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BRIEF COMMUNICATION

Methyl salicylate fumigation increases monoterpene emission rates

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Abstract

We aimed to assess the potential effects of fumigation by methyl salicylate (MeSA) on plant monoterpene production and emissions. We evaluated monoterpene production and emissions both by chromatographic and proton transfer reaction mass spectrometry at the whole plant- and leaf-scales, in MeSa-fumigated (*ca.* 60 mm^3 m^{-3} in air) and control (without MeSa fumigation) holm oak (*Quercus ilex* L.) plants exposed to temperatures ranging from 25 to 50 ºC. The MeSa-fumigated plants showed *ca*. 3 - 4-fold greater leaf monoterpene concentrations and emission rates than the control plants between the temperatures of 25 to 45 ºC.

Additional key words: antioxidants, limonene, β-myrcene, α-pinene, β-pinene, PTR-MS, *Quercus ilex*, thermotolerance.

Methyl salicylate (MeSA) is known to be released by stressed plants. It is considered to be a signal involved in eliciting plant resistance to stressors such as chilling (Ding *et al*. 2002, Fung *et al*. 2004), salinity (Borsani *et al*. 2001), drought (Munné-Bosch and Peñuelas 2003), heat (Clarke *et al*. 2004), and the gall midge *Dasineura marginemtorquens* in the willow (Ollerstam and Larsson 2003). It is also known that plants respond to biotic and abiotic stresses by emitting terpenes, a fact that seem to serve diverse physiological and ecological functions including possible thermotolerance enhan-cement (Peñuelas and Llusià 2001, 2002, 2003, 2004).

Fumigation with isoprene (10 cm^3 m^{-3}) has been found to confer thermotolerance on *Quercus ilex* seedlings and at the same time to suppress the activation of antioxidants in the leaf. Among these changes, monoterpene production and emission have been found to be reduced by 70 % (Peñuelas *et al*. 2005a). In another study, no clear thermotolerance or consistent changes in the emission of other monoterpenes occurred in response to limonene fumigation (7.5 cm^3 m^{-3}) (Llusià *et al*. 2005a). Finally, in a recent study, MeSA fumigation (60 mm^3 m^{-3}) was found to cause sustained accumulation of MeSA in leaves, reduced antioxidant levels (ascorbate and tocopherol) and increased oxidative damage in the holm oak at temperatures ranging from 25 to 50 ºC (Llusià *et al*. 2005b). We aimed to answer the question whether or not this decreased thermotolerance and antioxidant performance in response to the sustained accumulation of MeSA produced by MeSa fumigation would be accompanied by any increase in monoterpene production and emission. If this was the case, we would have another indication that airborne MeSA may activate this monoterpene response just as it does with the expression of defense-related or stress-related genes in other plants (Shulaev *et al*. 1997, Fung *et al*. 2004).

We studied the above-mentioned question in *Quercus ilex* L., a dominant Mediterranean forest species (Rodà *et al*. 1999) whose leaves may suffer from thermal stress at temperature above 35 ºC (Larcher 2000, Peñuelas *et al*. 1998). Usually, CO_2 uptake suddenly decreases at temperature between 40 and 50 ºC, although *Q. ilex* grows in sites where maximum air temperatures reach this range (*e.g*. Seville, Southern Spain). We exposed *Q. ilex* seedlings to temperature increases from 25 to 50 ºC in 5 ºC steps in atmospheres fumigated with MeSA (*ca.* 60 mm^3 m^{-3}). This represents a high air MeSA concentration, but it is only twice the concentration described in air surrounding tomato plants (Deng *et al*. 2004) and is much lower than the 100 cm^3 m^{-3} used in studies of gene expression enhancement by MeSa (Fung *et al*. 2004).

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Abbreviations: MeSA - methyl salicylate; PTR-MS - proton transfer reaction mass spectrometry.
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Two-year-old holm oak plants (*Quercus ilex* L.), obtained from a nursery (*Forestal Catalana S.A.*, Breda, Spain), were grown in a greenhouse in 2 dm^3 pots with peat and sand (2:1, v/v) containing slow-release fertilizer (2 g dm^{-3}, 15:8:11 N,P,K + 2 Mg) under irrigated conditions. For each treatment, twenty plants were transferred into a 1.59 m^3 (1.75 m high × 1.25 m wide × 0.725 m deep) chamber (*Vötsch-Industrietechnik, Bio Line model VB 1014*, Balingen-Frommern, Germany). Eight 400-W halogen lamps (model *HQI-T*, Balingen-Frommern, Germany) supplied photon flux density (PPFD) of 500 - 600 μmol m^{-2} s^{-1} at a height of 0.9 m during a 12-h photoperiod. The relative humidity was kept around 50 % throughout the experiment. Plants of the same height (*ca*. 0.9 m) were exposed to temperature increases from 25 to 50 ºC. Plants were kept at specified temperatures for 1 d before measurements were taken.

MeSA treatment was applied by fumigating plants with air containing 60 mm^3 m^{-3} MeSA by means of a membrane pump that flushed the ambient air through a thermostated flask containing MeSA (*Fluka*, Buchs, Switzerland). The airborne MeSA concentrations in the fumigated chamber were kept at 60 ± 10 mm^3 m^{-3} by regulating the flow of the MeSA flux into the chamber, whereas concentrations in the control chamber were kept below 1 mm^3 m^{-3} throughout the experiment, as monitored by gas chromatography coupled to mass spectrometry (GC-MS, see below). Airflow in the chamber was 0.125 m^3 min^{-1} and air in the chamber was completely renewed every 12.7 min.

Foliar MeSA and monoterpene contents were determined in fully developed young leaves of randomly chosen plants grown in non-fumigated (control) and MeSA-treated atmospheres. Leaves were collected around 2 h after the start of the light period, frozen in liquid nitrogen and then stored at -20 ºC until analysis. Measurements at each temperature were made on 4 - 6 individuals for each assayed atmosphere. The sampling and analysis of foliar concentrations and airborne MeSA and monoterpenes in the growth chamber was conducted as described in Peñuelas *et al*. (2005a)

To complement the test, we repeated the experiment at a foliar scale in a leaf chamber and measured monoterpene emissions "on-line" with a Proton Transfer Reaction Mass Spectroscopic (PTR-MS) system as described Copolovici *et al*. (2005) and Peñuelas *et al*. (2005b). Leaves of another batch of seedlings obtained from the same nursery were either fumigated with MeSA or maintained in the ambient atmosphere. Air with 60 mm^3 m^{-3} MeSA concentrations was prepared with a membrane pump that flushed the ambient air through a thermostated flask containing liquid gas-chromatography grade MeSA (purity ≥ 99 %, *Sigma-Aldrich*, Barcelona, Spain). The MeSA concentration in the leaf chamber was monitored with PTR-MS as described in Peñuelas *et al*. (2005b) and in Copolovici *et al*. (2005), and the evaporating surface area and the thermostat temperature were empirically adjusted to obtain an air MeSA concentration of 60 mm^3 m^{-3}.

The PTR-MS instrument (*IONICON Analytik GmbH*, Innsbruck, Austria) was described in detail in Lindiger *et al.* (1998) and the experimental setup used in our laboratory in Peñuelas *et al*. (2005b) and Copolovici *et al.* (2005). The measuring method is based on a proton-transfer reaction from hydronium ions (H_3O^+) to compounds with a higher proton affinity than water. The subsequent detection of the product ions is carried out in a quadrupole mass spectrometer. Volatile compounds that have proton affinities larger than water are ionized by proton transfer from H_3O^+, and the protonated volatiles are mass analyzed. The ion source produces nearly exclusively H_3O^+ ions (> 98 %), that are extracted and transferred into the drift tube (Mayr *et al*. 2003).

PTR-MS technique has two to three orders of magnitude greater sensitivity than conventional GC-MS and thus, emission rates of organic compounds can be estimated in real time (Cao and Hewitt 1995, Tani *et al*. 2003). However, the PTR-MS technique cannot distinguish between volatile compounds of the same molecular mass (for example, the different monoterpenes). Monoterpene concentrations can be estimated from the protonated molecular ions at mass 137 kDa (Tani *et al.* 2003). However, a certain fractionation of non-oxygenated monoterpenes in the drift tube occurs during ionization even under 'soft' conditions, resulting in masses 67, 81 and 95 among others (Tani *et al*. 2003). To take into account this fractionation, the total concentration of monoterpenes emitted by *Q. ilex* was calculated as the concentration of ions with mass 137 multiplied by 2.17 following Tani *et al*. (2003). This correction factor was corroborated by our simultaneous PTR-MS and GC-MS (carbotrap/carbosieve trapping) measurements and by calibration with α-pinene and limonene standards.

Valves situated before and after the leaf chamber were employed to allow the measurement of the monoterpene concentrations in the air entering and leaving the leaf chamber and thus the determination of the monoterpene emission rates. At every temperature, steady-state emission rates were monitored for 24 h and averages of the final 2 h (from 08:00 to 10:00) were calculated to reproduce the procedure of the plant level experimental system.

Analyses of variance (*ANOVA*) were conducted using *STATISTICA* version *6.0* for Windows (*StatSoft, Inc*., Tulsa, OK, USA). Statistical differences between measurements of different treatments at each temperature were also analyzed following the Student's *t*-test. Differences were considered significant at a probability level of $P < 0.05$.

Both plant-level and leaf-level experiments showed that MeSa-fumigated plants and leaves increased *ca*. 3 to-4-fold their production and emission of monoterpenes at 25 - 45 ºC (Figs. 1, 2). Of all monoterpenes, α-pinene, β-myrcene, and limonene increased by the greatest amount (Fig. 1). Foliar concentrations ranged between

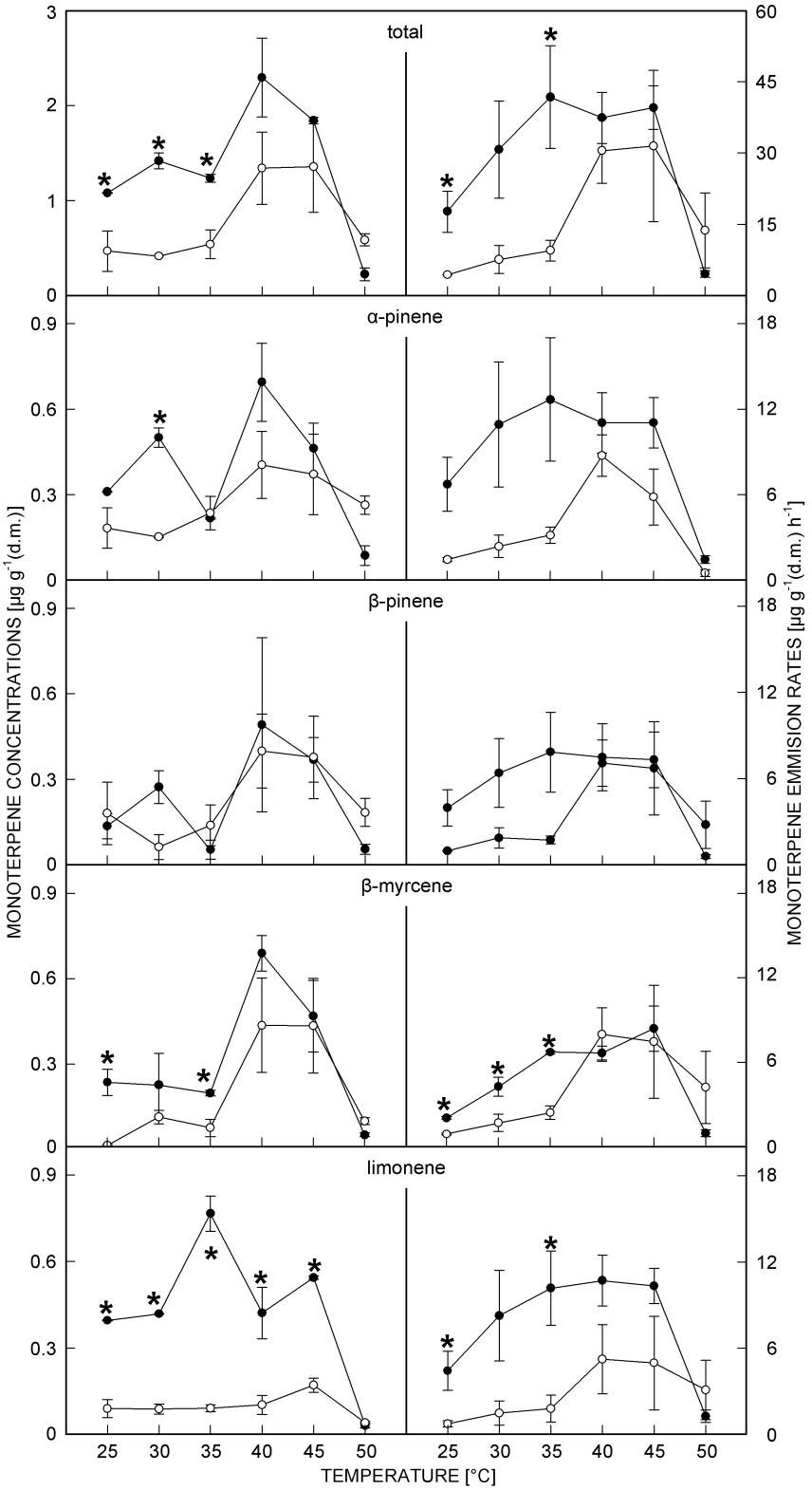

Fig. 1. Foliar monoterpene concentrations and emission rates of non-fumigated (control, *empty symbols*) and of MeSA-fumigated (*black symbols*) plants progressively exposed to increasing temperatures (from 25 to 50 ºC). The measurements were conducted under PPFD of 500 - 600 µmol m^{-2} s^{-1} and were conducted every morning for each temperature after temperature conditions were maintained constant for 24 h. *Error bars* indicate SE of $n = 2$ cycles per temperature. For each cycle and temperature, the mean of three chamber measurements was considered; * - significant differences (Student's *t*-test, $P < 0.05$) between MeSA-fumigated and control plants.

0.5 and 2.5 μg g^{-1}(d.m.) and they are similar to those found by previous studies of thermotolerance in the same species (Llusià *et al*. 2005a, Peñuelas *et al*. 2005a). Emission rates ranged between 1 and 40 μg g^{-1} (d.m.) h^{-1}, which are also similar to those found in previous studies with the same species (Llusià *et al*. 2005a, Peñuelas *et al*. 2005a). In those two articles a typing error occurred on the y-axis labels of the corresponding figures erroneously leading to 10-times higher values than those that were actually measured.

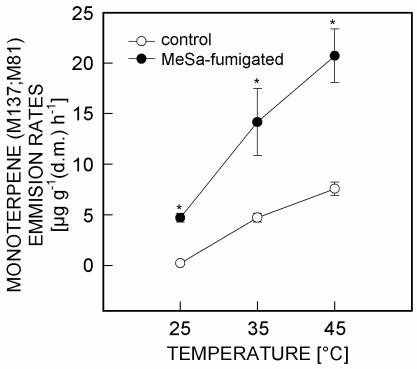

Fig. 2. Foliar emission rates of non-fumigated (control, empty symbols) and of MeSA-fumigated (black symbols) leaves progressively exposed to increasing temperatures (from 25 to 45 ºC) and measured with a PTR-MS system. The measurements were conducted under a saturating PPFD of 1000 μmol m^{-2} s^{-1}. They were conducted every morning for each temperature after temperature conditions were maintained constant for 24 h. *Error bars* indicate SE of n = 3 cycles per temperature. For each cycle the mean of the measurements of the final 2 h (n = 120) was considered; * indicates significant differences (Student's t-test, P < 0.05) between MeSA-fumigated and control leaves.

The foliar MeSA concentrations of MeSA-fumigated plants (which might also include MeSA deposited on the leaf surface or adsorbed into the waxy cuticle) were between 10 and 23 nmol g^{-1}(d.m.) throughout the experiment and showed a slight downward trend as the temperature increased (Llusià *et al*. 2005b). In control (non-fumigated) plants, foliar MeSA contents increased progressively as temperature increased, reaching maximum of 1.8 nmol g^{-1}(d.m.) at 45 ºC (Llusià *et al*. 2005b). Thus, transient increases in foliar MeSA levels occurred in control (non-fumigated) plants, while sustained foliar accumulation of MeSA occurred in fumigated ones. In our previous study (Llusià *et al*. 2005b), we found that the sustained accumulation of MeSA may lead to the uncontrolled production of reacive oxygen substances and overwhelm antioxidant defense and induce cell death under stress in MeSA-fumigated plants. Under those conditions increasing monoterpene synthesis and emissions may add antioxidant capacity to foliar tissues (Mimica-Dukić *et al*. 2003, Loreto *et al*. 2004). This possibility warrants further research. The results shown here indicate at least that airborne MeSa may drive plants to produce and emit monoterpenes in a similar way to what occurs with the expression of defense-related genes in other plants (Shulaev *et al*. 1997), with the expression of genes involved in defense against oxidative stress during chilling (Fung *et al*. 2004) or with the increase in antioxidant enzymes activities (Agarwal *et al*. 2005).

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