents. Whole leaves were cut and rapidly frozen under liquid nitrogen. Leaves were stored at −80 °C until processed. Hydrogen peroxide content was determined according to Velikova, Yordanov & Edreva (2000). Leaf tissues (0.07 g) were homogenized in an ice bath with 5 mL 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12 000 *g* for 15 min and 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance of the superna-

tant was measured at 390 nm. The content of H_2O_2 was calculated by comparison with a standard calibration curve previously made by using different concentrations of H_2O_2. Pigment contents (chlorophylls and xanthophylls) were measured by high performance liquid chromatography on 3 cm^2 leaf discs, as outlined by Brugnoli *et al.* (1994). The de-epoxidation status of xanthophylls was calculated as the ratio between zeaxanthin + antheraxanthin and the sum of zeaxanthin, antheraxanthin and violaxanthin.

Isoprene synthase analysis

Phragmites leaves were homogenized with a mortar and pestle in liquid N_2. All further steps were performed at 0–4 °C. The fine leaf powder (250 mg fresh weight) was suspended in 5 mL plant extraction buffer (PEB; 100 mM Tris/HCl, pH 7.0, 20 mM $MgCl_2$, 100 mM $CaCl_2$, 5% (v/v) glycerol, 0.1% (v/v) Tween 80, 20 mM dithiothreitol), with 200 mg polyvinylpolypirrolidone added directly prior to use of the buffer, and stirred for 15 min. The homogenate was centrifuged at 18 000 *g* for 20 min, and 2.5 mL each of the clear supernatant were desalted on PD-10 columns (Amersham-Pharmacia, Freiburg, Germany) with IsoS buffer (ISB; Lehning *et al.* 1999), resulting in a total of 3.5 mL leaf protein extract. IsoS activity was assayed as previously described by Lehning *et al.* (1999). Protein concentrations were determined by the Bradford assay with bovine serum albumin as a standard.

Data analysis and statistics

Gas exchange and isoprene emission measurements were replicated on at least three leaves of different plants, and biochemical assays were replicated on five samples. Data are shown as means ± standard errors unless otherwise noted. Mean separation and statistical differences between treatments (ambient versus elevated CO_2) were assessed with ANOVA using a Duncan's test and differences statistically significant at $P < 0.10$, 0.05 and 0.01, are shown by *, ** and ***, respectively, when comparing two means. Differences at $P < 0.05$ are shown by different letters when comparing several means (see Fig. 4).

RESULTS

System I

The basal emission of isoprene was higher in *Phragmites* plants growing at ambient CO_2 at the control site than in

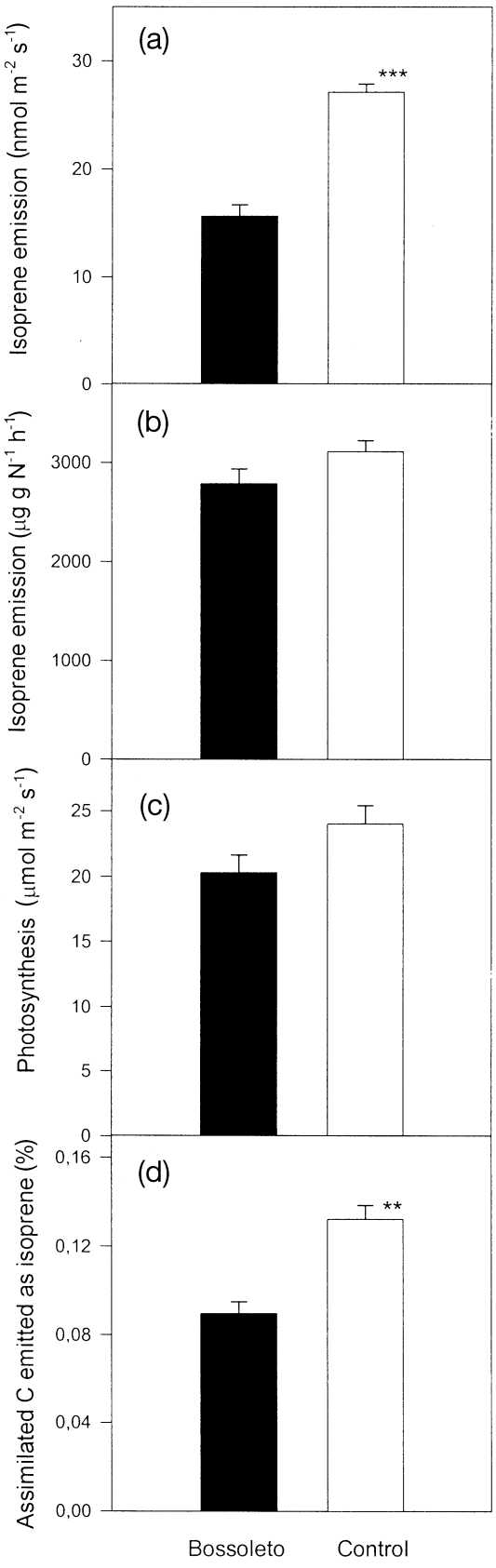

Figure 1. Isoprene emission rates expressed in terms of (a) leaf area basis, and (b) leaf nitrogen, (c) photosynthesis, and (d) percentage of photosynthetically fixed carbon emitted as isoprene for *Phragmites* leaves grown in the Bossoleto site at elevated CO_2 (black bars) and in the control site at ambient CO_2 (white bars). Mean ± SE, ($n = 5$) is reported and asterisks represent statistical significance of mean separation at $P < 0.01$ (***) or $P < 0.05$ (**) level.

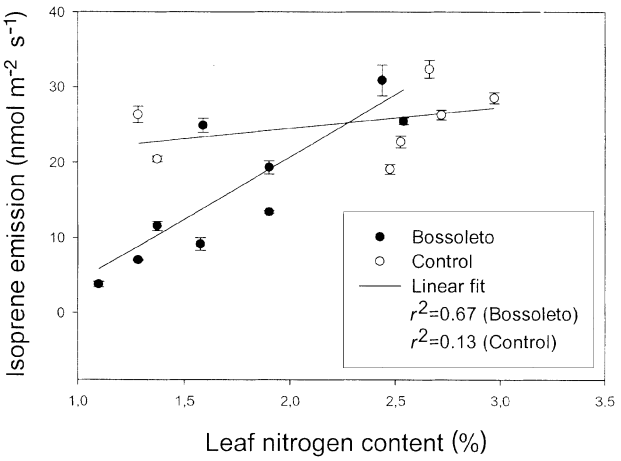

Figure 2. Relationship between isoprene emission rate and leaf nitrogen content in plants grown at ambient CO_2 (control) and at elevated CO_2 in the Bossoleto spring. Each data point is an average of five measurements on different leaves and error bars represent SE. Linear regression lines and regression coefficients for the two data-sets are also shown.

those growing at elevated CO_2 in the Bossoleto site (Fig. 1). This difference was found whether we expressed the emission rate on the basis of leaf area (Fig. 1a), or dry weight (not shown) or leaf nitrogen (Fig. 1b). However, the inhibition of isoprene emission was attenuated (and became not statistically significant) when expressed on a nitrogen basis. Photosynthesis was not significantly different in the plants grown at the Bossoleto spring relative to the control site (Fig. 1c).

Leaf nitrogen content was generally lower in plants growing in the Bossoleto site than in those of the control site. For plants of the Bossoleto site isoprene emission showed a good correlation with leaf nitrogen content ($r^2 = 0.67$), whereas this correlation was not found at the control site (Fig. 2). Expressed as a fraction of the carbon assimilated, isoprene emissions at the control site were $0.66 \pm 0.03\%$. At the elevated CO_2 site, isoprene emissions were $0.45 \pm 0.03\%$ of the assimilated C. The difference is significant at the $P < 0.05$ level (Fig. 1d)

Isoprene emissions were measured at five sampling stations of the Bossoleto site as mentioned previously, with typically two plants sampled at each sampling station. There was a clear increase of the emission with increasing distances from the vent and with decreasing average CO_2 concentration (Fig. 3). This was also confirmed by the experiment made with on-site, on-line analysis (see below).

System II

Isoprene emission was also measured with a portable gas-chromatograph at different distances from the CO_2 vent. In Fig. 4b, the emission of isoprene is plotted versus three different *vertical* distances from the vent. Also this set of measurements indicates that the emission of isoprene is reduced in the Bossoleto site with respect to the control site and that this reduction is stronger when plants are grown at increasingly high CO_2 concentration. In contrast, measurements of photosynthesis do not show any significant change of the rate of CO_2 fixation dependent on the concentration of CO_2, although a small reduction of photosynthesis was observed at the bottom of the spring (Fig. 4a).

In a second experiment, we rapidly switched the CO_2 concentration of the air flowing over the leaf in the gas-exchange cuvette and recorded immediate changes in isoprene emission rate, while photosynthesis and stomatal conductance were recorded 1 h after the switch. In the Bossoleto spring, a rapid switch from elevated to ambient CO_2 induced a progressive increase in isoprene emission (Fig. 5a). In the control site, a rapid switch from ambient to elevated CO_2 caused a rapid reduction of the emission. Switching back to ambient CO_2 again increased isoprene emission although the emission did not reach the same elevated level recorded at the beginning of the experiment (Fig. 5b).

In a third experiment, we wanted to see if isoprene basal emission of *Phragmites* leaves growing at elevated CO_2 was stable during the day. Measurements repeated in the morning, at midday and in the afternoon showed a distinct daily change for both isoprene basal emission and photosynthesis, but the change was opposite for the two parameters, with isoprene emission reaching its maximum during the central hours of the day, when photosynthesis was at its minimum (Fig. 6).

Destructive samplings: photochemical, biochemical and enzymological parameters

Physiological, *in vivo* measurements were supported by analytical assays of the photochemical, biochemical and enzymological properties of the *Phragmites* stands growing at elevated and ambient CO_2. These measurements revealed no significant differences attributable to CO_2 in

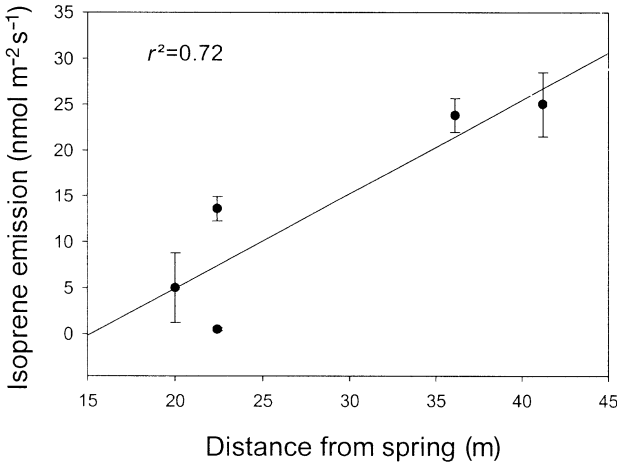

Figure 3. Relationship between isoprene emission rates measured with the CIRAS system and distance from the main CO_2 vent. Each data point represents mean ± SE. ($n = 5$) and the solid line represents a linear correlation fitted to the data ($r^2 = 0.72$).

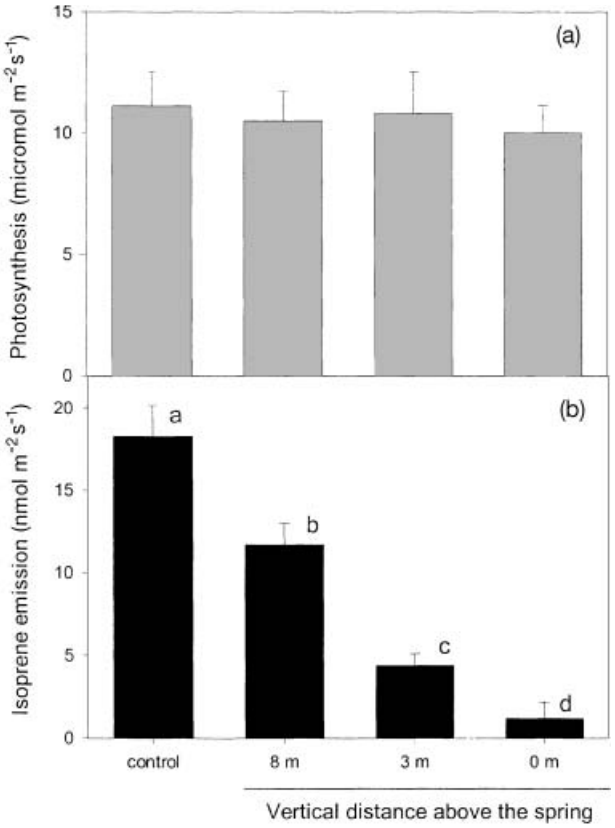

Figure 4. Photosynthesis (a) and isoprene emission (b) from leaves of *Phragmites* plants in the control site and in the Bossoleto site at different vertical distances from the CO_2 vent. Measurements were collected at 1200 h and at the CO_2 concentrations found in the environment at the moment (control: 350; Bossoleto: 400, 550, 800 p.p.m. at different altitudes from the vent). Other conditions as for basal emission measurements (30 °C and 1000 μmol photons m^{-2} s^{-1}). Means ± SE, (n = 5) are shown. When statistically different at $P < 0.05$ level, bars are labelled with different letters.

total amount of chlorophyll, ratio of chlorophyll *a*/*b*, heat dissipation through non-photochemical quenching, de-epoxidation status of xanthophylls, and formation of hydrogen peroxide (data not shown).

Isoprene synthase activity was measured in leaves of the control site and in leaves of the Bossoleto site growing on the slope or at the bottom of the spring. The enzyme activity was clearly reduced in leaves exposed to elevated CO_2 with respect to control and the reduction was strongest in leaves growing at the bottom of the spring, where an extremely elevated CO_2 concentration was experienced by plants for most of the day (Fig. 7a) The reduction of IsoS correlated with the reduction of isoprene emission at increasing CO_2 levels (Fig. 7b).

DISCUSSION

Field experiments conducted by two different groups using two different analytical instruments show that isoprene basal emission is significantly depressed in *Phragmites* plants grown at elevated CO_2 when compared with plants growing at the control site. Emission rates measured using system I and the off-site analysis of isoprene were slightly higher than those measured using system II and the on-site analysis, but in both data-sets the emissions of control plants exceeded the emission of plants growing in the Bossoleto.

An even stronger evidence of the inhibitory effect of elevated CO_2 on isoprene emission was given by measurements at different distance from the CO_2 vent in the Bossoleto spring. The isoprene emission by peripheral stands of *Phragmites* was higher than the emission of plants growing at the bottom of the spring. This presumably correlated inversely with the average CO_2 concentration at which plants were grown. Access to the bottom of the spring was prevented by the extremely high CO_2 concentration early in the morning and late in the evening (see cover picture of the spring). Van Gardingen *et al.* (1995) showed that the effective mean CO_2 concentration, i.e. mean CO_2 exposure during photosynthesis, integrated over a plant's lifetime, correlates with displacement from the main CO_2 vent. It is therefore reasonable to use distance from the vents at the bottom of the spring as a proxy for effective average CO_2 concentration. The same authors concluded that the bottom

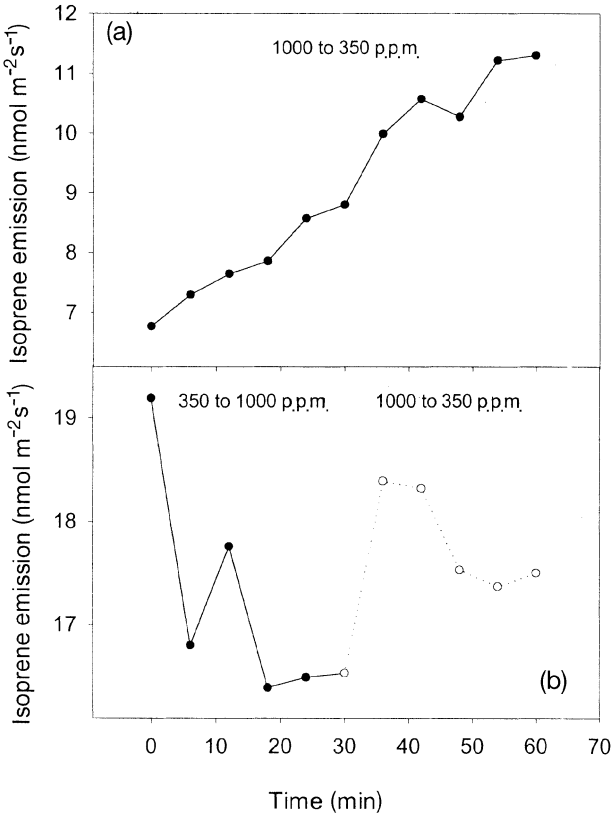

Figure 5. Response of isoprene emission to CO_2 switches. CO_2 was suddenly decreased from 1000 to 350 p.p.m. for plants growing in the Bossoleto site (a) while it was suddenly increased from 350 to 1000 p.p.m., and after 30 min again decreased, for plants growing in the control site (b). Single leaf measurements are shown as representative of the experiment which was replicated five times.

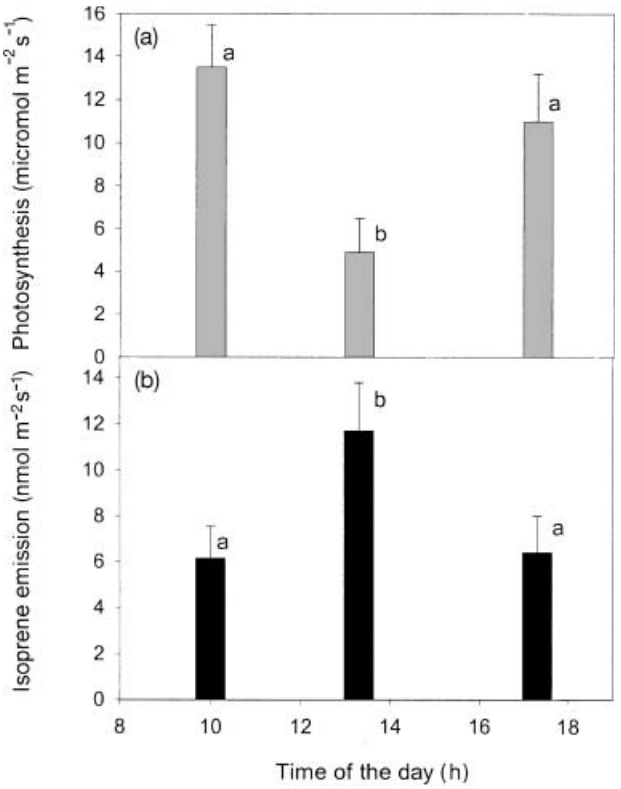

Figure 6. Diurnal trend of basal emission of photosynthesis (a) and isoprene (b) from *Phragmites* leaves. Measurements taken in the Bossoleto site during July 2001. Means ± SE, (n = 3) are shown. When measurements during the day were statistically different at $P < 0.05$ level, bars are labelled with different letters.

of the spring exhibits a CO_2 concentration averaged over the day of approximately 900–1100 μmol mol^{-1}, compared to peripheral concentrations of 700 μmol mol^{-1}. *Phragmites* plants at the peripheral stations thus grow under CO_2 concentrations that are both physiologically relevant and within the range of future predicted concentrations and are the most relevant to studies related to global change.

A negative effect of elevated CO_2 on isoprene emission has been often reported in laboratory studies (e.g. Loreto & Sharkey 1990; for plants *exposed* to elevated CO_2, and Sharkey *et al.* (1991) for plants *grown* at elevated CO_2 in growth cabinets, but see also contrasting results reported in the latter paper for different plant species). Results from field studies are very complex to interpret. Isoprene emission was reported to be higher in *Quercus pubescens* leaves grown at elevated CO_2 in the Bossoleto spring than in leaves of plants growing in a control site at ambient CO_2 (Tognetti *et al.* 1998), whereas experiments on a FACE facility did not show any effect of CO_2 on the emission of poplar clones (Loreto *et al.* 2001a) and recent experiments on controlled mesocosms indicated a clear inhibition of isoprene emission in poplar plants grown at elevated CO_2 (Rosenstiel *et al.* 2003). Isoprenoids more complex than isoprene can be emitted by Mediterranean oaks in an isoprene-like manner (Loreto *et al.* 1996). Two field experiments were made on *Quercus ilex* grown at elevated CO_2 and came to opposite conclusions, indicating inhibition (Loreto *et al.* 2001b) or stimulation (Staudt *et al.* 2001) of the emission of monoterpenes in elevated CO_2 with respect to ambient CO_2.

To better understand the causes of the observed CO_2-dependent inhibition of isoprene emission in *Phragmites* leaves, we carried out physiological and biochemical analyses regarding carbon metabolism components. CO_2-dependent differences of isoprene basal emission were not associated with changes in photosynthesis, indicating that the amount of fixed carbon was not limiting isoprene formation and emission at elevated CO_2. This observation also indicates that a different fraction of the fixed carbon was diverted into the isoprene biosynthetic pathway at the two CO_2 concentrations. Only at the bottom of the spring, where plants experienced very high CO_2 concentrations for part of the day, was photosynthesis slightly reduced. This reduction was not related to a reduction of chlorophyll but may have been caused by the consistent reduction in leaf nitrogen generally found in the Bossoleto spring.

Interestingly, leaf nitrogen reduction in the Bossoleto was also associated with a reduction in the rate of isoprene

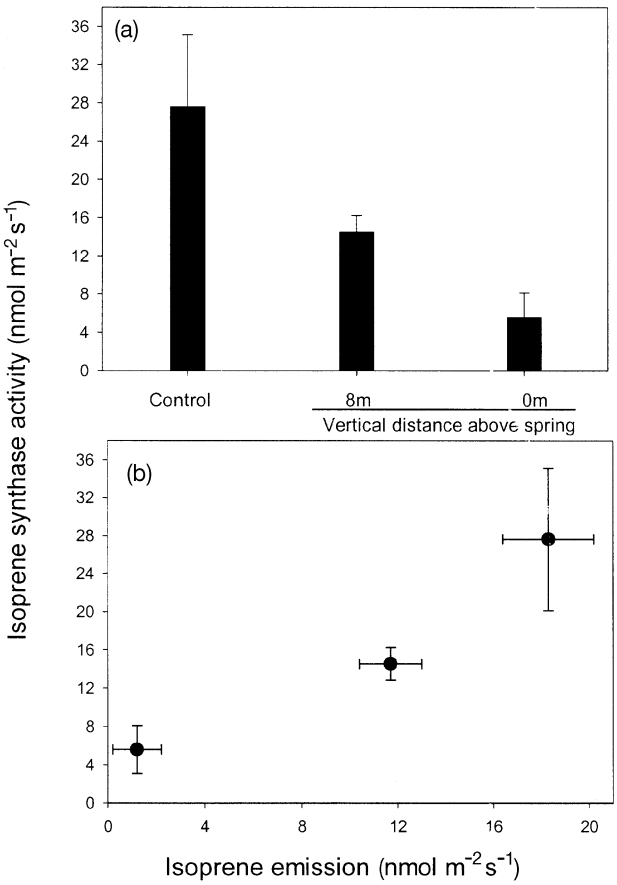

Figure 7. Isoprene synthase activity from leaves of *Phragmites* plants in the control site and in the Bossoleto site at different vertical distances from the CO_2 vent (a, details and statistical treatment as in legend of Fig. 4). Isoprene synthase activity is also plotted versus the isoprene emission measured at the corresponding sites (b).

emission. This suggests that nitrogen may be a regulating factor for isoprene formation. There have been reports that isoprene emission is related to nitrogen fertilization in poplar plants (Litvak *et al*. 1996) and that low nitrogen can limit the formation of other antioxidants Logan *et al*. 1999). Low nitrogen can limit the formation of nitrogen-rich molecules such as enzymes. Enzymological analysis revealed a progressive decrease of isoprene synthase activity at increasing level of CO_2. However, since the amount of soluble protein in the leaves from the different sampling sites are quite similar (data not shown), the observed levels of IsoS activity apparently are not reduced by a direct nitrogen limitation. A more likely explanation might be that enhanced CO_2 led to a reduced substrate availability (Rosenstiel *et al*. 2003) as a result of a metabolic competition for phosphoenolpyruvate. A similar negative CO_2 effect on isoprenoid-forming enzymes result was observed by Loreto *et al*. (2001b) in a study on *Quercus ilex* plants growing in open top chambers at different CO_2 concentrations. In plants growing at elevated CO_2, monoterpene synthase activities were significantly lower than in plants growing at ambient CO_2. Our result suggests that elevated CO_2 generally inhibits the expression of isoprenoid synthesis genes and isoprene synthase activity which may, in turn, limit formation of every chloroplast-derived isoprenoid.

We found that rapid switches from elevated to ambient CO_2 caused an immediate and steady increase in the emission for about 1 h. On the other hand, rapid switches from ambient to elevated CO_2 reduced the emission in less than 30 min. Interestingly, switching back to ambient CO_2 caused a partial recovery of the original emission at ambient CO_2. We interpret the velocity of the observed changes in isoprene emission after switching CO_2 as inconsistent with changes of nitrogen metabolism. Fast changes may however, be consistent with post-translational modification of enzymes operating in the biosynthetic pathway of isoprene formation, including IsoS, or to the availability of the isoprene precursor dimetylallylpyrophosphate (DMAPP), whose content has been shown to rapidly drop under elevated CO_2 (Rosenstiel *et al*. 2003). The response of isoprene emission to rapid switches of CO_2 was consistent with that observed in the stands permanently growing at ambient or elevated CO_2, namely, elevated CO_2 reduced isoprene emission. This suggests that other possible differences between the two sites (either in the genetics of the two populations or in the growth conditions) did not relevantly affect our experiments. However, as the spring environment is somewhat unique and difficult to replicate, we cannot exclude altogether that other environmental factors could also have somehow influenced the observed responses during our experiments.

There is growing evidence that basal emission of isoprenoids is not constant during the season (Lehning *et al*. 2001; Fischbach *et al*. 2002) and may also change during the day (Rapparini *et al*. 2004). We have seen a strong daily change of isoprene emission by *Phragmites* plants growing in the spring, with the emission peaking during the central hours of the day, in coincidence with a depression of photosynthesis. The daily trend observed in isoprene emission rate should not be directly attributed to changes of temperature and light intensity since basal emission is measured while maintaining these parameters at constant levels. We hypothesize that isoprene basal emission rate responds to the temperature contemporaneously experienced by other parts of the plants or by the whole stand of *Phragmites*. This explains why the emission is highest during the hottest hours of the day. Furthermore, the drop in CO_2 concentration occurring during the central hours of the day inside the spring may explain the observed increase in the emission of isoprene. The emission becomes low again when CO_2 starts to build-up at very elevated levels such as during the evening. If one (or both) explanations are valid, then basal emissions recorded under environmental conditions far different than those set for the leaf may not be adequate to correctly estimate emissions at the leaf or canopy level with current algorithms (Guenther *et al*. 1993, 1995). In any case the basal emission cannot be regarded as a fixed factor even for the same leaf and may need to be corrected for a series of variables perhaps including past temperatures (Sharkey & Yeh 2001), plant stand temperatures, and CO_2 concentration.

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