Methanol as a signal triggering isoprenoid emissions and photosynthetic performance in *Quercus ilex*

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Abstract

Several Volatile Organic Compounds (VOCs) have been reported as having a communication role between plants and also between plants and animals. We aimed to test whether methanol, a short-chain oxygenated VOC, could also have a signalling role between plants. We monitored photosynthetic performance and VOC exchange rates of *Quercus ilex* L. saplings before and after two different treatments: a) clipping of some leaves to simulate an attack by herbivores and b) fumigation with gaseous methanol during 5 hours to simulate the amount of methanol a plant could receive from surrounding plants if those had been already attacked by herbivores. The clipping treatment enhanced the photosynthetic rates, the chlorophyll a to b ratio, and the carotenoid to chlorophyll ratio of non-clipped leaves, suggesting an activation of plant protective metabolism. Also, a small but interesting systemic (in non-clipped leaves) increase in methanol emission rates was observed, which agrees with the possibility that methanol may act as a signalling cue. The methanol fumigation treatment induced an increase in the actual photochemical efficiency of PSII and also in the carotenoid to chlorophyll ratio. Methanol fumigation also promoted a 14% increase in the monoterpene emission rate, one day after the treatment, a similar response to the ones induced by other signalling VOCs. The enhanced monoterpene emissions could add to the blend of VOCs emitted after stress and be part of further signalling pathways, thus forwarding the message started by methanol. This study suggests that clipping and methanol fumigation at natural concentrations elicit significant neighbour plant physiological responses and further BVOC emissions.

Keywords: methanol, *Quercus ilex*, monoterpenes, emission rates, plant-plant communication, signal, BVOCs, photosynthetic rates, photosynthetic pigments

Introduction

Biogenic Volatile Organic Compounds (BVOCs) are recognized as having many roles in plant physiology and ecology (Peñuelas and Llusià 2002, Peñuelas and Staudt 2010, Singsaas and Sharkey 1998, Velikova et al. 2005) as well as in atmospheric chemistry (Atkinson 2000, Holzinger et al. 2005, Kavouras et al. 1998, Peñuelas and Llusià 2003), and consequently their emissions by plants have been measured and modeled extensively during recent years (Guenther et al. 2006, Keenan et al. 2009, Kesselmeier and Staudt 1999, Seco et al. 2007). In addition, BVOCs are implicated in plant-animal communication (Gershenzon 2007, Peñuelas et al. 1995b). Plant-animal relationships mediated by BVOCs do not only involve plant-pollinator relationships (Wright and Schiestl 2009) or direct defense against herbivores, but also indirect defense through the attraction of natural enemies of the herbivores (Kessler and Baldwin 2001, Llusià and Peñuelas 2001, Pichersky and Gershenzon 2002).

BVOCs can also play an important role in plant-plant communication by transmitting information between plants (Peñuelas et al. 1995b). One example is the sensing of proximate neighbour plants mediated by the phytohormone ethylene, which has been reported for *Nicotiana tabacum* (Pierik et al. 2003). Most recent BVOC-mediated plant-plant communication studies have dealt with response to herbivory and priming. In this context, priming refers to the fact that a plant gets ready for the possible attack of a pathogen or an herbivore based on the BVOC signals that attacked plants send to the atmosphere (Heil and Kost 2006), so the primed plant is then able to respond quicker and/or stronger to the herbivore when attacked. This phenomenon has been observed even between different parts of the same individual (Frost et al. 2007, Heil and Silva Bueno 2007). Great attention has been focused on certain C6 compounds (aldehydes, alcohols, and their acetates) biosynthesized via the lipoxygenase/hydroperoxide lyase pathway, called green leaf volatiles (GLV, Matsui 2006). GLV are emitted after mechanical stress (Fall et al. 1999) and herbivore attack (Schaub et al. 2010) and have shown to be responsible for priming effects on other plants (Engelberth et al. 2004, Frost et al. 2008, Heil and Kost 2006, Kost and Heil 2006). Other plant-emitted BVOCs such as methyl jasmonate (MeJA) and methyl salicylate (MeSA) are also involved in defense responses (Laothawornkitkul et al. 2009, Park et al. 2007), at least partly in interaction with the aforementioned ethylene (Arimura et al. 2008, Ruther and Kleier 2005, von Dahl and Baldwin 2007).

Methanol is the smallest alcohol and is among a group of short-chain oxygenated BVOCs which are becoming more studied in recent years (Filella et al. 2009, Seco et al. 2007). Under non-perturbed situations, it is released from plants to the atmosphere by demethylation of pectins during cell wall formation, e.g. during leaf growth (Harley et al. 2007, Hüve et al. 2007). Globally, it is the second most abundant organic gas in the atmosphere after methane (Jacob et al. 2005). But despite being somewhat abundant in the air, it can be emitted by plants in such high amounts during stress episodes –like herbivore attacks (Peñuelas et al. 2005a, von Dahl et al. 2006) and crop harvesting (Karl et al. 2001)– that it might play a communication role by being detectable as a signal by plants themselves or by other organisms in the near area. It has been suggested that plants may rely on more

long-lived compounds such as methanol for long distance communication, since molecules like GLV are very reactive in the atmosphere and may be rapidly degraded by oxidants like ozone (Pinto et al. 2007a).

Previously published experiments dealing with methanol effects on plants mainly consisted of spraying liquid methanol solutions on the leaves of several plant species. Some reported an increase of photosynthesis (Faver and Gerik 1996) and growth (Madhaiyan et al. 2006, Ramirez et al. 2006), as well as no effect on growth (Rajala et al. 1998). One experiment that used gaseous methanol, at a high concentration (1% in air), focused on the effect on the photosynthetic apparatus and found an inhibition of its biochemical reactions (Loreto et al. 1999). Sprayed methanol treatment also induced the expression of hundred of genes in *Arabidopsis thaliana* and activated multiple detoxification and signalling pathways (Downie et al. 2004), facts that agree with a possible signalling function of methanol.

We aimed to discern whether methanol in natural concentrations in the air could act as a signalling cue in plant-plant interactions, especially with regard to the triggering of further BVOC emissions by the receiving plant. We studied the widespread Mediterranean tree *Quercus ilex* L. (holm oak), one of the most abundant species in Mediterranean forests, and whose physiology has been broadly studied from different points of view, including BVOC exchange (Blanch et al. 2007, Peñuelas and Llusià 2002, Peñuelas et al. 2009, Seco et al. 2008). This species has, moreover, been reported to suffer from episodic outbreaks of herbivores like *Lymantria dispar* L. (Staudt and Lhoutellier 2007) which could potentially lead to high local methanol emissions and, consequently, to possible signalling to neighbour plants. We measured the physiological and BVOC emission responses of *Q. ilex* seedlings to i) a simulated herbivore attack by clipping leaves and ii) a fumigation with gaseous methanol during several hours.

Materials and methods

Plant material

We used 3-year-old potted *Quercus ilex* L. plants grown in a nursery (Tres Turons S.C.P., Castellar del Vallès, Catalonia, Spain), maintained under Mediterranean ambient conditions outdoors. They were grown in 2 L pots with a substrate composed of peat and sand (2:1), prior to being brought into the laboratory, where they were allowed to acclimate to laboratory conditions for several days before starting the experiment.

Plant and leaf chambers

We used two different chambers (Fig. 1). The whole aerial part of the monitored plant was enclosed in a 65 L cylindrical chamber made of transparent teflon film. This big plant chamber (PC) was illuminated from the top by fluorescent lights (Agrolite CFL, Barcelona) giving about 300 µmol m^{-2} s^{-1} of photosynthetic active radiation (PAR) flux inside the chamber, with a 12:12h light:dark photoperiod. Ambient air from outside the building was introduced into the PC at a rate of about 15 L

min^{-1} by means of an oil-free pump. A fan stirred the air inside the PC, and there was always an excess of air escaping from the PC through the tiny space between the trunk of the tree and the PC teflon film, thus preventing external air from entering the PC. CO_2 was added from a bottle (Abelló Linde S.A., Barcelona, Catalunya) through a mass flow controller (Bronkhorst High-Tech B.V., Ruurlo, Netherlands) during high photosynthetic activity periods, to keep atmospheric CO_2 concentrations at about 385 μmol mol^{-1}.

A second, small leaf chamber (LC) was introduced inside the PC and clamped a leaf. This leaf cuvette was part of a LCpro+ Photosynthesis System (ADC BioScientific Ltd., Herts, England), which recorded photosynthesis (net CO_2 uptake), stomatal conductance, air humidity, and temperature data, while controlling the light radiation and the flow of air entering the leaf cuvette. A light diurnal cycle was programmed in the LC to simulate a typical sunny day, ranging from 0 to 1500 μmol m^{-2} s^{-1} of PAR and with the same 12:12h photoperiod as the PC. The air entering the LC was taken from inside the PC: this way the air reaching all the leaves of the plant was the same.

Experimental design

Two groups (*A* and *B*) of plants were used, corresponding to two experiments. In *A* plants (n=3) herbivore damage was simulated by clipping some leaves, leaving the petiole and a little part of the leaf still attached to the plant. *B* plants (n=3) were submitted to a methanol fumigation treatment.

This experimental setup resulted in two sampling and measuring times for *A* plants: Control1 (control before any treatment) and Cut (after cutting leaves). Each one of these measures was taken on different consecutive days. On day 1, the plant was left untouched to acclimate to the chamber conditions. On day 2, at 15h, the PC was open from the top and in vivo, non-destructive measurements of reflectance and fluorescence were taken (Control1 measurement). After these measurements, some leaves were clipped with scissors to simulate herbivory, and the cut fragments were stored in liquid N_2 as leaf samples for further processing (pigment analysis, see later). On day 3, the PC was opened again at 15h and the same measurements and leaf samples were taken from non-clipped leaves, corresponding to the Cut measurement. Gas (BVOCs, H_2O and CO_2) exchange was monitored continuously during all the days, but for comparison between treatments we mostly focused on the average exchange rate from 14h to 15h of the leaf inside the LC, before the PC was opened (on day 2 for Control1, and day 3 for Cut). Additionally, *A* plants were further submitted to a methanol fumigation treatment the day after the clipping, to simulate the situation when an already attached plant receives an airborne signal from neighbour attacked plants. The fumigation was identical to the one described for *B* plants.

B plants had also two measures: Control2 and Fumigated (fumigated with methanol). The treatment consisted of five hours of whole-plant methanol fumigation, starting around 16h the day 2. On day 2, before fumigation, only non-destructive measurements were taken (i.e. no cutting of leaves was done), and on day 3, the same measures and leaf samples were taken as for *A* plants.

Fumigation with methanol

Gaseous methanol introduced into the chambers was obtained from a calibration mix (100 ppmv in N_2 from Abelló Linde S.A., Barcelona, Catalunya) and diluted into the PC inlet line by means of a mass flow controller (Environics 4000, Environics Inc., Tolland, CT, USA). Methanol fumigation lasted for five hours for each plant and the LC and PC methanol mixing ratio during such episodes reached values around 500-600 ppbv. This amount is in the range of what we calculated that a plant would receive, assuming that the surrounding plants would emit methanol at the 24 nmol m^{-2} s^{-1} high rates reported by Peñuelas et al. (2005a) for *Succisa pratensis* after being attacked by caterpillars of *Euphydryas aurinia*. We assumed a typical holm oak forest of 5 m^2 m^{-2} LAI (Leaf Area Index) and 5 m height and a typical moderate wind.

Plant reflectance and fluorescence measurements

Leaf-based reflectance was measured with a UniSpec Spectral Analysis System/ Reflectometer (PP Systems, Haverhill, MA, USA) operated with a palmtop PC. Single leaves were sampled by clamping them into a leaf clip (adaxial side upwards) attached to a fibreoptic halogen light source and detector. Four scans per sample were integrated (integration time 10 ms). Reflectance measurements were preceded by a dark scan, and were compared with reflectance from a Spectralon (Labsphere Inc., North Sutton, NH, USA) white standard to obtain percent reflectance. From the reflectance data, SIPI was calculated as (R445-R800)/(R680-R800) (Peñuelas et al. 1995a, Peñuelas and Filella 1998).

Chlorophyll fluorescence was determined using a portable modulated fluorometer PAM-2000, and a leaf clip holder 2030-B (Heinz Walz GmbH, Effeltrich, Germany). The actual photochemical efficiency of PSII in light adapted state was estimated as $(F_m'-F_s):F_m'$ or $\Delta F:F_m'$ as defined by Genty et al. (1989), where F_s is the steady-state fluorescence yield under the given environmental conditions, and F_m' is the maximum level of fluorescence obtained during a saturating flash of light (when all the PSII traps are closed) under the same environmental conditions (Genty et al. 1989).

Plant pigment analysis

Randomly collected cut leaves were immediately frozen in liquid nitrogen each sampling day. Samples were subsequently stored at –80 ºC until extraction. Chlorophyll a and total carotenoids were extracted in 80% acetone. The concentration was determined according to Lichtenthaler (1987).

CO_2, H_2O and BVOC exchange measurements

CO_2 and H_2O exchange were measured in the LC connected to the LCpro+ Photosynthesis System (ADC BioScientific Ltd., Herts, England). For BVOC exchange determination and quantification, both the air entering and exiting the PC and LC were monitored with flow meters and analysed with proton transfer reaction–mass spectrometry (PTR–MS from Ionicon Analytik, Innsbruck, Austria) at alternative intervals. The difference between the concentration of BVOCs before and after passing through the chambers, along with the flow rates, was used to calculate the BVOC exchange. The tubing used to connect PC and LC with the PTR–MS system was made of Siltek-passivated stainless steel (Restek, Bellefonte, PA, USA). In addition, the output and input air flowing from both chambers

was sampled by means of glass tubes filled with terpene adsorbents, and thereafter analyzed by thermal desorption and gas chromatography–mass spectrometry.

The PTR–MS technique. PTR–MS is based on chemical ionisation, specifically non-dissociative proton transfer from H_3O^+ ions to most of the common BVOCs, and has been fully described elsewhere (Lindinger et al. 1998). In our experiment the PTR–MS drift tube was operated at 2.1 mbar and 60ºC, with an E/N (electric field/molecule number density) of around 130 Td (townsend) (1 Td = 10^{-17} V cm^2). The primary ion signal (H_3O^+) was maintained at c. 4×10^6 counts per second. The instrument sensitivity and the transmission efficiency of its detection system were obtained using an aromatic mix standard gas (TO-14A, Restek, Bellefonte, PA, USA). BVOC mixing ratios were calculated taking into account the BVOC relative transmission, their measured fragmentation pattern and their proton transfer reaction rate coefficients reported elsewhere (Zhao and Zhang 2004) as described by de Gouw and Warneke (2007).

Terpene sampling and analysis by GC–MS. Exhaust air of the chambers was pumped through a glass tube (8 cm long and 0.3 cm internal diameter) manually filled with terpene adsorbents Carbopack B, Carboxen 1003, and Carbopack Y (Supelco, Bellefonte, Pennsylvania) separated by plugs of quartz wool. Samples were taken using a Qmax air sampling pump (Supelco Inc., Bellefonte, PE, USA). For more details see Peñuelas et al. (2005b) The sampling time was 10 min, and the flow varied between 470 and 500 mL/min depending on the glass tube adsorbent and quartz wool packing. Glass tubes were stored at -28 ºC until the analysis. The cartridges were then analyzed in the laboratory by a GC-MS system (Hewlett Packard HP59822B, Palo Alto, CA, USA) as described in Llusià et al. (2010).

Plant harvest

At the end of each individual experiment, the leaf inside the LC as well as all the leaves inside the PC were harvested, their area measured with a LI-3100 Area Meter (LI-COR, Lincoln, NE, USA), and dried at 70ºC until constant weight for dry weight calculation.

Data treatment

Variables were measured on each individual plant before and after each treatment, and thus paired t-test were performed to minimize the effect of inter-individual variation in all the parameters. The signification level was $P < 0.05$ but values below 0.1 were also considered. T-tests were conducted using Statistica 6.0 software (StatSoft Inc., Tulsa, OK; USA).

Results

Clipping

Physiological status. The clipping (Cut) treatment increased, on average, 19% the photosynthetic assimilation rate ($p=0.07$), 7% the chlorophyll a to chlorophyll b (Chl a/b) ratio ($p=0.03$) and 3% the SIPI (index for carotenoid to chorophyll ratio, $p=0.056$) (Fig. 2). The different plants presented different values of these ecophysiological variables but in all cases they responded with similar increases after clipping (Fig. 2). Although not significantly, the actual photochemical efficiency of

PSII ($\Delta F:F_m'$) also increased in all three tested plants (data not shown). Stomatal conductance did not change significantly by the clipping treatment, and showed high inter-individual variation, with values in the range 0.04-0.28 mol m^{-2} s^{-1} (data not shown).

BVOC exchange. Plants submitted to the clipping treatment to simulate herbivory did not show detectable or different methanol emissions from the damaged leaves, maybe due to the low accuracy of the PC (plant chamber) BVOC flux measurements. However, an interesting increase in methanol emission was observed from the undamaged leaves enclosed in the LC (leaf cuvette). Control emissions averaged 0.5 nmol m^{-2} s^{-1} while Cut emissions averaged 0.53 nmol m^{-2} s^{-1} (Fig. 2), in what represents a small (6% in average) but significant increase in methanol emissions (paired t-test: t=4.45, p=0.046). Again, inter-individual variation was high but all plants showed a similar increase in emissions (Fig. 2). No other significant change in BVOC emissions was detected in Cut plants, including monoterpene emissions (data not shown).

Methanol fumigation

Physiological status. Methanol fumigation induced no significant change in net photosynthetic rates but induced a significant 3% increase of the actual photochemical efficiency of PSII (p=0.07) and a significant 2% in the SIPI (index for Car/Chl ratio, p=0.04) of fumigated plants (Fig. 3). Stomatal conductance did not change significantly by the fumigation treatment, and showed high inter-individual variation, with values in the range 0.11-0.67 mol m^{-2} s^{-1} (data not shown).

BVOC exchange. Uptake of methanol during fumigation episodes reached values up to 15 nmol m^{-2} s^{-1} in the LC. Methanol fumigation induced an increase in monoterpene emissions (Fig. 4), as measured by the PTR-MS signals of the protonated masses M81 and M137 which correspond to monoterpenes (de Gouw and Warneke 2007). Fumigation of unattacked plants led to an average increase of about 1.8 nmol m^{-2} s^{-1}, from 12.2 to 14 nmol m^{-2} s^{-1} of monoterpene release (~14% more). Although monoterpene fluxes of the different plants were very different in magnitude, ranging from 0.25 to 22 nmol m^{-2} s^{-1}, all of them increased significantly (independent t-test for each plant, $p<0.01$). This increase in monoterpene emission after methanol fumigation in *B* –undamaged– plants was also found in *A* –previously clipped– plants when they were further submitted to an additional methanol fumigation treatment, and their monoterpene emission rate increased on average by 1.1 nmol m^{-2} s^{-1} (~8% increase, paired t-test p=0.02). We did not observe an effect of combined treatment (clipping + methanol fumigation) on terpene emissions: the increase in monoterpene emissions of the combined treatment was similar to that of fumigated-only plants. Analysis by GC-MS revealed that the released monoterpenes in all the cases were mainly composed of α-pinene and β-pinene, with minor contribution of sabinene, myrcene, and limonene. The relative contribution of each monoterpene to the sum of them was not changed substantially after fumigation.

Discussion

The clipping treatment increased the photosynthetic rates, the plant's chlorophyll a to chlorophyll b (Chl a/b) ratio, and the SIPI (carotenoid to chorophyll ratio) in non-clipped leaves (Fig.2), suggesting an activation of the physiological activity together with an activation of the plant protective metabolism (Young and Britton 1990). These responses agree with the enhanced expression of genes related to wounding, hormones, secondary signalling messengers, and photosynthesis, reported by Arimura and colleagues (2000) for lima bean (*Phaseolus lunatus*) after herbivory. Similarly, regarding the increase in photosynthetic rates, plants submitted to herbivory and wounding have been reported to compensate the loss of tissue with an increase in net CO_2 assimilation in non-injured leaves (Syvertsen 1994, Trumble et al. 1993). However, there are also, several published studies reporting rather the opposite: photosynthesis is downregulated by herbivory (Mantyla et al. 2008, Mitra and Baldwin 2008, Schmidt et al. 2009, Zangerl et al. 2002). In this experiment with *A* plants, we aimed to simulate the mechanical damage of herbivory. Nevertheless, the leaf clipping treatment does not completely simulate herbivore attacks, at least not those by chewing herbivore insects. It has been shown that a slow long-lasting wounding treatment is necessary to induce defense reactions in plants rather than a sudden removal of leaves (Mithöfer et al. 2005) –as was the case in this experiment. Furthermore, insect oral secretions absent in a clipping treatment may play a role in eliciting the plant responses to herbivory (Bricchi et al. 2010, Peiffer and Felton 2009). In any case, the increased methanol release after clipping agrees with previous studies showing local increased emissions after biotic and abiotic mechanical damage (Karl et al. 2001, Peñuelas et al. 2005a, von Dahl et al. 2006). But, furthermore, in this case the increased methanol emission after the clipping treatment, although only slight, was found in non-clipped leaves, indicating a systemic response of the plant to the herbivore attack, i.e. occurring in the whole plant –beyond the local emission at the wound–. These results suggest that methanol may act as a signal from the attacked plants.

This possibility was tested with the methanol fumigation treatment. This fumigation induced an increase in the actual photochemical efficiency of PSII ($\Delta F:F_m'$) and the carotenoid to chlorophyll ratio (SIPI), similarly to what happened to the cut plants in the clipping treatment. This similarity suggests a possible signalling function of airborne methanol, priming the plant to be able to cope with a likely imminent attack or stress. Pointing in the same direction, the higher monoterpene release by both damaged and undamaged plants after methanol fumigation further supports that methanol could have a role in plant-plant interaction. A previous experiment that reported the effects of methanol fumigation on horticultural plants showed that the treatment inhibited the plant's photosynthetic reactions (Loreto et al. 1999). However, in that occasion the high concentration of methanol used (1% in air) was about four orders of magnitude bigger that what we used in this paper. This fact may have caused the negative effects on photosynthesis, opposite to what we report here. In fact, stimulatory effects on growth at low concentrations of methanol have been reported to become inhibitory effects

as liquid methanol concentration was increased in spraying experiments with *Lemna gibba* (Dewez et al. 2003).

The increase in monoterpene emissions shown is not of a big magnitude (14%). However, it should be considered in the context of the complex mixtures of BVOCs released by plants, in which the effect of every individual BVOC could add to or complement the effects of other BVOCs. Similar increased monoterpene emissions by *Q. ilex* have also been reported as induced by other volatile signals like jasmonic acid (JA) (Filella et al. 2006) and MeSA (Peñuelas et al. 2007), with increases that were in the range of those described in this paper. Increases in terpenoid emissions induced by herbivore-induced plant volatiles (HIPV) have been reported in *Zea mays* (Ton et al. 2007). Likewise, terpenoid increased emissions have been reported also as a consequence of the concerted action of several other signalling BVOCs like JA, ethylene, and GLV (Arimura et al. 2008, Engelberth et al. 2004, Farag and Pare 2002, Ruther and Kleier 2005).

Monoterpenes emitted following a signalling cue (e.g. methanol or other BVOCs) have, in their turn, some possible functions in plant physiology and ecology. For example, it has been proposed that they may act as deterrents against herbivores (Raffa et al. 1985) in addition to become antioxidants (Loreto et al. 2004, Peñuelas and Llusià 2002) or cell membrane stabilizers (Sharkey and Singsaas 1995). Moreover, they may act as signals to parasitoids (Mumm et al. 2008, Pinto et al. 2007b), –or maybe, in the opposite sense, to attract more herbivores (Brilli et al. 2009, Halitschke et al. 2008). Hence this terpene signalling role in plant-animal communication, and also in plant-plant communication (Godard et al. 2008) forwards the message started by methanol.

The possible roles of each of these BVOCs found to be increasingly emitted in response to clipping or to methanol fumigation (methanol and monoterpenes) in the mixtures released by plants will be more clear when the roles of these and the other compounds of the BVOCs bouquet become gradually known and the modulation by biotic and abiotic factors are taken into account (Baldwin et al. 2006). Further research in this field should also address the mechanisms by which plants would distinguish between a "normal" and an "alarm" BVOC blend. Meanwhile, the results of this study suggest that clipping and methanol fumigation at natural concentrations elicit significant neighbour plant physiological responses and further BVOC emissions.

Acknowledgements

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Figures

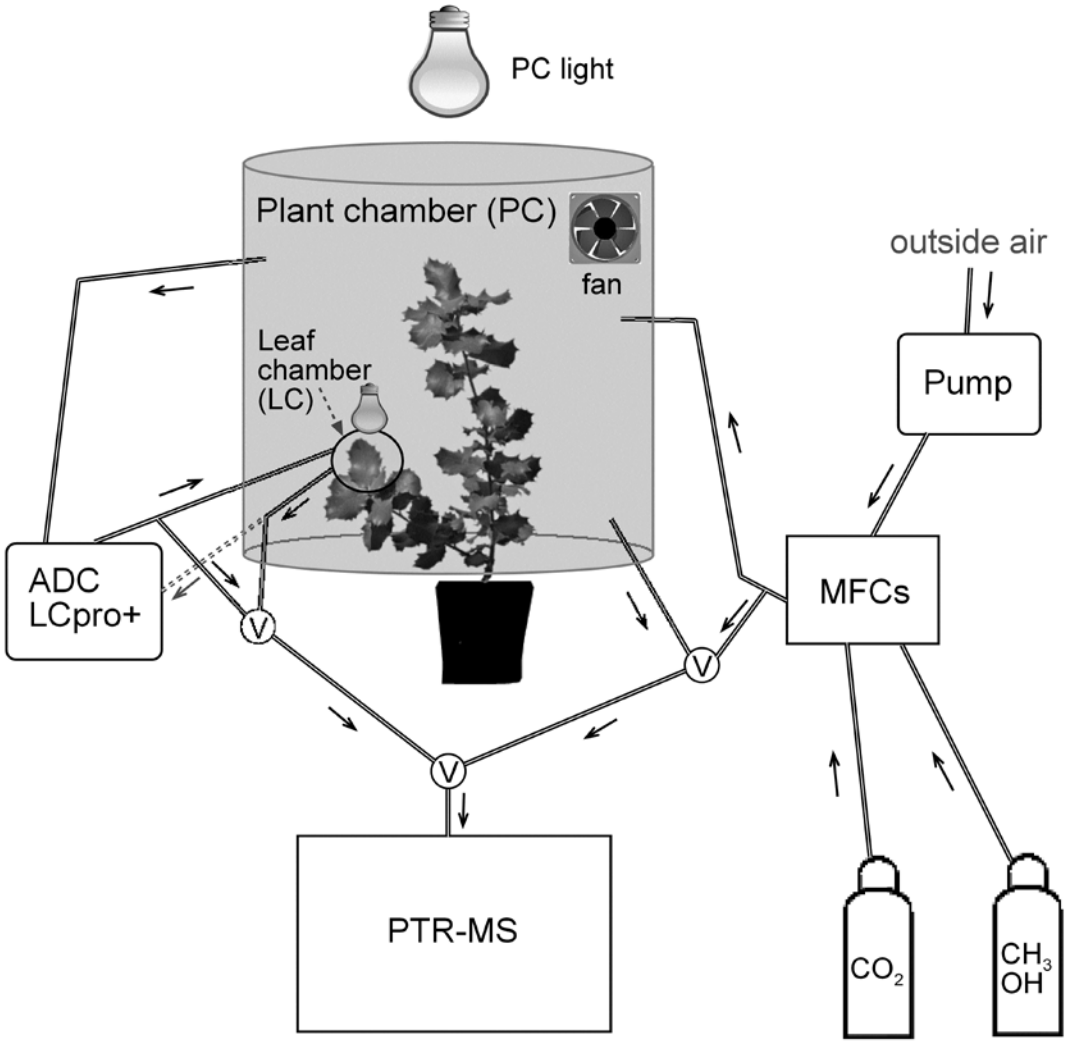

Fig. 1. Scheme showing the chamber setup for this experiment. *Quercus ilex* seedlings were enclosed in a 65L teflon chamber (Plant Chamber, PC). Outside air was flowing into the PC by means of an oil-free pump, at a flow regulated by a Mass Flow Controller (MFC). CO_2 from a bottle was used to keep concentration inside the PC at ca. 385 µmol mol^{-1}. An additional MFC regulated the flow of methanol (CH_3OH) coming from a bottle during fumigation episodes. A fan inside the PC assured that the air was well mixed. Inside the PC, a small Leaf Chamber (LC, part of an ADC LCpro+ photosynthesis system) enclosed one leaf, and controlled its illumination by means of a separate light source. It also gathered information on photosynthetic activity and stomatal conductance. The air coming in and out from both the PC and the LC was sampled by a PTR-MS system through the use of teflon solenoid valves (V). The solid black arrows show the direction of the flow of gas inside the passivated stainless steel tubing.

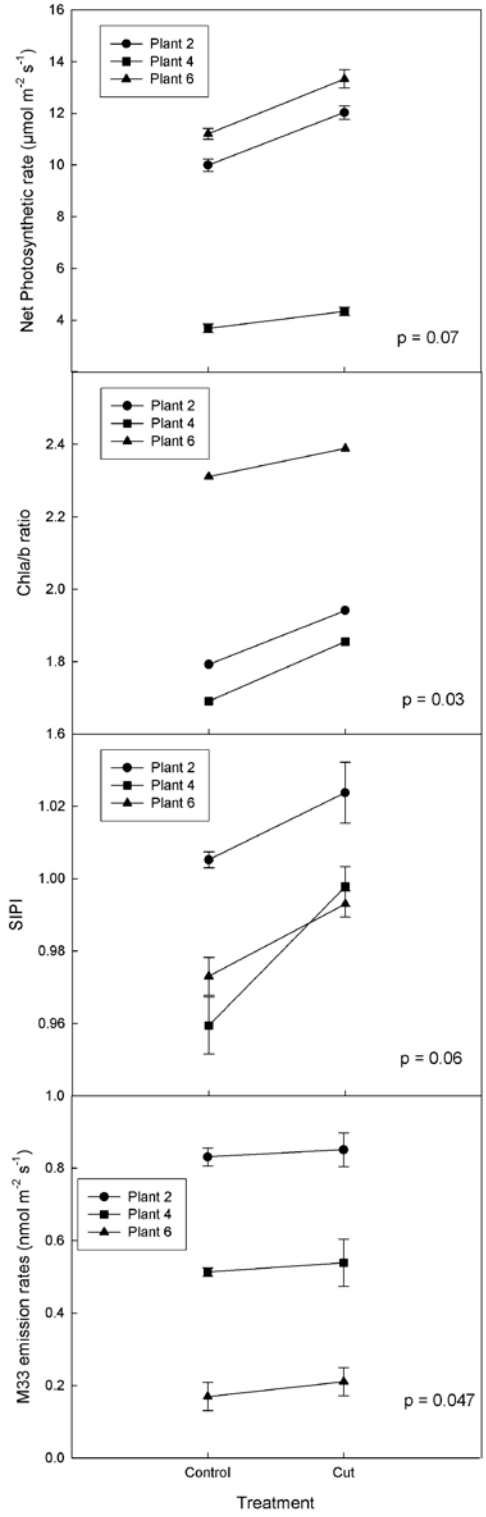

Fig. 2. Increases in the net photosynthetic rate, Chla/b ratio, SIPI (Car/Chl ratio), and M33 (methanol) emission, in response to clipping leaves in *A* plants. Values shown are the mean values ± standard error (n=10 measurements of the leaf in the LC for photosynthetic rate; n=7 different leaves measured from the PC for SIPI; n=4 measurements of the leaf in the LC for methanol between 14:00h and 15:00h; for the Chla/b ratio, a pool of 4-5 leaves from the PC was used for the measurement). The p value is the significance level of the paired t-test for the 3 plants.

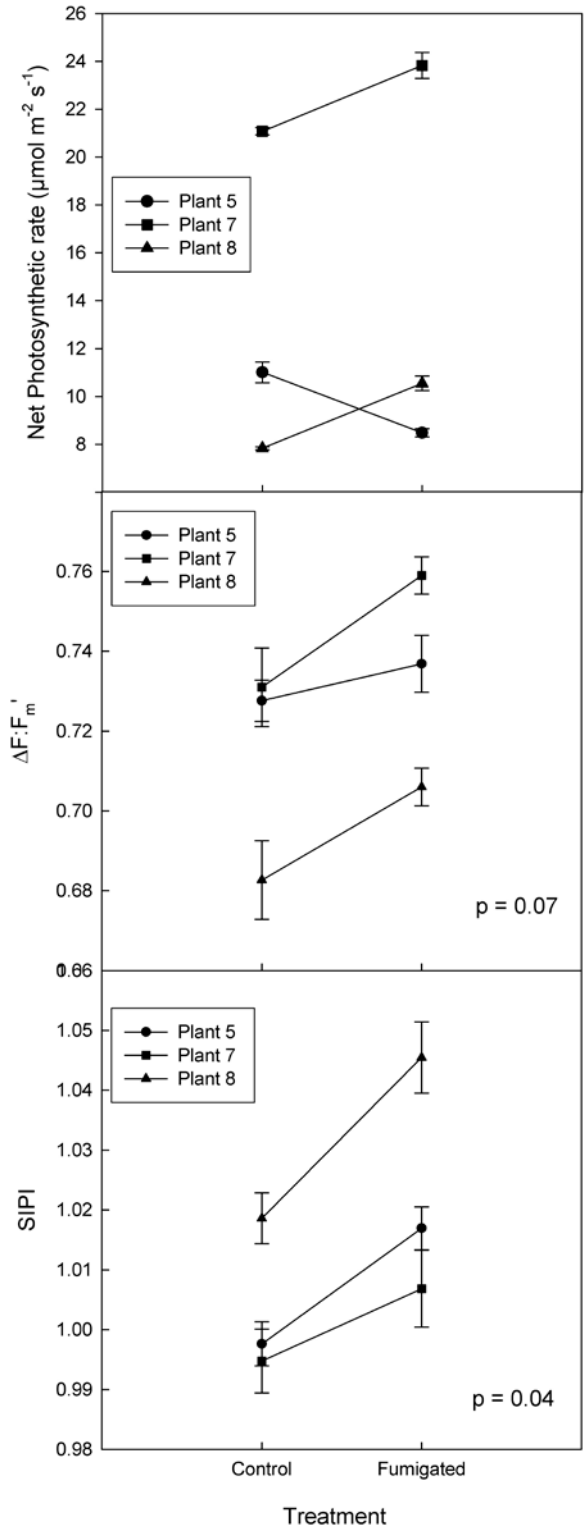

Fig. 3. Changes in net photosynthetic rate and increases in actual photochemical efficiency of PSII ($\Delta F:F_m'$) and SIPI (Car/Chl ratio) after the methanol fumigation in *B* (fumigated-only) plants. Values shown are the mean values ± standard error (n=10 measurements of the leaf in the LC for photosynthetic rate; n=7 different leaves measured from the PC for $\Delta F:F_m'$ and SIPI). The p value is the significance level of the paired t-test for the 3 plants.

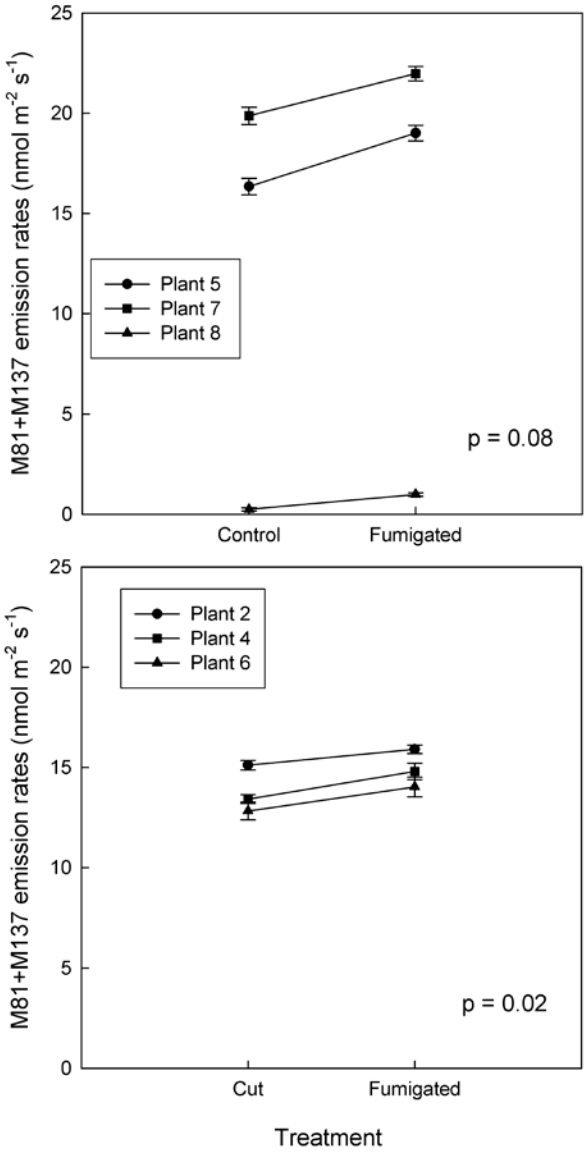

Fig. 4. Increases in the M81+M137 (monoterpenes) emission rate in the leaf chamber (LC), after the methanol fumigation treatment, in the case of *A* plants (previously clipped plants, bottom panel) and *B* plants (fumigated-only plants, top panel). Values shown are the mean values for the 14:00h-15:00h of each day ± standard error (n=4 measurements of the leaf). The p value is the significance level of the paired t-test for the 3 plants.

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