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^{13}C labelling study of constitutive and stress-induced terpenoid emissions from Norway spruce and Scots pine

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Abstract. Due to their large source strengths, biogenic volatile organic compounds (BVOCs) are important for atmospheric chemistry. Terpenoids, mainly consisting of isoprene, monoterpenes and sesquiterpenes, are the dominant BVOC class. There are two general mechanisms for their emissions: emissions directly from de novo biosynthesis (de novo emissions) and emissions from organs wherein the terpenoids are stored (pool emissions). While isoprene emissions are pure de novo emissions, the mechanism for monoterpene and sesquiterpene emissions is not always distinct. In particular, conifers have large storage pools and both mechanisms may contribute to the emissions.

To obtain more insight into the mechanisms of the terpenoid emissions from Eurasian conifers, we conducted $^{13}CO_2$ and ^{13}C-glucose labelling studies with Norway spruce (*Picea abies* L.) and Scots pine (*Pinus sylvestris* L.). The results from the labelling experiments were further compared to diurnal modulations measured for the emission fluxes of the respective terpenoids, as well as to their release from reservoirs in needles and bark tissue.

The comparison allowed the following comprehensive statements for the investigated conifers. Consistent to other studies, we found that constitutive monoterpene emissions mainly originate from storage pools but with compound-specific fractions of de novo emissions. In contrast, stress-induced monoterpene and sesquiterpene emissions are entirely of de novo nature. We also found at least three different carbon sources for monoterpene and sesquiterpene biosynthesis. These sources differ with respect to the timescale after which the recently assimilated carbon reappears in the emitted terpenoids. Carbon directly obtained from assimilated CO_2 has a short turnover time of few hours, while carbon from other alternative carbon sources has intermediate turnover times of few days and even longer. Terpenoid biosynthesis is not restricted to the presence of light and the carbon for terpenoid biosynthesis can be delivered from the alternative carbon sources. In particular for sesquiterpenes, there can be substantial de novo emissions in darkness reaching up to around 60 % of the daytime emissions. The use of the alternative carbon sources for sesquiterpene synthesis is probably linked to the mevalonic acid (MVA) pathway. The higher the contribution of the MVA pathway to terpenoid synthesis, the higher is the nocturnal de novo emission.

In general, the emission mechanisms of monoterpene and sesquiterpene are more complex than assumed so far. Besides pools for terpenoids themselves, there are also pools for terpenoids precursors. Terpenoid synthesis from alternative carbon

sources leads to nighttime emissions and hence the amplitude of diurnal modulations of terpenoid emissions may be determined by an overlap of three mechanisms involved: emissions from storage pools, emissions in parallel to CO_2 uptake and emissions from alternative carbon sources.

1 Introduction

5   The boreal forest is the world's largest biome and acts as an important source of biogenic volatile organic compound (BVOC) emissions, with terpenoids, i.e. isoprene, monoterpenes and sesquiterpenes, being the dominant class (Kesselmeier and Staudt, 1999). Different from most deciduous species, conifers, the dominant tree species of the boreal forest, have storage organs of BVOCs (resin ducts). Traditionally, the emissions from conifers have been assumed to originate from the evaporation of the compounds out of these storage pools. Such emissions exponentially depend on temperature but do not

10 depend on light intensity (Tingey et al., 1991). However, many recent studies have shown that de novo biosynthesis, which is regulated by both temperature and light (Guenther et al., 1993), also contributes to a certain amount of the total emissions from conifers (Shao et al., 2001; Ghirardo et al., 2010; Taipale et al., 2011). Furthermore, biotic stresses such as insect outbreaks, may significantly increase stress-induced emissions, including novel monoterpenes and sesquiterpenes (Blande et al., 2009; Ghimire et al., 2013; Niinemets et al., 2013; Ghimire et al., 2016).

15 Depending on how the terpenoids are produced and emitted, and whether the emissions are constitutive or induced by stresses, these terpenoid emissions have different responses to environmental conditions (Blande et al., 2007; Loreto and Schnitzler, 2010; Kleist et al., 2012). In order to better estimate the total amount of terpenoid emissions from the boreal forests and the tendency under future climate conditions, it is essential to classify them according to the mechanisms of synthesis and emission, and to know more about the regulation of different classes, especially the class of stress-induced

20 emissions (Grote and Niinemets, 2008; Arneth and Niinemets, 2010).

[13]C labelling techniques are a useful tool to trace carbon allocation and to study mechanisms of BVOC emissions. Experiments with feeding ^{13}C-enriched carbon sources such as [13]CO_2 and ^{13}C-glucose allow studying the contributions of different carbon sources to the terpenoid synthesis (Karl et al., 2002; Kreuzwieser et al., 2002; Loreto et al., 2004; Schnitzler et al., 2004; Ghirardo et al., 2011). Besides atmospheric CO_2, alternative carbon sources contribute simultaneously to

25 isoprene and monoterpene biosynthesis and their contribution become especially important when photosynthesis is limited (Brilli et al., 2007). [13]CO_2 labelling experiments have been also used to determine the fraction of de novo biosynthesis (Shao et al., 2001; Ghirardo et al., 2010). By feeding plants with 99 % [13]CO_2, most of the isoprene molecules, as well as those of monoterpenes emitted directly from de novo biosynthesis, are fully or partly labelled with ^{13}C-atom(s) and thus can be identified as de novo emissions (Delwiche and Sharkey, 1993; Loreto et al., 1996).

30 So far, most of the ^{13}C labelling studies regarding the multiple carbon sources have focused on isoprene in deciduous species. For monoterpenes, the study of Loreto et al. (1996) shows that the constitutive monoterpenes from Holm oak (*Quercus ilex* L.) are mainly de novo emissions and share the same carbon sources as isoprene. Ghirardo et al. (2010)

determined the fraction of de novo biosynthesis for the total monoterpene mixtures from both conifers and deciduous species by using Proton Transfer Mass Spectrometry (PTR–MS), but without differentiating between isomers. To the best of our knowledge, there are no ^{13}C labelling studies on quantitative determination of the contributions of the multiple carbon sources for constitutive and stress-induced monoterpenes and sesquiterpenes, nor the separation of de novo and pool
5   emissions for the individual compounds.

We conducted ^{13}C labelling studies on two dominant boreal species, namely Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L.). Both species emitted small amounts of isoprene, constitutive monoterpenes, and in some cases stress-induced terpenoids including some monoterpenes and sesquiterpenes. The aim of this study was (1) to distinguish between the emissions directly from de novo biosynthesis (de novo emissions) and the emissions from storage pools (pool
10   emissions) for individual compounds; (2) to quantify the contributions of the different carbon sources to the de novo synthesis by feeding $^{13}CO_2$ and ^{13}C-glucose; (3) to check the classification and the regulation of terpenoid emissions according to the mechanisms of their synthesis and emission.

2 Experiments

2.1 Plant material

15   Six plants were used for the experiments: three individuals of Scots pine (P1, P2 and P3) and three individuals of Norway spruce (S1, S2 and S3). Two to three years old seedlings were taken from forest, potted in buckets (10–20 L), and filled with the same soil that was a mix of peat (Einheitserde ED73) with quartz sand (volumetric ratio 3:1, density 0.34 ± 0.07 kg L^{-1}). They were stored outside under natural conditions for one to two years before they were used for the experiments. The experiments were carried out between May and October except for one spruce (S2) which was introduced in the chamber at
20   the beginning of November. This plant had been stored in a growth room (temperature = 20 °C) before the experiment since late summer. For all individuals, the total needle area was calculated using the projected area as described in Shao et al. (2001).

Before the experiments, P1 and S2 were found to be infested by aphids, although we could not differentiate the exact species. The other plants looked healthy and there was no significant defoliation or obvious damages. During the experiment,
25   white floccules were found for P2 and spiders were found in the chamber for S1 indicating that both plants may have suffered from stresses.

2.2 Chamber set up

The experiments were performed at the Jülich Plant Atmosphere Chamber facility (JPAC). The chamber setup has been described in detail elsewhere (Mentel et al., 2009; Mentel et al., 2013; Wu et al., 2015). In brief, the chambers were made of
30   borosilicate glass and operated as continuous stirred tank reactors (CSTRs). They were mounted in separate walk-in climate chambers to keep the temperature constant. In this study, two plant chambers with volumes of 1150 L and 164 L were used.

The analytical equipment has been described in detail in previous publications (Wildt et al., 1997; Schimang et al., 2006). Absolute H_2O concentrations of in- and outlet flows were determined with dew point mirrors (Walz, Effeltrich, Germany). Differences in mixing ratios of H_2O and CO_2 between chamber inlet and outlet were measured by IR absorption (Binos, Rosemount, Hanau, Germany).

2.3 Labelling studies with $^{13}CO_2$

The plants were introduced into the plant chamber at least 5 days before the $^{13}CO_2$ labelling experiments. The light intensity and the chamber temperature were in the range of 400–800 µmol m^{-2} s^{-1} and 18–30 °C, respectively (details see Supplement Table S1). A diurnal light rhythm was applied to the plants: they were exposed to 12 h illumination, 10 h darkness, 1 h twilight in the morning and 1 h twilight in the afternoon. In parallel to the variations of the illumination, a 4–9 °C variation of needle temperature was observed by the measurement using micro leaf temperature sensors (Type K, Ni-CrNi, Newport Sensors, USA).

On the testing day, the $^{13}CO_2$ exposure started in the morning 2–3 h after the lamps were switched on. The normal inlet air containing CO_2 with a natural ^{13}C abundance (1.1 % ^{13}C) was exchanged by a second inlet flow combining a flow of synthetic air (CO_2 < 10 ppm) and a small flow of $^{13}CO_2$ (99 % ^{13}C). The CO_2 mixing ratios at the chamber outlet were kept at levels of 330–380 parts per million (ppm). The typical inlet flows of the 164 L chamber and the 1150 L chamber were in the range of 8–20 L min^{-1} and 20–40 L min^{-1}, respectively, causing the residence times of the air in the chambers between 8–20 min in the 164 L chamber and between 30–60 min in the 1150 L chamber. The $^{13}CO_2$ feeding was carried out for 4–8 h until the incorporation of ^{13}C-atoms into terpenoids was near to steady state.

Thereafter, the inlet flow was switched back to the air containing CO_2 with the natural ^{13}C abundance. To follow the labelling of the emissions after the exposure, the lamps were kept on for at least 3–4 h (for S1 and S2 the whole night) and adjusted back to the normal diurnal light rhythm before the second night. The measurements of the terpenoid emissions and their labelling were continued for 4 days in total.

2.4 Labelling studies with ^{13}C-glucose

The labelling experiments with D-Glucose-$^{13}C_6$ (≥ 99 % ^{13}C, Sigma-Aldrich, Germany) were made for three individuals (S1, S2 and S3) to test the contribution of xylem transported sugar as an alternative carbon source to the de novo synthesis. On the day before the ^{13}C-glucose experiment, small branches of the individual plants were carefully cut and immediately transferred into a small glass chamber of 3 L volume. They were re-cut under water and initially fed with 10mM glucose solution (Kreuzwieser et al., 2002; Ghirardo et al., 2011) containing the natural abundance of ^{13}C. The small chamber was placed in the same walk-in climate chamber as the tested plants and equipped with an extra discharge lamp causing a light intensity of approximately 600 µmol m^{-2} s^{-1}, similar as for the living plants. Between 1.5–2 L min^{-1} of the air containing 350 ppm CO_2 with the natural ^{13}C abundance was pumped through the small chamber. The residence time of the air in the chamber was 1.5–2 min. After measuring the emissions from the branch for one day/night cycle, the glucose solution was

exchanged by another solution with 10 mM ^{13}C-glucose. The measurements of the terpenoid emissions and their labelling were continued for the next 72 h at permanent illumination.

2.5 Terpenoid storage in needles and bark tissue

Terpenoids stored in needles and bark tissue were characterized for three individuals (S1, S2 and P3). Twigs were taken from the plants, and they were crashed and transferred into the same chamber as used for the ^{13}C-glucose experiments. The release of terpenoids was measured in the dark for at least 8 h after putting the crashed tissue in the chamber. The emission pattern was calculated by using the release of β-pinene as the reference.

2.6 Measurement of stable ^{13}C-isotopes of terpenoids with GC–MS

The emission strengths and the isotope abundance in the emitted terpenoids were monitored continuously and analyzed by gas chromatography–mass spectrometry (GC HP 5890 Serie II + MSD HP 5972A, GC HP 6890 + MSD HP 5973 or GC HP 7890 + MSD HP 5975C, Agilent; equipped with thermal desorption, TDSG, Gerstel, Mülheim, Germany). The measurements were made every 60 to 80 min. For details on the GC systems and the calibration procedure see, e.g. Heiden et al. (2003).

Since isoprene was not the major component of the emissions from both species (and has been already well studied), we focused here on monoterpenes and sesquiterpenes. From the mass spectra, we determined the isotopic abundance patterns of the individual terpenoids. For a compound of molecular mass M containing n carbon atoms, $n + 1$ molecular peaks are observable during labelling with ^{13}C (m/z = M + i, i = 0, 1…n, where i is the number of ^{13}C-atoms in the molecule). Assuming an equimolar response for all isotopic forms of the respective compound molecule, the relative amounts of isotopes were quantified using the count rates (C_{M+i}) of the corresponding molecular peaks. For each isotopic form, the relative ion intensity (P_{i_meas}) was calculated by dividing the count rate of this molecular peak by the sum of count rates of all peaks of the molecular ion. The relative error caused by this method was less than 1 %. Table 1 lists the symbols and the abbreviations used for the quantities determined for the labelling experiments.

2.7 Prediction of isotopic abundance patterns

In principle, all emitted terpenoids, no matter whether originating from storage pools or de novo biosynthesis, are naturally labelled before the labelling experiments with the natural abundance of ^{13}C (1.1 %). During the ^{13}CO$_2$ or ^{13}C-glucose feeding, the labelling of pool emissions is not changed since emissions and synthesis are decoupled in time due to the large capacity of storage pools. De novo emitted terpenoids are released immediately after their production and are therefore extra labelled by the freshly incorporated ^{13}C. If a compound is emitted both via storage pools and directly from de novo biosynthesis, a mixed labelling pattern is expected, with the fraction emitted from storage pools showing natural ^{13}C content and the fraction from de novo biosynthesis showing a higher labelling.

We simulated the isotope abundance patterns for the individual compounds naturally labelled with the abundance $R_{13C}^{natural} = 0.011$, and originating from a carbon source with an elevated ^{13}C abundance R_{13C}^{j} (j = de_novo1, de_novo2…). We assumed that instantaneous mixing of ^{13}C-atoms and ^{12}C-atoms occurs throughout the carbon sources. Based on findings from previous studies (Delwiche and Sharkey, 1993; Loreto et al., 1996), we also assumed that all carbon atoms of the isoprene,

5 monoterpene and sesquiterpene molecule skeleton have equal probability to be labelled by ^{13}C-atoms from the carbon source. The predicted relative ion intensity (P_{i_cal}) of the isotope mass M + i (i = 0, 1…n) of the compound can be calculated by a binomial distribution (Karl et al., 2002) (Eq. (1)). It reflects the probability that the molecule is labelled by i carbon(s).

$$P_{i\_cal} = \binom{n}{i} \cdot \left(R_{13C}^{j} \right)^{i} \cdot \left(1 - R_{13C}^{j} \right)^{n-i} \tag{1}$$

Where

10 $$\binom{n}{i} = \frac{n!}{i!(n-i)!} \tag{2}$$

As shown in Fig. 1, for a C$_{10}$ compound synthesized from the carbon atoms with the natural labelling abundance ($R_{13C}^{natural} = 0.011$), 11 % of the total molecules are labelled, mainly with a single ^{13}C-atom (black bars). When the de novo pathway has a high $R_{13C}^{de\_novo3}$ of 0.99, about 90 % of the molecules are completely labelled and the rest are mainly labelled with nine ^{13}C-

15 atoms (red bars). When they are less labelled, e.g. with $R_{13C}^{de\_novo2} = 0.9$, all of the molecules are still partly or fully labelled with ^{13}C-atoms (blue bars), but the most abundant mass peak is M + 9. P_{7_cal}, P_{8_cal}, P_{9_cal} and P_{10_cal} contribute together to 99 %. If $R_{13C}^{de\_novo1} = 0.2$, most of the molecules are labelled with one to four ^{13}C atoms with M + 2 as the most abundant peak. As an example, if a compound is emitted from storage pools as well as directly after de novo biosynthesis using only the freshly assimilated carbon atoms from 99 % ^{13}CO$_2$, the pattern would be bimodal and represented by the sum of the black

20 bars ($R_{13C}^{natural} = 0.011$) and red bars ($R_{13C}^{de\_novo3} = 0.99$) in Fig. 1, just with the normalization that the sum of the relative ion intensity of all molecular peaks is 100 %.

2.8 Calculation of labelling ratios and the fraction of de novo biosynthesis

The ratio of ^{13}C-atoms over total carbon atoms (R_{13C_meas}) was calculated by summing the labelled carbon and dividing it by the total carbon (Eq. (3)),

25 $$R_{13C\_meas} = \frac{C_{M+1} \cdot 1 + C_{M+2} \cdot 2 \dots + C_{M+n} \cdot n}{n \cdot (C_M + C_{M+1} \dots + C_{M+n})} \tag{3}$$

while the ratio of labelled molecules over all molecules was calculated using Eq. (4).

$$R_{iso\_meas} = \frac{C_{M+1} + C_{M+2} + .. + C_{M+n}}{C_M + C_{M+1} \dots + C_{M+n}} \tag{4}$$

We furthermore assumed that all molecules with excess ^{13}C-atoms have been synthesized during the time of ^{13}CO$_2$ exposure, i.e. they are de novo emissions. Therefore, the fraction of de novo biosynthesis (f_{synth}) is defined here according to Eq. (5):

30 $$f_{synth} = \frac{C_{M+1} - C_M \cdot \gamma + C_{M+2} + .. + C_{M+n}}{C_M + C_{M+1} \dots + C_{M+n}} \tag{5}$$

As shown in Fig. 1 with black bars, the natural labelling brings a small fraction of the labelled molecules at M + 1. Based on Eq. (1), the ratio (γ) of the naturally labelled C_{M+1} over the naturally labelled C_M is fixed ($\gamma = {C_{M+1}}/{C_M} = {P_{1\_cal}}/{P_{0\_cal}}$).

Thus, the baseline caused by the natural labelling was considered by subtracting the amount of naturally labelled M + 1 ($C_M \cdot \gamma$ in Eq. (5)) from the sum of labelled molecules. The number of the carbon atoms in a molecule increases the

5   probability of the entire molecule to contain at least one ^{13}C-atom. Hence, γ is 11.1 % for C_{10} compounds (monoterpenes) and 16.7 % for C_{15} compounds (sesquiterpenes). The amount of the molecules naturally labelled with two or more ^{13}C-atoms was neglected.

3 Results

3.1 Emission patterns

10   Figure 2 shows the constitutive and the stress-induced emissions of all tested individuals. At the example of S1, the emission fluxes of the individual terpenoids are listed as well. The classification of the constitutive and the stress-induced emissions was based on previous studies (Guenther et al., 2012; Niinemets et al., 2013) and on the comparison of the compounds detected for all individual plants and those found only for the infested ones.

The daily emission fluxes of the constitutive emissions were 10.2 ± 4.7 nmolC m^{-2} s^{-1} for the pines and 1.4 ± 0.2 nmolC m^{-2} s^{-1}

15   for the spruces (Fig. 2a). Biotic stresses induced strong stress-induced emissions from P1, P2, S1 and S2, causing 4.5, 0.3, 48 and 1.6-fold higher carbon release, respectively, than the carbon release in form of their constitutive emissions.

For Scots pine, constitutive isoprene and sesquiterpene emissions were low compared to their constitutive monoterpene emissions. The major constitutive monoterpene emissions were α-pinene, camphene, β-pinene, myrcene, Δ^3-carene, β-phellandrene and 1,8-cineole, which together contributed to 95 % of total monoterpene emissions. The detailed emission

20   patterns were different for the individual plants. The major constitutive emissions from Norway spruce were similar to those of Scots pine, but with a higher contribution from limonene, and lower contributions from Δ^3-carene and 1,8-cineole. The stress-induced emissions from both plant species were mainly those of α-farnesene and (*E*)-β-farnesene. For S1, linalool and (*E*)-β-ocimene were measured additionally as stress-induced monoterpenes. Detailed emission fluxes of the individual compounds are listed in the Supplement Table S2.

25   The emission pattern observed for the spruce S1 was then compared with the pattern of compounds released from the crashed needles and bark tissue of the same plant (Table 2). The temporal changes of the released emissions are shown in Supplement Fig. S2. In addition, the diurnal variations of the individual emission compounds from the living plant are listed as well (Table 2).

Different from the emission pattern of the living plant, which was dominated by the stress-induced emissions, only several

30   constitutively emitted monoterpenes like α-pinene, camphene, β-pinene, myrcene, and limonene were released from the

storage pools in needles and bark. Taking the dominating compound β-pinene as the reference, the normalized release of isoprene and all stress-induced terpenoids (including two sesquiterpenes (*E*)-β-farnesene and α-farnesene and two monoterpenes linalool and (*E*)-β-ocimene) were extremely low, indicating there were no specific storage pools for them and they were de novo emissions. The results from two other individuals showed similar results that all stress-induced emissions were of de novo nature.

However, the measured diurnal modulation showed that all of these de novo compounds were still released from the plant in the dark. With a temperature difference (ΔT) of 6 °C between day and night, the ratios of nighttime to daytime emissions were 0.03 to 0.06 for isoprene, (*E*)-β-ocimene and linalool, but 0.18 for the two sesquiterpenes. For other plants (not shown here), such ratios for the de novo monoterpenes were relative low and up to 0.06; for sesquiterpenes, the value ranged from 0.10 to 0.20 for S2 and P1 (ΔT = 8 °C and 4 °C), and 0.50 to 0.58 for P2 (ΔT = 4 °C).

3.2 Labelling exposure with $^{13}CO_2$

At the example of the spruce S1, Fig. 3 shows typical temporal labelling behavior of the constitutive compounds β-pinene and myrcene, and the stress-induced compounds (*E*)-β-ocimene and (*E*)-β-farnesene during and after the $^{13}CO_2$ labelling exposure.

Before the $^{13}CO_2$ exposure (-3 to 0 h), R_{13C_meas} of all compounds was close to the natural ^{13}C abundance. R_{iso_meas} was around 0.1 for three monoterpenes, and 0.16 for (*E*)-β-farnesene. Within the error limits, these values are the same as predicted with the natural ^{13}C abundance.

During the whole experiment, the absolute emission strengths of the individual compounds were relative stable and didn't show impacts from the procedure of the exposure. Different degrees of labelling were found for the individual terpenoids. While only slight labelling was observed for β-pinene, strong and fast labelling was found for myrcene, (*E*)-β-ocimene and (*E*)-β-farnesene. For the latter terpenoids, there was a fast increase in labelling within the first 3–4 h of the exposure followed by a slower increase during the rest of the exposure time. R_{iso_meas} reached nearly steady state at the end of the exposure (7 h) with values of 0.54, 0.81 and 0.93 for myrcene, (*E*)-β-ocimene and (*E*)-β-farnesene, respectively.

An enhancement of labelling was also observed for other constitutive and stress induced terpenoids, showing a general contribution of de novo emissions to the total. Overall, constitutive monoterpenes were much less labelled than stress-induced monoterpene and sesquiterpene emissions.

Switching back to CO_2 with the natural ^{13}C abundance (Fig. 3, 7 h) led to a fast decrease of the labelling of myrcene, (*E*)-β-ocimene and (*E*)-β-farnesene for the first 3–4 h. Thereafter, the labelling degree deceased on a much longer time scale and remained higher than the pre-exposure level. Shortly before the first dark period (33 h), R_{13C_meas} fell to 0.04 and R_{iso_meas} was still 0.35 for (*E*)-β-farnesene and 0.22 for (*E*)-β-ocimene and myrcene.

After switching off the light, the needle temperature and the emission fluxes of all compounds decreased and reached steady state again after 3–4 h. The signals of the labelled molecular peaks for myrcene and (*E*)-β-ocimene were too low for isotopic analysis and thus not shown in the figure. The absolute emission fluxes of β-pinene and (*E*)-β-farnesene during darkness

were 45 % and 18 % of those during the period of illumination, respectively. However, the labelling pattern of β-pinene showed no significant variation with changing light conditions, whereas the labelling of (*E*)-β-farnesene significantly increased. Until the end of the first dark period, R_{iso_meas} and R_{13C_meas} reached 0.64 and 0.11, respectively, and were much higher than that shortly before the darkness. During the second light and dark cycle, the labelling of (*E*)-β-farnesene showed

5   a similar behavior as that during the first cycle, but with an overall slight decrease in labelling.

3.3 Fraction of de novo biosynthesis

Based on the labelling results during the $^{13}CO_2$ exposure, we calculated the fraction of de novo biosynthesis (f_{synth}) for the individual terpenoids (Table 3). Overall, there are no obvious differences between the constitutive emissions from healthy plants (S3 & P3) and the plants affected by stresses (S1, S2, P1 & P2). For most of constitutive monoterpenes, f_{synth} was

10   less than 0.2, except myrcene and limonene whose f_{synth} was up to 0.49 and 1,8-cineole whose f_{synth} was above 0.8. For stress-induced monoterpenes, f_{synth} was around 0.8, similar as that of 1,8-cineole. f_{synth} of the two sesquiterpenes ranged from 0.82 to 0.97 for S1, S2 and P1. For P2, f_{synth} was lower (around 0.5) indicating a high variability of this quantity.

3.3.1 Isotopic abundance patterns with feeding $^{13}CO_2$

The isotopic abundance patterns of monoterpenes and sesquiterpenes are shown in Fig. 4 and Fig. 5, respectively. β-pinene,

15   as a terpenoid dominantly emitted from the storage pools, nearly retained the natural labelling pattern as the predicted pattern in Fig. 1 (black bars), i.e., no substantial labelling was found. As neither (*E*)-β-ocimene nor linalool was released from the crashed needles and bark tissue (Table 2), they were considered as pure de novo emissions. Both compounds showed strong labelling. For example, the pattern of linalool (Fig. 4d) was dominated by highly labelled peaks. P_{7_meas}, P_{8_meas}, P_{9_meas} and P_{10_meas} contributed 62 % to the total and the pattern fitted well with the predicted pattern of a C_{10} compound with

20    $R_{13C}^{de\_novo2} = 0.9$ (Fig. 1, blue bars). The rest of the pattern of linalool included the unlabelled peak P_{0_meas} (15 %) and some less labelled peaks. Looking at the pattern of P_{1_meas}, P_{2_meas}, P_{3_meas} and P_{4_meas}, there was a slight maximum at P_{2_meas} (5 %). The pattern of these four peaks was similar to that predicted for a C_{10} compound with $R_{13C}^{de\_novo1} = 0.2$ (Fig. 1, yellow bars). Myrcene had an overwhelming part of de novo emissions, only a very small fraction was found to be released from needle reservoirs (Table 2). Its isotopic abundance pattern was similar as that of (*E*)-β-ocimene and linalool, but with higher

25   unlabelled peak P_{0_meas} (46 %).

The isotope abundance patterns clearly showed the naturally labelled pattern for a terpenoid predominantly emitted from pools (Fig. 4a), a mixed pattern for compounds emitted via both mechanisms, i.e., de novo and pool emissions (Fig. 4b), and the patterns for pure de novo emissions (Fig 4c&d) dominated by highly labelled peaks along with some less labelled peaks and a peak containing only ^{12}C-atoms. Such labelling patterns of de novo emitted terpenoids were also observed for the other

30   plants.

For sesquiterpenes, the isotope abundance patterns were always similar for α-farnesene and (*E*)-β-farnesene emitted from the same individual plant. However, different labelling patterns were observed for different individual plants. The patterns observed for S1, S2 and P1 included highly labelled, less labelled and unlabelled peaks. For (*E*)-β-farnesene emitted from S1 (Fig. 5a), P_{12_meas}, P_{13_meas}, P_{14_meas} and P_{15_meas} contributed to 53 % in total and the pattern of the highly labelled peaks

5 reflecting a ^{13}C abundance of around 90 %, similar as the pattern of the highly labelled peaks observed for monoterpenes (Fig. 4). The slight maximum at P_{2_meas} for monoterpenes (Fig. 4b, c&d) seemed to be shifted to higher labelled peaks. In the case of P2 (Fig. 5b), on the contrary, the highly labelled peaks were extremely low. Though more than 60 % of the molecules were still labelled, they were mainly labelled with one to six ^{13}C-atoms.

3.3.2 Dynamic changes of isotopic abundance patterns

10 Figure 6 illustrates the dynamic changes of signal intensities for some isotopes of (*E*)-β-farnesene. The data was taken from S1 during and after the ^{13}CO$_2$ exposure, including two light and dark cycles. The peaks M, M + 2 and M + 15 represent unlabelled molecules, molecules with low numbers of ^{13}C atoms and the highly labelled molecules, respectively.

Switching from ^{13}CO$_2$ to the normal CO$_2$ at 7 h caused a rapid disappearance of the peak M + 15 and a successive increase of the peaks M and M + 2. After 5–6 h, the count rate of the peak M + 15 fell below 5 % of its highest value. Though the highly

15 labelled molecules vanished, there were still 33 % of the total molecules remaining labelled shortly before the first dark period (32 h).

Switching off light caused the reductions of all peak intensities and also substantial changes of the labelling pattern. Compared to the count rates shortly before darkness, the count rate of the peak M decreased to 10 % (43 h), while the peak M + 2 decreased by about one half. Though the total emission strength decreased, the ratio of the labelled molecules over the

20 total molecules increased from 33 % to 64 %. During the second light and dark cycle, the changes of these peaks were similar as during the first circle with an overall decrease of ^{13}C labelling (represented by the peak M + 2).

3.4 Alternative carbon source: xylem transported glucose

During the exposure to ^{13}C glucose, the overall labelling of all compounds increased slowly and the time to reach steady state was much longer than that with ^{13}CO$_2$ feeding. The absolute emission fluxes of both de novo and pool emission showed

25 some decreases during the whole exposure period, probably because of the cutting. The data shown in Table 4 was taken shortly before the de novo emissions showed significant drops (48 h for S1 and S3, and 36 h for S2).

With feeding ^{13}C-glucose, the incorporation of ^{13}C was observed for all emitted terpenoids including β-pinene. Similar as observed with feeding ^{13}CO$_2$, the labelling ratios were quite low for the constitutively emitted monoterpenes and higher for the stress-induced emissions. Overall, the labelling degrees were lower than that during the ^{13}CO$_2$ experiments. For example,

30 (*E*)-β-farnesene was labelled with R_{iso_meas} = 0.65 ± 0.08 and R_{13C_meas} = 0.10 ± 0.02 (mean ± SD, n = 3) by the ^{13}C-glucose feeding. For the same compound during the ^{13}CO$_2$ feeding, we measured R_{iso_meas} = 0.82 ± 0.20 and R_{13C_meas}=

0.51 ± 0.26 (mean ± SD, n = 4, details see Supplement, Table S3–S6). The isotopic abundance patterns during the ^{13}C-glucose exposure also indicated that though we added fully labelled glucose via the xylem sap, the emissions were mainly labelled with one to four ^{13}C atoms for monoterpenes, and one to six ^{13}C atoms for sesquiterpenes (Supplement, Fig. S1). Highly labelled molecules containing n or n - 1 ^{13}C atoms were not observed.

5 **4 Discussion**

4.1 Different methods for separating de novo and pool emissions

According to the current knowledge, there are two different mechanisms of BVOC emissions: light- and temperature dependent emissions in parallel to biosynthesis without storage pools and temperature-dependent emissions from storage pools (Guenther et al., 2012). In principle, de novo and pool emissions can be distinguished regarding the way that the

10 respective compounds are stored within leaves (Grote and Niinemets, 2008). There are two major methods to separate de novo and pool emissions: (1) $^{13}CO_2$ labelling exposure (Shao et al., 2001; Ghirardo et al., 2010); (2) based on the different light responses, separation can be obtained by correlation analysis of day- and nighttime emissions or comparing daytime emissions with nighttime emissions made at elevated temperature (Helmig et al., 2007; Taipale et al., 2011).

We tested these two methods and the results indicated that both methods led to an underestimation of the fraction of de novo

15 synthesis, especially for sesquiterpenes.

The stress-induced emissions of monoterpenes and sesquiterpenes were pure de novo emissions because none of these compounds was released from the crashed needles and bark. Therefore, the fraction of de novo biosynthesis f_{synth} should be equal to 1. Nevertheless, none of these molecules was completely labelled even after 7–8 h of $^{13}CO_2$ exposure. Thus, f_{synth} calculated based on R_{iso_meas} from the $^{13}CO_2$ exposure gives a lower limit for the fraction of de novo biosynthesis.

20 Ghirardo et al. (2010) calculated f_{synth} of monoterpene mixtures based on R_{13C_meas} from $^{13}CO_2$ exposure. Different from R_{iso_meas}, R_{13C_meas} gives the ratio of ^{13}C-atoms over the total carbon and has always lower values than R_{iso_meas}. For example, the de novo (E)-β-ocimene emission from S1 was labelled by $^{13}CO_2$ with $R_{13C\_meas} = 0.61$ and $R_{iso\_meas} = 0.82$ (Fig. 3). In Ghirardo et al. (2010), they considered the uncomplete labelling of de novo monoterpenes by assuming that R_{13C_meas} of de novo monoterpenes is equal to that for the pure de novo isoprene emission, since monoterpenes share the

25 same carbon sources as isoprene (Delwiche and Sharkey, 1993; Loreto et al., 1996). We determined the values of R_{13C_meas} 0.62–0.77 for de novo monoterpenes and 0.13–0.68 for sesquiterpenes, indicating their assumption of equal R_{13C_meas} may not hold for other terpenoids than isoprene and monoterpenes. Rather, f_{synth} calculated using R_{iso_meas} is based on the fundamental assumption that all emitted molecules containing excess ^{13}C atoms during ^{13}C exposure must have been synthesized during the exposure. Such emissions therefore must be de novo emissions.

30 When comparing the diurnal variation of the emission fluxes to the release of the respective terpenoids from the crashed needles, we observed that several terpenoids were emitted during darkness from living plants but there was no significant

release from reservoirs. This demonstrates de novo emissions in darkness and shows some difference to the traditional classification: besides emissions from storage pools and de novo emissions with zero nighttime emission, there is another class of de novo emissions. The respective terpenoids can be synthesized and emitted in substantial amounts during darkness.

5   The nocturnal emission fluxes of the de novo emitted monoterpenes were relative low and up to 6 % of the daytime fluxes at a PPFD of 400–800 µmol m^{-2} s^{-1} with a temperature difference of 4–9 °C between day and night. For sesquiterpenes, the de novo nocturnal emission fluxes were much higher. For P2, they reached up to 58 % of the daytime fluxes, and for the other three individuals they ranged from 10 % to 20 % of the daytime fluxes. With the assumption that de novo emission fluxes are zero in the dark, such nocturnal emissions can easily be mistaken for pool emissions.

10 So far, the light intensity dependence of sesquiterpene emissions has not been clear (Sakulyanontvittaya et al., 2008; Guenther et al., 2012). Hansen and Seufert (2003) compared the prediction of short-term variations of β-caryophyllene emissions from orange trees (*Citrus sinensis* L.) with an algorithm accounting exclusively for temperature dependence and with another including both light and temperature dependence. Using the second algorithm they obtained better accordance to the experimental data. Helmig et al. (2007) studied the light intensity dependence of sesquiterpene emissions from a

15 loblolly pine tree (*Pinus taeda* L.) and found that the nighttime emissions under elevated temperature conditions were only slightly lower than the daytime emissions at similar temperatures, i.e., the light dependence was not significant. Similarly, nocturnal emissions of around 30 % of the daytime fluxes were observed for the herbivore induced sesquiterpenes from Norway spruce (Martin et al., 2003). These results together with our study strongly suggest that the light intensity dependence of sesquiterpene emissions is different from that described for isoprene and de novo emitted monoterpenes, and

20 may vary from individual to individual of the same species.

Obviously neither the results from labelling studies with ^{13}C nor the determinations based on light intensity dependencies alone allow clear separation into de novo and pool emissions. For a better clarification of the underlying mechanisms, we now discuss possible impacts of multiple carbon sources on the emissions and their possible regulation by different synthesis pathways.

25 **4.2 Multiple carbon sources for de novo synthesis**

The contributions of multiple carbon sources to isoprene biosynthesis are well discussed in previous studies (Kreuzwieser et al., 2002; Schnitzler et al., 2004; Ghirardo et al., 2011): carbon taken from atmospheric CO_2, xylem transported glucose and other leaf internal carbon pools, e.g. starch, can contribute together to isoprene biosynthesis and cause the uncomplete labelling of the emitted molecules. By feeding $^{13}CO_2$ to the plants and ^{13}C-glucose to their cut branches, we also obtained the

30 contributions of the different carbon sources for the de novo biosynthesis of monoterpenes and sesquiterpenes.

With feeding $^{13}CO_2$ (99 % ^{13}C), the de novo emitted monoterpenes 1,8-cineole, (*E*)-β-ocimene and linalool contained 62–77 % ^{13}C-atoms (details see Supplement Table S3–S6). This finding supports results from previous studies on isoprene and monoterpenes from deciduous species (Loreto et al., 1996; Kreuzwieser et al., 2002; Schnitzler et al., 2004; Ghirardo et al.,

2011), in which the contribution of freshly fixed CO_2 to the total emitted carbon was determined to 70–80 %. For sesquiterpenes, the contribution from the assimilated CO_2 was in the range of 13 % to 68 %, showing higher contributions from alternative carbon sources.

In principle, all the carbon atoms stored in alternative carbon sources originate from atmospheric CO_2. Depending on the
5 timescale for the replacement, the ^{13}C-atoms from $^{13}CO_2$ can be temporary stored in the respective alternative carbon sources. Such storage was found during our $^{13}CO_2$ labelling experiments, where ^{13}C-atoms were detected even several days after the exposure (e.g. Fig. 3). Hence, there must be at least a second carbon source, which was partly filled up with ^{13}C-atoms during the $^{13}CO_2$ exposure. This carbon source had an intermediate carbon turnover rate on the time scale of few days. Moreover, the completely unlabelled molecular peak M was observed for all de novo emissions (e.g. Fig. 4 and Fig. 5). This
10 indicates a third carbon source with a very slow carbon turnover rate. We therefore conclude that terpenoid synthesis in Scots pine and Norway spruce uses carbon atoms from sources with fast, intermediate and very slow carbon turnover rates.

Alternative carbon sources are suggested to be refixation of unlabelled respiratory CO_2 (Loreto et al., 2004), xylem-transported glucose (Kreuzwieser et al., 2002) or starch-breakdown (Schnitzler et al., 2004). The labelling during feeding ^{13}C-glucose showed that this well-known alternative carbon source provided ^{13}C-atoms for all tested terpenoids. Consistent
15 with previous studies on isoprene (Kreuzwieser et al., 2002; Schnitzler et al., 2004), we determined a maximal contribution of 10 % to the total carbon emitted as monoterpenes and sesquiterpenes.

4.3 Different biosynthesis pathways of monoterpenes and sesquiterpenes

As mentioned above, the contribution of alternative carbon sources to sesquiterpene synthesis were higher than that to de novo emitted monoterpenes. Meanwhile the sesquiterpene emission fluxes in darkness were also found to be higher than
20 those of the monoterpenes. These observations raise the question whether there are different pathways for the synthesis of monoterpenes and sesquiterpenes, which are supported differently by different carbon sources and regulated differently by environmental conditions, such as light intensity.

It is well known that all terpenoids are derived from C_5 isoprene building units dimethyl-allyl-pyrophosphate (DMAPP) and its isomer isopentenyl pyrophosphate (IPP). In higher plants, there are two independent pathways for the DMAPP formation:
25 the mevalonic acid (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway (Lichtenthaler et al., 1997b). They have different subcellular localizations, begin with different precursors, and have different regulation mechanisms (reviewed by Dudareva et al. (2013)).

The MEP pathway is exclusively plastidic and formed from glyceraldehyde-3-phosphate, an intermediate in the photosynthetic carbon reduction cycle (Calvin cycle), and pyruvate, which can be formed within the plastid from 3-
30 phosphoglyceric acid (Lichtenthaler et al., 1997a). It is thus not surprising that the synthesis of isoprene, which is considered to originate mainly from the MEP pathway (Lichtenthaler et al., 1997a), is closely related to photosynthesis and regulated by light intensity (Delwiche and Sharkey, 1993; Sharkey and Yeh, 2001). The light intensity dependence of isoprene synthesis is explained by light-dependent activation of terpenoid synthesis and/or activation of the MEP pathway providing

chloroplastic DMAPP (Fall and Wildermuth, 1998; Lehning et al., 1999). By contrast, the MVA pathway is extrachloroplastic and distributed between cytosol, endoplasmic reticulum and peroxisomes; it is derived from acetyl-CoA, whose origins are distinct in different subcellular compartments (Oliver et al., 2009; Dudareva et al., 2013) and the nature of its regulatory mechanisms remains largely unknown.

5   It was previously assumed that monoterpenes use the DMAPP through the MEP pathway, while sesquiterpene synthesis is from the DMAPP synthesized from the MVA pathway (McGarvey and Croteau, 1995; Lichtenthaler et al., 1997a). Recent studies report that monoterpenes and sesquiterpenes share precursors from both the MEP and the MVA pathways (Karl et al., 2002; Loreto et al., 2004; Dudareva et al., 2005; May et al., 2013). With $^{13}CO_2$ labelling study on *Populus nigra* L. and *Phragmites australis* L., Loreto et al. (2004) proved that feeding with $^{13}CO_2$ brings high labelling of the chloroplastic

10   DMAPP but low labelling of the extrachloroplastic DMAPP. By adapting all these findings to the investigated conifers, it should be possible to use the results from $^{13}CO_2$ labelling to distinguish between the DMAPP synthesized through different pathways.

In our study, the labelling patterns of de novo emitted monoterpenes were similar for all individual plants and showed a multimodal distribution (Fig. 4c&d). It was dominated by the highly labelled peaks synthesized from the highly labelled

15   DMAPP. There was a good agreement between the patterns of the highly labelled molecular peaks and the predicted isotopic abundance pattern of a C_{10} compound with a ^{13}C abundance of 90 % (Fig. 1, blue bars). Hence, the DMAPP used for the synthesis of these highly labelled molecules was very likely from the biosynthetic pathway closely related to photosynthesis, i.e. the MEP pathway. Meanwhile, the whole labelling pattern also included the completely unlabelled molecules as well as molecules labelled with less ^{13}C-atoms ($i \leq 6$). These were clearly from the DMAPP not closely related to photosynthesis.

20   The DMAPP used for the synthesis of the less labelled molecules was very likely formed through the MVA pathway, rather than the MEP pathway. Our observation on the monoterpene labelling was consistent with the study of Loreto et al. (2000) reporting that α-pinene emission from *Quercus ilex* L. is mainly but not exclusively formed from the DMAPP directly originating from photosynthesis intermediates.

The labelling patterns of sesquiterpenes had higher variability than that of monoterpenes. For P2 (Fig. 5b), highly labelled

25   molecular peaks were missing and most of the molecules were labelled with one to six ^{13}C-atoms. This labelling pattern reflects that sesquiterpenes can be synthesized exclusively by the alternative carbon sources with a low ^{13}C abundance and very likely through the MVA pathway. Nevertheless, the isotopic abundance patterns of S1 (Fig. 5a), S2 and P1 showed a multimodal distribution. The pattern of the highly labelled molecular peaks reflected a similar ^{13}C abundance of 90 % as that observed for monoterpenes, indicating that sesquiterpenes can also share the DMAPP derived mainly from photosynthesis

30   and through the MEP pathway. Our observation is well supported by the studies reporting that sesquiterpene synthesis is usually formed from the cytosolic MVA-derived DMAPP, but in some cases, also share the precursors from both pathways (Dudareva et al., 2005; Hampel et al., 2005). The higher contribution from the MVA pathway for sesquiterpene synthesis can explain the higher contribution from alternative carbon sources compared to monoterpene synthesis. It also can explain

that among all plants, the sesquiterpene emissions from P2, where it was predominantly synthesized from the MVA pathway, had the lowest $R_{13C\_meas} = 0.15$ during the $^{13}CO_2$ exposure.

With its extrachloroplastic localization and more diverse carbon sources, the different response of the MVA pathway to the light intensity is understandable. The higher contribution from the MVA pathway explains that the sesquiterpene emissions from P2 had the highest ratios of darkness emissions to daytime emissions. The values ranged from 0.50 to 0.58 and were much higher than those of the sesquiterpenes emitted from other plants where the ratios were from 0.10 to 0.20. It can also explain the increase in the degree of labelling of (E)-β-farnesene emissions during darkness (Fig. 6). In this case, the MEP pathway was limited by the low delivery of the carbon atoms from photosynthesis. Hence, the de novo synthesis of sesquiterpenes could only rely on the pathway supported by the alternative carbon sources which was still partly labelled because of the preceding $^{13}CO_2$ exposure. As the unlabeled fraction through the MEP pathway decreased, the degree of labelling increased.

4.4 Comparison of the stress-induced and constitutive emissions

In our study, 0.1 % to 2 % of the carbon atoms fixed by the tested plants during photosynthesis were emitted back into the atmosphere as constitutive emissions. The additional stress-induced emissions increased the total emissions and therewith the carbon loss up to 6 % (details see Supplement, Table S1).

Based on the missing release of the stress-induced terpenoid emissions from reservoirs in needles and bark (Table 2) and f_{synth} calculated based on the $^{13}CO_2$ labelling exposure (Table 3), the stress-induced emissions were assigned to the class of de novo emissions. This conclusion agrees with other studies on stress-induced emissions (Loughrin et al., 1994; Pare and Tumlinson, 1997; Martin et al., 2003; Brilli et al., 2009) wherein it is shown that insect induced terpenoid emissions as well as induced emissions from the shikimate pathway are mainly de novo emissions. In contrast, the constitutive monoterpene emissions from both conifers had much lower de novo fractions (Table 3) with values quite consistent to the empirical light dependent factor (LDF) given by Guenther et al. (2012) with values ranging from 0.05 to 0.1 for most monoterpenes.

For the fraction of de novo synthesis of the total terpenoid emissions from conifer species, various results have been reported in previous studies. In Tarvainen et al. (2005), most of monoterpenes (except 1,8-cineole) from Scots pine followed well with temperature dependence, i.e. they are mainly pool emissions. Taipale et al. (2011) give the contributions of de novo emissions to the total emissions from a boreal Scots pine dominated forest between 30 % and 46 %. Ghirardo et al. (2010) report fractions of de novo monoterpene emissions of 33 % from Norway spruce and of 58 % from Scots pine. In all these studies, the constitutive and the stress-induced emissions were not separated which might cause arbitrary and therefore inconsistent results. For example, the average f_{synth} calculated for all constitutive monoterpene emissions from S1 (sum of α-pinene, β-pinene, camphene, limonene, and myrcene) was 15 %. Including the two stress-induced monoterpene emissions, linalool and (E)-β-ocimene, the average f_{synth} increased to 59 %. The stress-induced emissions can strongly increase the fraction of de novo biosynthesis of the total emission mixtures.

5 Summary

Depending on the plants' living conditions, the stress-induced emissions can contribute largely to the total terpenoid emissions from conifer species (Fig. 2) (Blande et al., 2009; Bouvier-Brown et al., 2009; Bourtsoukidis et al., 2014). They play a crucial role in plant signaling and plant defence against stresses, especially herbivore attacks (Kessler and Baldwin,
5   2001; Holopainen, 2004). They cause higher release of carbon back into the atmosphere and thus strongly affect the carbon balance of ecosystem. The stress-induced emissions are also important for secondary organic aerosol formation (Mentel et al., 2013; Bergström et al., 2014) and cloud droplet formation (Zhao et al., 2017). Thus, they also influence air quality and climate.

Different from the constitutive emissions from conifers, which had a big fraction from pool storage, we determined that the
10   stress-induced emissions are mainly from de novo biosynthesis. Thus, it is imperative to introduce the fraction of de novo biosynthesis for predicting the emission fluxes from conifer forests, especially when the stress-induced emissions are dominant.

We further investigated the regulation of de novo synthesis, and the contributions from multiple carbon sources. A second class of de novo emissions was observed. The respective terpenoids were also synthesized during darkness and the regulation
15   mechanisms were different from the classic light-dependent de novo emissions. Though the nocturnal de novo emissions of monoterpenes were not very high, they were substantial for sesquiterpenes. Such emissions of sesquiterpenes in darkness seem to be linked with the alternative carbon sources with slow carbon turnover rates and with the MVA synthesis pathway.

During the past decade, studies of plant terpenoid emissions have been focused on the MEP pathway with neglecting the role of the MVA pathway (Hemmerlin et al., 2012). So far, empirical algorithms (Guenther et al., 1993; Guenther et al., 1995;
20   Guenther et al., 2006; Guenther et al., 2012) as well as process-based models (Niinemets et al., 1999; Arneth et al., 2007) have mainly focused on constitutive isoprene and monoterpenes, which mainly originate from the MEP pathway. Our finding of the substantial contributions of alternative carbon sources to monoterpene and sesquiterpene synthesis that probably are synthesized through the MVA pathway shows that this pathway should not be neglected. However, the current understanding of the regulation of the MVA pathway is still quite limited, and requires further investigation (Dudareva et al.,
25   2013). A better understanding of the mechanisms of emission and regulation is not only important for simulating the diurnal variation in terpenoid emission fluxes but also essential for predicting the different responses of terpenoid emissions to changing environmental conditions.

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Table 1: List of symbols and abbreviations.

C_{M+i}	Count rate of molecular peak M + i
P_{i_meas}	Measured relative ion intensity of molecule M + i
R_{iso_meas}	Measured ratio of labelled molecules over total molecules
R_{13C_meas}	Measured ratio of ^{13}C atoms over total carbon atoms
R_{13C}^{j}	^{13}C abundance of carbon source j
P_{i_cal}	Calculated ion intensity of molecule M + i
f_{synth}	Fraction of de novo biosynthesis

5 **Table 2: Ratios of nighttime (needle temperature = 25 °C) to daytime (needle temperature = 31 °C) emissions of the individual compounds from S1 and their normalized emission fluxes from the living plant (daytime) and from the crushed needles and bark relative to β-pinene. Data were taken after the crushed needles had been in the dark for 8 h.**

Compound	C/S[a]	Type[b]	Nighttime-/daytime emission fluxes	Normalized emission fluxes[c]	
				Living plant	Needles and bark
isoprene	C	HT	0.03	1.8	< DL
α-pinene	C	MT	0.37	0.9	0.4
camphene	C	MT	0.21	0.7	0.1
β-pinene	C	MT	0.45	1.0	1.0
myrcene	C	MT	0.11	1.8	0.03
limonene	C	MT	0.25	6.2	0.3
(*E*)-β-ocimene	S	MT	0.03	1.2	< DL
linalool	S	MT	0.06	15	< DL
(*E*)-β-farnesene	S	SQT	0.18	215	< DL
α-farnesene	S	SQT	0.18	157	< DL

[a] C = constitutive emissions; S = stress-induced emissions
[b] HT= hemiterpene; MT = monoterpene; SQT = sesquiterpene
[c] The normalization was conducted by dividing the release of individual compounds by the release of β-pinene.

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Table 3: Fraction of de novo biosynthesis (f_{synth}) of the major compounds from spruces and pines.

Compounds	C/S[a]	P1	P2	P3	S1	S2	S3
α-pinene	C	0.18	0.02	0.03	0.06	0.05	0
β-pinene	C	[b]	0.02	0.03	0.02	0.01	0
camphene	C	0.13	0.01	0.01	0.02	0.02	0
limonene	C	0.41	-	-	0.09	0.03	0
myrcene	C	[b]	0.18	0.54	0.49	-	0.02
Δ^3-carene	C	0.04	0.01	0.01	-	0.06	0.01
β-phellandrene	C	0.21	0	-	-	0.02	-
1,8-cineole	C	0.92	0.86	0.84	-	-	-
(E)-β-ocimene	S	-	-	-	0.79	-	-
linalool	S	-	-	-	0.83	-	-
α-farnesene	S	0.95	0.45	-	0.91	0.87	-
(E)-β-farnesene	S	0.97	0.55	-	0.92	0.82	-

[a]C = constitutive emissions; S = stress-induced emissions
[b]For P1, the peaks of β-pinene and myrcene from the chromatogram were overlapping, and f_{synth} = 0.45 for the sum of these two compounds.

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Table 4: R_{13C_meas} and R_{iso_meas} of individual compounds during ^{13}glucose exposure. Data are expressed as mean ± SD (n = 3).
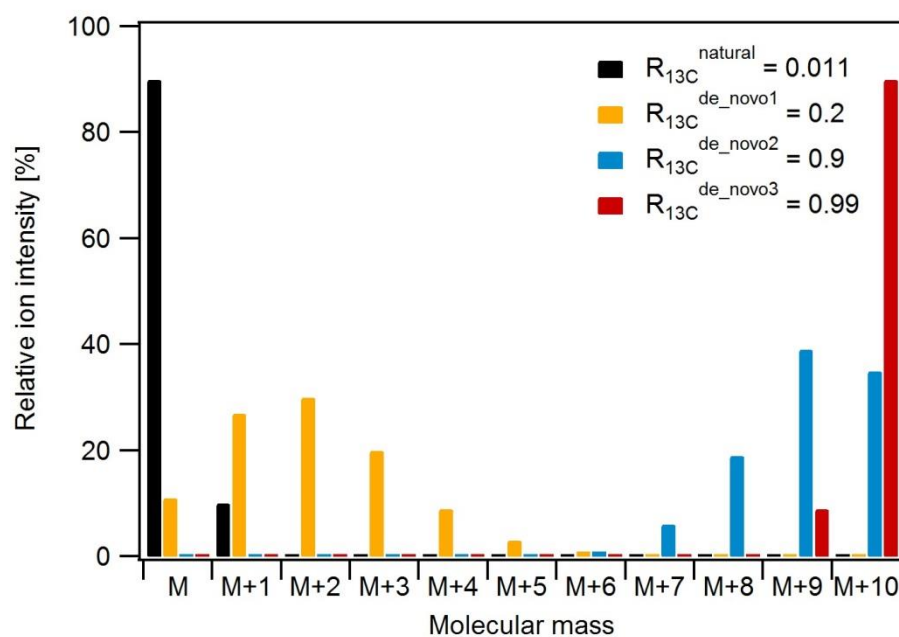
Compounds	R_{iso_meas}	R_{13C_meas}
α-pinene	0.27 ± 0.14	0.05 ± 0.03
camphene	0.14 ± 0.04	0.02 ± 0.01
β-pinene	0.13 ± 0.01	0.02 ± 0.01
myrcene	0.26 ± 0.15	0.05 ± 0.03
limonene	0.14 ± 0.05	0.03 ± 0.02
α-farnesene	0.63 ± 0.11	0.10 ± 0.03
(E)-β-farnesene	0.65 ± 0.08	0.10 ± 0.02

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Fig. 1: Simulated isotope abundance patterns of a C_{10} compound with $R_{13C}^{natural}$ = 0.011 (black bars), $R_{13C}^{de\_novo1}$ = 0.2 (yellow bars),
$R_{13C}^{de\_novo2}$ = 0.9 (blue bars) and $R_{13C}^{de\_novo3}$ = 0.99 (red bars).

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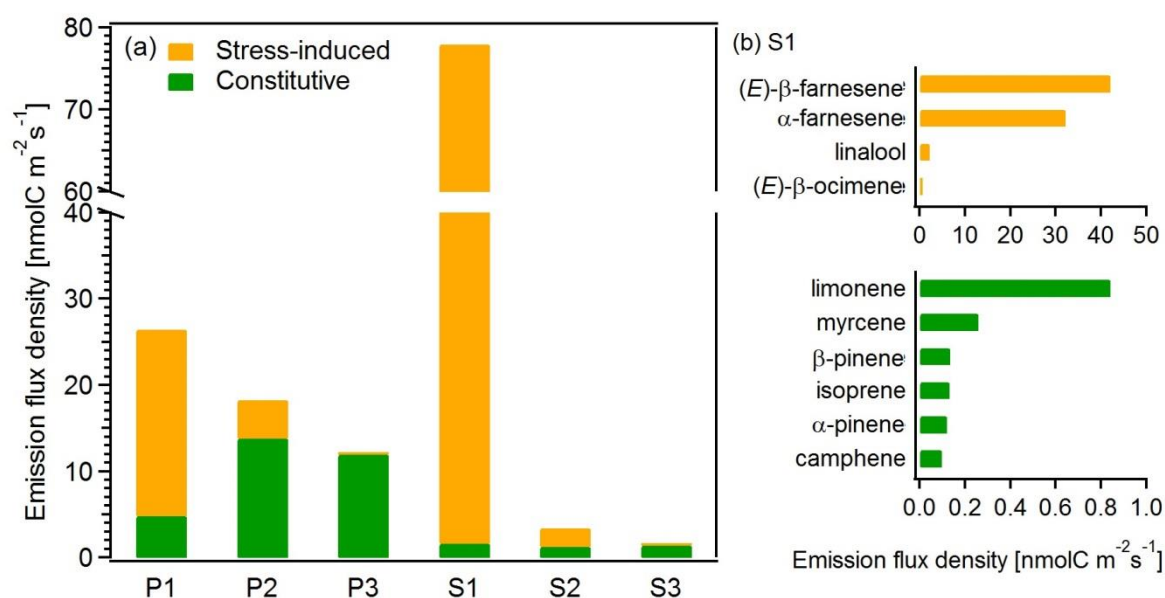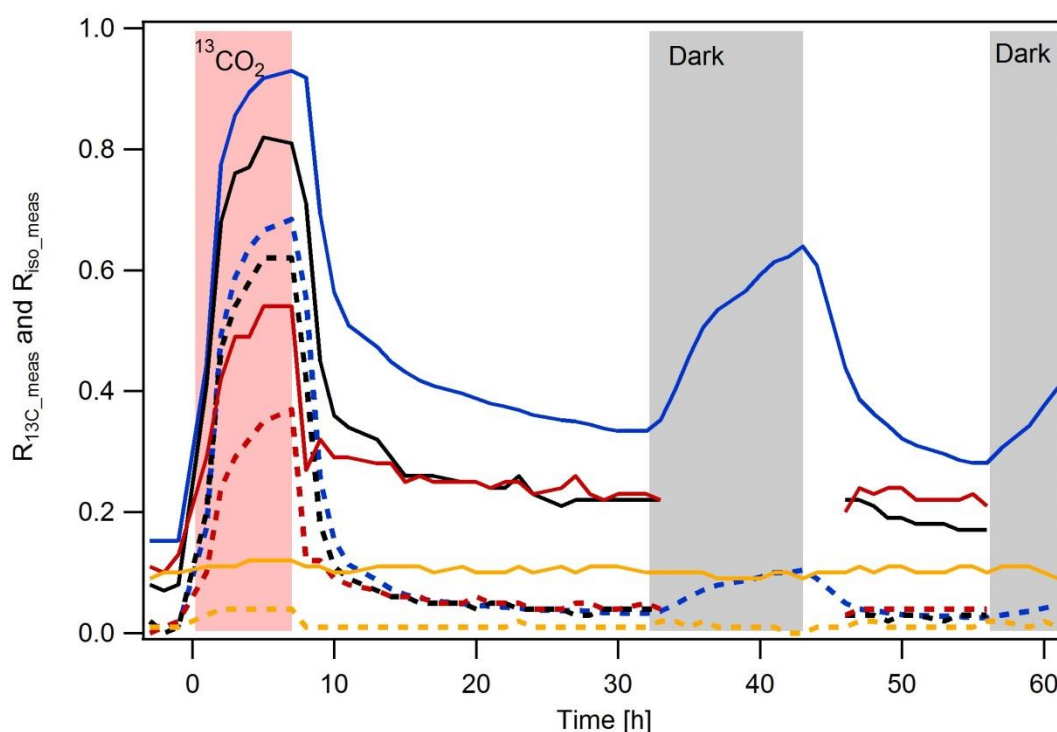20

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Fig. 2: (a) Emission flux densities of the total constitutive (green bars) and stress-induced emissions (yellow bars) from individual plants and (b) composition of the emissions from S1 at a light intensity of 700 μmol m^{-2} s^{-1} and a chamber temperature of 25 °C.

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Fig. 3: Temporal shapes of R_{iso_meas} (solid lines) and R_{13C_meas} (dashed lines) for β-pinene (yellow), myrcene (red), (*E*)-β-ocimene (black) and (*E*)-β-farnesene (blue) emitted by S1. The time of starting the $^{13}CO_2$ exposure was defined as time 0. The red shaded area shows the time period of the $^{13}CO_2$ exposure; the grey shaded areas represent the periods of darkness; and the white background shows the time periods when the normal CO_2 with the natural ^{13}C abundance was added.
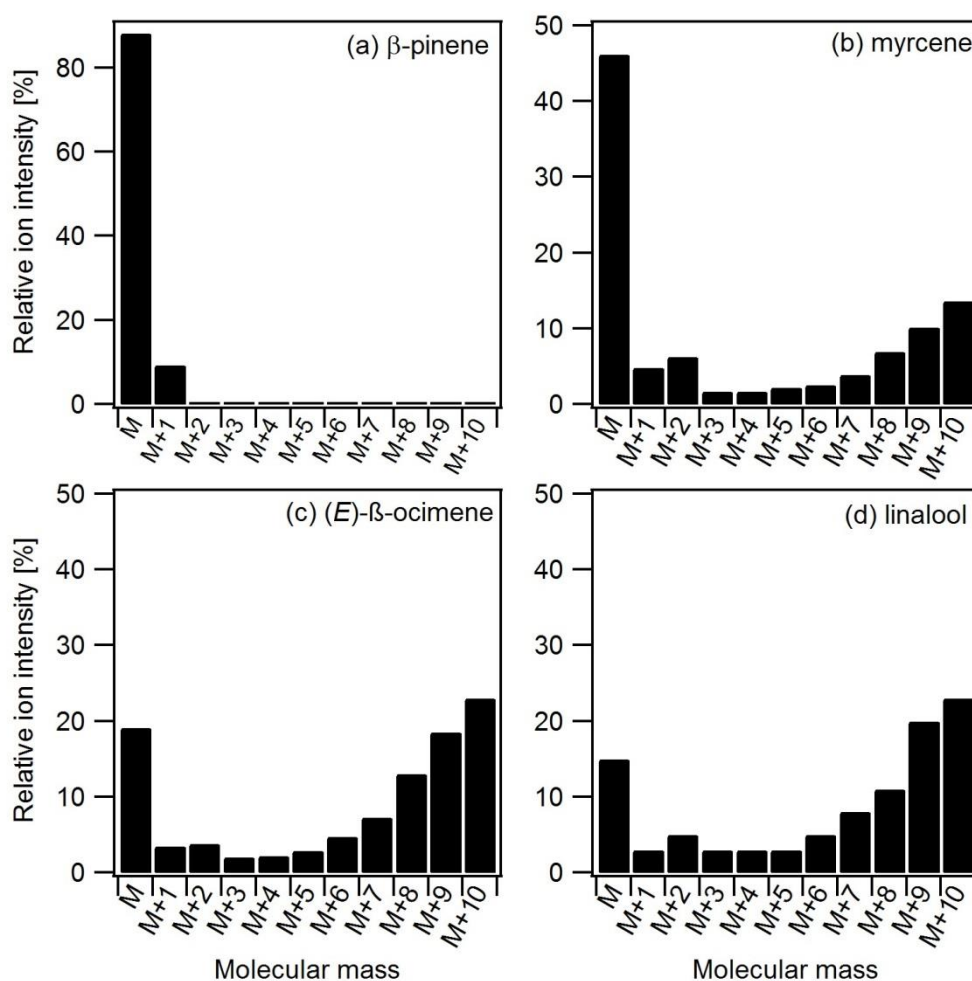
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Fig. 4: Measured isotope abundance patterns of (a) β-pinene, (b) myrcene, (c) (E)-β-ocimene, (d) linalool from S1. The plotted data were taken shortly before the end of the $^{13}CO_2$ exposure of S1 (7 h in Fig. 3) when the labelling ratios were close to steady state.
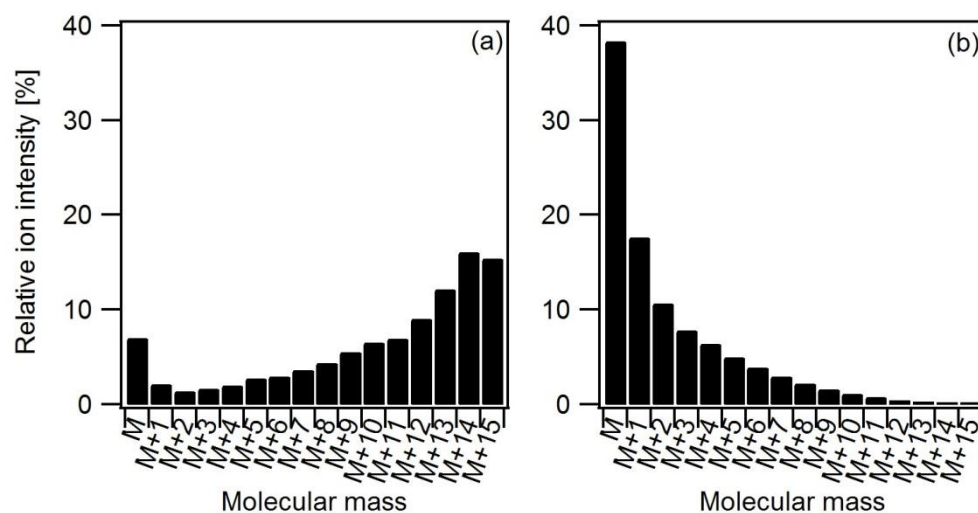
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Fig. 5: Measured isotope abundance patterns of (*E*)-β-farnesene from (a) S1 and (b) P2 at the end of the $^{13}CO_2$ exposure. The plotted data were taken shortly before the end of the $^{13}CO_2$ exposure when the labelling ratios were close to steady state.
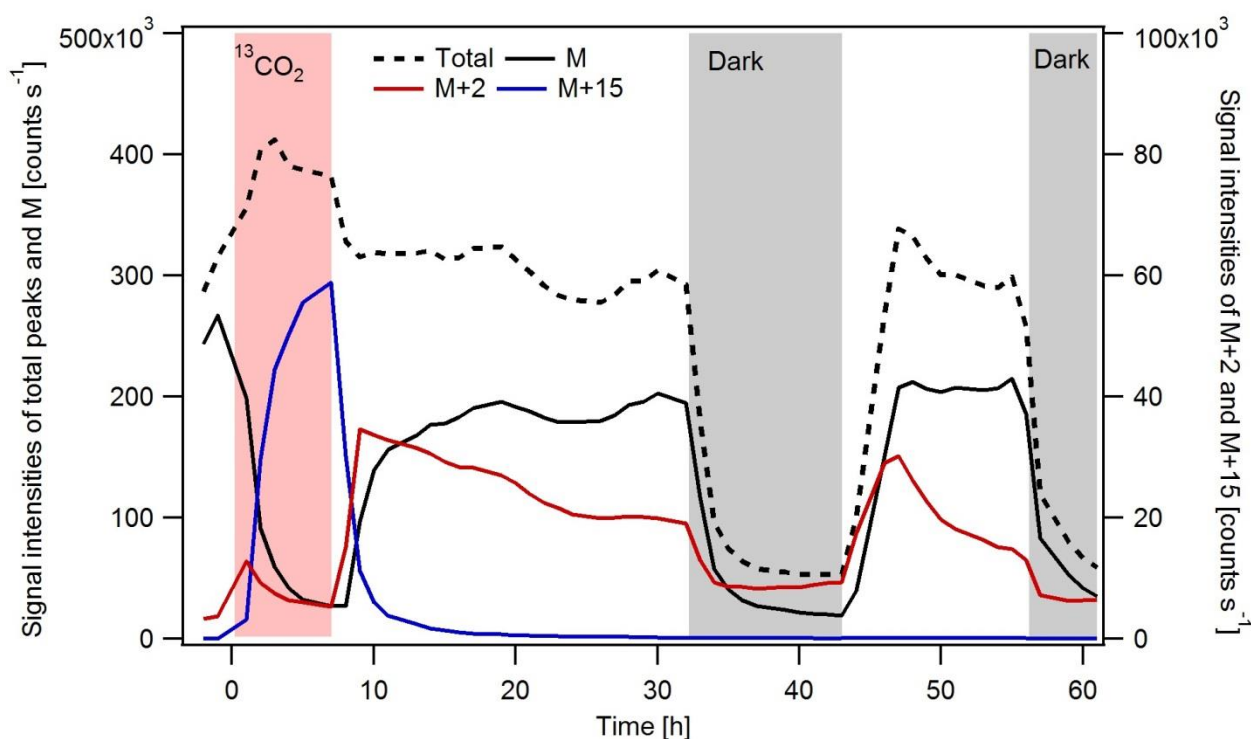
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Fig. 6: Temporal changes of the signal intensity of (left y-axis) total peaks, the peak M, (right y-axis) the peak M + 2 and the peak
10 **M + 15 of (*E*)-β-farnesene from S1 during and after $^{13}CO_2$ exposure. The time of starting the $^{13}CO_2$ exposure was defined as time 0. The red shaded area shows the time period of $^{13}CO_2$ exposure; the grey shaded areas represent the periods of darkness; and the white background shows the time periods when CO_2 with the natural ^{13}C abundance was added.**

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