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Linking isoprene with plant thermotolerance, antioxidants and monoterpene emissions

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ABSTRACT

The purpose of the present study was to test the possible plant thermotolerance role of isoprene and to study its relationship with non-enzymatic antioxidants and terpene emissions. The gas exchange, chlorophyll fluorescence, extent of photo- and oxidative stress, leaf damage, mechanisms of photo- and antioxidant protection, and terpene emission were measured in leaves of *Quercus ilex* seedlings exposed to a ramp of temperatures of 5 °C steps from 25 to 50 °C growing with and without isoprene (10 μL L^{-1}) fumigation. The results showed that isoprene actually conferred thermotolerance (shifted the decrease of net photosynthetic rates from 35 to 45 °C, increased F_v/F_m at 50 °C from 0.38 to 0.65, and decreased the leaf area damaged from 27 to 15%), that it precluded or delayed the enhancement of the antioxidant non-enzymatic defence conferred by α-tocopherol, ascorbic acid or β-carotene consumption in response to increasing temperatures, and that it decreased by approximately 70% the emissions of monoterpenes at the highest temperatures. This suggests that there are inducible mechanisms triggered by the initial stages of thermal damage that up-regulate these antioxidant compounds at high temperatures and that these mechanisms are somehow suppressed in the presence of exogenous isoprene, which seems to already exert an antioxidant-like behaviour.

Key-words: antioxidants; ascorbic acid; α-tocopherol; β-carotene; fumigation; F_v/F_m; high temperatures; isoprene; monoterpenes; photosynthetic rates; thermotolerance; zeaxanthin.

INTRODUCTION

All plants emit a substantial fraction of their assimilated carbon into the atmosphere in the form of phytogenic volatile organic compounds (PVOCs). Biologists have always wondered about their function, if there is any (Peñuelas & Llusià 2004). Among these PVOCs, isoprene is the most frequntly emitted (Geron *et al*. 2000) and its possible function one of the most studied (Sharkey & Yeh 2001; Rosenstiel *et al*. 2004). However, the role of isoprene in plants is still unclear. Several experiments have shown that isoprene may have a role in protecting plants from thermal damage (Sharkey & Singsaas 1995; Singsaas *et al*. 1997; reviewed by Sharkey & Yeh 2001). However, such enhancement of thermotolerance has not always been found (Logan & Monson 1999).

One potential thermal protection role of isoprene is the stabilization and protection of plant membranes against high temperatures (Sharkey & Singsaas 1995; Singsaas 2000; Loreto & Velikova 2001), which has even been described in neighbouring non-emitting species, although at very high air concentrations (Delfine *et al*. 2000). A second hypothesis for a protective role of isoprene and monoterpenes is that they serve as an antioxidant in leaves (Loreto & Velikova 2001). Isoprene may confer protection against singlet oxygen in leaves (Affeck & Yakir 2002) but the antioxidant effect may be a general hydrocarbon effect and related to the double bonds in the isoprene molecule. This more general antioxidant action has been hypothesized on the basis of the protection offered by exogenous isoprene in non-emitting plants exposed to acute ozone doses. Loreto & Velikova (2001) inhibited the synthesis of endogenous isoprene by feeding fosmidomycin and observed that *Phragmites australis* leaves became more sensitive to ozone than those leaves forming isoprene. Their results indicated that isoprene may exert its protective action at the membrane level, although a similar effect could be obtained if isoprene reacted with ozone before forming activated oxygen species (AOS). Irrespective of the mechanism, results suggest that endogenous isoprene has an important antioxidant role in plants. Furthermore, Loreto *et al*. (2004) have suggested that all volatile isoprenoids may have similar antioxidant properties and may be stimulated by the same stress-inducing conditions.

High temperatures may lead to an imbalance between antioxidant defences and the amount of AOS resulting in oxidative stress (Gong *et al*. 1997; Larkindale & Knight 2002). Accumulation of AOS can cause damage at various levels of organization, including chloroplasts (Halliwell & Gutteridge 1989; Asada 1999). Apart from the xanthophyll cycle, photorespiration and other changes in metabolic activity that may protect the chloroplasts from oxidative damage (Demmig-Adams & Adams 1996; Kozaki & Takeba 1996; Eskling, Arvidsson & Akerlund 1997;

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Osmond *et al*. 1997; Peñuelas & Llusià 2002), there are a number of enzymatic and non-enzymatic antioxidants present in chloroplasts that serve to control oxygen toxicity (Smirnoff 1993; Foyer, Lelandais & Kunert 1994; Asada 1999). Among the latter, tocopherols (vitamin E) and carotenoids play an important role maintaining the integrity of the photosynthetic membranes under oxidative stress (Havaux 1998; Asada 1999; Smirnoff 2000; Munné-Bosch & Alegre 2002a).

As there are inducible mechanisms triggered by the initial stages of thermal damage that up-regulate antioxidant compounds at high temperatures, we hypothesized that if isoprene fumigation confers thermotolerance to plants, then antioxidant defences such as α-tocopherol or ascorbic acid, or the consumption of β-carotene, or the emissions of monoterpenes, would not increase as much in isoprene-fumigated plants than in plants not fumigated with isoprene. To test this hypothesis, we studied an apparently non-isoprene emitting but monoterpene-emitting plant species, *Quercus ilex* (Loreto *et al*. 1996). In this way, we avoided interference with internally produced isoprene, and moreover we could test the responses of monoterpene emissions and their likely antioxidant thermotolerant effect. *Quercus ilex* is one of the most typical dominant Mediterranean forest species. Its leaves may suffer from thermal stress above 35 °C (Larcher 2000). Usually CO_2 uptake suddenly decreases at 40–45 °C, but *Q. ilex* still grows on sites where the maximum air temperatures reach 40–50 °C (Sevilla, Spain). Its emissions of biogenic terpenes might have an enhancing effect on the thermotolerance of leaves (Loreto *et al*. 1998; Peñuelas & Llusià 1999) although some times only in particular conditions such as in the absence of photorespiration (Peñuelas & Llusià 2002). We measured leaf net photosynthetic rates, chlorophyll fluorescence, visual leaf damage, lipid peroxidation, antioxidant concentrations, and monoterpene emissions and concentrations of *Q. ilex* seedlings exposed to temperature increases from 25 to 50 °C in 5 °C steps in atmospheres fumigated with isoprene (10 μL L^{-1}) or under control atmospheric conditions. Our general aims were: (1) to assess the hypothesis of thermotolerance induced by isoprene; and (2) to determine its links to oxidative stress, antioxidants and monoterpene emissions.

MATERIALS AND METHODS

Experimental system, isoprene fumigation and variables measured

Two-year-old plants of *Quercus ilex* L previously grown in a nursery (Forestal Catalana, S.A., Breda, Spain) in Mediterranean-like environmental conditions were transplanted to 2 L pots with a substrate composed of peat and sand (2 : 1), and were well watered, and maintained in Mediterranean-like environmental conditions in a greenhouse until the beginning of the experiments. They were then placed inside a 1.68-m^3 chamber (Bio Line Mod. VB 1014; Vötsch-industrietechnik, Balingen-Frommern, Germany). Chamber environmental conditions [photosynthetic photon flux density (PPFD), relative humidity and temperature] were programmed independently by an automated control mechanism. Light was supplied by eight 400-W halogen lamps (Model HQI-T 400 W; Vötsch-industrietechnik, Balingen, Germany), each supplying a PPFD of about 500–600 μmol m^{-2} s^{-1} on the upper canopy during a 12-h photoperiod. Relative humidity in the chamber was maintained around 50%. Plants were exposed to a temperature ramp of 5 °C steps from 25 to 50 °C. A final 25 °C temperature was tested to assess recovery. Two different atmospheres were assayed as a result of isoprene-fumigation and non-fumigation. Isoprene fumigation was applied from a liquid standard with a membrane pump that fluxed air through a flask containing liquid isoprene (Fluka, Buchs, Switzerland). This flask was placed in a thermostatic bath at 25 °C. The concentration in the fumigated chamber was 10 ± 1.3 μL L^{-1} ($n = 21$), whereas the concentration in the control chamber was 0.05 ± 0.016 μL L^{-1} ($n = 21$). The system was allowed to equilibrate for 1 d before starting the measurements. The plants were maintained at each temperature for 24 h before measuring leaf net photosynthetic rates, chlorophyll fluorescence, extent of oxidative stress, leaf damage, photo- and antioxidative protection, terpene emissions and concentrations, leaf area and dry weight in fully developed, apparently healthy, green current-year leaves sampled at similar height (close to the top of the canopy) and exposed to a similar light intensity. For measurements of terpene concentrations, malondialdehyde (MDA), α-tocopherol, ascorbic acid, and photosynthetic pigments, the leaves were collected, frozen in liquid nitrogen and stored at –30 °C until analysis.

Photosynthetic rates and stomatal conductance

A calibrated Ciras-2 porometer (PP Systems, Hitchin, Hertfordshire, UK) was used for determination of CO_2 and H_2O exchange. Intact leaves were clamped in Parkinson leaf cuvettes (Std Broad 2.5). Leaf temperature was also measured with this IRGA-porometer. It was approximately 2 °C higher than the temperature of the growth chamber.

Fluorescence measurements

The variable to maximum fluorescence ratio, F_v/F_m in the non-energized state after darkness is a reliable measure of the potential efficiency of PSII photochemistry. It is used as an estimate of the functional state of the photosynthetic apparatus at a given environmental situation. A decrease in the F_v/F_m indicates photo-inhibition of PSII (Oliveira & Peñuelas 2001).

At each temperature of the experimental ramp, leaf chlorophyll fluorescence was determined using a portable modulated fluorometer PAM-2000, including the leaf clip holder part 2030-B (Heinz Walz GmbH, Effeltrich, Germany). Maximal (potential) photochemical efficiency of PSII (given by F_v/F_m) was estimated in leaves after dark adaptation for 20 min

Estimation of lipid peroxidation

The extent of lipid peroxidation in leaves was estimated by measuring the amount of MDA by the method described by Hodges *et al*. (1999), which takes into account the possible influence of interfering compounds in the assay for thiobarbituric acid (TBA)-reactive substances. In short, samples were repeatedly extracted with 80 : 20 (v/v) ethanol : water containing 1 p.p.m. butylated hydroxytoluene (BHT) using sonication. After centrifugation, supernatants were pooled and an aliquot of appropriately diluted sample was added to a test tube with an equal volume of either: (1) –TBA solution containing 20% (w/v) trichloroacetic acid and 0.01% (w/v) BHT; or (2) +TBA solution containing the above plus 0.65% (w/v) TBA. Samples were heated at 95 °C for 25 min and, after cooling, absorbance was read at 440, 532 and 600 nm. MDA equivalents (nmol mL^{-1}) were calculated as $10^6 \times ((A - B)/157000)$, where $A = [(Abs\ 532_{+TBA}) - (Abs\ 600_{+TBA}) - (Abs\ 532_{-TBA} - Abs\ 600_{-TBA})]$, and $B = [(Abs\ 440_{+TBA} - Abs\ 600_{+TBA}) \times 0.0571]$.

Photosynthetic pigments, α-tocopherol and ascorbic acid

The extraction and high-performance liquid chromatography (HPLC) analyses of photosynthetic pigments and α-tocopherol were carried out essentially as described by Munné-Bosch & Alegre (2000). In short, leaves were repeatedly extracted with ice-cold 85 (v/v) and 100% acetone using sonication. Pigments were separated on a Dupont non-endcapped Zorbax ODS-5 μm column (250 × 4.6 mm, 20%C; Scharlau, Barcelona, Spain) at 30 °C at a flow rate of 1 mL min^{-1}. The solvents consisted of (A) acetonitrile/methanol (85 : 15, v/v) and (B) methanol/ethyl acetate (68 : 32, v/v). The gradient used was: 0–14 min 100% A, 14–16 min decreasing to 0% A, 16–28 min 0% A, 28–30 min increasing to 100% A, and 30–38 min 100% A. Detection was carried out at 445 nm (Diode array detector 1000S; Applied Biosystems, Foster City, CA, USA). Purified standards of chlorophyll *a* and *b* were purchased from Fluka, and lutein, zeaxanthin and β-carotene were provided by Hoffman-La Roche (Basel, Switzerland). Neoxanthin, violaxanthin and antheraxanthin were identified by their spectra in hexane and ethanol.

The α-T was separated on a Partisil 10 ODS-3 column (250 × 4.6 mm; Scharlau,) at a flow rate of 1 mL min^{-1}. The solvents consisted of (A) methanol/water (95: 5, v/v) and (B) methanol. The gradient used was: 0–10 min 100% A, 10–15 min decreasing to 0% A, 15–20 min 0% A, 20–23 min increasing to 100% A, and 23–28 min 100% A. The α-T was quantified through its absorbance at 283 nm (Diode array detector 1000S; Applied Biosystems). The α-T was identified by its characteristic spectra and by co-elution with an authentic standard, which was obtained from Fluka.

The extraction and HPLC analysis of reduced and oxidized ascorbic acid in leaves was performed as previously described (Munné-Bosch & Alegre 2002b) In short, leaves were ground in liquid nitrogen and repeatedly extracted with ice-cold extraction buffer [40% (v/v) methanol, 0.75% (w/v) *m*-phosphoric acid, 16.7 mM oxalic acid, 0.127 mM diethylenetriaminepentaacetic acid] using ultrasonication (Vibra-Cell Ultrasonic Processor). After centrifugation, 0.1 mL of the supernatant was transferred to 0.9 mL of the mobile phase [24.25 Na-acetate/acetic acid, pH 4.8; 0.1 mM diethylenetriaminepentaacetic acid; 0.015% (w/v) *m*-phosphoric acid; 0.04% (w/v) octylamine; 15% (v/v) methanol] for determination of reduced ascorbic acid. For determination of total ascorbic acid (reduced plus oxidized) 0.1 mL of the supernatant was incubated for 10 min at room temperature in darkness with 0.25 mL of 2% (w/v) dithiothreitol and 0.5 mL of 200 mM $NaHCO_3$. The reaction was stopped by adding 0.25 mL of 2% (v/v) sulphuric acid and 0.8 mL of the mobile phase. Ascorbic acid was isocratically separated on a Spherisorb ODS C_8 column (Teknokroma, St. Cugat, Spain) at a flow rate of 0.8 mL min^{-1}. Detection was carried out at 255 nm (Diode array detector 1000S; Applied Biosystems). Ascorbic acid was identified by its characteristic spectrum and by co-elution with an authentic standard from Sigma (Steinheim, Germany).

Terpene emission analysis

Part of the air exiting the chamber flowed through a 'T' system to a glass tube (11.5 cm long and 0.4 cm internal diameter) manually filled with terpene adsorbents Carbotrap C (300 mg), Carbotrap B (200 mg), and Carbosieve S-III (125 mg) (Supelco Inc., Bellefonte, PA, USA) separated by plugs of quartz wool. The hydrophobic properties of the tubes were supposed to minimize sample displacement by water. In these tubes terpenes did not suffer chemical transformations as checked with standards (α-pinene, camphene, β-pinene, myrcene, *p*-cymene, limonene, α-phellandrene, and dodecane). Prior to use, these tubes were conditioned for 3 min at 350 °C with a stream of purified helium. The sampling time was 5 min, and the flow varied between 100 and 200 cm^3 min^{-1} depending on the glass tube adsorbent and quartz wool packing. The flow passing through the volatile organic compound (VOC) adsorbents was measured with a bubbler flowmeter. The trapping and desorption efficiency of liquid and volatilized standards such as α-pinene, β-pinene or limonene was practically 100%.

After VOC sampling, the adsorbent tubes were stored at –30 °C until analysis (within 24–48 h). There were no observable changes in terpene concentrations after storage of the tubes as checked by analysing replicate samples immediately and after 48-h storage. Isoprene and terpene analyses were conducted in a GC-MS (Hewlett Packard HP59822B; Palo Alto, CA, USA). They were desorbed (Thermal Desorption Unit, Model 890/891; Supelco Inc) at 250 °C during 2 min and injected into a 30 m × 0.25 mm × 0.25 mm film thickness capillary column (HP-5, Crosslinked 5% pH Me Silicone; Supelco Inc.).

After sample injection, the initial temperature (46 °C) was increased at 30 °C min^{-1} up to 70 °C, and thereafter at 10 °C min^{-1} up to 150 °C, temperature that was maintained for another 5 min. Helium flow was 1 cm^3 min^{-1}. The identification of isoprene and monoterpenes was conducted by GC-MS and comparison with standards from Fluka, literature spectra, and GCD Chemstation G1074A HP. Internal standard dodecane, which did not mask any terpene, together with frequent calibration with common terpene α-pinene, 3-carene, β-pinene, β-myrcene, *p*-cymene, limonene and sabinene standards once every five analyses were used for quantification. Terpene calibration curves ($n = 4$ different terpene concentrations) were always highly significant ($r^2 > 0.99$) in the relationship between signal and terpene concentration. The most abundant terpenes had very similar sensitivity (differences were 5%).

Terpene foliar concentration analysis

Individual leaves were sampled at each temperature after the measurement of fluorescence and were immediately submerged in liquid nitrogen. For extraction of leaf terpenes, these leaves were submerged in liquid nitrogen in Teflon tubes. They were afterwards heated in a water bath at 100 °C while a flow of 166 cm^3 min^{-1} of nitrogen drew volatiles towards an adsorbent tube such as those described above for analysis of terpenes in the atmospheres of the different treatments. The absence of breakthrough was checked by placing two traps in series and by verifying that no monoterpenes were collected in the second one. Standards of the different monoterpenes were also frozen and extracted with the same method to check for absence of losses. Monoterpenes trapped in the adsorbent tubes were desorbed and measured by gas-chromatography as described above.

Leaf measurements: damage, area and dry weight

The percentage of damaged (brown) leaves was measured 24 h after the end of each temperature-ramp experiment by measuring the damage area of all the plant leaves. Leaf area (separating damaged and healthy) was measured in the laboratory using a Li-Cor LI-3100 area metre (Li-Cor Inc., Lincoln, NE, USA). The leaf dry mass was determined after drying at 60 °C until mass constancy.

Statistical analyses

Repeated measures analyses of variance (ANOVAs), and regression analyses were conducted using STATISTICA versus 5.0 for Windows (StatSoft, Inc. Tulsa, OK, USA; 1996). Statistical differences between measurements on different treatments in each temperature were also analysed following the Student's *t*-test. Differences were considered significant at a probability level of $P < 0.05$. These analyses and additional correlation and regression analyses were conducted using SPSS (Chicago, IL, USA).

RESULTS

The net photosynthetic rates of isoprene-fumigated plants (IF) did not significantly decreased until 50 °C whereas those of control (C) plants had already started to decrease at 35 °C (Fig. 1). The recovery at 25 °C was higher in IF plants than in C plants (Fig. 1). The F_v/F_m, fluorescence measure of the potential photochemical efficiency of PSII (Oliveira & Peñuelas 2001) of IF plants was higher in IF plants and decreased at 50 °C only up to 0.65, whereas it decreased up to 0.38 in C plants (Fig. 2). The recovery at 25 °C was also higher in IF plants than in C plants (Fig. 2). In agreement with these differences, IF plants presented lower visual leaf damage (browning) than C plants (15 ± 2.3% of total leaf area was damaged in IF plants versus 27 ± 6.3% in C plants) (Fig. 3).

The Z/Chl (zeaxanthin/chlorophyll ratio) and the DPS [de-epoxidation state of the xanthophyll cycle calculated as (Z + 0.5 A)/(V + Z + A) where Z is zeaxanthin, A is antheraxanthin and V is violaxanthin] increased with temperature in both IF and C plants. The differences between the Z/Chl and the DPS of IF plants and C plants were minimal; there were only slightly lower values of Z/Chl and DPS at 50 °C in IF plants than in C plants and the recovery was worse in IF than in C plants (Fig. 4).

The amounts of the antioxidant α-tocopherol did not change or even tended to decrease at high temperatures in IF plants, whereas they tended to increase in C plants (Fig. 5), and as a result the responses to high temperatures were different in IF plants than in C plants (Fig. 5). The

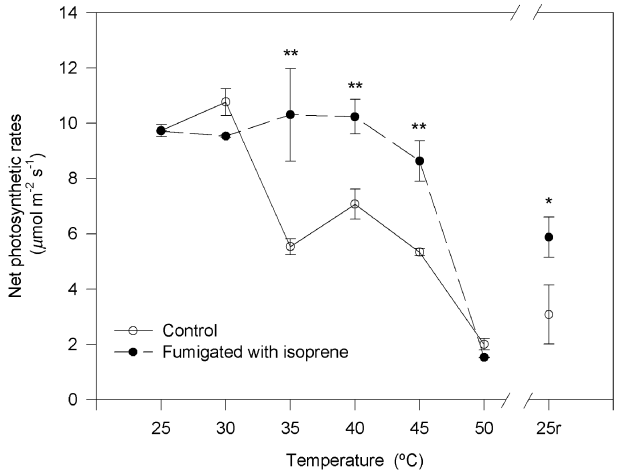

Figure 1. Effect of increasing air temperature (foliar temperatures were approximately 2 °C higher) on *Quercus ilex* foliar net photosynthetic rates under isoprene fumigation (10 μL L^{-1}) and non fumigation conditions. Control (white symbols) and isoprene-fumigated atmospheres (black symbols). Error bars indicate SEM; $n = 5$–6 plants. 25r = 25 °C recovery. * indicates statistically significant difference between control and isoprene-fumigated plants at each tested temperature (Student's *t*-test, $P < 0.05$).

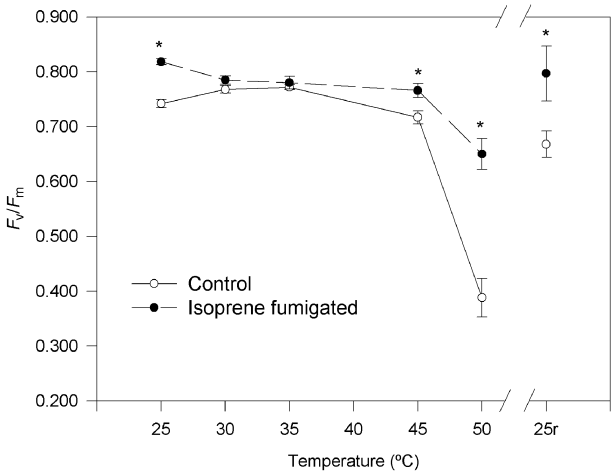

Figure 2. Effect of increasing air temperature (foliar temperatures were approximately 2 °C higher) on *Quercus ilex* F_v/F_m (potential photochemical efficiency of the PSII) under isoprene fumigation (10 μL L^{-1}) and non fumigation conditions. Control (white symbols) and isoprene-fumigated atmospheres (black symbols). Error bars indicate SEM; $n = 5$–6 plants. 25r = 25 °C recovery. * indicates statistically significant difference between control and isoprene-fumigated plants at each tested temperature (Student's *t*-test, $P < 0.05$).

antioxidant ascorbic acid reached its maximum concentration only at 45 °C in IF plants, whereas it reached its maximum at 35 °C in C plants (Fig. 5). The concentration of β-carotene did not start to decrease until 50 °C in IF plants whereas it decreased 30% from maximum at 25–35 °C to minimum at 50 °C in C plants (Fig. 5). Therefore, isoprene fumigation produced a delay in the responses of antioxidant defences to high temperatures, namely higher temperatures were needed to enhance the activation of antioxidant mechanisms such as β-carotene decrease or ascorbic acid increases in IF plants. For the other antioxidant studied, α-tocopherol, there was no increase in IF plants at 50 °C.

IF plants increased their monoterpene (α-pinene, α-phellandrene, β-pinene, β-myrcene and 2-carene) emissions approximately 70% less than C plants at highest temperatures, 40–50 °C (Fig. 6). Most monoterpene foliar concentrations were also approximately 50–70% lower at highest temperatures (45 and 50 °C) in IF plants but they were so low (between 1 and 2 μg g^{-1} DM at the most) that they only seemed able to support emissions for approximately 0.5 min (Fig. 7). Most monoterpenes (α-phellandrene, β-pinene, β-myrcene) reached maximum emissions at 50 °C; although, some of them (α-pinene and 2-carene) reached maximum emission at 45 °C (Fig. 6). Nevertheless, the latter also had their maximum emission at 50 °C in IF plants. Emissions decreased again rapidly in the recovery test at 25 °C.

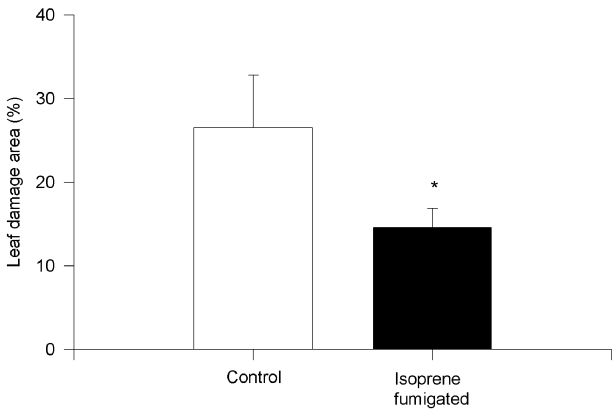

Figure 3. Leaf visual damage after the temperature treatment (ramp increase up to 50 °C) under isoprene fumigation (10 μL L^{-1}) and control conditions. Control (white symbols) and isoprene-fumigated atmospheres (black symbols). Error bars indicate SEM; $n = 5$–6 different plants. * indicates statistically significant difference between control and isoprene-fumigated plants at each tested temperature (Student's *t*-test, $P < 0.05$).

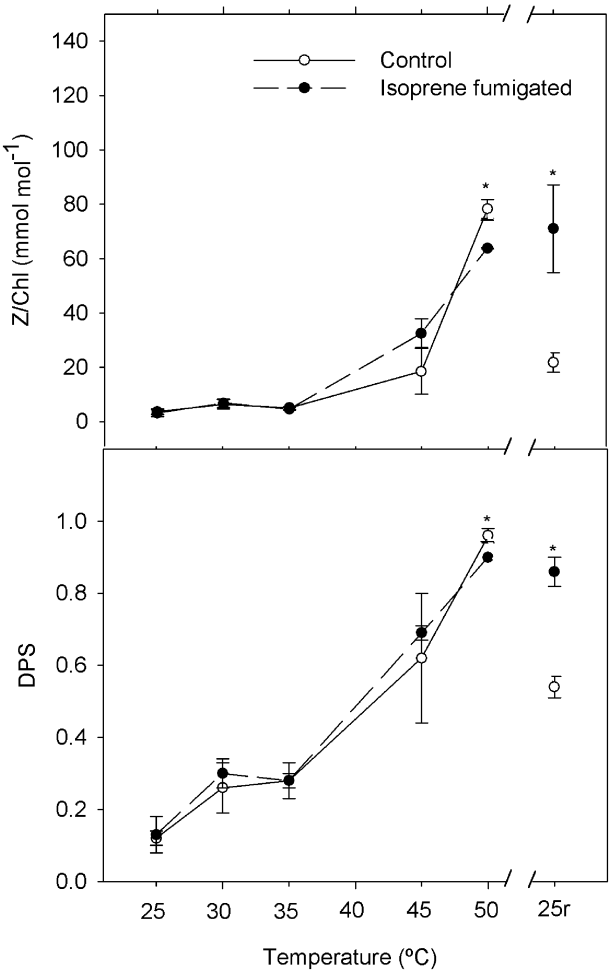

Figure 4. Zeaxanthin/chlorophyll ratio (Z/chl), and de-epoxidation state of the xanthophyll cycle (DPS) in leaves of isoprene fumigated (IF) plants and control (C) plants. DPS was calculated as (Z + 0.5 A)/(V + Z + A), where Z is zeaxanthin, A is antheraxanthin and V is violaxanthin. Control (white symbols) and isoprene-fumigated atmospheres (black symbols). Error bars indicate SEM; $n = 5$–6 different plants. 25r = 25 °C recovery

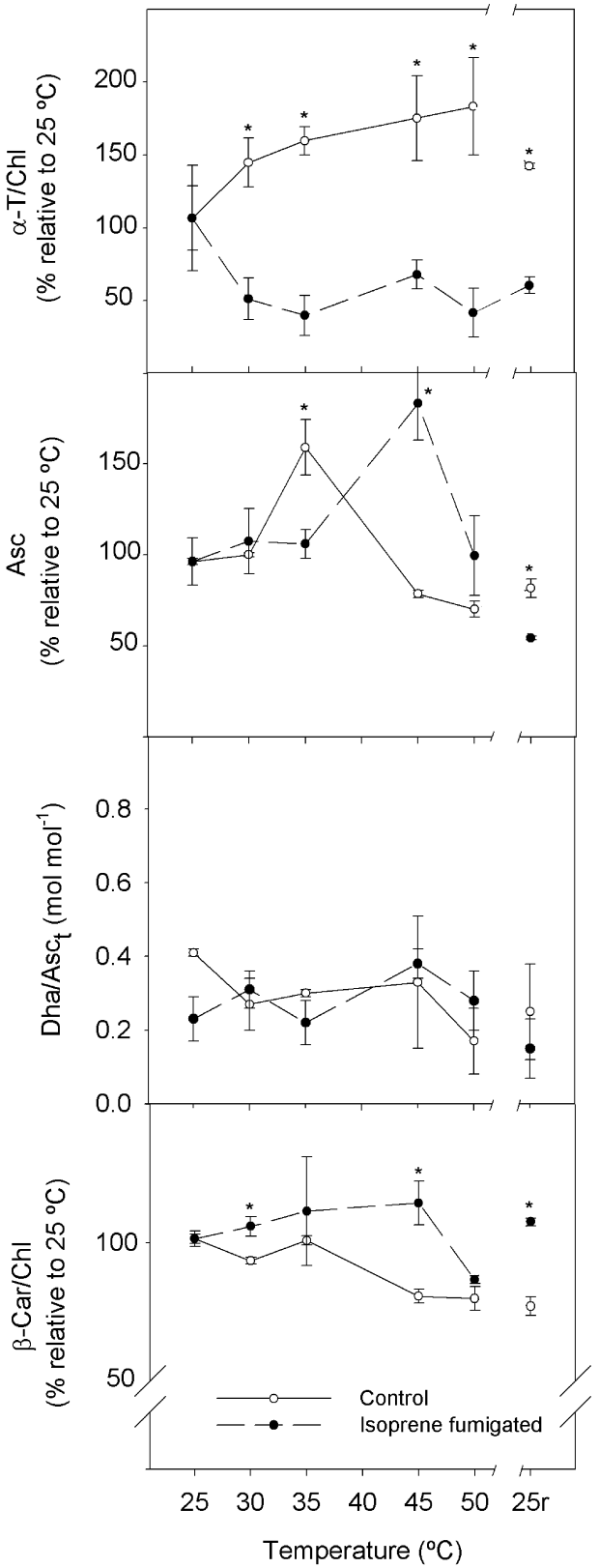

Figure 5. α-tocopherol relative to chlorophyll (α-T/Chl), ascorbic acid (Asc), redox state of ascorbic acid [estimated as dehydroascorbic acid/total ascorbic acid (Dha/Asc$_t$, where Asc$_t$ = Dha + Asc)] and β-carotene relative to chlorophyll (β-Car/Chl), in leaves of isoprene-fumigated plants (IF) and control plants (C). The values are expressed relative to 25 °C. Control (white symbols) and isoprene-fumigated atmospheres (black symbols). Error bars indicate SEM; n = 5–6 different plants. 25r = 25 °C recovery. * indicates statistically significant difference between control and isoprene-fumigated plants at each tested temperature (Student's t-test, $P < 0.05$).

DISCUSSION

These results show that isoprene fumigation (1) actually conferred thermo tolerance to *Quercus ilex* seedlings; (2) precluded, or delayed until higher temperatures, the increase of some antioxidants such as α-tocopherol or ascorbic acid, or the consumption of others such β-carotene; (3) decreased the emissions of monoterpenes; and therefore (4) seems to present an antioxidant-like behaviour that makes the action of the other mentioned non-enzymatic antioxidants unnecessary.

The results of this study are in agreement with the conclusion of Sharkey, Chen & Yeh (2001) that thermotolerance of photosynthesis is a substantial benefit of isoprene. The net photosynthetic rates already started to clearly decrease in C plants at 35 °C whereas in IF plants no decrease was found until 45 °C, and even then it was only a slight decrease (less than 10% versus 50% in C plants). The F_v/F_m of these IF plants at 50 °C also decreased much less (16%) than that of C plants (62%)and the recovery at 25 °C was also much better in IF plants than in C plants.

These results also confirm that one of the primary effects of high-temperature stress is the damage to photosynthetic electron transport through PSII (Berry & Björkman 1980). Heat damage arises from inactivation of the highly sensitive water-splitting reaction, disconnection of PSII centres from the bulk pigments, thermal uncoupling of photophosphorylation, and biomembrane lesions (Berry & Björkman 1980). Necrosis after the exposure at 50 °C appeared in several leaves (27% of total leaf area in C plants but only 15% in IF plants).

In the present study, dissipation of excess excitation energy by the xanthophyll cycle, and detoxification of AOS and lipid peroxyl radicals by increased α-T and ascorbate levels, by consumption of β-carotene and by production-emission of monoterpenes seem to have afforded certain protection to the photosynthetic apparatus in C plants as shown by the absence of changes in redox state of ascorbic acid (Fig. 5), MDA (data not shown), and chlorophyll (data not shown) in green, well-developed leaves that were still photosynthetically active at 45 °C. The photo- and antioxidant protection exerted by carotenoids, tocopherols and ascorbic acid has been previously reported in sun leaves of holm oak exposed to low temperatures in the field (García-Plazaola, Artexte & Becerril 1999). Our results show that the concerted action of these antioxidants may also enhance the tolerance of this species to heat stress.

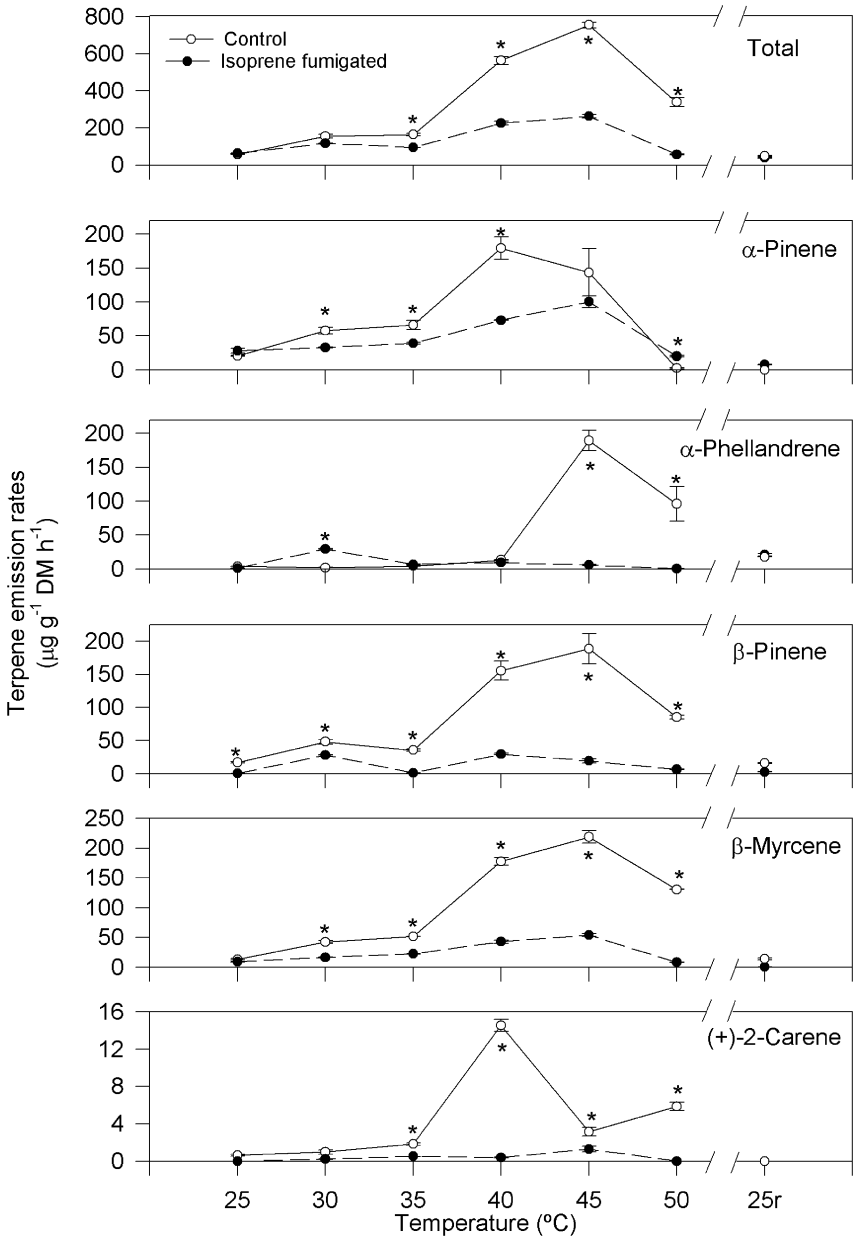

Figure 6. Effect of increasing air temperature (foliar temperatures were approximately 2 °C higher) on emission rates of total monoterpenes, α-pinene, α-phellandrene, β-pinene, β-myrcene, and carene under isoprene fumigation (10 μL L^{-1}) and control conditions. Control (white symbols) and isoprene-fumigated atmospheres (black symbols). Error bars indicate SEM; n = 5–6 chamber measurements. 25r = 25 °C recovery. * indicates statistically significant difference between control and isoprene-fumigated plants at each tested temperature (Student's t-test, $P < 0.05$).

In spite of presenting lower or delayed photo and antioxidant protection by xanthophylls, α-T, ascorbic acid and β-carotene compounds, the photosynthetic apparatus of IF plants suffered even less photo-inhibitory damage than that of C plants as indicated by smaller changes in net photosynthetic rates and F_v/F_m and by less visual leaf damage. These results therefore indicate that *Q. ilex* leaves can reduce the damage to the photosynthetic apparatus produced by high temperature-induced oxidative stress as a result of an isoprene fumigation that did not seem far from natural. Although the tested concentrations of isoprene were higher than usual in the atmosphere, they were still slightly lower than intercellular isoprene concentrations reported in water-limited saplings of oak species (up to approximately 11–16 μL L^{-1}) where isoprene seems to serve as a short-term thermoprotective agent in isoprene-emitting plant species (Bruggemann & Schnitzler 2002). However, it is very likely that higher concentrations of AOS are present in water-stressed plants than in tested plants.

There is not enough evidence of the mechanism or mechanisms and the interactions involved in this phenomenon of enhanced plant thermotolerance by isoprene. It is possible that the achieved thermotolerance results from the isoprene-induced adjustment of membrane lipid fluidity (Sharkey *et al.* 2001). However, our data on isoprene enhancement of thermotolerance accompanied by the decreased response of the other antioxidants are also in accordance with a mechanism linked to isoprene capacity of scavenging the photosynthetic-derived AOS (Loreto & Velikova 2001; Affeck & Yakir 2002; Peñuelas & Llusià 2002). These AOS raise their concentration inside the leaf during periods of high temperature and light (Gong *et al.*

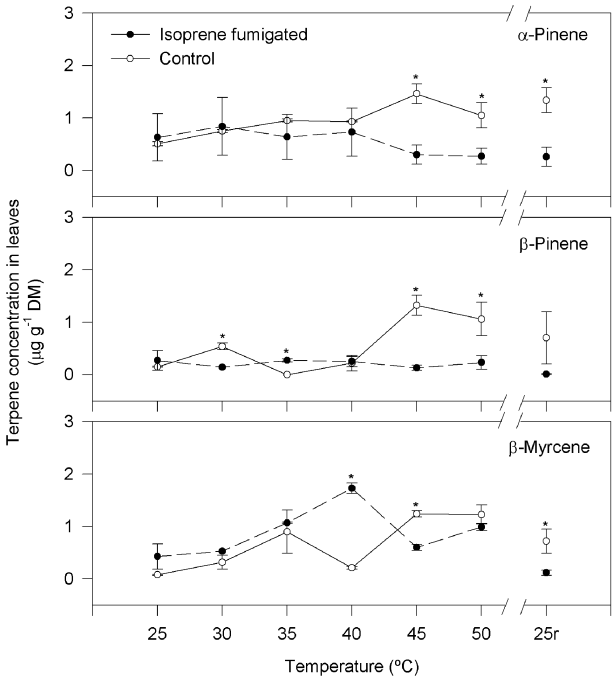

Figure 7. Changes in α-pinene, β-pinene, and β-myrcene leaf concentrations in *Quercus ilex* seedlings at isoprene-fumigated and control atmospheres in response to increasing air temperatures (foliar temperatures were approximately 2 °C higher). Control (white symbols) and isoprene-fumigated atmospheres (black symbols). Error bars indicate SEM; $n = 5$–6 different plants. 25r = 25 °C recovery. * indicates statistically significant difference between control and isoprene-fumigated plants at each tested temperature (Student's t-test, $P < 0.05$).

1997; Larkindale & Knight 2002). The isoprene would use its capacity for scavenging the AOS produced under high temperatures and therefore would make the increase of photo and antioxidant defences in IF plants less necessary.

Another novel result of this study was to find that the monoterpene (α-pinene, β-phellandrene, β-pinene, β-myrcene and 2-carene) emissions increased up to eight times at 40–50 °C relative to 25 °C in C plants whereas they only increased twice in IF plants (Fig. 6). Most monoterpene foliar concentrations were also lower in IF plants (Fig. 7). Therefore, the protective effect of isoprene was accompanied by these approximately three times lower terpene emissions and concentrations at the highest temperatures. This suggests that isoprene was somehow inhibiting production and emission of the other isoprenoids. Further studies are needed to determine the mechanisms of this inhibition. Isoprene might interfere with the methylerythritol 4-phosphate (MEP) pathway for the synthesis of the other isoprenoids including carotenoids (Rohmer 1999) but this inhibitory effect of monoterpenes has not been found for the carotenoids (β-carotene and the xanthophylls). In any case, isoprene fumigation enhanced thermotolerance in IF plants even though there were more monoterpene emissions in the control plants, which suggests a less effective thermotolerance of monoterpenes than isoprene in agreement with previous results (Peñuelas & Llusià 2002).

All these results suggest that there are inducible mechanisms triggered by the initial stages of thermal damage that up-regulate antioxidant compounds at high temperatures and that these mechanisms are somehow suppressed in the presence of exogenous isoprene, which seems to already exert an antioxidant-like behaviour. These results also suggest a likely unifying explanation for the various observations about isoprene fumigation effects that have been reported in the past. Past observations of exogenous isoprene as an antioxidant agent (Loreto & Velikova 2001; Affeck & Yakir 2002) and as a thermotolerance agent (Sharkey & Singsaas 1995; Loreto *et al*. 1998; Singsaas 2000; Delfine *et al*. 2000) may share a common explanation since the high temperature effects seem to be due at least in part to AOS effects (Aro, Vergin & Anderson 1993; Gong *et al*. 1997; Larkindale & Knight 2002).

CONCLUSIONS

In conclusion, we have demonstrated (1) that the evergreen *Q. ilex* plants activate several mechanisms of photo- and antioxidant protection to withstand high temperatures; (2) that, when the temperature rise is not extremely severe, the mechanisms that include monoterpene production and emission are able to protect the tissues from heat damage, and to allow the maintenance of significant photosynthetic rates; (3) that fumigation with isoprene confers thermotolerance enhancement to these IF plants; and (4) that it seems to suppress the activation of other antioxidants in the leaf including monoterpene production and emission. All these results suggest that the antioxidant capacity and thermotolerant effects of isoprene can have a common explanation as the high-temperature effects seem to be due at least in part to AOS effects. Therefore, the antioxidant capacity appears as an additional explanation for the enhanced thermotolerance induced by isoprene.

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REFERENCES

Affeck H.P. & Yakir D. (2002) Protection by isoprene against singlet oxygen in leaves.. *Plant Physiology* **129,** 269–277.

Aro E.M., Vergin I. & Anderson B. (1993) Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. *Biochimica et Biophysica Acta* **1143,** 113–134.

Asada K. (1999) The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 601–639.

Berry J. & Björkman O. (1980) Photosynthetic response and adap-

tation to temperature in higher plants. *Annual Review of Plant Physiology* **31,** 491–543.

Bruggemann N. & Schnitzler J.P. (2002) Comparison of isoprene emission, intercellular isoprene concentration and photosynthetic performance in water-limited oak (*Quercus pubescens* Willd. *& Quercus robur* L.) saplings. *Plant Biology* **4,** 456–463.

Delfine S., Csiky O., Seufert G. & Loreto F. (2000) Fumigation with exogenous monoterpenes of a non-isoprenoid-emitting oak (*Quercus suber*): monoterpene acquisition, translocation, and effect on the photosynthetic properties at high temperatures. *New Phytologist* **146,** 27–36.

Demmig-Adams B. & Adams W.W. (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends in Plant Sciences* **1,** 21–26.

Eskling M., Arvidsson P.O. & Akerlund H.E. (1997) The xanthophyll cycle, its regulation and components. *Physiologia Plantarum* **100,** 806–816.

Foyer C.H., Lelandais M. & Kunert K.J. (1994) Photooxidative stress in plants. *Physiologia Plantarum* **92,** 696–717.

García-Plazaola J.I., Artexte U. & Becerril J.M. (1999) Diurnal changes in antioxidant and carotenoid composition in the Mediterranean schlerophyll tree *Quercus ilex* (L) during winter. *Plant Science* **143,** 125–133.

Geron C., Rasmussen R., Arnts R.R. & Guenther A. (2000) A review and synthesis of monoterpene speciation from forests in the United States. *Atmospheric Environment* **34,** 1761–1781.

Gong M., Chen S.N., Song Y.Q. & Li Z.G. (1997) Effect of calcium and calmodulin on intrinsic heat tolerance in relation to antioxidant systems in maize seedlings. *Australian Journal of Plant Physiology* **24,** 371–379.

Halliwell B. & Gutteridge J.M.C. (1989) *Free Radicals in Biology and Medicine*. Oxford University Press, Oxford, UK.

Havaux M. (1998) Carotenoids as membrane stabilizers in chloroplasts. *Trends in Plant Science* **3,** 147–151.

Hodges D.M., DeLong J.M., Forney C.F. & Prange R.K. (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* **207,** 604–611.

Kozaki A. & Takeba G. (1996) Photorespiration protects C3 plants from photooxidation. *Nature* **384,** 557–560.

Larcher W. (2000) Temperature stress and survival ability of Mediterranean sclerophyllous plants. *Plant Biosystems* **134,** 279–295.

Larkindale J. & Knight M.R. (2002) Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiology* **128,** 682–695.

Logan B.A. & Monson R.K. (1999) Thermotolerance of leaf discs from four isoprene-emitting species is not enhanced by exposure to exogenous isoprene. *Plant Physiology* **120,** 821–826.

Loreto F., Ciccioli P., Cecinato A., Bracaleoni E., Frattoni M. & Tricoli D. (1996) Influence of environmental α-pinene from factors and air composition on the emission of *Quercus ilex* leaves. *Plant Physiology* **110,** 267–275.

Loreto F., Förster A., Dürr M., Csiky O. & Seufert G. (1998) On the monoterpene emission under heat stress and on the increased thermotolerance of leaves of *Quercus ilex* L. fumigated with selected monoterpenes. *Plant, Cell and Environment* **21,** 101–107.

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

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.

Munné-Bosch S. & Alegre L. (2000) Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in *Rosmarinus officinalis* plants. *Planta* **210,** 925–931.

Munné-Bosch S. & Alegre L. (2002a) The function of tocopherols and tocotrienols in plants. *Critical Reviews in Plant Sciences* **21,** 31–57.

Munné-Bosch S. & Alegre L. (2002b) Interplay between ascorbic acid and lipophilic antioxidant defences in chloroplasts of water-stressed *Arabidopsis* plants. *FEBS Letters* **524,** 145–148.

Oliveira G. & Peñuelas J. (2001) Allocation of absorbed light energy into photochemistry and dissipation in a semi-deciduous and an evergreen Mediterranean woody species during winter. *Australian Journal of Plant Physiology* **28,** 471–480.

Osmond B., Badger M., Maxwell K., Björkman O. & Leegod R. (1997) Too many photons: photorespiration, photoinhibition and photooxidation. *Trends in Plant Science* **2,** 119–120.

Peñuelas J. & Llusià J. (1999) Seasonal emission of monoterpenes by the Mediterranean tree *Quercus ilex* in field conditions: Relations with photosynthetic rates, temperature and volatility. *Physiologia Plantarum* **105,** 641–647.

Peñuelas J. & Llusià J. (2002) Linking photorespiration, monoterpenes and thermotolerance in *Quercus*. *New Phytologist* **155,** 227–237.

Peñuelas J. & Llusià J. (2004) Plant VOC emissions: making use of the unavoidable. *Trends in Ecology and Evolution* **19,** 402–404.

Rohmer M. (1999) The mevalonate-independent methylerythritol 4-phosphate (MEP) pathway for isoprenoids biosynthesis, including carotenoids. *Pure and Applied Chemistry* **71,** 2279–2284.

Rosenstiel T.N., Ebbets A.L., Khatri W.C., Fall R. & Monson R.K. (2004) Induction of poplar leaf nitrate reductase: a test of extrachloroplastic control of isoprene emission rate. *Plant Biology* **6,** 12–21.

Sharkey T.D., Chen X.Y. & Yeh S. (2001) Isoprene increases thermotolerance of fosmidomycin-fed leaves. *Plant Physiology* **125,** 2001–2006.

Sharkey T.D. & Singsaas E.L. (1995) Why plants emit isoprene? *Nature* **374,** 769.

Sharkey T.D. & Yeh S. (2001) Isoprene emission from plants. *Annual Review of Plant Physiology and Plant Molecular Biology.* **52,** 407–436.

Singsaas E.L. (2000) Terpenes and the thermotolerance of photosynthesis. Forum commentary. *New Phytologist* **146,** 1–4.

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

Smirnoff N. (1993) The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist* **125,** 27–58.

Smirnoff N. (2000) Ascorbate biosynthesis and function in photoprotection. *Philosophical Transactions of the Royal Society of London Series B-Biology Sciences* **355,** 1455–1464.

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