BVOC emissions, photosynthetic characteristics and changes in chloroplast ultrastructure of *Platanus orientalis* L. exposed to elevated CO₂ and high temperature

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Isoprene biosynthesis has a protective role on the photosynthetic machinery of Platanus plants exposed to changing environment (i.e., interaction between rising [CO₂] and heat wave).

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**A B S T R A C T**

To investigate the interactive effects of increasing [CO₂] and heat wave occurrence on isoprene (IE) and methanol (ME) emissions, *Platanus orientalis* was grown for one month in ambient (380 μmol mol⁻¹) or elevated (800 μmol mol⁻¹) [CO₂] and exposed to high temperature (HT) (38 °C/4 h). In pre-existing leaves, IE emissions were always higher but ME emissions lower as compared to newly-emerged leaves. They were both stimulated by HT. Elevated [CO₂] significantly reduced IE in both leaf types, whereas it increased ME in newly-emerged leaves only. In newly-emerged leaves, elevated [CO₂] decreased photosynthesis and altered the chloroplast ultrastructure and membrane integrity. These harmful effects were amplified by HT. HT did not cause any unfavorable effects in pre-existing leaves, which were characterized by inherently higher IE rates. We conclude that: (1) these results further prove the isoprene's putative thermo-protective role of membranes; (2) HT may likely outweigh the inhibitory effects of elevated [CO₂] on IE in the future.

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1. Introduction

Isoprene, the most important biogenic volatile organic compound (BVOC) released by leaves into the atmosphere (Guenther et al., 1995), is a key player in plant–environment interactions, as well as in atmospheric reactions. Because isoprene emission (IE) modulates plant tolerance to heat (Sharkey and Singsaaas, 1995), pollutant (Loreto and Velikova, 2001), and oxidative stresses (Loreto et al., 2001; Velikova et al., 2004), variations in its emission fluxes caused by continuously changing environment may significantly alter the biological and physico-chemical processes (Chameides et al., 1988; Grote and Niinemets, 2008). Given the importance of BVOC in the biosphere–atmosphere interactions, it is essential to understand whether current and future environmental changes, which include increased [CO₂] and incidence of extreme climatic events (Ciais et al., 2005; IPCC, 2007), will affect BVOC emissions by plants.

The effect of CO₂ fertilization on primary physiological processes of C₃ species, i.e. photosynthesis and stomata movement, is now firmly established (Curtis and Wang, 1998; Ainsworth and Long, 2005). The stimulation of photosynthesis and the reduction in stomata conductance, which result in increased water-use efficiency (Centritto et al., 2002) and delayed leaf senescence (Ainsworth and Long, 2005), are the basis of the beneficial effects of elevated [CO₂] on C₃ plant growth, and especially on forest trees that show the larger growth response to elevated [CO₂] (Ainsworth and Long, 2005). In addition, elevated [CO₂] also causes a wide range of secondary responses, which includes isoprene biosynthesis. In fact, while IE is closely linked to photosynthesis in ambient [CO₂], because 72–91% of the carbon in the isoprene molecule originates from recently fixed carbon (Delwiche and Sharkey, 1993; Ferrieri et al., 2005; Brilli et al., 2007) via the methylerythriol 4-phosphate (MEP) chloroplastic pathway (Lichtenthaler, 1999), it has been shown that, despite the stimulation in photosynthesis, rising [CO₂]...
negatively affects IE (Monson and Fall, 1989; Sharkey et al., 1991; Rosenstiel et al., 2003; Centritto et al., 2004; Scholtefeld et al., 2004). This reduction can be due to restricted availability of cytosolic phosphoenolpyruvate, necessary for the synthesis in the chloroplast of dimethylallyl-diphosphate, the immediate precursor in isoprene biosynthesis (Rosenstiel et al., 2003; Loreto et al., 2007).

Temperature is another major factor controlling IE. The rate of IE increases exponentially with temperature, and the trend of this response depends on plant species and on the temperature at which plants were grown or exposed (Monson and Fall, 1989; Monson et al., 1992; Sharkey and Loreto, 1993; Filella et al., 2007). Thus, because of the contrasting effects of elevated [CO2] and high temperature (HT) on IE, predicting plant IE and related changes in adaptive capacity to a changing environment becomes very difficult.

The interest in studying IE changes is also enhanced because of its proposed protective role under different stress conditions. The mechanisms by which isoprene protects plants are still unknown. One of the most studied aspects of isoprene protection is the thermotolerance hypothesis introduced for the first time by Sharkey and Singsaas (1995), and isoprene thermophot protection was demonstrated in different species (Singsaas et al., 1997; Hanson 1999; Velikova and Loreto, 2005).

The objective of the present research was to investigate the impact of the simultaneous occurrence of rising CO2 and transient heat waves on IE in 2-year-old Platanus orientalis plants. We focused on the response of two leaf types developed and matured in either ambient or elevated [CO2]. We specifically examined the relationships between carbon assimilation, carbon metabolism and IE, and on indicators of ultrastructural damage to chloroplasts after the exposure to heat stress. It was hypothesized that (1) the exposure to elevated [CO2] might positively affect photosynthesis and that this could reduce the negative effect of heat on carbon metabolism; and (2) CO2 enrichment might inhibit isoprene biosynthesis, which in turn could reduce thermotolerance and worsen the heat stress for photosynthesis.

2. Material and methods

2.1. Plant material and growing conditions

Two-year-old P. orientalis L plants were grown in 1.5 dm3 pots in a climate chamber under controlled conditions of 350 μmol m–2 s–1 photon flux density, 25°C day/night temperature, 65% relative humidity, and 12 h photoperiod. Plants were regularly watered to pot water capacity and fertilized once a week with full-strength Hoagland solution.

2.2. CO2 and high-temperature treatments

Plants were divided into two groups, ten saplings each, and transferred to two climatic chambers supplied with two different [CO2] amounts: 380 μmol mol–1 (ambient [CO2]) and 800 μmol mol–1 (elevated [CO2]). To simulate exposure to a heat wave, plants were exposed once to HT (38 °C) for 4 h at the end of 1 month of treatment with ambient or elevated [CO2] by increasing the temperature in climatic chambers. All analyses were done immediately after 4-h exposure to HT. Two types of leaves were used in the following experiments. Leaves that had already reached their fully expanded state before the onset of the CO2 fumigations are termed as “pre-existing” leaves, while leaves that developed during the CO2 fumigations are termed as “newly-emerged” leaves (Fig. 1).

2.3. Gas exchange and fluorescence measurements

Steady-state photosynthesis and stomatal conductance (gs) were measured by a portable gas exchange system (Li-6400-40 LiCor, Lincoln, NE, USA) equipped with a fluorescence probe. Measurements were made at the [CO2] of 380 and 800 μmol mol–1 on individual leaves enclosed into a leaf cuvette at a rate of 0.3 L min–1 air flow, relative humidity within the cuvette at 60–70% and growth-intensity illumination. Gas exchange and chlorophyll fluorescence were measured in unstressed control plants for each [CO2] treatment and on the same plants after the HT treatment. Gas exchange and fluorescence measurements were performed at 25 °C for control plants and 38 °C for temperature treated plants. Mesophyll conductance (gms) to CO2 was calculated by assuming that the electron transport rates calculated by gas exchange and fluorescence match in the absence of internal resistances and photosynthesis (Loreto et al., 1992). The actual [CO2] at the chloroplast site (ɛCO2) was then calculated from the gms value as shown elsewhere (Harley et al., 1992).

2.4. VOCs measurements

Isoprene and methanol (ME) emissions from leaves were measured in parallel with physiological parameters by diverting part of the cuvette outflow to a Proton Transfer Reaction-Mass Spectrometer (PTR-MS, Ionicon, Innsbruck, Austria), that allows sensitive quantification of BVOC emissions (Lindinger et al., 1998). The PTR-MS was calibrated before measurements against an isoprene and methanol gas standards (60 nL L–1) (Rivoira S.p.A. Milan, Italy). Isoprene and methanol were detected as parent ions at protonated m/z = 69 and 33, respectively (see for details Loreto et al., 2006; Filella et al., 2007). Then IE and ME rates (ER) were calculated as:

\[
\text{ER} = \frac{(E/26)/(F/LA)}
\]

where E is the gas concentration detected by PTR-MS (i.e. the ratio of count number s–1 to gas standard), 26 is the gas volume (L) at 30 °C, F is the air flow rate through the cuvette clamping a leaf of known area (LA).

2.5. Phosphoenolpyruvate carboxylase (PEPc) activity measurements

Flash frozen 0.2 g midrib-free leaf material was ground in a pre-chilled mortar with 1.5 ml extraction buffer containing 100 mM HEPES–KO H (pH 7.2), 10 mM DTT, 0.3% (v/v) Triton-X 100, 5 mM MgCl2 and PVP-40. Extracts were centrifuged at 11 000 × g for 5 min at 4 °C. Then 20 μl of the supernatant was immediately used for PEPc activity in an assay mix containing 25 mM Tricine KOH (pH 8.1), 5 mM MgSO4,
5 mM NaHCO₃, 5 mM DTT, 0.2 mM NADH and 5 U MDH (malic dehydrogenase). The reaction was initiated by the addition of 2 mM PEP and NADH. Absorbance changes were recorded for 5 min at 520 nm, at room temperature, according to Rosenstiel et al. (2004). Total sample protein content was determined according to Bradford (1976). Assays were performed on triplicate samples per leaf type and treatment.

2.6. Transmission electron microscopy (TEM)

Leaf segments (1 mm²) were cut from the middle part of the leaves for TEM analyses. These segments were then prefixed in 3% glutaraldehyde (m/v) in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4 °C and postfixed in 2% (m/v) O₃O₃₂ in the same buffer for 4 h at room temperature. After dehydration the material was embedded in Durcupan (Fluka). Ultra-thin sections were cut from palisade parenchyma using a Reichert ultra-microtome and were examined using electron microscope (JEOL 1200EX, Japan). Leaf segments from five plants of each treatment were investigated and at least ten micrographs of different palisade cells were taken.

2.7. Isolation of thylakoid membranes and measurements of low-temperature fluorescence

Thylakoid membranes were isolated as described by Harrison and Melis (1992) and were suspended in a medium containing 350 mM sorbitol, 5 mM MgCl₂, 10 mM NaCl and 10 mM Tris (pH 7.8). Low temperature (77 K) chlorophyll fluorescence emission spectra were recorded with a Jobin Yvon JY3 spectrofluorimeter (Jobin Yvon ISA, Longjumeau, France) supplied with a low-temperature device. Additional methodology can be found in Apostolova et al. (2006). To determine the relative contribution of specific chlorophyll-protein complexes to the overall fluorescence pattern, 0.5 μM fluorescein (sodium salt) was added as an internal standard to the medium. The fluorescence spectra were normalized to the same area (100) under the spectrum (Andreeva et al., 2003). The chlorophyll content of the samples was adjusted to 10 μg ml⁻¹ (Lichtenthaler, 1987).

2.8. Statistics

All data shown in the figures are means derived from replicates on different plants. The treatment means were statistically compared by Tukey’s test. Significantly different means (P < 0.05), derived from 6 to 8 plants per treatment, are shown by different letters among groups.

3. Results and discussion

3.1. Impact of high temperature on photosynthesis and emission of volatiles in elevated [CO₂]

Photosynthesis and the electron transport rate (ETR) were not significantly different in newly-emerged and pre-existing leaves in ambient [CO₂] (Fig. 2). Growth in elevated [CO₂] often stimulates net photosynthetic rate in C₃ plants due to enhanced supply of substrate to the CO₂ fixing enzymes and depression of photorespiration (Moore et al., 1999; Ainsworth and Long, 2005). However, we did not observe a significant stimulation of photosynthesis in pre-existing leaves, whereas photosynthesis of newly-emerged leaves grown in elevated [CO₂] was even reduced in comparison to that measured in ambient [CO₂] (Fig. 2a,b). ETR of both types of leaves was significantly lower in elevated [CO₂] (Fig. 2d), due to the reduction of photorespiratory electron transport. CO₂ insensitivity or reversed sensitivity can be due to a downward acclimation of photosynthetic capacity (Curtis and Wang, 1998; Moore et al., 1999). This process, which is likely related to unbalanced source-sink relations, often occurs when plants exposed to elevated [CO₂] are grown in small pots. In our study, the rather small pot volume in which the 2-year-old saplings were grown have likely affected the sink size by restricting root growth. This may have induced a decrease in the amount of the photosynthetic pigments and enzymes, for instance amount and activation state of Rubisco and, thus, acclimation of the photosynthetic apparatus (Centritto and Jarvis, 1999; Moore et al., 1999; Stitt and Krapp, 1999; Ainsworth and Long, 2005). However, negative feedback on enzymes involved in sucrose synthesis and transport (Micallef et al., 1996), or direct damage to chloroplast membranes (Cave et al., 1981) may also cause loss of photosynthetic capacity in C₃ species in elevated [CO₂].

Moderate heat stress was shown to induce photochemical damage, stimulating the reduction of the plastoquinone pool in the dark and increasing cyclic electron flow around PSI in the light (Pastenes and Horton, 1996), and inducing changes in thylakoid structure that increase membrane leakiness (Havaux et al., 1996). In our experiments, a 4-h exposure to HT caused a significant decline of photosynthesis and especially in photosynthetic (linear) ETR only in newly-emerged leaves under elevated [CO₂], further indicating a downward acclimation of Rubisco amount and/or activation state in elevated [CO₂], while it did not significantly affect these parameters in all other cases (Fig. 2c,d). We hypothesized that photosynthesis stimulation in elevated [CO₂] could reduce the negative effect of HT on the photochemical apparatus, mainly by keeping a high linear ETR. Inefficient photosynthetic ETR contributes to direct oxygen photoreduction and to the accumulation of dangerous reactive oxygen species in the mesophyll (Sharkey, 2005). However, photosynthesis was not stimulated by elevated [CO₂] in Platanus plants and our hypothesis could not be successfully tested.

We verified whether reduction of photosynthesis of newly-emerged leaves in elevated [CO₂] after the HT treatment was due to diffusive limitations, i.e. to reduced CO₂ availability for photosynthesis. As expected (Ainsworth and Long, 2005), gs, which was clearly higher in newly-emerged than in pre-existing leaves in ambient [CO₂], was decreased in both type of leaves in response to...
elevated [CO\textsubscript{2}] (Fig. 3a,b). After the HT treatment, \( g_s \) of newly-emerged leaves grown in ambient [CO\textsubscript{2}] decreased significantly while no changes were observed in pre-existing leaves grown in the same CO\textsubscript{2} level (Fig. 3a). A slight but not significant increase of \( g_s \) was observed in both leaf types grown in elevated [CO\textsubscript{2}] after the HT treatment (Fig. 3b). However, stomatal limitation to photosynthesis did not increase during heat stress as revealed by the generally steady intercellular CO\textsubscript{2} concentration (\( C_i \)) (Fig. 3c,d). In the case of newly-emerged leaves, in particular, a higher \( C_i \) was observed, suggesting that biochemical or photochemical constraints limited the use of CO\textsubscript{2} acquired by the leaves (Flexas et al., 2004). Numerous experiments have shown that CO\textsubscript{2} drawn down in the mesophyll can largely reduce CO\textsubscript{2} concentration available at chloroplasts. The mesophyll therefore constitutes an additional diffusive resistance that should be accounted for, especially when limiting photosynthesis, such as in tree species (Loreto et al., 1992), and under stress conditions (Centritto et al., 2003; Flexas et al., 2004).

Mesophyll conductance was indeed strongly reduced in leaves growing in elevated [CO\textsubscript{2}] (Fig. 3e,f). The reduction of \( g_m \) upon short-term exposure to elevated [CO\textsubscript{2}] was observed first in Quercus rubra leaves by Harley et al. (1992) and then repeatedly, e.g. by Centritto et al. (2003) and Flexas et al. (2007). However, acclimatory responses to high [CO\textsubscript{2}] showed controversial results. Bernacchi et al. (2005) did not find any significant difference in \( g_m \) in soybean under FACE conditions, whereas Singsaas et al. (2003) found a small, but significant effect of elevated [CO\textsubscript{2}] on \( g_m \) in almost all species studied. They also found a linear relationship between \( g_m \) and photosynthetic capacity. Our finding reveals that \( g_m \) reduction in high [CO\textsubscript{2}] can be an acclimatory response when plants are grown under enhanced [CO\textsubscript{2}]. In addition, we also showed that the effect of elevated [CO\textsubscript{2}] on \( g_m \) was much higher in newly-emerged leaves, suggesting that \( g_m \) is more influenced by changes occurring during leaf development. The anatomical features are indeed the main determinants of \( g_m \) (Evans, 1999) and the impact of CO\textsubscript{2} on mesophyll assembly and packaging, and associated gaseous and liquid phase resistances to CO\textsubscript{2} diffusion may be responsible for the observed acclimatory response. Interestingly, the HT treatment associated with growth in elevated [CO\textsubscript{2}] had contrasting consequences for \( g_m \) of the two leaf types. While the heat stress slightly reversed the CO\textsubscript{2}-induced reduction of \( g_m \) in pre-existing leaves, \( g_m \) of newly-emerged leaves was further reduced. \( C_i \) was not affected in ambient [CO\textsubscript{2}] (Fig. 3g), whereas \( C_i \) of pre-existing leaves was higher than in newly-emerged leaves in elevated [CO\textsubscript{2}] (Fig. 3h). However, irrespective of the impact of HT on the leaf types, \( C_i \) increased in leaves grown in elevated [CO\textsubscript{2}] and after the HT treatment (Fig. 3h). This further confirms that diffusive limitations are not responsible for photosynthesis inhibition caused by HT in the newly-emerged leaves in elevated [CO\textsubscript{2}].

The second question that we asked was whether CO\textsubscript{2}-dependent inhibition of IE could make worse the impact of HT on photosynthesis. Isoprene emission was inherently higher in pre-existing than newly-emerged leaves in both CO\textsubscript{2} treatments (Fig. 4a,b). IE was stimulated by HT exposure in both leaf types and in both CO\textsubscript{2} treatments, but this stimulation was much lower in elevated [CO\textsubscript{2}] (Fig. 4b) than in ambient [CO\textsubscript{2}] (Fig. 4a). Indeed our data show that newly-emerged leaves in elevated [CO\textsubscript{2}], which are characterized by the lowest IE, were the most sensitive leaves to HT (compare Figs. 2 and 4). Thus, isoprene thermo-protective function remains once more, though indirectly, confirmed. It is known that young leaves become slowly competent to emit isoprene and emit less than mature leaves (Sharkey and Loreto, 1993; Centritto et al., 2004; Loreto et al., 2007). The lower capacity of young leaves to emit isoprene was attributed to isoprene synthase activity and quantity as the enzyme features developed similarly to the emission rates (Kuzma and Fall, 1993), and it has been suggested that the biosynthesis of isoprene is developmentally regulated at the level of gene expression (Mayrhofer et al., 2005; Wiberley et al., 2005).

Growth in elevated [CO\textsubscript{2}] also caused the expected decrease in IE in both newly-emerged and pre-existing P. orientalis leaves (Fig. 4b). In elevated [CO\textsubscript{2}] a substantial decline in IE is commonly observed (Rosenstiel et al., 2003; Centritto et al., 2004; Scholefield et al., 2004), even though photosynthesis, which provides the bulk of the carbon needed for isoprene biosynthesis (Sharkey and Yeh, 2001), is often stimulated, as previously commented. Such
a surprising effect of elevated [CO₂] was attributed to either a decrease of isoprene synthase activity (Schollefeld et al., 2004), or the metabolic competition for phosphoenolpyruvate (PEP), the common substrate of isoprene biosynthesis and mitochondrial respiration (Rosenstiel et al., 2003). Rosenstiel et al. (2003, 2004) showed that inhibition of the activity of phosphoenolpyruvate carboxylase (PEPc), the enzyme that provides substrate for respiration and nitrate assimilation, stimulated IE. In our experimental conditions, PEPc activity was almost two-fold higher in pre-existing leaves compared to newly-emerged leaves in ambient [CO₂] (Fig. 4c) and, consequently, a direct relationship was generally found between IE and PEPc activity when comparing newly-emerged and pre-existing leaves grown in ambient [CO₂] (compare Fig. 4a,c). This suggests that the lower emission of newly-emerged leaves can also be caused by a low contribution of cytosolic pyruvate. The lower emission of isoprene in leaves grown in elevated [CO₂] was also associated with a lower activity of PEPc (Fig. 4b,d), thus confirming the hypothesis by Rosenstiel et al. (2003) that isoprene inhibition in elevated [CO₂] may be due to insufficient pyruvate supply. However, in leaves exposed to HT treatment the relationship between IE and PEPc was not straight. In particular, in elevated [CO₂] PEPc activity, which was largely stimulated by the HT treatment (Fig. 4d), did not sustain high rates of IE. In contrast, in ambient [CO₂], the large increase of IE after the HT treatment was associated with reduced PEPc activity (Fig. 4c) with respect to that measured before heat stress. It is therefore suggested that PEPc-generated cytosolic pyruvate may not limit IE or regulate isoprene stimulation upon HT treatments.

The activity of isoprene synthase and IE are temperature-dependent processes (Loreto and Sharkey, 1990; Monson et al., 1992). Consistently, the stimulation of IE after the HT treatment, observed in both newly-emerged and pre-existing leaves, and in both [CO₂] levels, is attributed to higher isoprene synthase activity. It should be noted, however, that the stimulation of IE was lower in elevated [CO₂], and especially in newly-emerged leaves. As we discussed before, the low stimulation of IE may be due to constitutively low levels of this enzyme in newly-emerged leaves and/or to inhibition of enzyme activity in elevated [CO₂]. It is suggested that a down-regulation of isoprene synthase, often occurring upon prolonged exposure to HT (Singsaas and Sharkey, 2000; Loreto et al., 2006) might be also responsible for the loss of temperature stimulation of IE and could therefore indirectly influence leaf sensitivity to HT.

Fig. 4. Isoprene emission (a, b), PEPc activity (c, d) and methanol emission (e, f) in newly-emerged and pre-existing Platanus orientalis leaves, before (white bars) and after a 4-h heat stress (38 °C) (hatched bars). Bar assignment, treatments, replications and statistical treatment as in Fig. 2.
ultrastructure and functionality. Well-differentiated chloroplasts characterized the mesophyll of pre-existing (Fig. 5a) and newly-emerged leaves (Fig. 5b) at ambient [CO$_2$], containing a well-developed inner membrane system, composed of grana of different sizes (from 5–10 up to 30 thylakoids) and long stromal thylakoids. Single midsize starch grains were rarely discerned. Investigation of chloroplast ultrastructure, however, revealed that it was affected by the [CO$_2$] treatment, especially in newly-emerged leaves. Chloroplasts of newly-emerged leaves in elevated [CO$_2$] were characterized by an unusually large peristromium located toward the cell wall without destruction of the inner membrane system (Fig. 5c). These chloroplasts were also in close structural contact with mitochondria through relatively large typically structured associative regions. Changes in the chloroplast structure organization of newly-emerged leaves in elevated [CO$_2$] are unlikely stress-induced traits and rather represent adaptive changes at subcellular level to our experimental conditions (Mostowska, 1997). The increase of stroma volume and the reduction of part of

Fig. 5. Electron micrographs of *Platanus orientalis* leaves, illustrating chloroplast structural changes in leaf mesophyll cells in pre-existing (A, D) and newly-emerged (B, C, E) leaves under two regimes of CO$_2$ (380 and 800 μmol mol$^{-1}$) and after exposure to high temperature (38 °C) for 4 h, applied alone and in combination. Scale bars = 500 nm. CH, chloroplast; G, grana; ST, stroma thylakoid; S, starch grain; P, peristromium; T, tannins. (A) Intact chloroplast of pre-existing leaves at ambient CO$_2$ and 25 °C; (B) intact chloroplast of newly-emerged leaves at ambient CO$_2$ and 25 °C; (C) elevated-CO$_2$-induced structural changes in newly-emerged leaves at 25 °C; (D) combined effect of elevated CO$_2$ and 38 °C/4 h on chloroplast structure of pre-existing leaves; (E) combined effect of elevated CO$_2$ and 38 °C/4 h on chloroplast structure of newly-emerged leaves.
the thylakoid system, accompanied by a reduction of thylakoid electron density and increased number of plastoglobules, have been previously observed after treatments with elevated \([\text{CO}_2]\) (Kutí´k et al., 1995). It has been hypothesized that these changes in chloroplast ultrastructure in plants grown in elevated \([\text{CO}_2]\) may be needed to meet the higher energy demand required for faster growth (Griffin et al., 2001). In fact, similar changes were only observed in newly-emerged, fast-growing leaves, and not in pre-existing leaves in our experiment. It should be noted, however, that these ultrastructural changes did not cause a stimulation of photosynthesis in Plananus leaves.

TEM analysis also showed that exposure to elevated \([\text{CO}_2]\) caused the accumulation of tannin-like vacuolar deposition in both types (Fig. 5c,d). This was observed both in newly-emerged and pre-existing leaves and therefore is not associated to growth requirements. Increased tannin depositions were also observed in aspen plants exposed to increasing \([\text{CO}_2]\), suggesting a \([\text{CO}_2]\)-induced activation of the phenylpropanoid pathway (Oksanen et al., 2001), with possible but yet largely unknown protective functions at cellular level.

Four-hour HT treatment did not cause any structural changes in the chloroplasts of both types of leaves grown in ambient \([\text{CO}_2]\) (data not shown). However, in leaves grown in elevated \([\text{CO}_2]\) the HT exposure affected chloroplast ultrastructure of both leaf types. HT treatment of pre-existing leaves grown in elevated \([\text{CO}_2]\) caused the accumulation of starch and structural changes of thylakoid membranes that became wavy (Fig. 5d). A large part of chloroplasts was occupied by 1–2 big starch grains, in which zones of saccharides and capsule, containing enzymes, were not typically structured. Starch accumulation in elevated \([\text{CO}_2]\) occurs when the production of new assimilates is larger than the capacity to handle them (Stitt and Krapp, 1999). Normally, starch accumulation in leaves, by maintaining the Pi cycling, allows photosynthesis to continue (Stitt and Krapp, 1999). However, when the source–sink relation is disrupted, starch accumulation may become excessive and Pi cycling results progressively inhibited; this is, in turn, usually related to feedback inhibition of photosynthesis (Pritchard et al., 1997). The thylakoid membranes of the same chloroplast were wavelike and parts of stroma thylakoids were fragmented. Several studies showed that heat stress and elevated \([\text{CO}_2]\) can cause thylakoid destruction and starch accumulation in woody and herbaceous plants (Mostowska, 1997; Oksanen et al., 2001; Sam et al., 2001; Lambreva et al., 2005).

Starch did not accumulate in the chloroplasts of newly-emerged leaves, probably because photosynthesis of these leaves was impaired before starch synthesis. In fact, when newly-emerged leaves grown in elevated \([\text{CO}_2]\) were exposed to HT, a destruction of part of the peripheral thylakoid membranes was observed (Fig. 5e). The unusually large peristromum was filled with membrane substance from fragmented peripheral thylakoids with swollen intrathylacoidal space (Fig. 5e, arrows). This is a typical ultrastructural damage induced by HT. Kislyuk et al. (2004) also found that a large volume of the stroma was occupied by sinuous thylakoids and fragments of thylakoid membranes in response to 30 min heating at 42 °C under high light. We speculate that the negative impact of HT on membrane structure of newly-emerged leaves that emit the lowest level of isoprene indicates that isoprene is indeed involved in a mechanism of thylakoid membrane protection, as previously hypothesized (Sharkey and Singsaas, 1995; Singsaas et al., 1997) and theoretically recently predicted (Siwko et al., 2007). Isoprene lipophilic properties may actually allow easy insertion of the molecule in thylakoidal membranes that are strengthened by such an insertion. However, localization of isoprene into chloroplast membranes is needed to conclusively prove the proposed mechanism of action.

77 K chlorophyll fluorescence emission spectra were measured in order to understand if isoprene presence can modulate the integrity and energy transfer between both photosystems and whether it can protect photosynthetic apparatus against HT injuries. These analyses revealed that lower IE in newly-emerged leaves also considerably affected the functionality and integrity of pigment–protein complexes even under control conditions. Pre-existing and newly-emerged leaves grown at both \([\text{CO}_2]\) regimes had well-defined F685 and F735 peaks (Fig. 6). The differences occurred in amplitude ratio F735/F685 (Table 1), used to characterize the direct energy transfer from PSII to PSI complexes.

Fig. 6. 77 K emission spectra of thylakoids isolated from: (a) newly-emerged leaves at ambient \([\text{CO}_2]\); (b) pre-existing leaves at ambient \([\text{CO}_2]\); (c) newly-emerged leaves at elevated \([\text{CO}_2]\); (d) pre-existing leaves at elevated \([\text{CO}_2]\).
and it is likely to be reduced under future elevated [CO₂] levels. On climate-induced damage than in the present climate. This investigation supports our second hypothesis that leaves with inhibited IE are more sensitive to heat stress. Thus, in a warmer and elevated [CO₂] environment, Picea abies, grown at ambient or elevated [CO₂] a t2 5 0.03a 1.05b 0.02d 1.04 0.09b 0.86 ± 0.03b
Treatments 25 °C 38 °C
Newly-emerged Pre-existing Newly-emerged Pre-existing
Ambient [CO₂] 1.03 ± 0.02a 1.43 ± 0.03a 1.05 ± 0.05a 1.56 ± 0.07b
Elevated [CO₂] 0.74 ± 0.02c 0.78 ± 0.02c 1.04 ± 0.09b 0.86 ± 0.03b

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