Volatile organic compounds in the roots and rhizosphere of *Pinus* spp.

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**Abstract**

Plant roots normally release a complex mixture of chemicals which have important effects in the rhizosphere. Among these different root-emitted compounds, volatile isoprenoids have received very little attention, yet they may play important and diverse roles in the rhizosphere, contributing to the regulation of microbial activity and nutrient availability. It is therefore important to estimate their abundance in the rhizosphere, but so far, there is no reliable sampling method that can be used to measure realistic rates of root emissions from plants growing in field conditions, or even in pots. Here, we measured root content of volatile isoprenoids (specifically monoterpenes) for *Pinus pinea*, and explored the feasibility of using a dynamic bag enclosure method to measure emissions from roots of intact pot-grown plants with different degrees of root cleaning. We also investigated a passive diffusion method for exploring monoterpenes in soil at incremental distances from mature *Pinus sylvestris* trees growing in field conditions. Total monoterpene content of *P. pinea* roots was 415±50 μg g⁻¹ fresh wt in an initial screening study, and between 688±103 and 1144±208 μg g⁻¹ dry wt in subsequent investigations. Emissions from shaken-clean roots of intact plants and roots of intact plants washed to remove remaining soil after shaken-clean experiments were 119±7 and 26±5 μg g⁻¹ dry wt h⁻¹, respectively. Emissions from intact roots in soil-balls were an order of magnitude lower than from shaken-clean roots, and probably reflected the amount of emitted compounds taken up by physical, chemical or biological processes in the soil matrix surrounding the roots. Although monoterpene content was not significantly different in droughted roots, emission rates from droughted roots were generally significantly lower than from well-watered roots. Finally, passive sampling of monoterpenes in the soil at different distances from mature *P. sylvestris* trees in field conditions showed significantly decreasing sampling rates with increasing distance from the trunk. We conclude that it is feasible to measure volatile isoprenoid emissions from roots but the method of root preparation affects magnitude of measured emissions and therefore must be decided according to the application. We also conclude that the rhizosphere of *Pinus* species is a strong and previously un-characterized source of volatile isoprenoid emissions and these are likely to impact significantly on rhizosphere function.

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1. Introduction

Our understanding of the biology, biochemistry and genetic development of plant roots has been greatly improved during the last decades (Benfey and Scheres, 2000; Flores et al., 1999; Smith and Fedoroff, 1995). It is now known that roots, besides the classical functions of mechanical support and water/nutrient uptake, also perform some specialized roles, including synthesizing, accumulating and secreting a vast range of organic compounds to the rhizosphere around them, possibly accounting for 5–21% of all photosynthetically fixed carbon (Flores et al., 1999; Marschner, 1995). These exudates include amino acids, organic acids, sugars, phenolics and various other secondary plant metabolites (Walker et al., 2003). They play complex and important roles in the rhizosphere, such as regulating the soil microbial community in their immediate vicinity, providing a defence mechanism against herbivores, encouraging beneficial symbioses, changing the chemical and...
physical properties of the soil, and inhibiting the growth of competing plant species (Nardi et al., 2000). Some of these compounds have been well studied, but volatile isoprenoids in the rhizosphere have so far received little attention. Because volatile isoprenoids are reactive, and are likely to undergo rapid changes and transformations (physical, chemical and/or biological) in the soil system, a considerable proportion of rhizosphere sources of volatile isoprenoids may not diffuse through soil to the atmosphere.

In this study, we focus mainly on monoterpenes, which are volatile members of the large group of isoprenoids whose carbon skeletons are composed of characteristic C$_5$-units (McGarvey and Croteau, 1995). They have characteristic odours, they are hardly soluble in water, they are synthesized in foliage of many plant species, and they are also synthesized by microorganisms (Kesselmeier and Staudt, 1999). They can be utilized by some soil microflora as growth substrates (Vokou et al., 2002), and can act as co-metabolites for the degradation of persistent organic pollutants (Singer et al., 2003). In addition, there are reports of monoterpenes inhibiting soil nitrification and mineralization processes (White, 1991; Smolander et al., 2006). However, the source strength (i.e. potential abundance) and specific effects of monoterpenes in the rhizosphere environment are still not known, not least because sampling and quantifying exudation or emission of these compounds from roots is challenging. Meanwhile, due to the lack of reliable sampling, there have been few direct measurements of monoterpenes emissions or exudations from root systems in natural environments, or even from roots of plants growing in pots. Here, an initial screening exercise was carried out before the main investigation to test roots of a range of plants for monoterpane content. In particular, Pinus spp. were expected to have high root monoterpane content because their needles and resin contain high concentrations of these compounds (Peñuelas and Llusia, 1999; Iason et al., 2005).

Our aims were to (1) quantify monoterpane content of roots of the screened species to confirm that Pinus spp. are a good experimental subject for investigating monoterpenes in the rhizosphere, (2) measure monoterpane emission rates from roots of small pot-grown Pinus pinea plants subject to different preparation and watering treatments, testing a simple dynamic sampling system, and (3) estimate monoterpane content in rhizosphere soils of Pinus sylvestris in field conditions, testing a passive diffusion method.

2. Materials and methods

2.1. Plant materials and growth conditions

Some of the plant species used in this study were grown from seed (Table 1). They were sown in John Innes M3 potting compost in individual pots and incubated in a temperature controlled growth cabinet in the dark to encourage germination. After 7–10 days, seedlings were transferred to John Innes M3 compost in 150 mm (1.8 L) pots. Plants were sampled when they were between 12 and 20 weeks old. Other species were obtained as small nursery grown plants (Table 1). Young pine trees were ~50 cm tall, and plants of peppermint, sage, lavender, thyme and rosemary were about 10 cm tall. Plants obtained from nurseries were re-potted in Levingtons M3 compost (Fisons PLC, Ipswich, UK) in 1 L containers and allowed to acclimate for 1 week in the laboratory in well-watered conditions before measurements commenced. They were maintained under artificial illumination (14 h light at 250 µmol m$^{-2}$s$^{-1}$/10 h dark) with the plants illuminated between the hours of 06:00 and 20:00, at ambient temperatures of ~30°C day and ~20°C night.

2.2. Volatile isoprenoid extraction from roots

Three solvents (dichloromethane, diethyl ether and methanol) were used in a preliminary trial to test extraction efficiency for screening the roots for monoterpe content. Diethyl ether extracted the greatest amount of monoterpane compounds from root materials, but its volatility resulted in large variability in recovery. It is also a strong oxidant, which could lead to degradation and loss of extractable compounds. Therefore methanol, the second most efficient solvent, was used in subsequent experiments.

Eleven plant species were tested in the screening exercise, including one pine species (Pinus halepensis; Table 1). Plants were removed from pots and their roots washed thoroughly in water to remove the soil. The washed roots were cut into small pieces and placed in a mortar. The pieces were quickly submerged in liquid nitrogen and ground with a pestle to a coarse, frozen powder. Aliquots of 1 g were weighed into small glass vials with Teflon-lined caps, and 2 mL methanol was added. For some species, less root material was used due to the small size of their root systems, in which case the volumes of solvent were reduced accordingly, maintaining approximately the same root:solvent ratio.

The vials were sealed with Teflon-lined caps, and were incubated for 3 days on a heated shaker at 45°C. After incubation, 5 µL of the extracts were injected in a stream of helium onto pre-conditioned dual bed stainless steel sample tubes (Perkin Elmer, UK), packed with solid phase adsorbents Tenax TA (200 mg) and Carbosieve (100 mg) for thermal desorption and GC-MS analysis. Injection onto sample tubes was followed by thermal desorption, affording a technique comparable with subsequent emission sampling and analyses.

2.3. Volatile isoprenoid emissions from pine roots in soil-balls

Three young P. pinea plants were gently removed from their pots with the whole soil (soil-ball) retained around the roots. Needle litter was carefully removed from the soil surface without disturbing the root system. Each plant was
then fixed on a tripod and the whole root/soil-ball was enclosed in a Teflon bag supplied with a mass-flow controlled inflow of air at a rate of 1 L min\(^{-1}\) via a Teflon tube through a tightly fastened inlet port. Inflow air was filtered through activated charcoal to remove potential background volatile isoprenoid in the ambient air. The inlet air flow was started 60 min before actual sampling to allow sufficient time for the system to recover from installation and to reach emission equilibrium. This is a normal equilibration times for foliage after installation in branch enclosure systems (Owen et al., 1997; Street et al., 1997). The outlet of the bag was loosely fastened, which allowed inflow air to escape, maintaining a slight positive pressure within the bag, and also to allow insertion of sample tubes. After equilibration, sample tubes (described in Section 2.2) connected to mass flow controlled sampling pumps (SKC, UK) were inserted through the outlet opening, and samples of enclosure air were taken at 100 mL min\(^{-1}\). Two samples were taken simultaneously from each of three replicate plants for 10 min, giving six 1 L samples for each treatment.

Two pairs of thermocouples were also inserted into the bag through the inlet opening. Enclosure temperature was recorded every 30 s giving an average enclosure temperature of 24.8 ± 0.3 °C. After sampling, tubes were capped and stored at 4 °C prior to thermal desorption and GC-MS analysis, and the plants were gently re-potted for recovery for 24 h before subsequent measurements.

### 2.4. Volatile isoprenoid emissions from shaken-clean pine roots of intact plants

After recovery for 24 h following shaken-clean root emission sampling, the remaining soil and vermiculite on the roots of the three pine plants were removed by washing thoroughly in water. The clean roots were dried gently with laboratory tissue then enclosed in the sampling system and monoterpene emission samples were taken for GC-MS analysis. The average enclosure temperature was 25.7 ± 0.7 °C. Pine plants were repotted in watered vermiculite for 1 day to recover for measuring monoterpene content.

To investigate the possibility that emissions and content from washed-clean roots might be adversely affected by two previous preparations and emission measurements, roots of three further *P. pinea* plants, which had not been sampled previously in soil-balls, nor shaken-clean, were also washed, gently dried, and sampled for emissions for GC-MS analysis (1-day process).

### 2.5. Volatile isoprenoid emissions from washed-clean pine roots of intact plants

After 24 h recovery following shaken-clean root emission sampling, the remaining soil and vermiculite on the roots of the three pine plants were removed by washing thoroughly in water. The clean roots were dried gently with laboratory tissue then enclosed in the sampling system and monoterpene emission samples were taken for GC-MS analysis. The average enclosure temperature was 25.7 ± 0.7 °C. Pine plants were repotted in watered vermiculite for 1 day to recover for measuring monoterpene content.

### 2.6. Monoterpene emissions from intact pine roots under drought conditions

A third group of three *P. pinea* plants were grown in the same laboratory conditions, but water was withheld for 7 days. After this period of drought, monoterpene emissions and content were sampled from soil-balls, shaken-clean roots and washed roots as described above for well-watered plants. Between measurements, the droughted pine trees were re-potted in dry vermiculite. The recorded average enclosure temperatures for soil-ball, shaken-clean and washed-clean root experiments under drought
condition were 22.3 ± 0.3, 22.6 ± 0.2, 22.0 ± 0.5 °C, respectively. These temperatures were up to 4 °C lower than the temperatures in the bag enclosures for the well-watered experiments.

2.7. Monoterpene content of the pine roots

After recovery for 24 h in vermiculite following washed-clean emission sampling, the roots of each of the pine trees were washed again to remove vermiculite, gently dried with laboratory tissue, removed from the stem, and weighed (total fresh weight $M_{\text{fresh, total}}$). Three sub-samples of the fine roots were prepared for root content analysis by grinding in liquid N₂ (see above). The total weight of the fresh root sub-samples was recorded ($M_{\text{assayed, total}}$). The remaining root materials were dried at 60 °C for 3 days and dry root mass recorded ($M_{\text{dry, rest}}$). Dried weight of the total root system, $M_{\text{dry, total}}$, is estimated by

$$M_{\text{dry, total}} = \frac{M_{\text{dry, rest}} \times M_{\text{fresh, total}}}{M_{\text{fresh, total}} - M_{\text{assayed, total}}}.$$  

The estimated dry weight was then used for calculating monoterpene emission rates and concentrations in the roots.

2.8. Passive sampling of monoterpens in rhizosphere soils in field conditions

The field experiments were carried out in the rhizosphere soil of two mature P. sylvestris trees (~20 m tall) growing 20 m apart on the edge of a grass field near Bailrigg House, Lancaster University, UK. Sampling was performed on sunny days between 25 and 27 July 2005. Sample tubes used for sampling emissions from roots (described above) were used here as passive diffusion sampling devices. Six sample locations were chosen, one at 0, 5 and 20 m from trunks of the two P. sylvestris trees. At each sample location, three un-capped sample tubes were buried within a single clean cotton bag to prevent ingress of larger soil particles and fauna into the tubes at a depth of 10 cm. This provided three replicate analyses of two replicate samples of soil volatile compounds at three distances from the tree trunks. After 2 days, the tubes were recovered for analysis. Thermal desorption of samples was preceded by a dry purge with helium to remove water.

2.9. GC-MS methods

A Perkin-Elmer AutoSystem XL gas chromatograph coupled to a Perkin-Elmer TurboMass Gold quadrupletype mass selective detector was used for the GC-MS analyses, with helium as the carrier gas at a flow rate of 1 mL min⁻¹. Ionization potential of the MS unit was 70 eV and the scan range was 40–250 amu. A Perkin-Elmer TurboMatrix Automated Thermal Desorber was used to perform two-stage sample desorption. Primary desorption was at 10 mL min⁻¹ for 6 min: compounds were desorbed from the sample tubes at 280 °C to cryofocus onto a cold trap at −20 °C. Secondary desorption occurred when the cold trap was flash-heated and held at 300 °C for 5 min transferring compounds from the cold trap to the GC column via a transfer line at 250 °C. Compounds were then separated on an Ultra-2 GC column (crosslinked 5% phenyl methyl silica, 50 m × 0.2 mm × 0.11 µm) with a temperature programme starting at 40 °C for 2 min, rising to 165 °C at 4 °C min⁻¹, then to 300 °C at 45 °C min⁻¹.

2.10. Compound identification and quantification

Identification of compounds in both root extracts and emissions was by comparison of retention times on the chromatograms of the collected samples with those of authentic standards (Fluka UK and Aldrich UK; 95–99% purity, diluted to 10 ng µL⁻¹ in methanol), and by comparison of sample ion spectra with NIST and WILEY Mass Spectral libraries.

Ion 93 was selected for quantification of compounds. Quantification was by comparison with calibrations using standards for α-pinene, β-pinene, myrcene, limonene and γ-terpinene. Other compounds were quantified by deriving a response factor based on the % contribution of ion 93 to total ions in the sample compound compared with the % contribution of ion 93 to total ions in the α-pinene standard. Although a variety of compounds were present in the samples collected, we focussed on eight firmly identified monoterpene compounds for comparison of sampling method efficiencies and treatments (α-pinene, camphene, β-pinene, β-myrcene, 1-phellandrene, α-terpinene, limonene, and γ-terpinene). Previous work has shown that the concentration–response relationship is linear for the range of concentrations of compounds analysed in this study (S.M. Owen, Ph.D. thesis, Lancaster University, 1998). A monoterpene quality control standard mixture was analysed for every six samples.

2.11. Statistical analyses

Results are expressed as mean ± standard error of the mean. Two-way analysis of variance (on transformed data when needed to achieve normality and equal variance) was used to explore significance of differences in content and emissions from different preparations of pine roots, and between control and drought treatments. The Tukey test was used for pairwise comparisons of mean responses to different treatment groups, to identify significantly different pairs of treatments. Where data were not normally distributed, one-way ANOVA on ranks (Kruskall-Wallace with Dunn’s test) was used to test significance of differences in amounts of compound sampled passively from rhizosphere soil at different distances from a tree trunk.
3. Results

3.1. Screening eleven species for monoterpene content in roots

Large amounts of monoterpenes were found in the initial extracts from P. halepensis roots, with a total of $415 \pm 50 \mu g^{-1}$ fresh weight for eight main monoterpenes compounds (Table 1). The major extracted compounds were $\alpha$-pinene, $229 \pm 54 \mu g^{-1}$ fresh wt and $\beta$-pinene, $152 \pm 22 \mu g^{-1}$ fresh wt. A total of $643 \pm 25 \mu g^{-1}$ fresh wt of monoterpenes were also detected in ginger roots, with camphene alone present at a dominant concentration of $370 \pm 16 \mu g^{-1}$ fresh wt. Up to 0.5 and 0.4 $\mu g^{-1}$ fresh wt of $\alpha$- and $\beta$-pinene, respectively, were found in mint. The other screened species contained no significant concentrations of monoterpenes in their roots, perhaps because the plants were very young. However, the foliage of some of the species is known to contain monoterpenes (tomato, Winer et al., 1992; eucalyptus, King et al., 2004; mint, Ringer et al., 2005; sage, Novak et al., 2006; peppermint, Ringer et al., 2005; lavender, Owen et al., 2001; thyme, Owen et al., 2001; and rosemary, Hansen et al., 1997). The leaves were already fragrant at the time of sampling roots, indicating that synthesis of monoterpenes was already underway in the leaf tissue. Because roots of P. halepensis contained significant amounts of monoterpenes (Table 1) and because many different Pinus spp. are important forest and plantation species throughout the world, the Pinus genus was chosen for subsequent experiments.

3.2. Total monoterpene emissions from roots

Roots of P. pinea plants were sampled for emissions of monoterpenes, first within soil-balls, then after cleaning by shaking the soil from the root system, and finally after cleaning by washing in water. Total monoterpene emissions from roots of well-watered and droughted plants prepared in this way are shown in Table 2. Total monoterpene emissions from well-watered roots in soil-balls were an order of magnitude higher than total monoterpene emissions from droughted roots in soil-balls ($P<0.002$; Table 2). Total emissions from well-watered shaken-clean roots were around four times higher than total emissions from droughted shaken-clean roots ($P<0.001$; Table 2). There was no significant difference in total monoterpene emissions from well-watered and droughted washed-clean roots. Differences between watered and drought treatments were not observed in total monoterpene content of roots (Section 3.3).

3.3. Root contents after emission experiments

After the emission experiments were completed, there was no significant difference in amount of total monoterpenes extracted from P. pinea roots following the different treatments and preparations ($688 \pm 103, 940 \pm 43$ and $1144 \pm 208 \mu g^{-1}$ dry wt, from roots after the 1-day process, 3-day process and droughted 3-day preparation process, respectively; Fig. 1; Table 3). There was also no significant difference between treatments and preparations in amount of total compounds extracted ($1950 \pm 770$ and $3940 \pm 770 \mu g^{-1}$ dry wt, from roots after the 1-day process, 3-day process and droughted 3-day preparation process, respectively; Table 3).

The relative contributions of different compounds to the total extractable from roots are presented according to treatment sequences and watering regime. While emission experiments focused on monoterpenes, a range of compounds was subsequently detected in root extracts, and therefore some sesquiterpenes as well as two non-isoprenoid compounds are reported (Table 3). Limonene and $\beta$-pinene contributed the largest part to total content (between 27.5 and 33.1%, respectively). Alpha-pinene also contributed a large part to total content (between 4.8 and 5.2%, respectively). The sesquiterpenes trans-caryophyllene and $\alpha$-humulene...
lylbenzene, the compound tentatively identified as methoxy-4,2-propenyl benzene (anisole), 2,4-dimethoxyallylbenzene (\(\gamma\)-allylbenzene) were significantly reduced in the droughted treatments (Table 3), however there were some significant individual root systems was similar for all individuals and compared with the 1-day process (\(P<0.05\)). Concentrations of anisole were significantly reduced in well-watered samples subject to the 3-day preparation, compared with the 1-day preparation (\(P<0.05\)).

3.4. Individual monoterpene emission rates from roots

Different methods of root preparation affected absolute emission rates of the three main monoterpene compounds (\(\alpha\)-pinene, \(\beta\)-pinene and limonene; Fig. 2). Emission rates from well-watered shaken-clean roots were significantly higher (up to a factor of \(~20\)) than emissions measured from well-watered roots in soil-balls (\(P<0.05\), all compounds). Emissions of the three compounds from shaken-clean roots were also larger (up to a factor of \(~4\)) than emissions from roots after washing (\(P<0.05\)).

There was no significant difference in emissions between watered and droughted washed-clean roots. However, emissions of all compounds from roots of droughted plants were significantly lower (an order of magnitude) than those from well-watered plants when considering all preparation treatments together (\(P<0.05\)).

3.5. Relative contributions of monoterpene compounds to total emissions from roots

The relative contributions of different monoterpene compounds to total emissions from roots of plants undergoing different preparation regimes and watering treatments are shown in Fig. 3. Limonene, \(\beta\)-pinene and \(\alpha\)-pinene were the main contributing compounds to total emissions. In general, emission rates of limonene tended to be higher than \(\beta\)-pinene, which in turn tended to be higher than \(\alpha\)-pinene, although differences were not always significant. Not all of the compounds detected in root extracts occurred in the root emissions. Relative amounts of different monoterpene compounds in emissions varied between different individual pines and different treatments, e.g., contributions from limonene, \(\alpha\)-pinene and \(\beta\)-pinene ranged from 36% to 72%, from 5% to 11%, and from

Table 3
Relative contribution of most abundant compounds (monoterpenes, sesquiterpenes and non-isoprenoids) to total content of Pinus pinea roots after sequences of preparations for sampling for emissions; \(n=3\); 3-day preparation = soil-ball, shaken-clean, washed-clean; 1-day preparation = washed-clean only.

<table>
<thead>
<tr>
<th>Compound</th>
<th>One-day preparation well-watered</th>
<th>Three-day preparation well-watered</th>
<th>Three-day preparation droughted</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-pinene(^{1,4})</td>
<td>4.8 ± 0.2</td>
<td>5.2 ± 0.1</td>
<td>5.1 ± 1.0</td>
</tr>
<tr>
<td>Camphene(^{1,4})</td>
<td>0.1 ± 0.01</td>
<td>0.1 ± 0.02</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>(\beta)-pinene(^{1,4})</td>
<td>28.8 ± 1.8</td>
<td>29.2 ± 1.0</td>
<td>33.1 ± 5.4</td>
</tr>
<tr>
<td>Myrcene(^{1,4})</td>
<td>0.9 ± 0.06</td>
<td>0.8 ± 0.03</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>(\alpha)-phellandrene(^{1,4})</td>
<td>0.01 ± 0.0</td>
<td>0.01 ± 0.0</td>
<td>0.01 ± 0.0</td>
</tr>
<tr>
<td>(\alpha)-terpinene(^{1,4})</td>
<td>0.1 ± 0.01</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>Limonene(^{1})</td>
<td>35.8 ± 2.0(^{a})</td>
<td>37.1 ± 0.5(^{a})</td>
<td>27.5 ± 0.9(^{b})</td>
</tr>
<tr>
<td>(\gamma)-terpinene(^{1})</td>
<td>0.01 ± 0.0</td>
<td>0.01 ± 0.0</td>
<td>0.02 ± 0.0</td>
</tr>
<tr>
<td>1-methoxy-4,2-propenyl benzene(^{2,4}) (anisole)</td>
<td>13.7 ± 3.4(^{a})</td>
<td>2.3 ± 1.1(^{b})</td>
<td>6.8 ± 6.8</td>
</tr>
<tr>
<td>A caryophyllene oxide(^{3,4})</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>(\alpha)-longipene(^{3,4})</td>
<td>2.2 ± 0.2</td>
<td>3.4 ± 0.6</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>2,4-dimethoxyallylbenzene(^{2,4})</td>
<td>7.6 ± 0.9(^{a})</td>
<td>9.4 ± 1.5(^{a})</td>
<td>4.7 ± 0.3(^{b})</td>
</tr>
<tr>
<td>(\beta)-selinene/aromadendrene(^{3,4})</td>
<td>0.0 ± 0.0(^{a})</td>
<td>0.0 ± 0.0(^{a})</td>
<td>10.7 ± 0.5(^{b})</td>
</tr>
<tr>
<td>Transcaryophyllene(^{3,4})</td>
<td>4.7 ± 0.3(^{a})</td>
<td>10.1 ± 1.4(^{b})</td>
<td>5.8 ± 0.6(^{a})</td>
</tr>
<tr>
<td>(\alpha)-bergamotenol(^{3,4})</td>
<td>0.1 ± 0.02</td>
<td>0.1 ± 0.04</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>(\alpha)-humulene(^{3,4})</td>
<td>0.9 ± 0.06(^{a})</td>
<td>2.0 ± 0.3(^{b})</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Total monoterpenes ((\mu)g g(^{-1}) dry wt)</td>
<td>688 ± 103</td>
<td>940 ± 43</td>
<td>1144 ± 208</td>
</tr>
<tr>
<td>Total compounds ((\mu)g g(^{-1}) dry wt)</td>
<td>1950 ± 300</td>
<td>2890 ± 170</td>
<td>3940 ± 770</td>
</tr>
</tbody>
</table>

Superscripts of different letters indicate significant differences between treatments for that compound.

1Monoterpenes.
2Non-isoprenoid volatiles.
3Sesquiterpenes.
4Based on quantification derived from relative contribution of ion 93 to total ion count.
22% to 47%, respectively, while other compounds contributed relatively consistently (e.g. β-myrcene below 1–2%). Of the major compounds contributing to total emissions, the contribution of limonene was significantly less in droughted roots than in well-watered roots \((P<0.02)\). Limonene contribution to total emissions from washed-clean roots (1-day process) was significantly greater than limonene contribution to emissions after re-potting in vermiculite \((P<0.05)\). In contrast, β-pinene contributed significantly more to total emissions in droughted roots than in well-watered roots \((P<0.05)\) but there was no significant difference in contribution of this compound between preparations (soil-ball, shaken-clean and washed-clean). Contribution of α-pinene to total emissions was not significantly affected by watering regime, but was significantly different between soil-ball emission samples, and shaken-clean and washed-clean samples \((P<0.05; \text{Fig. 3})\).

3.6. Sampling monoterpenes in rhizosphere soils in field conditions

There was a difference in total monoterpenes concentrations between distances 0 and 5, and 0 and 20 m from the trunk, which was not significant \((0.210\pm0.210, 0.014\pm0.010, 0.005\pm0.001 \mu\text{g h}^{-1}\) at 0, 5 and 10 m, respectively; Fig. 4). The trend of decreasing concentrations of individual compounds with distance from trunk was also clear, but not significant.

Relative contribution from different monoterpenes compounds to samples obtained at different distances from the mature \(P. sylvestris\) trunks are shown in Fig. 5. The most abundant compounds in each sample were α-pinene, camphene and limonene. Relative contribution of limonene to total monoterpenes in the samples decreased significantly from \(\approx11\%\) to \(\approx2\%\) with distance from the trunk \((P<0.005)\). In contrast, there was a trend of increasing α-pinene contribution from \(\approx65\%\) to \(\approx88\%\) with increasing distance from the trunk, but this was not significant. Relative percentage of camphene tended to be larger at the edge of the canopy, but this was also not significant.

4. Discussion and conclusions

The composition of monoterpenes emissions from roots did not totally reflect the composition of root content. Compounds present in the extracts represented a bulk
content of the whole root, while monoterpenes compounds in emissions were probably from a variable outer structure or layer, as discussed below. In both emissions and content, α-pinene and limonene were important components, but camphene was present in content to a larger extent than in emissions, and β-pinene figured more prominently in emissions. For the three major emitted monoterpenes compounds, emission rates from shaken-clean pine roots were, on average, around ten times higher than emissions from soil-balls (Table 2; Fig. 2). It is possible that microorganisms in the soil-ball might be consuming the root-emitted monoterpenes as a carbon source. Alternatively, the non-biotic matrix of the soil-ball itself might absorb emitted monoterpenes, or there might be chemical removal in the soil, e.g. by oxidation (Harms et al., 1999). Removal of soil from the pine roots by shaking greatly stimulated monoterpane root emissions, and yet washing the roots with tap water resulted in much lower emissions. An outer monoterpane-emitting structure or layer was presumably removed completely with washing in water. This layer might be cells, exudates, microorganisms or mycorrhizal hyphae. Interestingly, Chen et al. (2004) discovered a monoterpane synthase in Arabidopsis roots which produces cineole, but they could not detect cineole content in roots which they had prepared by washing rigorously in tap water. However, Steeghs et al. (2004) reported that cineole is an Arabidopsis root volatile. It is possible that cineole is not stored in Arabidopsis root tissue, or it could be stored in an outer layer of Arabidopsis roots which was removed by washing.

Various functions of different types of root exudation have been proposed, including the maintenance of root-soil contact, protection of roots from desiccation, stabilization of soil micro-aggregates and selective adsorption and storage of ions (Bengough and McKenzie, 1997; Griffin et al., 1976; Hawes et al., 2000; Rougier, 1981; Walker et al., 2003). Here, removing soil by shaking the roots broke root-soil contact and disturbed the outer structure of the root system which resulted in high emissions. Hayward et al. (2001), in their study on monoterpane soil emissions in a Sitka spruce forest, also observed significant monoterpane emission increases after installing a soil enclosure into the ground, which they attributed to the damage of the fine root system a few centimetres beneath the soil surface. In droughted pines, emissions from shaken-clean roots were similar to emissions from washed-clean roots, and were significantly lower (by up to an order of magnitude) than emissions from roots of well-watered shaken-clean plants. This could be attributable to lower temperatures in the enclosure for sampling droughted pine roots. However, monoterpane emissions from control and droughted roots, standardized to 30 °C by applying an algorithm which assumes exponential response of vaporization of monoterpenes with increasing temperature (Guenther et al., 1993), were also significantly different (P<0.05; data not shown). We conclude that drought inhibited the synthesis or maintenance of the monoterpane-emitting outer structure or layer of the root system (including mycorrhizal fungi, and associated microbial populations), although it is also possible that the lower shaken-clean emissions from droughted roots was because less vigorous shaking was needed to remove the much drier soil from droughted roots. However, extrapolation from plant seedlings in laboratory conditions to forest trees in field plots is difficult. In contrast to our findings, Asensio et al. (2006) found larger soil monoterpane emissions in summer in droughted plots compared with control plots in a southern Catalunyan oak forest, which they suggested might be linked to autotrophic (roots) metabolism. However, they were measuring flux from soil surfaces, which may not reflect rhizosphere source strength (as discussed in the Introduction). Alternatively, or additionally, emissions from droughted soil plots measured by Asensio et al. (2006) may have reflected the higher (but non-significant) monoterpane root content observed here, perhaps via degradation of roots killed by the severity of the drought.
The decreasing absolute emission rates of total monoterpenes with increasing distance from the pine trees in field conditions (Fig. 4) suggest that the source of these compounds in the rhizosphere soil might be the root system of the \textit{P. sylvestris} trees, or incorporation of fallen litter into the soil. Currently, there is little published work on concentrations of monoterpenes in soils. White (1991) investigated monoterpenes in the rhizosphere soil of \textit{Pinus ponderosa}, and their role in soil nitrogen cycling processes. Monoterpene concentrations were quantified by ether extraction from known soil sample masses, and varied from 35 to 42 \( \mu \)g g\(^{-1}\) dry wt for the F-H horizon (organic) and from 0.03 to 0.23 \( \mu \)g g\(^{-1}\) dry wt (mineral soil, equivalent to the soil sampled in our investigation), according to season of extraction. There was considerable variability within season, so inter-seasonal differences were not significant. Recently, Smolander et al. (2006) reported concentrations of \( \sim 9 \) mg m\(^{-3}\) (soil air) for monoterpenes in the gas phase in rhizosphere soil under \textit{P. sylvestris}. However, as the authors acknowledge, their sampling methods involved considerable disturbance of the root system, which Hayward et al. (2001) show can cause highly elevated concentrations of monoterpenes in the soil air phase.

In conclusion, this study shows that measuring emissions of volatile isoprenoids from roots is feasible in laboratory and natural field conditions, but the preparation method for dynamic enclosure sampling affected magnitude and composition of emission from root systems of potted \textit{P. pinea}. Emissions from \textit{P. pinea} roots were also affected by drought. We have shown that emissions from soil-balls can vary according to water status of the soil, with lower emissions from dry soil-balls. Lower emissions from shaken-clean droughted roots in the laboratory indicate that the water status of the root system itself, or that of the soil closest to the root system, may control emission rates of monoterpenes from roots. It is possible that monoterpene emissions from living roots are largely from some structure at the root/soil interface, and that a different quantitative and qualitative fingerprint of emissions is generated from degradation of dead roots within the soil. Further work is needed to investigate source strength, controls and mechanisms of volatile isoprenoid emissions or exudations from roots of \textit{Pinus} spp.

The decrease in amount of compounds sampled with increasing distance from \textit{P. sylvestris} trunks indicates the possibility of quantification and distribution modelling studies in the future. We conclude that the rhizosphere of \textit{Pinus} species is a considerable and measurable source of volatile isoprenoids which are likely to impact widely and profoundly on rhizosphere function and biodiversity below and above ground.

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