# *$^{13}$C labelling study of constitutive and stress-induced terpenoid emissions from Norway spruce and Scots pine by Cheng Wu et al.*

## *Final response of the authors*

We would like to express our appreciation to reviewer #2 for her/his efforts and for many valuable comments and suggestions. We have carefully addressed all the comments. The corresponding changes and refinements made in the revised paper are summarized in our response below.

To allow easy discrimination between the reviewer's remarks and our responses, the remarks are set in bold. When citing the manuscript, text is written in italic. All page- and line numbers of the new text are based on the revised paper.

**Anonymous Referee #2**

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**In the present work the authors aimed to contribute to the understanding of the origin carbon ending up in the emission of volatile terpenoids in conifers, i.e. Scots pine and Norway spruce. In particular the authors aimed to separate constitutive emissions coming from storage pools (resin ducts) in needles and bark tissue from constitutive and stress-induced emissions originating from recently fixed carbon intermediates (de novo). Studying the carbon origin of isoprene and monoterpene emission not novel. It started in the 90ties of the last century and was driven by the interest in understanding the link between light-dependent isoprene and monoterpene emission of trees with no distinct storage structure for terpenoids. For understanding the origin of carbon ending up in terpenoid emissions the situation in conifers is rather complex due to the presence of these molecules in storage structure in needles and bark tissue where the can be released by temperature-dependent evaporation of mechanical destruction of resin reservoirs. Hence only a few studies on this topic exists. The present work uses 13CO2 and 13C-glucose feeding to determine the amount of 13C in monoterpenes and sesquiterpenes released from needles (bark) of tree coincidentally infested by insects and pests when grown under natural conditions. This arbitrary selection of plant material resulted in an individual response pattern of each plant reflected in different emission rates and pattern of i.e. stress-induced mono- and sesquiterpenes. Despite this limitation in homogeneity the experiments were performed with high accuracy enabling some conclusions about the carbon origin in terpenoids. Overall, the data are not novel, nevertheless adding new and welcome information on the origin of terpenoid compounds in the emission conifers. Understanding of this trait is still of great importance for the development of new/better emission algorithms to be implemented into BVOC emission models.**

**Overall the work is technically performed very well and the methods are comprehensively described. In particular the description of the calculation of 13C incorporation into terpenoid compounds is important to understand and interpret the results. However, due to the uncontrolled treatment /plant cultivation and hence the**

**very individual response of each plant, overall conclusions on the fractions of constitutive and de novo-synthesized terpenoid cannot be drawn seriously, weakening strongly its relevance to the field.**

Our response with respect to the selection of plants:

We used plant individuals stored at natural conditions. Only when studying plants stored at natural conditions it is possible to obtain data relevant with respect to the field. This "uncontrolled" plant cultivation allowed finding the variability of the plant's responses but nevertheless the systematics in the plant's behaviour:

We looked at basic mechanisms such as de novo emissions during darkness or different carbon sources for BVOC biosynthesis. We found evidence for such mechanisms from all plants and we also show the variability of the plant's behaviour (e.g. stress-induced emissions). We are convinced that the relevance of our data to the real environment is enhanced by using plants grown under natural emissions. The generality of our results would be much lower if we would have used only plants grown up under artificial conditions.

**Specific comments:**

**Concerning night emissions of de-novo synthesized mono- and sesquiterpenes. I agree that is very interesting and not well documented. However, not surprising: E.g. In some floral tissues (see e.g. papers of Dudareva and colleagues) sesquiterpene emissions peak during night, indicative of a highly active MVA pathway, which is circadian regulated. Also monoterpene biosynthesis in roots and during resin duct formation in wood is not depending on light. Moreover, many sesquiterpene synthases are bifunctional, either using C10 or C15 precursors depending on substrate availability. Gene expression analyses show that MVA pathway genes are more expressed during night while transcription of MEP pathways genes is higher during the light phase. There are many indications that the MVA pathway is more active during darkness, while the MEP pathway mostly works in the light. Your discussion is very general. Please check more actual literature on that issue. I agree that the labeling of sesquiterpenes with 13C is more variable probably due to the multiple sources of carbon ending up in the cytosol, compared to the situation in the plastids, therefore the light-dependency of sesquiterpene emission cannot be as tighly linked to photosynthesis as is the case for de novo synthesized monoterpenes. The regulation of the MVA pathway is not widely unknown. Please check literature and update your discussion.**

Our response: We agree and we therefore added a more detailed discussion of the regulation of the MVA pathway in Section 4.3 (page 14, lines 22–26).

Instead of

"*... subcellular compartments (Oliver et al., 2009; Dudareva et al., 2013) and the nature of its regulatory mechanisms remains largely unknown*",

the new text reads:

"*... subcellular compartments (Oliver et al., 2009; Dudareva et al., 2013). Different from the isoprenoid synthesis through the MEP pathway, the isoprenoid synthesis through the MVA pathway is negatively affected by light, since light is responsible for the downregulation of rate-controlling enzyme of the MVA pathway, HMG-CoA reductase (HMGR), and the most*

*other MVA pathway enzymes (Rodríguez-Concepción et al. 2006; Vranová et al. 2013). Thus, the MVA pathway is not tightly linked to photosynthesis.*"

**p.14 line 31: must be MVA derived IDP (isopentyl pyrohosphate) and DMAPP**

Our response: Thank you for the hint. This is now corrected and the new sentence reads (page 15, line 21): *"from the cytosolic MVA-derived IPP and DMAPP"*. We also substituted *"IPP and DMAPP"* for *"DMAPP"* in other places when *"DMAPP"* was mentioned.

**P15 line 26/27: In Taipale et al 2011 and Ghirardo et al. 2010, no stress-induced emissions were observed. Therefore, they couldn't be taken into account in these studies.**

Our response: Whether or not Taipale et al. (2011) observed stress-induced emissions is not clear. Their measurements in a Boreal Scots pine forest were made with a PTR-MS that cannot distinguish different monoterpenes such as the constitutively emitted $\alpha$-pinene and the stress-induced emission (*E*)-$\beta$-ocimene. Thus, we deleted this reference.

In Ghirardo et al. (2010), the emission patterns were shown. Most of the monoterpene emissions from Scots pine and Norway spruce were constitutive emissions, but with a small fraction of stress-induced ocimene emissions for Norway spruce. Therefore, we still took Ghirardo et al. (2010) for the comparison with the constitutive monoterpene emissions.

Meanwhile, there are two very new manuscripts reporting on labelling results (Lüpke et al. 2017) and field measurements (Wang et al., 2017). We also used these new results for comparison now.

Instead of

"*... 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."*,

the new text reads (page 16, lines 10–28):

"*... mainly de novo emissions.*

*In contrast, the constitutive monoterpene emissions from both conifers had much lower de novo fractions than the stress-induced emissions (Table 3). There are several other laboratory $^{13}C$ labelling studies reporting the fraction of de novo biosynthesis of constitutive monoterpenes: Ghirardo et al. (2010) report fractions of de novo monoterpene emissions of 58 ± 4 % from Scots pine (all constitutive) and of 34 ± 8 % from Norway spruce (mainly constitutive with a small fraction of stress-induced ocimenes). Lüpke et al. (2017) measured the fraction of de novo biosynthesis for individual compounds from Scots pine seedlings and the values were 37 ± 5 %, 9 ± 4 %, 32 ± 12 %, and 85 ± 4 % for α-pinene, β-pinene, limonene and myrcene, respectively. Our values were somewhat lower than reported in these studies. Reasons therefore maybe plant to plant variations, the differences in the growing conditions or the different ways how these fractions were calculated.*

*However, as shown in Table 3, even for the same compound, the fraction of de novo biosynthesis can vary a lot. For example, the range of the fractions was 0 – 18 % and 0 – 41% for α-pinene and limonene, respectively. Such range is much wider than those obtained in the labelling studies using plants cultivated under controlled conditions. From field measurements, the variability observed for fractions of de novo emissions is even wider than that found in our laboratory studies: In Tarvainen et al. (2005), most of constitutive monoterpene emissions (except 1,8-cineole) from Scots pine growing in a natural forest environment followed well with temperature dependence, i.e. they were mainly pool emissions. However, using their results from field measurements with Norway spruce Wang et al. (2017) showed that the contribution of the de novo emissions to the constitutive emissions was higher than 50 % throughout all the months from June to September with almost 100% in June.*

*Overall, considering the different contributions of de novo fractions to constitutive and stress-induced emissions, the stress-induced emissions can strongly increase the fraction of de novo biosynthesis of the total emission mixtures.*".

**There are a few more studies giving a ration between constitutive and de novo based emissions in conifers. The values in the present work are somehow lower (excluding the stress-induced emissions). Do you think it can be related to your culture conditions and the time analysis? It might be that the expression of terpene synthases responsible for the constitutive de novo emissions are highly variable and therefore the values are more scattering and lower compared to other studies. Please be more precise in your discussion.**

Our response: Thanks for the hint. There may be a lot of reasons for such different ratios and wide ranges, such as plant to plant variations, the mentioned differences in the growing conditions or the different ways how these fractions were calculated (see the above updated discussion).

**Table 4: Please explain in the legend the abbreviation of RC-meas and Riso_meas**

Our response: We followed the reviewer's suggestion and the text now reads: "*Ratios of $^{13}C$-atoms over total carbon atoms ($R_{13C\_meas}$) and ratios of labelled molecules over all molecules ($R_{iso\_meas}$) of individual ...*".

**Figure 1: Please make the legend more explicit describing that the scenarios 1 and 2 reflect the bi-modular overlay of unlabeled (likely from storage or old carbon) and completely de novo synthesized compounds.**

Our response: We add more words and it now reads: "*... of a $C_{10}$ compound synthesized from the carbon atoms with the naturel labelling abundance, $R_{13C}^{natural} = 0.011$, and from the carbon atoms with three elevated labelling abundances, $R_{13C}^{de\_novo1} = 0.2$, $R_{13C}^{de\_novo1} = 0.9$ and $R_{13C}^{de\_novo1} = 0.99$.*".