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Volatile isoprenoid emission potentials are correlated with essential isoprenoid concentrations in five plant species

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Running title: Volatile and essential isoprenoids are correlated

Abstract

This study offers new insight and data in support of the "opportunist hypothesis". Five species of volatile isoprenoid-emitting plants (*Eucalyptus globulus*, *Eucalyptus gunnii*, *Mucuna pruriens*, *Lycopersicon esculentum* and *Quercus ilex*) were exposed to a wide range of imposed and natural stress conditions over a period of a few weeks in order to generate different levels of isoprenoid production potential. Volatile isoprenoid emissions and carotenoid concentrations were measured in all species and dimethylallyl diphosphate (DMAPP) concentrations were measured in *E. globulus*, *E. gunnii*, *M. pruriens* and *L. esculentum.* Generally, instantaneously emitted isoprenoid emissions were positively correlated with carotenoid concentrations, and were negatively correlated with DMAPP concentrations. In contrast, stored monoterpene emission potentials were negatively correlated with carotenoid concentrations, and positively correlated with DMAPP concentrations. These results support the possibility of a direct or indirect control of volatile isoprenoid emission potential via carotenoid synthesis at time scales of days to weeks.

Key words: Opportunist theory, stress, VOCs, isoprene, monoterpenes, biogenic emissions, carotenoids, DMAPP.

1. Introduction It is well known that vegetation emits a wide range of volatile isoprenoid compounds into the atmosphere, where they contribute to the chemistry associated with air quality e.g. ozone and particle formation (Fehsenfeld *et al*., 1992). Volatile isoprenoid compounds are a special group of metabolites which are not synthesised and emitted by all plant species, but for emitting species, they have important roles in plant function and ecophysiology, including protection in conditions of high temperature or light intensity stress, oxidative stress, and herbivore stress (e.g. Kesselmeier and Staudt, 1999; Loreto and Velikova, 2001; Peñuelas and Llusià, 2002; Llusià *et al.*, 2005; Peñuelas and Munne-Bosch, 2005; Peñuelas et al., 2005a, Peñuelas et al., 2005b). Volatile isoprenoids share the same biochemical precursors as essential isoprenoids such as carotenoids, abscisic acid, and sterols which have vital roles in plant protection and development (Owen and Peñuelas, 2005; Figure 1).

The magnitude and composition of volatile isoprenoid emissions from individual leaves, plants, species and canopies depend on emission potentials of each compound. The main abiotic controls which modify the potential for emission in the short-term (i.e., 24 hours) are temperature (Tingey *et al*., 1980; Guenther *et al*., 1991), and for isoprene-emitters and some monoterpene-emitting tropical and Mediteranean oak species, PAR (Guenther *et al*., 1995; Baker *et al*., 2005). Biotic controls such as insect herbivory can also significantly modify emission potential at the time-scale of 24 hours (e.g. Peñuelas *et al*., 2005a). In the longer term (> days), emission potentials (*per se*) of volatile isoprenoids from leaves, whole plants and canopies are affected by herbivory, pollution and other abiotic stresses, carbon dioxide concentration, phenology and season (e.g. Kesselmeier and Staudt, 1999; Litvak *et al*., 1999). It has been suggested that many of these longer term controls on volatile isoprenoid emissions potentials may in fact be a result of biochemical demands of essential carotenoid biosynthesis (the "Opportunist Hypothesis", Owen and Peñuelas, 2005). Owen and Peñuelas (2005) remind us that carotenoid production is ubiquitous and that plants can not survive in the absence of these compounds. They suggest that this group of compounds is therefore a more important product of the isoprenoid synthesis pathways than volatile isoprenoid production, and that volatile isoprenoid production is coincidental or "opportunistic", perhaps taking advantage of a surplus of substrate. From the shared early biochemical pathway for the volatile and the essential isoprenoids (Figure 1), a stress that creates a demand for synthesis of essential carotenoid compounds might increase production of volatile isoprenoids if the demand produced excess biochemical precursor dimethylallyl

diphosphate (DMAPP) and the volatile isoprenoid synthase enzymes were active. On the other hand, a carotenoid production stress response might exhaust DMAPP supply, resulting in a substrate limited production of volatile isoprenoid.

Owen, Hewitt and Rowland (2013) review the different effects of different plant stresses on emissions of volatile isoprenoids. A modified summary of this is provided in Table 1, which also shows examples of the effects of stresses on photosynthesis. Clearly, the substrates for the volatile isoprenoid pathway depend upon the products of photosynthesis. In addition, the biotic and abiotic controls of isoprenoid production (including stresses) also affect photosythesis processes. The responses for volatile isoprenoids are not consistant across all plant taxa and across all types of stress, and combinations of stresses. Whatever the magnitude and direction of the response to stresses in different taxa, because of the shared early stages of the biosynthesis pathways, and because of the shared functionality of volatile isoprenoids and carotenoids, we propose that it might be possible to see a relationship between concentrations and emissions of these compounds within species subject to different levels of different stresses.

The aim of the work described here was to investigate this hypothesis, in the context that essential isoprenoid biosynthesis might affect volatile isoprenoid synthesis and emission over a temporal scale of weeks to months. At this temporal scale, time itself can be considered as a source of stress to plants, as growth, development and senescence take place. We measured volatile isoprenoid emissions, total carotenoid concentrations and in some cases, DMAPP concetrations from different species in different stress conditions, at different phenological stages. We used the data to investigate correlations between essential and volatile isoprenoids in plants whose emissions were expected to vary either due to phenology, or to biotic or abiotic stress. Significant correlations were considered to provide support for the Opportunistic Hypothesis.

2 Materials and methods

The relationships between volatile isoprenoids and carotenoid concentrations were investigated in five different plant species (*Quercus ilex*, *Eucalyptus globulus*, *Eucalyptus gunnii*, *Mucuna pruriens* and *Lycopersicon esculentum*) experiencing different conditions of growth and stress. Two relatively fast-growing plant species (*L. esculentum* and *M. pruriens*) were grown to obtain samples at different stages of development over a period of weeks. *L. esculentum* emits stored monoterpenes (e.g. Winer *et al*., 1992) and *M. pruriens* emits isoprene (e.g. Harley *et al*., 1996a). In this study we refer to "emission potential". This is the emission rate at standard environmental conditions, which can vary from study to study. Here we follow a widely accepted convention of 30 °C and 1000 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR) (Guenther *et al.* 1995). It is known that leaf age can affect emission potentials (Kuzma and Fall, 1993; Staudt *et al*., 2003), so we expected to see changes over the relatively short life cycle of these plants.

Two further plant species were used (*E. globulus* and *E. gunnii*) which require a longer time to reach maturity. These species were subject to ozone and water stresses which are known to affect the magnitude of emission potentials (e.g. Llusià *et al*., 2002). The fraction of *Eucalyptus spp.* that have been screened emit large amounts of volatile isoprenoids (He *et al*., 2000). *E. globulus* and *E. gunnii* emit both isoprene and stored monoterpenes.

Q. ilex is widespread and common in Mediterranean Europe (Michaud *et al*., 1995) and emits light-dependent (non-stored) monoterpenes (Staudt and Seufert, 1995; Peñuelas and Llusia, 1999b). Here, measurements were made on trees of *Q. ilex* growing naturally in field conditions, where different conditions of growth and stress were provided by sampling trees growing at different altitudes, affording different degrees of exposure and water stress, and different degrees of sun and shade. Young and old leaves were sampled to provide another dimension of variability.

2.1 Plants and plant material

All plants except *Q. ilex* were grown in a greenhouse, either on open staging or within fumigation chambers constructed in the greenhouse, under artificial light at 14/10 photoperiod at 500 μmol m^{-2} s^{-1} and with partial temperature control (to ensure sufficiently high minimum temperatures) with temperatures varying between 18 and 28 ºC. Tomato plants (*L. esculentum*) were raised from seed (Chiltern seeds, UK) in John

Innes seedling compost between January and March. When large enough to handle, they were transferred to 9 cm plastic pots containing Levington M3 Scott compost. Velvet bean seed (*Mucuna pruriens)*, (B & T World Seeds, France) were also raised from seed during April 2005. They were soaked in warm water overnight, and germinated in individual pots containing vermiculite. When large enough to handle, they were transferred to Levington M3 Scott compost in 5 litre pots. During the experiment, plant pots stood on greenhouse mesh shelving, which allowed thorough watering and free draining of the compost in the pots.

Plants of *E. globulus* were grown from seed (Chiltern seeds, UK) in February 2005, in trays containing John Innes seedling compost. When large enough to handle, they were pricked out into individual pots (9 cm in diameter) filled with Levington M3 Scott compost to grow into young plants ~ 50 cm tall. Just before measurements commenced, the *E. globulus* plants were diagnosed with an infestation of *Aulacorthum solani*. This did not preclude use of the data from these plants in this report, as the aim was to investigate relationships between isoprenoids in plants subject to a range of different stress conditions.

E. gunnii plants were obtained from a commercial nursery ("Cath's garden plants", Cumbria, UK). Plants were placed in fumigation chambers for the ozone fumigation and drought treatments. Plant pots were placed on small inverted trays within the chambers to enable free draining of excess irrigation water from the compost in the pots. Except when undergoing drought treatment, all plants were watered to saturation daily, and twice daily in warm weather. *E. globulus* plants were sampled between April and June (~2-4 months old), and *E. gunnii* were sampled during July and August (4-6 months old).

Full grown *Q. ilex* trees were growing in natural conditions in the Collserola park around 5 km north west of Barcelona (central Catalonia, NE Spain, 41°27'N, 2°7.7'E). The climate is Mediterranean, with cool winters and hot dry summers. Mean annual temperature is 14.5 °C and mean annual precipitation is 610 mm. Different conditions of growth and stress were provided by different altitudes which resulted in different degrees of drought stress and sun exposure, and different aged leaves. The trees growing at the highest altitude were more exposed to sunlight and drought stress, and were more stunted in growth than the trees growing at lower altitudes.

2.2 Experimental conditions and sampling strategy

2.2.1 L. esculentum and M. pruriens.

Emissions, carotenoids and DMAPP concentrations were measured when the plants were 4 and 6 weeks old for *L. esculentum*, and when plants were 6, 8 and 10 weeks old for *M. pruriens*. Leaves were selected of equivalent size and maturity, usually corresponding to node 4 for *L. esculentum*, and node 10 for *M. pruriens*, but this varied if leaves at those particular nodes were too small, or damaged in any way. At each sampling session, a leaf cuvette (ADC, UK) was installed on the leaf, allowed to equilibrate for one hour before three consecutive samples of volatile isoprenoid emissions were taken. Three replicate plants were measured in this way for each time period. Leaf samples were taken at the same time of day for each plant (between 11:00 and 14:00) from the same node from three plants of the same age and in the same growth conditions, and flash frozen in liquid nitrogen for storage at -20 ºC prior to analyses for DMAPP and carotenoid concentrations. These analyses were carried out within a few days of the emissions samples.

2.2.2 Eucalyptus spp.

Six fumigation chambers were used (0.75m x 0.75m x 0.75m), constructed of plasticised chipboard. The chambers were ventilated with ambient air at a rate of 0.4 m^3 min^{-1}. In all, there were 12 plants of each *Eucalyptus spp.* Two plants were placed in each of the six chambers, one of each pair as a drought control, and the other droughted (at a later time). Three chambers were control (i.e. non-ozone fumigated), and the inflow air to the other three chambers was supplemented with ozone generated from clean air with a Triogen TOG B1 (1g h^{-1}) to produce a concentration inside the chambers of between 70 and 90 ppb above ambient. Thus there were three replicate plants for each treatment. The chambers were located in a greenhouse and were therefore subject to the influence of ambient light and temperature fluctuations, typically ranging from 16 to 33 ºC. Artificial light from "Plantastar" 600W sodium lamps maintained a minimum PAR of 250 μmol m^{-2} s^{-1} during the photoperiod of 14 hours light (between 06:00 and 20:00 local time) and 10 hours dark. Leaves of equivalent maturity and size were used for each plant at each sampling time-point. Leaves positioned at nodes 3 to 5 from the apex of the *Eucalyptus* plants were used for measurement, because these were the best size with easiest accessibility for installing the leaf cuvette.

Volatile isoprenoid emissions, carotenoid and DMAPP analyses were performed in triplicate before ozone fumigation started. The ozone-treatment plants were then fumigated for 4 weeks, and the chemical analyses were repeated. *E. globulus* plants

were then left for a further 15 weeks, and then water was withheld from the drought-treatment plants (one in each chamber, both control and ozone fumigated). *E. gunnii* plants were left for a further 2 weeks before water was withheld from the drought-treatment plants. In each case, the water deprivation lasted one week, after which volatile isoprenoid emissions, carotenoid and DMAPP sampling were carried out for the third time.

At each sampling session, three consecutive samples of volatile isoprenoid emissions were taken from each plant. Plants were sampled in the same order at each session, to avoid confounding the results with a variable emission potential which some plant species exhibit throughout the course of a day (Dudavera *et al*., 2005). It was possible to sample only six plants each day, and so plants destined for drought-treatment (in both ozone fumigated and non-ozone fumigated chambers) were sampled on day 1, and plants destined as drought controls were sampled on day 2. A leaf cuvette was installed on each plant in turn, beginning on day 1 at 08:30 with the installation in the cuvette of the plant for drought treatment in the first control chamber, followed by the plant for drought treatment in the first ozone-treatment chamber, then alternating between control and and ozone chamber plants for drought treatment, until each of the six plants had been sampled. On day 2, the sampling pattern was repeated with the plants destined as drought controls. After each emission sample, the leaf was harvested, along with the leaf growing opposite, and flash frozen in liquid nitrogen for storage at -20 ºC prior to analyses for DMAPP and carotenoid concentrations. These were carried out within a few days of the emissions samples.

2.2.3 Q. ilex

Emission and leaf samples for carotenoid analyses were collected during a hot dry period of 6 days in July from trees growing at three altitudes (100 m, 350 m, 500 m). A leaf was sampled from a total of 24 different trees. Sampled leaves were growing in a wide range of conditions ranging from sun-exposed to shaded, from water stressed to well supplied with water, from exposed to sheltered, and from healthy to infected or herbivored. A leaf cuvette was installed on each leaf in turn, and the leaf was allowed to equilibrate for half an hour before emissions were sampled. After each emission sample, the leaf was harvested, along with the leaf growing opposite. These were flash frozen and stored in liquid nitrogen for transport to storage in a laboratory freezer (-20 ºC) prior to analysing carotenoid concentrations. These were carried out within a few days of harvesting the leaf.

2.3 Sampling volatile isoprenoid emissions

2.3.1 L. esculentum, M. pruriens and Eucalyptus spp.

For these species, the leaf cuvette was left to equilibrate for 45 min on the leaf to avoid abnormally high emissions resulting from installation of the cuvette (Owen *et al*., 1997), and then 3 consecutive emission samples were taken over a period of 0.5 h. The leaf cuvette was supplied with ambient air filtered through charcoal. For these species, inflow air was supplied at a constant rate of 350 mL min^{-1} to maintain a positive flow of air such that any gases sampled from the cuvette were solely from within the cuvette system and not from outside. Samples were collected onto preconditioned dual bed stainless steel sample tubes (Perkin Elmer, UK), packed with solid phase adsorbents Tenax TA (200 mg) and Carbotrap (100 mg) using a mass flow controlled sampling pump (SKC, UK), at a rate of 100 mL min^{-1} for 10 min (Owen *et al*., 1997). Sampled tubes were stored at 4 ºC prior to analysis with GC-MS.

2.3.2 Q. ilex

For *Q. ilex*, the cuvette was installed for 30 min prior to sampling emissions. Flow through the cuvette was approx. 560 mL min^{-1} (the exact flow was recorded every minute). Two consecutive samples were taken at 500 mL min^{-1} for 4 min (total sample volume of 2 litres). Sampling was by means of a peristaltic pump (BUCK I.H. Pump™, Orlando, MI) drawing air from the cuvette through preconditioned triple bed glass sample tubes (8 cm long and 0.4 cm internal diameter), packed with solid phase adsorbents Carbotrap C (250 mg), Carbotrap B (180 mg) and Carbosieve S-III (100 mg) from Supelco (Bellefonte, PA, USA) separated by plugs of quartz wool. Prior to use, they were conditioned for 10 min at 350°C with a stream of purified helium. This sampling system has been checked for hydrophobicity and stability for the compounds of interest (Peñuelas and Llusia, 1999a). Sampled tubes were stored at 4 ºC in the field, and at -20 ºC in the lab prior to analysis with GC-MS.

2.4 GC-MS analysis of volatile isoprenoids

2.4.1 L. esculentum, M. pruriens and Eucalyptus spp.

For these species, GC-MS analysis was performed using a Perkin-Elmer AutoSystem XL gas chromatograph, with helium carrier gas at 1 mL min^{-1}, coupled to a TurboMass Gold quadrupole-type mass selective detector, with transfer line temperature 250 ºC, ionization potential 70 eV and a scan range of 40 to 250 amu. The sample tubes were desorbed using an automatic Perkin-Elmer Turbomatrix thermal desorption unit. Compounds were desorbed from the sample tube held at 280 ºC to the cold trap at -20

ºC for 6 min. Secondary desorption to the Ultra-2 GC column was by flash-heating of the cold trap to 300 ºC, which was sustained for 5 mins. The temperature profile for separating volatile isoprenoids was 40 ºC for 2 min, rising to 165 ºC at 4 ºC min^{-1}, then to 300 ºC at 45 ºC min^{-1}, which was held for 10 min. Ions 67 and 93 were used for quantification of isoprene and monoterpenes, respectively, which was carried out by comparison with commercial standard compounds (Sigma Aldrich, Linde UK), or by the contribution of ion 93 to total ion count for compounds where no standard was available. Identification was by comparison with commercial standard compounds, and by reference to the MS libraries (Wiley and NIST). Standards were analysed before every 6 samples for quality assurance and quantification.

2.4.2 Q. ilex

For *Q. ilex*, monoterpene analyses were conducted using a GC-MS (Hewlett Packard HP59822B, Palo Alto, USA). Sampled monoterpenes were desorbed from the tubes using an OPTIC3 injector system (ATAS GL International). The injector program started at 45 ºC, rising to 300 ºC at 5 ºC sec^{-1}. The transfer flow was 0.7 mL min^{-1}, and the split flow after 60 s transfer time was 20 mL min^{-1}. Desorbed samples were passed to a pre-column cold trap at -20 ºC held for 200 s before heating at 50 ºC min^{-1} to inject compounds into a 30 m x 0.25 mm x 0.25 mm film thickness capillary column (Supelco HP-5, Crosslinked 5% pH Me Silicone). After sample injection, the initial temperature of 45°C was increased to 60°C at 4°C min^{-1}, and thereafter up to 150°C at 10°C min^{-1}, followed by a final increase to 270°C at 40 °C min^{-1}; this temperature was maintained for 5 min. Helium flow (carrier gas) was 0.7 mL min^{-1}. The identification of monoterpenes was confirmed by comparison with standards from Fluka (Chemie AG, Buchs, Switzerland) and literature spectra. Frequent calibration was performed with the most common terpene standards (α-pinene, β-pinene, limonene) for every three analyses, and the responses of the standards were used for quantification based on the abundance of ion fragments m/V 93 and 67. The efficacy of this analytical system has been determined previously (Peñuelas and Llusia, 1999a). Emission rate calculations were made on mass balance basis and by subtracting the control samples without leaves from the samples with twigs. Monoterpene emission rates were expressed on leaf dry matter basis (mg g^{-1} h^{-1}).

2.5 DMAPP analyses

Analyses were performed in triplicate for each leaf sample using the method of Ficher *et al*. (2001). This method retrieves only 5% of total tissue DMAPP (Fisher *et al*., 2001;

Loreto *et al*., 2004), but it was used for all samples in the same way, and thus results were sufficient for correlations and comparing treatments as done in this study. Further, the method has been used by several studies in recent years (e.g. Bruggemann and Schnitzler, 2002; Rosenstiel *et al*, 2002; Wolfertz *et al*, 2003; Loreto *et al*, 2004; Nogues *et al*, 2006; Rasulov *et al*, 2009). The frozen leaf was ground to a powder with liquid nitrogen using a pestle and mortar, and 65 mg aliquots were weighed into 5 mL glass vials, which were kept at <4 ºC in ice. To each aliquot of ground frozen leaf tissue, 600 µL of distilled water was added followed by 600 µL of 8 M H_2SO_4. Each vial was then capped immediately with a screw top with a Teflon lined septum, and shaken. The vials were placed in an incubator at 30 ºC for one hour to allow hydrolysis of DMAPP to isoprene. After incubation, vials were removed from the incubator and placed immediately in a vial holder at 4 ºC in ice. A 1 mL headspace sample, containing the isoprene derived from the acid hydrolysis, was withdrawn and injected into a Perkin-Elmer sample tube (described above) in a flow of helium at ~150 mL min^{-1}. To quantify the DMAPP concentration in samples, standard DMAPP (prepared by J. Schnitzler, IMK-IFU, Garmisch-Partenkirchen), gave a response factor of 9.52 nmol isoprene from hydrolysis of 1 µmol DMAPP. Headspace samples were stored refrigerated until GC-MS analysis (described above).

2.6 Carotenoid analyses

The methods described by Lichtenthaler (1987) and Wellburn (1994) were used for the determination of carotenoid concentration. Work was carried out in low illumination to avoid photoreaction of the extracted pigments. About 40 mg frozen powdered leaf was weighed into glass centrifuge tubes, using a cold spatula. Ten mL 80% acetone was added, with vigorous shaking. The leaf material and solvent were then centrifuged for 13 mins at 4600 rpm to extract carotenoids and chlorophylls. At the end of centrifugation, the supernatant was decanted into centrifuge tubes held at 4 ºC in ice, which were then capped to avoid evaporation of solvent. A further 10 mL 80% acetone was added to each pellet, mixed well, and centrifuged for 13 mins as before. Absorption measurements were made at 470, 646 and 663 nm (CEAL CE 1010 spectrophotometer), with blank measurements, using 80% acetone alone, for each wavelength. The measurements at each wavelength were used in the following equations to calculate concentration of total carotenoids (Ctot) in each sample:

$$(Ctot) = \frac{(1000 \times A_{470}) - (1.82 \times C_a) - (85.02 \times C_b)}{} \quad (1)$$

where A_{470} = absorbance reading at 470 nm, and

$$C_a = 12.25\ A_{663} - 2.79\ A_{646} \quad (2)$$

$$C_b = 21.50\ A_{545} - 5.20\ A_{663} \quad (3)$$

Where A_{663} = absorbance reading at 663 nm, A_{545} = absorbance reading at 545 nm, and A_{646} = absorbance reading at 646 nm.

2.7 Statistical analyses

Statistical analyses were performed using Statistica 6 (StatSoft Inc). One-way analysis of variance was used to investigate the effect of ozone and drought treatments on volatile isoprenoid emission potentials, carotenoid and DMAPP concentrations for the *Eucalyptus* species. Pearson Product Moment Correlation was performed to investigate relationships between volatile isoprenoid emission potentials, carotenoid and DMAPP concentrations, and photosynthesis rates. Linear regression analysis was performed to determine trend lines between different variables.

3. Results

3.1 Isoprenoid compounds emitted by each species

L. esculentum emitted up to 0.6 μg g^{-1} h^{-1} of total monoterpenes, the major component being limonene. *M. pruriens* emitted isoprene at rates between 0.1 and 20 μg g^{-1} h^{-1}. *Q. ilex* emitted light dependent monoterpenes, whose total ranged between 3 and 49 μg g^{-1} h^{-1} . The major emitted compounds from *Q. ilex* were α-pinene, limonene and β-pinene. The two species of *Eucalyptus* emitted isoprenoids, but at different rates, and with different emission compositions (Table II). The minimum and maximum isoprene emission potentials for *E. globulus* and *E. gunnii* were 4 and37 μg g^{-1} h^{-1}, and 20 and 41 μg g^{-1} h^{-1} respectively (Table II). *E. globulus* emitted total monoterpenes at minimum and maximum rates of 17 and 185 μg g^{-1} h^{-1} , of which cineole was the major component. *E. gunnii* emitted total monoterpenes at mean rates between 0.04 and 1 μg g^{-1} h^{-1} , the major component of which was cis-ocimene (Table II).

3.2 The relationship between isoprenoids and photosynthesis rates

For *Eucalyptus spp.* and *Q. ilex*, the slope of the regressions between isoprene emission potentials, carotenoid concentrations and DMAPP concentrations, and photosynthesis rates were significantly greater than zero (*E.globulus* $P<0.00001$, Figure 2A; *E. gunnii* $P<0.02$ Figure 2B; *Q. ilex* $P<0.05$, Figure 2C). The positive correlations between carotenoid concentrations and photosynthesis rates were also significant for the two *Eucalyptus spp.* and *Q. ilex*, as were the negative correlations between DMAPP concentrations and photosynthesis rates for these species. Data for *M. pruriens* showed similar trends but there was no significance, perhaps due to insufficient data (Figure 2D). Emissions of stored monoterpenes were non-significantly negatively correlated with photosynthesis rates for *E. globulus*, *E. gunnii* and *L. esculentum* (data not shown).

3.3 Effect of ozone treatment on isoprene emissions, carotenoids and DMAPP concentrations in Eucalyptus spp.

Emissions of isoprene and monoterpenes declined for ozone fumigated and control plants as the experiment progressed for both species of *Eucalyptus* (Figure 3). After four weeks of fumigation with ozone at ~60 ppb above ambient, isoprene emissions from *E. globulus* were significantly higher than emissions from non-fumigated (control) plants (Figure 3A; 0.46±0.05 and 0.23±0.03 $\mu g\ m^{-2}\ s^{-1}$, respectively, $P<0.001$, n=6 replicates x 3 sequential measurements), and total monoterpene emissions were significantly lower than those from control plants (Figures 3C; 4.11±0.38 and 9.86±1.19 $\mu g\ m^{-2}\ s^{-1}$, respectively; $P<0.001$, n=6 replicates x 3 sequential measurements). However, there was no significant difference in isoprenoid emissions between treatment and control plants after four weeks of ozone fumigation of *E. gunnii* (Figures 3B, 3D). There was no significant difference in isoprenoid emissions between control and fumigated plants after 20 and seven weeks of ozone fumigation of *E. globulus* and *E. gunnii*, respectively (Figures 3A, 3B, 3C, 3D).

Concentrations of carotenoids in both species of *Eucalyptus* decreased significantly ($P<0.01$) from the pre-ozone sampling at week 0 to the final sampling after ozone fumigation at weeks 20 and 7, for *E. globulus* and *E. gunnii*, respectively, but there was no significant difference between ozone fumigated plants and controls (Figures 3E, 3F). DMAPP concentrations increased significantly ($P<0.01$) from week 0 to the final sampling date, but again there was no significant difference between ozone fumigated plants and controls (Figures 3G, 3H).

3.4 Effect of drought treatment on isoprenoid emissions, and on carotenoids and DMAPP concentrations.

Water was withheld from treatment plants for 1 week until the mean soil water potential was 30% and 20% lower than control soil water content for *E. globulus* and *E. gunnii*, respectively. No significant differences were found for carotenoid and DMAPP concentrations between treated and control plants. Emission rates of total monoterpenes were also unaffected by drought, probably because the drought was not severe enough. However, isoprene emission rates from *E. gunni* were significantly lower in droughted plants ($P<0.02$, n=3 plant replicates x 3 sequential measurements), but were not affected by drought in *E. globulus* (data not shown). Isoprenoid emissions, carotenoid and DMAPP concentrations were not significantly afected by combined ozone and drought stress (data not shown).

3.5 The relationship between isoprenoid emissions and carotenoids.

There were positive correlations between isoprenoid emission potentials and carotenoid concentrations for isoprene emissions from *M. Pruriens, E. globulus* ($P<0.05$) and *E. gunnii* ($P<0.05$), and for light-dependent monoterpene emissions from *Q. ilex* ($P<0.05$; Figure 4)In contrast, the relationships between the stored monoterpene emission potentials and carotenoid concentrations were negative (data not shown), with non-significant regression coefficient for *L. Esculentum, E. Globulus* and *E. gunnii*.

3.6 The relationship between isoprenoid emissions, carotenoid concentrations and DMAPP concentrations.

There was a negative correlation between isoprene emission potentials and DMAPP concentrations for both *M. Pruriens* (n.s.) and *Eucalyptus spp.* ($P<0.05$) (Figures 5A, 5B, 5C). Carotenoid concentrations were also significantly negatively correlated with DMAPP concentrations for *M. pruriens* and the *Eucalyptus spp.* ($P<0.05$; Figures 5D, 5E, 5F). Correlations between emission potentials of stored monoterpenes from *L. esculentum* and the *Eucalyptus* species were positive, but not significant (data not shown).

4. Discussion

Generally, our results show a strong positive relationship between instantaneously emitted volatile isoprenoid emission potential and carotenoid concentration in the studied species. Instantaneously emitted isoprenoid emissions were negatively correlated with DMAPP concentrations. In contrast, stored monoterpene emission potentials were negatively correlated with carotenoid concentrations, and positively correlated with DMAPP concentrations. DMAPP concentrations were non-significantly negatively correlated with photosynthesis rates at this time scale. These results support the possibility of a direct or indirect control of volatile isoprenoid emission potential via carotenoid synthesis at time scales of days to weeks.

Porcar-Castell *et al*. (2009) also showed a significant positive correlation between monoterpene emission potential and carotenoid content of *Q. ilex* subject to sun and shade treatments over a period of weeks. Examination of isoprene emission potentials and carotenoid concentration data from a study of the effect of ozone and elevated CO_2 on isoprene emissions from *Populus tremuloides* (Calfapietra *et al.*, 2008) showed a positive correlation at a time-scale of two weeks. In the data presented here, both carotenoid concentrations and isoprenoid emission potentials were positively correlated with photosynthesis rates, significantly so for *E. globulus, E. gunnii* and *Q. ilex*. This suggests that these isoprenoid compounds depend upon substrate supply over a time scale of a few weeks, and does not exclude the possibility of an indirect dependency on photosynthesis rate for volatile isoprenoids via the carotenoid demand. The slight but consistent negative correlation in all species between DMAPP concentrations and photosynthesis rates suggests that at time scales of weeks to months, there is higher turnover with higher demand on the DMAPP pools when photosynthesis rates are higher.

Concentrations of carotenoids were significantly negatively correlated with DMAPP concentrations for the two *Eucalyptus spp.* and *M. pruriens*. Isoprene emission potentials from the *Eucalyptus* species also showed significant negative relationships with DMAPP concentrations ($P<0.005$). This has also been shown for *Populus alba* and *Q. ilex* over time scales of weeks (Nogués *et al*., 2006), but Magel *et al*. (2006) found a non-significant positive correlation between isoprene emission rates and DMAPP content of *Populus canescens* over a shorter time scale of 24 hours. Rosenstiel *et al*. (2002) studied concentrations of DMAPP in dawn and midday leaf samples of *Populus deltoides*. They found that isoprene emitting species tended to have higher DMAPP

concentrations, which also showed diurnal variation similar to a typical diurnal isoprene emission trend. This suggests a positive relationship between DMAPP concentrations and isoprene emission rates. However, these authors did not measure isoprene emission rates, and did not compare magnitude of isoprene emission potential with DMAPP concentration within and between plant species. Loreto *et al*. (2004) studied ^{13}C labelling of DMAPP and isoprene emissions in *Phragmites australis* and *Populus nigra*. Generally, the ^{13}C label was taken up much more by isoprene than by DMAPP in both species, reflecting a chloroplastic and cytosolic pool for DMAPP. DMAPP concentrations and isoprene emissions were higher in old leaves than young leaves of *P australis,* suggesting a positive linear relationship between isoprene emissions and DMAPP concentrations. This is also contrary to the findings presented here. However, Loreto *et al.* (2004) did not follow the changing concentrations and emissions with time (as presented here), and in fact found a negative linear relationship between isoprene emissions and DMAPP concentrations in mature leaves of *P. nigra* untreated, and treated with fosmidomycin,

Carotenoids and isoprene are derived from the MEP pathway that operates in the chloroplasts, and their instantaneous production rate (assuming that all enzymes are in an activated state) should therefore be directly dependent on carbon dioxide fixation rate. However, biotic and abiotic conditions existing at the time of reference will cause variations in isoprene synthase activity, and hence actual emission rate, within these constraints. We assume that (1) an increase in need for carotenoids in the leaf will increase carotenoid synthesis rate, which results in at least a corresponding increase in DMAPP synthesis rate, (2) DMAPP synthesis rate is greater than the sum of essential isoprenoid synthesis rates making demands on the DMAPP pool. There is little information on the relative availability of DMAPP for synthesising different isoprenoid compounds, but these assumptions seem reasonable, otherwise the plant would be in danger of not producing adequate DMAPP for essential needs. A further assumption is that (3) the emission potential for isoprene at any time is often substrate limited (e.g. Magel *et al*., 2006), and this is supported by the very high Km for isoprene synthase (up to 9 mM; Datukishvili *et al*., 2001). These assumptions are supported by the results presented here, which show a direct relationship between carotenoid concentrations and instantaneously emitted volatile isoprenoid emission potential.

Stored monoterpene emission potentials from *Eucalyptus spp.* and *L. esculentum* were inversely correlated with carotenoids over a time scale of a few weeks (not

significant; data not shown). This could indicate competition for precursors (direct or indirect), or a common function in the plant tissue requiring either compound. At this stage of the isoprenoid biosynthesis pathway there are several routes to carotenoid production, and different controls operate to ensure maximum production rate of carotenoids when the need arises. Because monoterpenes and sesquiterpenes are usually stored in pools within leaf tissue, synthesis rates are not necessarily reflected by their rates of emissions and so the relationship between stored monoterpene and sesquiterpene emission rates and carotenoid pool sizes is difficult to predict.

Isoprene emission potentials for *E. globulus* were similar in magnitude to isoprene emissions reported for other *Eucalyptus spp.* (He *et al*., 2000; Street *et al*., 1997b). Monoterpene emission rates from *E. globulus* were very high, up to an order of magnitude greater than monoterpene emission rates reported by He *et al*. (2000) and Street *et al*. (1997b) for this species. There are no existing published reports of monoterpene emission rates from *E. gunnii*, but our results are similar to monoterpene emission rates reported for other *Eucalyptus spp.* (He *et al*., 2000). The reason for the extraordinarily high monoterpene emission rates observed from *E. globulus* might have been due to the infestation of *A. solani*.

Emission potentials measured from the other study species were comparable to published values. Winer *et al.* (1992) found rather high emissions of monoterpenes from tomatoes (12 - 30 $\mu g\ g^{-1}\ h^{-1}$), but it is possible these could have been caused by damage to the leaf during sampling. Emissions from *M. pruriens* were of the same order of magnitude as isoprene emissions reported from this species by Harley *et al.* (1996a) whose lower estimate is ~5 nmol $m^{-2}\ s^{-1}$, equivalent to 22 $\mu g\ g^{-1}\ h^{-1}$. The range of emission potentials measured from *Q. ilex* agrees with the speciation and range of emission rates from this species reported by Owen *et al*., (1997), Kesselmeier *et al*. (1996), and Peñuelas and Llusia (1999b).

The effect of the ozone and water stress conditions on emission potentials and carotenoid concentrations in the *Eucalyptus spp.* were not as great as the effect of time. It is possible that the stresses were not severe enough to result in large changes in these variables.

5. Conclusions and final remarks

Although literature shows that there can be a high intraspecific variability in enzyme activities and precursor concentrations in the isoprenoid pathway, with differences up to

a factor of 7 between different plants of the same species grown under the same conditions (Lehning *et al*., 1999), we show, remarkably, that instantaneously emitted volatile isoprenoid emission potentials were positively correlated with carotenoid pool size for different plant taxa subject to diverse biotic and abiotic stresses over a time period of weeks. Carotenoid pool size and instantaneously emitted volatile isoprenoid emission potentials decreased with time. Stored volatile isoprenoid emission potentials were negatively correlated with carotenoid pool size in three different plant taxa. In this case, carotenoid pool size decreased and stored volatile isoprenoid emission potentials increased over time.

DMAPP pools increased with time over timescales of a few weeks, and were inversely correlated with carotenoid pool size and instantaneously emitted volatile isoprenoid emission potentials. Stored monoterpene emission potentials from *L. esculentum* and the two *Eucalyptus spp.* were related in a different way to carotenoid pools than instantaneously emitted monoterpene emissions from *Q. ilex*. We therefore suggest that a synthase with high Km similar to isoprene synthase might exist for production of instantaneously emitted monoterpenes. Indeed, Andres-Montaner (2008) found three different monoterpene synthases extracted from *Q. ilex* tissue, with Km values ranging from 138 – 270 μmol, which are far higher values than previously found for monoterpene synthases.

In a review of isoprenoid synthesis, accumulation and emissions, Lichtenthaler (2007) summarised that, "depending on the light and temperature conditions, enormous amounts of freshly fixed photosynthetic carbon flow into various volatile and non-volatile isoprenoid compounds. Thus, the chloroplast isoprenoid biosynthesis via the IPP forming pathway appears to be a 'metabolic valve' for regulating photosynthetic carbon flow as well as a fine tuning for chloroplast and cell metabolism. This chloroplast isoprenoid pathway consumes large amounts of photosynthetically formed ATP and NADPH, and may also serve as a 'safety valve' in order to avoid overreduction and photoinhibition of the photosynthetic apparatus.". Our data presented here from laboratory and field experiments show that in this biochemical complexity, magnitude of light-dependent volatile isoprenoid emission potential is directly correlated with magnitude of carotenoid pool size at time scales of weeks to months. These findings go beyond supporting the metabolic safety valve theory, and support the opportunist hypothesis of volatile isoprenoid emissions (Owen and Peñuelas, 2005). It is worth extending these studies to other emitting species in different field and laboratory

conditions, especially to investigate the poperties of light-dependent monoterpene synthase enzymes. A rigorous modelling treatment would be enlightening, similar to that of Zimmer *et al*. (2000) which used process-based biochemistry and enzyme kinetics for modelling isoprene emissions alone. The Opportunist Hypothesis also merits further physiological and biochemical investigations to evaluate its limitations, ramifications and scope.

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Figure legends

Figure 1 The isoprenoid biosynthetic pathway

Figure 2 The dependencies of instantaneous emission rates of isoprene and monoterpenes and essential isoprenoids on photosynthesis rate in experiments conducted over time scales of weeks in *Eucalyptus globulus* (A), *Eucalyptus gunnii* (B), *Quercus ilex* (C) and *Mucuna pruriens* (D).

Figure 3 Changes in volatile isoprenoid emissions, carotenoid and DMAPP concentrations in *Eucalyptus* spp. with time; for all weeks, measurements were made for "control" plants and "treatment" plants; * significant difference ($P<0.001$) between control and ozone-treatment; different letters indicate significant difference between time points (control and ozone treatment considered together); n=6 for weeks 0 and 4, n=3 for weeks 7 and 20

Figure 4 Relationship between instananeously emitted isoprene and monoterpene emission potentials, and carotenoid content in *Eucalyptus globulus* (A), *Eucalyptus gunnii* (B), *Mucuna pruriens* (C) and *Quercus ilex* (D).. Closed symbols in A, B and C are means of each sampling date.

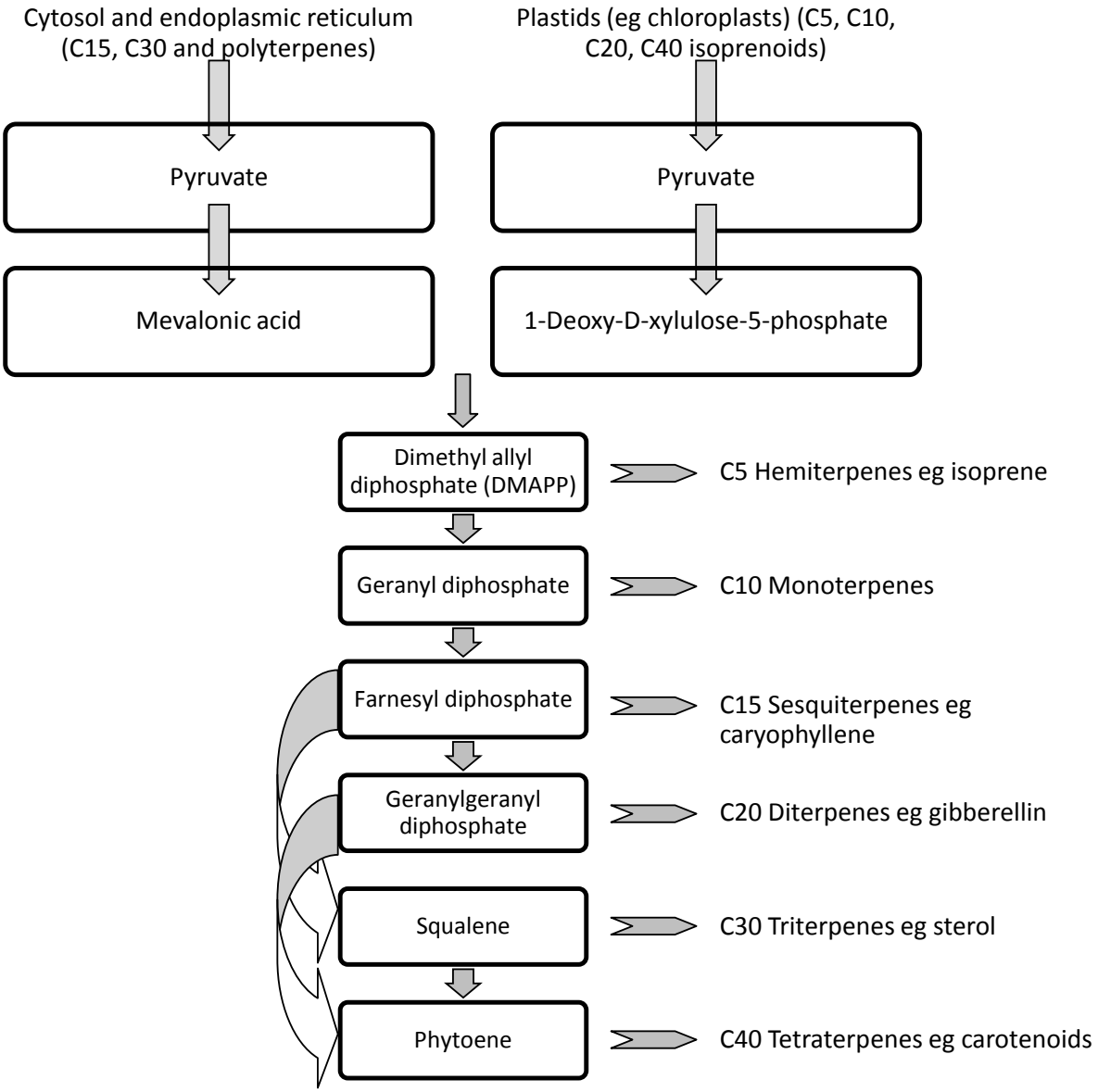
Figure 5 Relationship between instananeously emitted isoprene and monoterpene emission potentials, and DMAPP content in *Eucalyptus globulus* (A,D), *Eucalyptus gunnii* (B,E), and *Mucuna pruriens* (C,F). Closed symbols are mean of each sampling date.

1 Table I (Adapted from Owen, Hewitt and Rowland 2013)
2 Some responses of bVOC emissions from vegetation in response to stresses

Management practice/Stress	Isoprene/instantaneously emitted terpenes	Monoterpenes from stored tissue pools	Sesquiterpenes	Oxygenated compounds	Photosynthesis
Fertiliser	↑4, 5 no change 6	↓1 ↑2, 4	↑3, 4	↑ 13(soil)	↑50, 51
Irrigation	↑21	↑ 21	↑ 27; ↓ 27(depends on plant species)	↑25; no change 25 (depends on compound)	↑ 51, waterlogging ↓ 52
Cropping, felling, pruning or mowing	↑26	↑9	↑28	↑7, 8	No effect-f(pre-pruning light regime) 53, 54; grazing ↓ 55
Managed for young plant growth	↓12	↑10	No change 32; ↓33 (depends on species)	↑ 34; ↓34 (depends on compound and species)	↑ 56
Managed to encourage establishment of mature plants	↑12	↑10, 11	No change 32; ↑33 (depends on species)	↓ 34; ↑34 (depends on compound and species)	↓ 56
Drought/dessication stress	No change 14; ↓15, 16, 17, 18, 19, 22;	↓20, 23, 31; ↑ 31 (depends on severity of stress)	↓24; no change 31	↓ 25; no change 25 (depends on compound)	↓ 57
Herbivory stress in plantations	↑ short-term 35; ↓ long-term 35	↑29, 30	↑29, 30	↑29	↓ 58
Over-crowding/shading	↓ due to shading 37;	↓due to low light intensity 38, 39; no change 39 (depends on compound and whether from stored pools)	↓due to low light intensity 40	↓due to low light intensity 41	↓ 46
High light intensity	↑42	↑ if light-dependent, up to a saturated max. 47	↑ if light dependent 47	No effect? 50	↑ up to a saturated max. 46 ↓ beyond saturation 60
High temperature	↑43 (up to a species specific max~35º), then ↓43	↑46, 47	↑47	↑7	↓ above optimum 61
Exposure to ozone	↓44 due to degradation in atmosphere; ↑ in intersticial tissue spaces 44, ↑as induced response 59	Variable, but overall ↑45; no effect or ↑ 62	↑48	↑49	Variable, but overall ↑45

1 Blanch et al. 2007; 2 Blanch et al. (2012); 3 Rinnan et al. (2011); 4 Ormeno et al. (2009); 5 Possell et al. (2004); 6 Funk et al. (2006); 7 , Seco et al. (2007); 8 Davison et al, 2008; 9 Raisanen et al. (2008); 10 Kim et al. (2005); 11 Street et al. (1997a); 12 Street et al. (1997b); 13 Hörtnagl et al. (2011) ; 14 Steinbrecher et al. 1997; 15 Tingey et al.1981, 16 Sharkey and Loreto 1993, 17 Fang et al. (1996),18 Lerdau et al. (1997); 19 Brilli et al. (2007); 20 Lavoir et al. (2009); 21 Peñuelas et al. (2009); 22 Pegoraro et al. (2004); 23 Bertin & Staudt.(1996); 24 Ormeno et al. (2007); 25 Filella et at. (2009); 26 Brilli et al. (2011); 27 Lluisa et al. (1998); 28 Piesik et al. (2011); 29 Schaub et al. (2010); 30 Staudt and Lhoutellier (2007); 31 Staudt et al. (2008); 32 Agelopoulos et al. (2000); 33 Hakola et al. (2001); 34 Bracho-Nunes (2011); 35 Loreto et al. (2006); 36 Brilli et al. (2009); 37 Harley et al. (1996b); 38 Owen et al. (2002); 39 Tarvainen et al. (2005); 40 Staudt and Lhoutellier (2011); 41 Folkers et al. (2008); 42 Behnke et al. (2010); 43 Singsaas et al. (2000); 44 Yuan et al. (2009); 45 Llusia et al. (2002);46 Guenther et al (1995); 47 Vickers et al. (2009); 48 Bourtsoukidis et al. (2012); 49 Pellegrini et al. (2012); 50 Efthimiadou et al. (2010); 51 Murchie et al. (2009); 52 Dreyer et al. (1991); 53 Forrester et al. (2012); 54 Li et al. (2004); 55 Smetham (1995); 56 Bond (2000); 57 Chaves et al. (2003); 58 Zangerl et al. (2002); 59 Pinto et al.(2010); 60 Demmig-Adams et al. (2012); 61 Haldimann & Feller (2004); 62 Penuelas et al. (1999)

Table II Range of isoprenoid emission rates from the studied species										
	Emission potential (μg g^{-1} h^{-1})									
	E. globulus		*E. gunnii*		*L. esculentum*		*M. pruriens*		*Q. ilex*	
number of samples	12		12		8		8		24	
	max	min	max	min	max	min	max	min	max	min
isoprene	37.02	3.84	40.84	19.70			20.25	0.11		
unknown 1	0.17	0.01	0.00	0.00						
α-pinene	27.31	2.49	0.14	0.00	0.13	0.00			20.56	0.15
sabinene	0.20	0.01	0.00	0.00					6.17	0.00
β-pinene	1.18	0.10	0.00	0.00					14.78	0.69
myrcene	3.10	0.26	0.04	0.00					1.26	-0.02
a-phellandrene	0.36	0.03	0.00	0.00						
α-terpinene	0.22	0.00	0.00	0.00						
Δ-3-carene									0.69	-0.01
limonene	22.91	1.62	0.20	0.00	0.43	0.00			16.20	-0.03
cineole	133.41	11.47	0.36	0.00						
cis-ocimene	13.88	0.00	0.84	0.00						
γ-terpinene	2.15	0.07	0.00	0.00						
α-terpinolene	0.73	0.03	0.45	0.00						
α-terpineol	1.52	0.03	0.00	0.00						
α-longipene	0.86	0.26								
junipene	11.00	7.20	-	-	-	-	-	-	-	-
trans-caryophyllene	2.73	1.43	-	-	-	-	-	-	-	-
aromadendrene	0.77	0.14	-	-	-	-	-	-	-	-
alpha humulene	1.69	0.68	-	-	-	-	-	-	-	-
unknown 2	6.40	0.00	-	-	-	-	-	-	-	-
TOTAL monoterpenes	**184.72**	**17.07**	**0.97**	**0.04**	**0.56**	**0.00**	**0.00**	**0.00**	**49.31**	**3.01**



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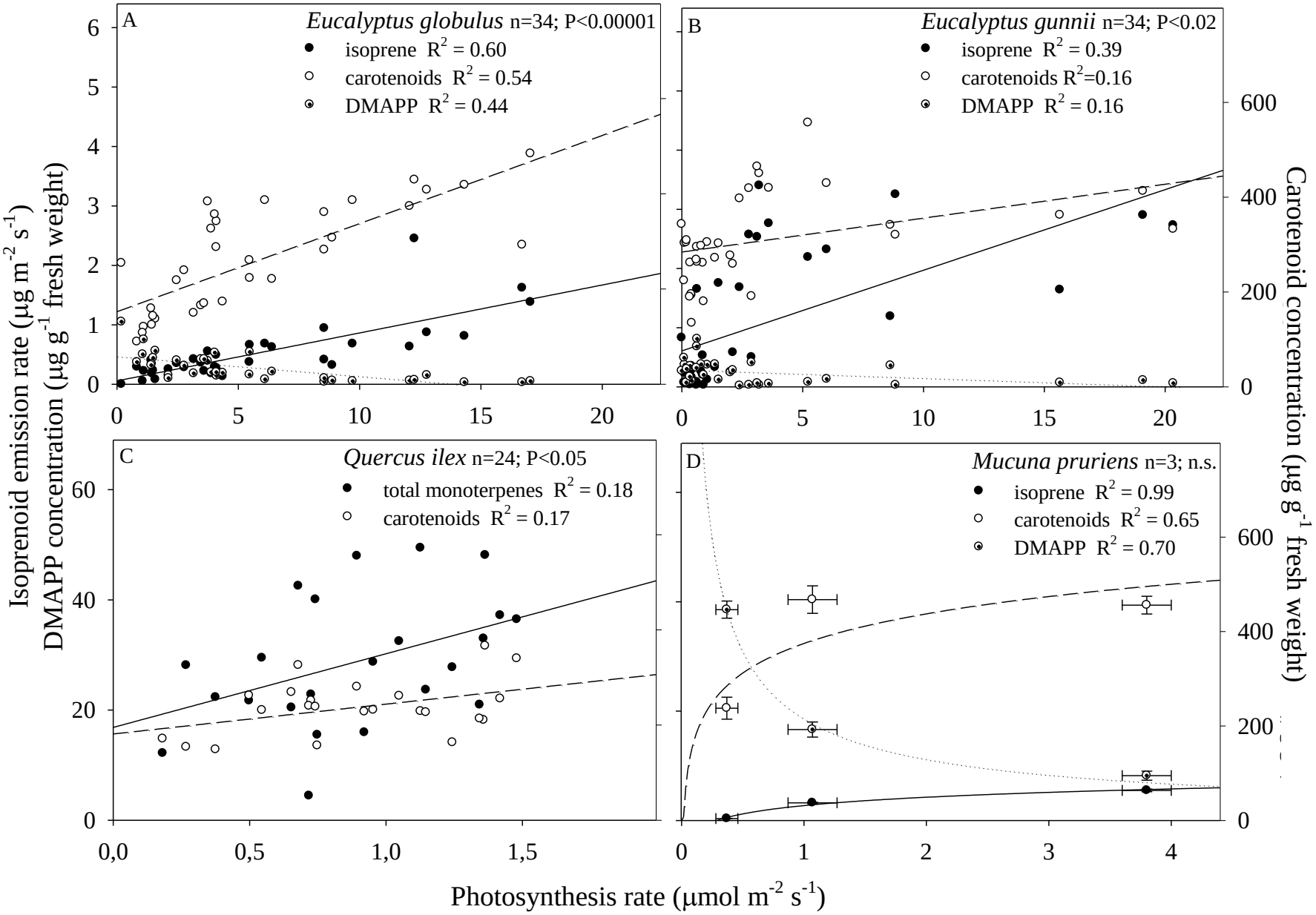

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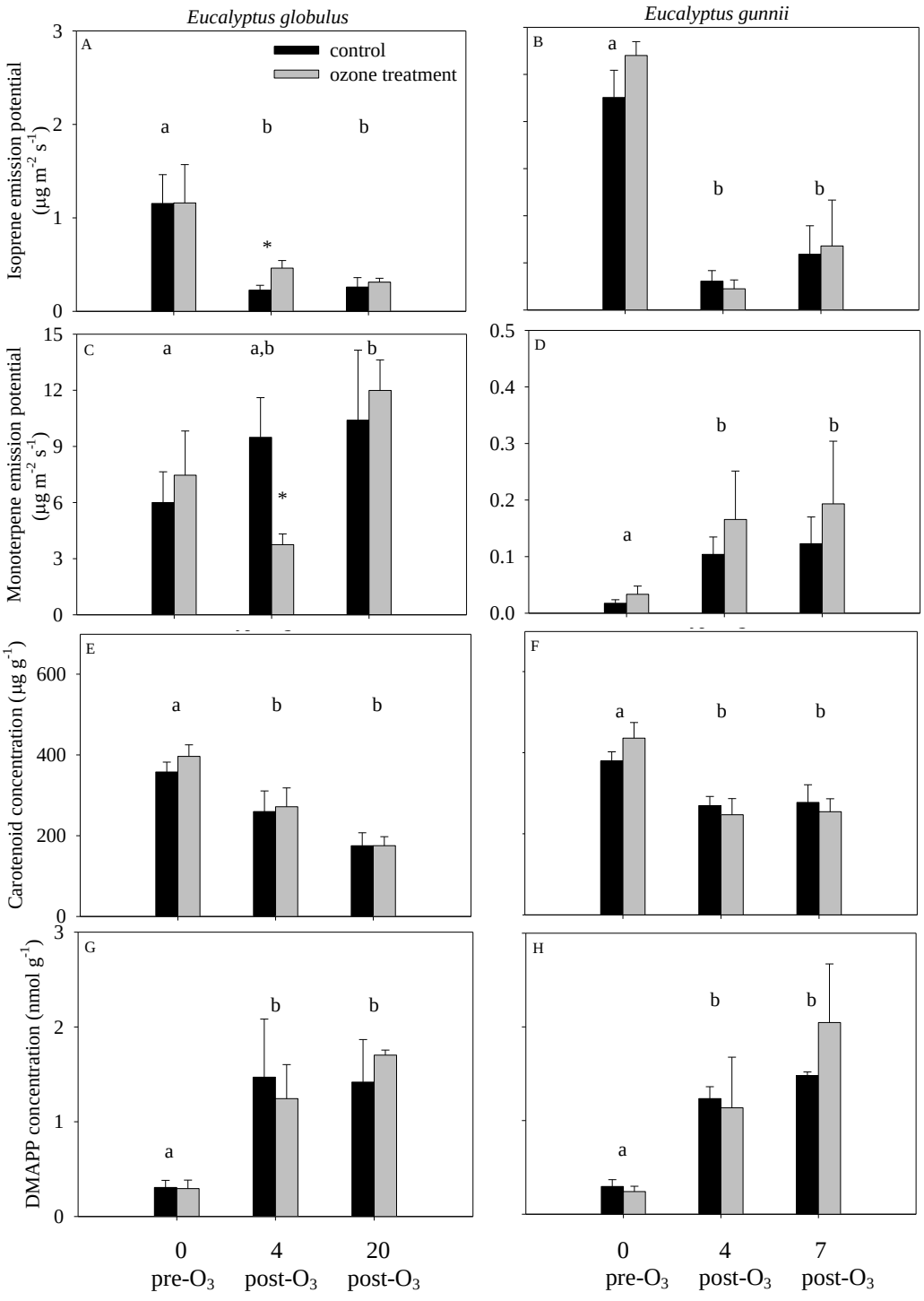

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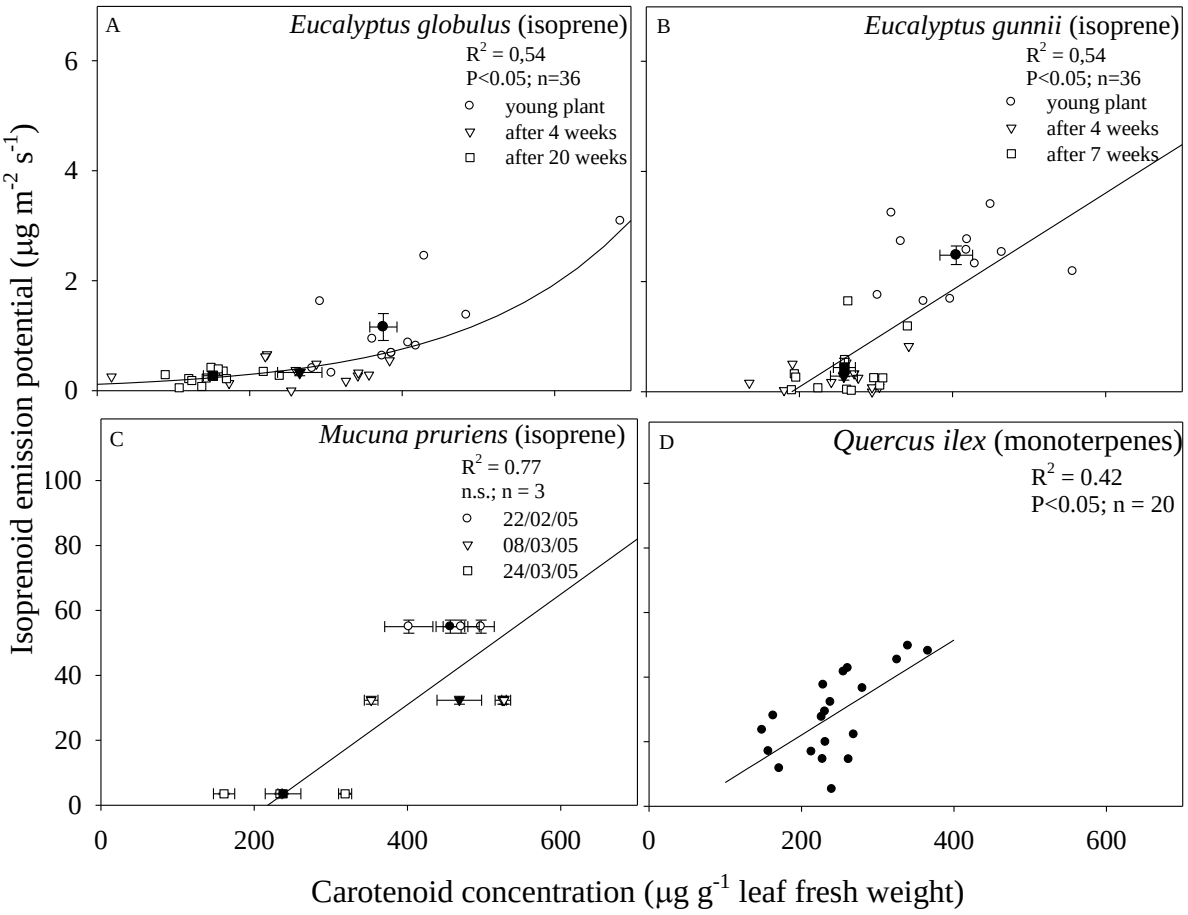

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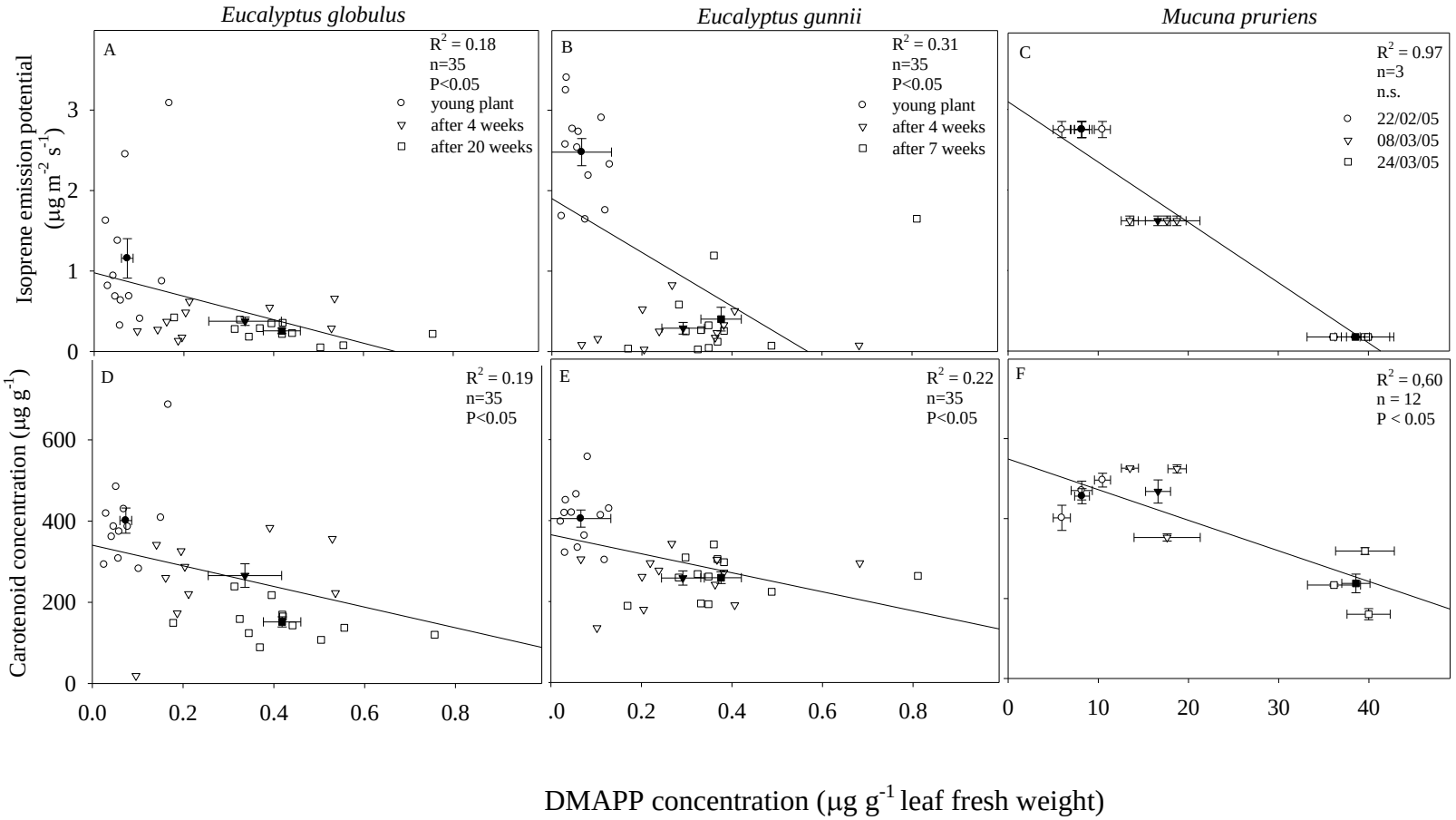

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