^{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 #1 for her/his efforts and for 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 #1

This study investigates de-novo and pool emissions of different terpenes in response to biotic stress. In general terpenoid emissions induced by biotic stress are a very important, timely and interesting topic and it is a good idea to investigate if and how de-novo and pool emissions differ in response to biotic stress. However my major concerns are that there are only three trees per species which are all at different states of healthiness before the start of the measurements. One of each species seemed to be healthy one per species infested with aphids, one of the trees seemed to be disturbed by spiders (which was attributed to stress), and for one of the trees white floccules were attributed to stress. This leads to a n=1 for each treatment and species. This point together with different climatic conditions within the chambers during measurements (e.g. different temperatures) makes the paper very problematic. Without replicates, information on the severity of stress, and without comparable conditions during the measurements I don't see in which way this paper would increase our understanding how trees respond to biotic stressors with de-novo and pool emissions. There is no information about tree to tree variability, the severity of stress, how the trees behaved before stress, and how different temperatures or seasonality during the measurements (taken between May and October) could have biased the measurements (additionally to biotic stress) and a proper statistic alanalysis is not possible. Additionally the stress response to biotic stressors is known to be dependent on severity of stress (Niinemets et al., 2013). Although more quantitative information about stress responses due to biotic stress is required to increase the scientific understanding the manuscript in the current state is unfortunately not able to deliver defendable information.

Our response:

In principle, we understand the concerns of reviewer #1 regarding statistics. However, they are not applicable in the context of our implications. We do not quantify stress responses, but address common mechanisms underlying BVOC emissions for several, different situations i.e. we investigated the basic mechanisms of BVOC emissions. We did this by labelling experiments and we made 6 exposures with $^{13}CO_2$ to different plants each lasting for several

hours. These basic mechanisms are termed as basic mechanisms because they hold independent of stresses, independent of temperature and independent of time of the year.

As an example: the definition of pool emission and de novo emission is not restricted to a certain temperature, infestation or seasonality. The same is true for our statement that different carbon sources contribute to BVOC synthesis and for our claim that there is biosynthesis and de novo emission during darkness. We made neither statements nor conclusions on specific impacts of temperatures, specific injuries, or stresses on these basic mechanisms. In contrast, the fact that the basics we found hold independent of plant-external variables clearly shows that we investigated such basics.

Major comments:

(1) There is no quantitative information about the severity of stress, how long the trees were influenced by biotic stress and how spiders or white floccules would influence stress. This makes it difficult to judge how severe stress was.

Our response: The basics of a pool emission being a pool emission, a de novo emission being a de novo emission as well as a stress-induced emission being a stress-induced emission is independent of the severity of the stress. There is no quantitative information on the severity of the stress needed on the basic level of our results.

(2) In principle the complete information given in this paper is based on one tree n=1 (one healthy tree, one infested with aphids, and one suffering from some other potential biotic stressor) and there are no time-replicates later on or before stress. Consequently it is not known how the infested trees were behaving before they were exposed to aphids (or other potential biotic stressors) and if there were potential differences between trees already before stress.

Our response: As we responded above, it is not our intention to quantify impacts of stress on the investigated basics. We only show that these basic mechanisms can be observed for a variety of plants grown up under natural conditions and with different amounts and types of stress-induced emissions.

(3) The experimental description is very short and important information is missing. There is neither information on temperature variations within the climate chambers nor if and how temperatures within the plant chambers were measured or kept constant. Additionally information about relative humidity or CO_2 within the chambers is not given. Also the time of measurement of single trees would be important. According to Table S1 temperatures during the measurements differed considerably (between 18°C and 30°C) for different trees. With the information currently given it is not possible to judge if changes between the trees are, at least partially, due to different climatic conditions, seasonality or history. At least the essential information to understand what was done and how this possibly affected the trees should be transparent.

Our response: We thank referee #1 for requesting some information which were indeed missing. We added necessary information concerning the temperature variations with in the

climate chambers, the way of measurement, relative humidity and CO_2 within the chambers in the first paragraph of Section 2.3 (page 4, lines 13–19). The new text reads:

"*... and 18–30 °C, respectively (details see Supplement Table S1). CO_2 concentrations at plant chamber outlet were kept at levels of 330–380 parts per million (ppm). Relative humidity was kept about 40–60 %. 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. At constant light intensity the temperature in the climate chambers was stable to ± 0.5 °C. Varying light intensity changed the temperature in the glass chambers. At full light intensity the glass chamber temperature was 7 °C and 4 °C higher than in darkness in the 164 L chamber and 1150 L chamber, respectively. In parallel to the variations of the illumination, also variation of needle temperature (4–9 °C) was observed. Temperature measurements were made using micro temperature sensors (Newport Omega, Germany).* "

(4) Between May and October is a quite long time frame which also means that the trees were additionally measured within different seasons. Was a single tree stored outside until the experiment was performed? This in turn would mean that (apart from season) trees possibly experienced different climatic conditions several weeks before they were measured)? Without this information it is not possible to judge if other conditions than biotic stress e.g. different temperatures or dry conditions could have led to changes in emissions.

Our response: Indeed, between May and October is a quite long time frame and most of the trees were stored outside in the environment. This was made to ensure that the plants experience the real environment and that their behaviour is not disturbed by keeping them in an artificial environment.

We investigated the basic mechanisms of BVOC emissions by labelling experiments and we do not discuss impacts of stresses or the time of the year on these basic mechanisms. Hence there is no need to give information regarding the impacts of stresses or season on the emissions and we also do not discuss this. For the generality of our mechanistic findings, it is more important that the basic processes leading to the emissions were always similar and independent of season.

As a summary of our response to the major comments of reviewer #1:

There seems to be some misunderstandings. We here show general results such as:

- different carbon sources are used for monoterpene- and sesquiterpene biosynthesis
- there are de novo emissions during darkness
- sesquiterpene emissions are pure de novo emissions although they are occurring in absence of light.

All these are new and important findings and we show that these basic mechanisms can be seen at very different conditions for the plant. Our main findings are clearly described in the text and in the abstract (page 1, lines 20–32). The reviewer is correct that in a next step quantification should be attempted, but this is outside the scope of this manuscript.

Specific comments:

(1) Page 1, Line 10-11: I agree that BVOCs are important for atmospheric chemistry but I doubt that it is due to their large source strength since the source strength is rather small compared to the source or sink of CO2, for example. It is rather the high reactivity which matters...

Our response: BVOC are important for atmospheric chemistry because their source strength is much larger than that of anthropogenic emitted VOC. From the view of carbon budget the contribution of BVOC is small, but the major contributor CO_2 is not reactive, thus is not considered for atmospheric *chemistry*.

As written the sentence on page 1, lines 10–11 is correct and we see no need to change it.

(2) Page1, Line17-19: It is hard to understand what you want to say with this sentence – consider rewriting.

Our response: The text is changed from:

"*...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.*"

To (page 1, lines 17–19):

"*…The mechanisms deduced from the labelling experiments were further compared to diurnal modulations of emission fluxes and to the release of the respective terpenoids from reservoirs in needles and bark tissue.*"

(3) Page 1, Line 20: 'The comparison allowed the following comprehensive statements...' This sentence seems to be redundant.

Our response: We do not think this sentence to be redundant. We therefore prefer to leave it as it is.

(4) Page 1, Line 23: You talk about three different carbon sources but you just refer to two 'assimilated CO2' and 'other alternative carbon sources'. The third carbon source is missing...

Our response: Correct, thanks for the hint. We added some words with this respect. Instead of "*while carbon from other alternative carbon sources has intermediate turnover times of few days and even longer*",

we now write (page 1, lines 25–27):

"*while the second carbon source has intermediate turnover times of few days and the third carbon source has an even longer turnover time. The observation of still completely unlabelled de novo molecules indicates that this third carbon source is uncoupled from the freshly assimilated carbon.*"

(5) Page 3, Line 8: 'and in some cases stress-induced terpenoids' is a very general statement. It would be better to specify at least that you are talking about biotic stress.

Our response: Thank you for the specific comment (5), (6), (9), (16). They are all related to the definition of stress-induced emissions. It is widely accepted that induced BVOC emissions mean those BVOC induced in plants in response to stress. Constitutive emissions mean that the respective BVOC are emitted under both, stress-free and stressed conditions. The emission strengths of constitutive emissions can be elevated by stress conditions. Nevertheless, these emissions are still termed "constitutive emissions" (Arneth and Niinemets, 2010; Niinemets, 2010). We clarify this and the new text is added after the first paragraph of Section 3.1 (page 7, lines 22–26).

The new text reads:

"*It is worth noting that stress conditions can also alter constitutive emissions. However, the stress-induced emissions considered here were those associated with novel synthesis of corresponding compounds due to biotic stresses. As an example, biotic stress can induce bursts of the constitutive pool emissions because of mechanical damages (e.g. Holopainen and Gershenzon (2010)). Such emissions were still considered as constitutive emissions in this study.*"

Based on this clarification, we hope that we now have well responded to the specific comments (5), (6), and (9). For the specific comment (16), we will give additional explanation.

(6) In the abstract it is stated that 'stress induced monoterpene and sesquiterpene emissions are entirely of de-novo nature' which is not reasonable since the storage pools are still present and it is more than likely that stress induced also changes in constitutive emissions which were not de-novo synthesized. I guess you wanted to refer to the compounds which were classified as stress compounds since they are known to be de-novo emitted in response to stress. In this case the argumentation is somewhat circular.

Our response: See the response to the specific comment (5).

(7) Materials and Methods: see Detailed comments above

Our response: See the response to the major comment (3).

(8) Page 6, Line 29: Is it justified to assume that all molecules with excess 13C-atoms are de novo emissions? What if de novo synthesized molecules are incorporated to storage pools?

Our response: Yes, this is justified because there was no excess ^{13}C in the plant before the ^{13}C exposure. Therefore, the BVOC molecules with excess ^{13}C-atoms must have been synthesized during the time of the ^{13}C exposure.

The size of pools is large enough to dilute the ^{13}C content of the stored BVOC near to the natural degree. The time scales for significant diffusion of labelled de novo compounds into the pools are too long to be important here. The situation is more comparable to a big lake with cold water where a litre of hot water is added and the water is mixt. After mixing the lake

will not have enhanced temperature. Please note that slow pool exchange is the base of all (not only our) $^{13}CO_2$ labelling experiments performed to distinguish between de novo and pool emissions.

(9) Page 7, Line 12-13 and Fig 2a: I am not sure if 'stress induced emissions' is the right terminology for the separation you chose... constitutive emissions are also very likely to change in response to stress and these changes are also stress induced. At least this needs to be discussed

Our response: See the response to the specific comment (5).

(10) Fig 2a: It is a bit tricky to draw any conclusions without proper statistics which is unfortunately not possible with one tree per treatment and without time-replicates see also the general comments above

Our response: Please see our response to the major comments. At no instance we attempt to quantify the strengths of stress-induced emissions. On contrast, we take advantage of the large variation of the strengths of the stress-induced emissions, as this supports the generality of our findings about the basic mechanisms behind.

(11) Page 7, Line 19-20: you state 'the detailed emission patters were different for individual plants' why were they different? Due to the stress or due to tree to tree variability? Another unknown factor without proper statistics.

Our response: The variability is certainly due to both, changes in kind and intensity of stress as well as tree to tree variability. However, we do not discuss or give comments on the reason of this variability. We just use diverse plant material to better cover a range of plant behaviour in the real environment.

(12) Page 8, Line 12- 13: Why did you choose the constitutive compounds β – pinene and myrcene but not the other constitutive compounds like α-pinene or limonene?

Our response: β-pinene had the lowest f_{synth} and myrcene had the highest value of f_{synth} of the constitutive monoterpene emissions (see Table 3). Showing data for the lowest and highest degree of labelling should be enough to show the typical behaviour.

We considered this comment and we added some words (page 8, lines 26–29). The new text reads:

"*At the example of the spruce S1, Fig. 3 shows typical labelling behavior of four representative compounds with different labelling ratios during and after the $^{13}CO_2$ labelling exposure: constitutive compounds β-pinene and myrcene and stress-induced compounds (E)-β-ocimene and (E)-β-farnesene. Qualitatively, all other BVOC behaved similar as those shown in Fig. 3.*"

(13) Section 3.2. You stated that you assume that labelled molecules are exclusively de novo emissions on the other hand you find constitutive emissions to be labelled.

Following this argumentation would mean that these constitutive emissions are also de novo and not from storage pools?

Our response: Yes, correct. Constitutive emissions from Norway spruce and Scots pine are partly from pools and partly de novo. This is correct and this is what we write.

(14) Section 3.3.2: Why do you show only (E)-β-farnesene? How do time-series of labelling patterns of other terpenes look like? Similar?

Our response: Correct, thanks for the hint. We did not mention that the time series were similar. In addition to the text added according to the specific comment (12), we added the following text after the first paragraph of Section 3.3.2 (page 10, lines 27–28):

"They also represent the typical time series of labelling."

(15) Page 11, Line 14-15: Was this shown in the results?

Our response: The results concerning method (1) i.e. $^{13}CO_2$ exposure please see Section 3.3, Fraction of de novo biosynthesis (page 9, line 22–28). For the results obtained with the method (2) i.e. testing the light response please see page 8, lines 20–24.

For better understanding, we changed the wording. Instead of

"*We tested these two methods and the results indicated that both methods led to an underestimation of the fraction of de novo synthesis, especially for sesquiterpenes.*",

we now write (page 11, lines 28–29):

"*In the following we compare the results obtained using these two methods.*"

And we move the conclusion (second part of this sentence) to the last paragraph of Section 4.1 (page 13, lines 5–6). The text now reads:

"*... de novo and pool emissions. Our results indicated that both methods led to an underestimation of the fraction of de novo synthesis, especially for sesquiterpenes. For a better clarification of the underlying ...*"

(16) Page 11, Line 16-17: To my opinion this is also a consequence of your definition of stress-induced monoterpenes.

Our response: This is only partly correct. On one hand, there are also constitutive emissions that are purely de novo (e.g. 1,8-cineole). Also these emissions are not enhanced when pools are destroyed. In this text part, the missing emission from pools is mentioned because this is our proof that the stress-induced emissions we discuss here are purely de novo emissions.

On the other hand, there may also be stress-induced constitutive emissions when storing structures are destroyed. These emissions are not considered here (see our response to the specific comment (5)). Therefore, we prefer to leave the sentence as it is.

(17) Page 12, Line 5: '...relatively low' Were this nocturnal fluxes above the limit of detection for fluxes or not?

Our response: Thanks for the hint, this was indeed very qualitative. We added some numbers here. The new text reads (page 12, line 21): "*The nocturnal emission fluxes of the de novo emitted monoterpenes were 1–6 % of the daytime fluxes ...*"

(18) Page 12, Line 5-25: An interesting question to discuss would also be if stress induced emissions of mono- or sesquiterpenes need to be light dependent or if their light dependency follows the same function as for isoprene (partly discussed in section 4.3). I guess it is not possible to extract light response curves from your measurements?

Our response: We agree it is very interesting. Unfortunately this was not studied in this study.

(19) Section 5: Summary or Conclusion?

Our response: Referee #1 is correct. Besides the summary there are also some conclusions, e.g. page 17, lines 17–19 "*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*."

We therefore changed the title to "*Summary and conclusions*".

References:

Arneth, A., and Niinemets, Ü.: Induced BVOCs: how to bug our models?, Trends in Plant Science, 15, 118-125, doi: 10.1016/j.tplants.2009.12.004, 2010.

Niinemets, Ü.: Mild versus severe stress and BVOCs: thresholds, priming and consequences, Trends in plant science, 15, 145-153, 2010.