Dear Editor,

We thank the two reviewers for their thoughtful and detailed comments on our manuscript. Please find below our response to the comments raised by the reviewers. We hope to have satisfactorily responded to all reviewer suggestions and comments, which have substantially improved the manuscript. Changes in the revised manuscript were made using the track-mode tool and are visible. Author comments below are in bold.

REVIEWER #2

Generally, I think that the study offers new knowledge in the area of transformation pathways of carbonyl compounds to 2-butanol and 3-buten-2-ol. The results are valuable and will inspire researchers to test the new hypothesis. For example, in this study with red oak, the authors did find a link between isoprene and methylvinylketone. Yet, I wonder, whether the link could be characteristic to some other plant species? In similar as in 2012 Jardine et al published a correlation between the emissions of isoprene and methacrolein, but in the present study, that correlation was missing. By the way, although testing of methacrolein did not give expected results, I still recommend adding its molecular structure to Figure 5.

We thank the reviewer for these comments. We tested the MVK transformation mechanism on two other plant species beside red oak (i.e. *Hedera helix and Vitis vinifera*) using GC-MS and did find analogous results to red oak. Such data is reported in the Supplementary Information of the revised manuscript. Since Figure 5 refers specifically to within-plant MVK transformation, we suggest not to add methacrolein. In the supplement, Figure S1 was added.

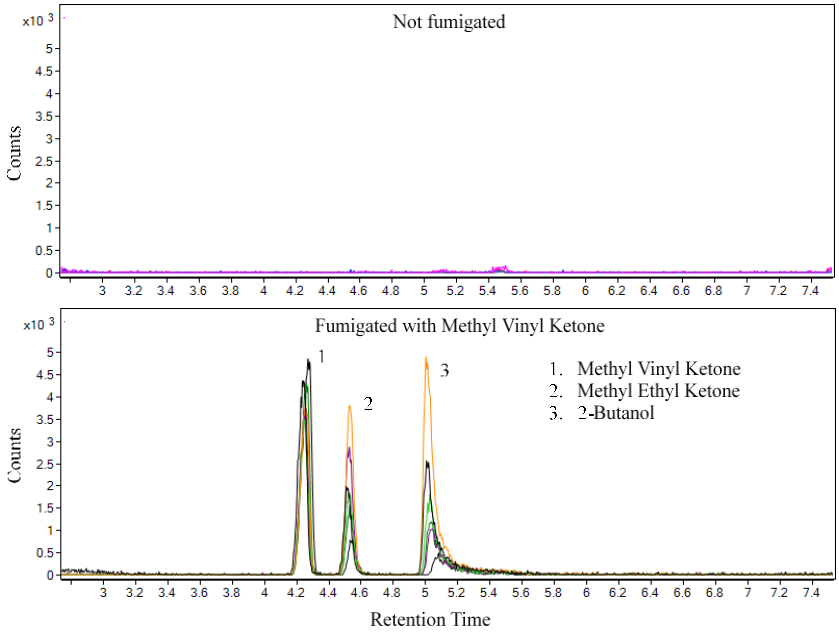

Figure S1. Excerpt of qualitative GC-MS analysis of six untreated plants (upper figure) and of the same plants fumigated with MVK (lower figure, n=6). The reported chromatograms correspond to m/z = 45 Th, so that all relevant peaks are clearly visible. Different colours indicate different plants (yellow for *Hedera helix*; black for *Vitis vinifera*; other colours for *Quercus rubra*). Upon MVK fumigation, MEK and 2-butanol were formed by all plants, while 3-buten-2-ol was below detection limit and could only be detected by PTR-ToF-MS (Table S1). No other MVK transformation compounds were detected by GC-MS. Such data is reported to support compound identification for the fumigation experiments reported in the main text (Figure 1).

In the supplement, the following paragraphs were added on page 1, line 1-30:

S.1 Complementary GC-MS analysis of MVK transformation products

In order to support compound identification of MVK transformation products by red oak plants, the MVK fumigation experiments described in the main text were repeated with some minor modifications to include qualitative analysis by solid phase microextraction - gas chromatography – mass spectrometry (SPME-GC-MS). As depicted in Figure S1, MEK and 2-butanol were unambiguously identified as MVK transformation products by red oaks, confirming the results obtained using PTR/SRI-ToF-MS (Figure 1 of the main text). Similar results were obtained for *Hedera helix* and *Vitis vinifera* (Figure S1). No other putative MVK transformation products were detected by the SPME-GC-MS analysis. In particular, 3-buten-2-ol was below detection limit. At the contrary, the corresponding PTR-ToF-MS analysis reported in Figure 1 and Table S1, besides MEK and 2-butanol, also detected a small but statistically significant emission of 3-buten-2-ol (Table S1). The identification of the PTR-ToF-MS signal corresponding to the ion $C_4H_7^+$ as 3-buten-2-ol, besides being consistent with measurements using the pure compound standard (3-buten-2-ol undergoes protonation followed by dehydration upon reaction with H_3O^+), has also theoretical reasons. Ketones have been reported to be transformed by plants via reduction reactions (Kergomard et al., 1988). Hence MEK and 3-buten-2-ol are expected as MVK transformation products. Moreover, reduction reactions occur for MACR, producing in particular isobutyraldehyde and 2-methallyl alcohol (Figure 1, Table S1, and Muramoto *et al.* (2015)).

S.1.1 Experimental setup for SPME-GC-MS analysis

The plant management and experimental setup was analogous to the one described in main text with the addition of a SPME fiber in the VOC-bag for collecting VOC for subsequent GC-MS analysis. No PTR/SRI-ToF-MS measurements were performed in this case. The experiment was repeated on six plants (four *Quercus rubra*, one *Hedera helix*, one *Vitis vinifera*). The GC-MS analysis was carried out as follows.
Headspace volatile compounds were collected by a 2 cm Solid Phase Microextration fibre coated with divinylbenzene/carboxen/polydimethylsiloxane 50/30 lm (DBV/CAR/PDMS, Sigma-Adrich, St. Lewis, USA), inserted through the VOC-bag using a manual holder. The fibre was exposed to the headspace air through the duration of the fumigation. Volatile compounds adsorbed on the SPME fibre were desorbed at 250°C in the injector port of a GC interfaced with a mass detector (GC Agilent 7820A with Agilent 5977B MSD, Agilent Technologies, Santa Clara CA, USA). The mass detector was operated in electron ionization mode (EI, internal ionization source; 70 eV) with scan range from m/z 25–200. Separation was achieved on a Supelco SPB-624 capillary column (20 m x 0.18 mm ID x 1 µm film thickness; Sigma-Adrich, St. Lewis, USA). The GC oven temperature program consisted in 40°C for 6 min, then 40–200°C at 40°C min^{-1}, and stable at 200°C for 5 min. Helium was used as the carrier gas with a constant column flow rate of 0.8 mL min^{-1}. Compound identification was based on mass spectra matching with the standard NIST libraries (NIST 2.2 2014) and retention times of authentic reference standards.

I find that the study is done by using suitable analytical methods and the conclusions are all appropriate. MS is well written and there are no serious flaws. According to the initial MS evaluation, the authors have improved the figures. The MS is well structured and the abstract provides a complete summary of the results.
We thank the reviewer for these comments.

Minor criticism

In the Abstract the abbreviation of MVK is not explained
This point has been corrected

In the Abstract L 7, please change '…we show that MVK and MEK emission caused by heat stress is…' to '…we show that MVK and MEK emissions caused by heat stress are…'
This point has been corrected.

Among Keywords and later in the text (P 3 L9 and 22) please change '2-butenol' to '2-butanol'
This point has been corrected.

At the beginning of the Introduction P1, I would change 'has' to 'have' in '…and particularly the latest studies employing genetic engineering to produce transgenic plants with modified isoprene emission, has…' P 2 L3 change 'there is plenty of evidence that isoprene positively influence… to 'there is plenty of evidence that isoprene positively influences…'
This point has been corrected.

At the beginning of Results L4 'MVK reduction generated either MEK or 3-buten-2-ol,'
- according to Figure 2 I would say 2-butanol instead of 3-buten-2-ol
It is true that the main final transformation products are either MEK or 2-butanol, as shown in the figure. However, in that sentence we are referring to the compounds generated upon reduction of the either the alkene moiety of MVK, leading to MEK, or the carbonyl moiety, leading to 3-buten-2-ol. We suggest not to change that sentence.

At the beginning of P4 starting from L2 'Emission of MVK+MACR+ISOPOOH was detected at a level of 0.001±0.002 nmol m-2s-1'- by comparing the text to the figure
it seems that the number should be multiplied with 1000, no? The same mistake is repeated in the following sentences.
A factor of 10^{-3} is reported at the top of the axis, this may have generated the confusion. We revised the figure (converting the y axis in pmol $m^{-2}s^{-1}$) in order to make it clearer.

Abbreviation of methylvinylketone, methylethylketone and ROS should be explained again at the beginning of the Discussion
This point has been corrected.

P5 L 7 to 9 I would use the plural instead of singular 'Emission of MVK and MEK has been rarely measured at leaf level and is challenging to measure because i) emission is small, especially in the case of MVK; ii) separation of leaf and atmospheric sources in the 10 presence of high isoprene emissions is difficult. Jardine and co-workers (Jardine et al., 2012, 2013) reported emission of methacrolein (MACR) and methyl vinyl ketone (MVK) in isoprene emitting trees.' to 'Emissions of MVK and MEK have been rarely measured at leaf level and are challenging to measure because i) emissions are small, especially in the case of MVK; ii) separation of leaf and atmospheric sources in the presence of high isoprene emissions is difficult. Jardine and co-workers (Jardine et al., 2012, 2013) reported the emissions of methacrolein (MACR) and methyl vinyl ketone (MVK) in isoprene emitting trees. '
We agree with the reviewer. This point has been corrected.

P9 L9 I would change '…repeated switching…' to 'repeated by switching'
This point has been corrected.

Figure 1 - please add titles to y-axes, change the unit to 'nmol m-2 s-1' and it is sufficient to write 'Time (min)' only under panel e
This point has been corrected

Also, I would change 'Uptake and transformation of MVK (a) and MACR (e) and of MVK transformation products, MEK (b), 2-butanol (c), 3-buten-e-ol (d) by red oak leaves. Negative values denote uptake, while positive values indicate emission. ' as follows
'Uptake of MVK (a), MEK (b), 2-butanol (c), 3-buten-e-ol (d) and MACR (e) and emission of their oxidized or reduced products in red oak leaves. Negative values denote the uptake - and positive values the emission of volatiles. '
We agree with the reviewer. This point has been corrected.

Please check also the rest of the figure legends
We checked the figure legends.

Figure 2 – Is it possible to show or mark in the text the proportion of methacrolein (MACR) in the mixture of MVK+MACR+ISOPOOH? Does the figure show mean ± SE or mean ± SD? I would delete negative error bars. Please use 'min' instead of 'minutes'.
It is not possible to mark the proportion of methacrolein as these data correspond to measurement using H3O+ as primary ions, therefore only the sum of MVK+MACR+ISOPOOH is measured. In the caption of figure 2, we clarified that "Results are reported as mean ± standard error (n=6)". In the Figure 2, we replaced "minutes" with "min". We delete negative error bars as suggested.

REVIEWER #1

General comments: Cappellin et al. describe a possible mechanism for the biogenic production of methyl ethyl ketone (MEK) from both exogenous and endogenous methyl vinyl ketone (MVK), which is decoupled from the plant's isoprene synthesis.

Earlier studies (even by one of the co-authors of this study) attributed isoprene an anti-oxidative role in plants, which was explained with its capability to capture reactive oxygen species (and thereby being oxidized to MVK/MACR). Over the last years, different studies have questioned this assumption. Cappellin et al. unequivocally show here that MVK production within plants under heat stress is not necessarily linked to the plant's capability to synthesize isoprene. Therefore, the manuscript has the potential to become an important contribution to the controversial discussion, whether isoprene exerts an antioxidant role in plants.
We thank the reviewer for these comments.

In general, the manuscript is very well written, in a clear and concise way. However, I'm struggling a bit with the experimental design and the data interpretation. The Methods part misses details on peak assignment in PTR/SRI-ToF-MS, and CO2 measurements/calibrations (see comments below). The number of replicates (3) in each experiment is borderline. This is also reflected in the large error bars in Figure 2.

We added details on peak assignment in PTR/SRI-ToF-MS and CO_2 measurements/calibrations (see answer to comments below). We revised Figure 2 using 6 replicates as we already had the data (see answer to comments below). For the transformation mechanism we added GC-MS data for 6 plants (see answer to comments below). The statistical significance of the conclusions is solid.

It is pretty brave to make statements on interconversion of in part isomeric compound using solely PTR-MS. Even when using NO+ ions for chemical ionization in the PTR/SRI-ToF-MS you have a lot of interfering ions from the compounds you were investigating. Moreover, natural isotopes of some of the investigated compounds could interfere with the parent ions of other compounds. The description of the data analysis in the methods section does not reveal if this effect was taken into account, nor does it explain satisfactorily how the ions signals were attributed to the different compounds. Especially in the case of the various alcohols a proper identification seems almost impossible with the instrumentation you used. I would expect to have a table containing all the different compounds and the associated ions in the two measurement modes of the PTR/SRI-ToF-MS. This would allow the reader to better judge whether the peak assignment is justified.

We added a table containing all the different compounds and the associated ions in the two measurement modes of the PTR/SRI-ToF-MS in the Supplementary Information.
In the supplement, the following table was added: Table S2.

Table S2. Spectral peaks and corresponding ions in PTR/SRI-TOF-MS for H_3O^+ mode and NO^+ mode. The mass resolving power of the PTR/SRI-ToF-MS (> 4000) and the peak deconvolution algorithm used (Cappellin et al., 2011) allowed to resolve the peaks reported in this table in each mode.

Compound	H3O+ mode		NO+ mode	
	m/z	Ion sum formula	*m/z*	Ion sum formula
Isoprene				
	69.07	$C_5H_8H^+$	68.062	$C_5H_8^+$
	70.0732	$^{13}CC_4H_8H^+$	69.0654	$^{13}CC_4H_8^+$
	71.0766	$^{13}C_2C_3H_8H^+$	70.069	$^{13}C_2C_3H_8^+$
	72.0844	$^{13}C_3C_2H_8H^+$	71.0721	$^{13}C_3C_2H_8^+$
	73.0833	$^{13}C_4CH_8H^+$	72.0754	$^{13}C_4CH_8^+$
	74.0866	$^{13}C_5H_8H^+$	73.0788	$^{13}C_5H_8^+$
			98.06	$C_5H_8{\cdot}NO^+$
			99.0634	$^{13}CC_4H_8{\cdot}NO^+$
			100.0667	$^{13}C_2C_3H_8{\cdot}NO^+$
			101.0701	$^{13}C_3C_2H_8{\cdot}NO^+$
			102.0734	$^{13}C_4CH_8{\cdot}NO^+$
			103.0768	$^{13}C_5H_8{\cdot}NO^+$
MVK				
	71.0491	$C_4H_6OH^+$	100.0393	$C_4H_6O{\cdot}NO^+$
	72.0525	$^{13}CC_3H_6OH^+$	101.0427	$^{13}CC_3H_6O{\cdot}NO^+$
	73.0558	$^{13}C_2C_2H_6OH^+$	102.046	$^{13}C_2C_2H_6O{\cdot}NO^+$
	74.0592	$^{13}C_3CH_6OH^+$	103.0494	$^{13}C_3CH_6O{\cdot}NO^+$
	75.0626	$^{13}C_4H_6OH^+$	104.0527	$^{13}C_4H_6O{\cdot}NO^+$
MEK				
	73.0648	$C_4H_8OH^+$	102.05495	C4H8O·NO+

	74.0681	$^{13}CC_3H_8OH^+$	103.0583	^{13}CC3H8O·NO+
	75.0715	$^{13}C_2C_2H_8OH^+$	104.0627	^{13}C2C2H8O·NO+
	76.0748	$^{13}C_3CH_8OH^+$	105.065	^{13}C3CH8O·NO+
	77.0782	$^{13}C_4H_8OH^+$	106.0684	^{13}C4H8O·NO+
3-buten-2-ol				
	55.0542	$C_4H_7^+$	71.0491	$C_4H_7O^+$
	56.0576	$^{13}CC_3H_7^+$	72.0525	$^{13}CC_3H_7O^+$
	57.0609	$^{13}C_2C_2H_7^+$	73.0558	$^{13}C_2C_2H_7O^+$
	58.0643	$^{13}C_3CH_7^+$	74.0592	$^{13}C_3CH_7O^+$
	59.0676	$^{13}C_4H_7^+$	75.0626	$^{13}C_4H_7O^+$
2-butanol				
	57.07	$C_4H_9^+$	73.0648	$C_4H_9O^+$
	58.0732	$^{13}CC_3H_9^+$	74.0681	$^{13}CCH_9O^+$
	59.0766	$^{13}C_2C_2H_9^+$	75.0715	$^{13}C_2C_2H_9O^+$
	60.0799	$^{13}C_3CH_9^+$	76.0748	$^{13}C_3CH_9O^+$
	61.0833	$^{13}C_4H_9^+$	77.0782	$^{13}C_4H_9O^+$

To my mind, such an experiment would have strongly benefited from additional analyses capable to distinguish isomeric compounds, such as GC-MS or similar.

Although I know it is a lot of work, I would recommend to perform additional experiments and to trap VOCs for GC-MS analyses in order to eliminate any doubt in the interpretation of the data.

We agree with the reviewer. Therefore, we carried out additional experiments using GC-MS. In the Supplementary Information we added the GC-MS data to support compound identification. Most importantly, methyl ethyl ketone and 2-butanol were unambiguously identified as transformation products of methyl vinyl ketone using both comparison to the NIST database of EI spectra and retention time of pure standards. 3-buten2-ol is a very minor product and was below detection limit in GC-MS. Only the high sensitivity of the PTR-TOF allowed detecting it. Its identification as 3-buten-2-ol among the possible isomers has theoretical reasons since ketones have been reported to be transformed by plants via reduction reactions (Kergomard et al., 1988), suggesting that MEK and 3-buten-2-ol are expected as MVK transformation products. No other transformation products were detected by GC-MS. The compound identification for the transformation products of methacrolein is supported by GC-MS data present within the literature (Muramoto *et al.*, 2015). In conclusion the additional GC-MS agree with the compound identification reported in the manuscript. We thank the reviewer for suggesting this improvement.

We added Sections S.1 and S.1.1 and Figure S1 in the Supplementary Information. They are also reported in an answer to the other reviewer.

The quality of some of the original figures was very bad (the updated ones submitted as Author Comment are OK).

Specific comments:

p. 2, line 18: was there really formed any 3-buten-2-ol? In Figure 1a, the 3-buten-2-ol seems to be zero throughout the whole experiment.

Yes, 3-buten-2-ol was formed as a minor product. Since this was not evident in Figure 1a, Table S1 was added in the Supplementary Information.
In the Supplement Table S1 was added.

Table S1. Total net flux in the fumigation experiments reported in Figure 1 (main text). Positive values denote net emission while negative values denote net uptake. Results are obtained by integrating the net flux curves reported in Figure 1. Results are reported as *mean* ± *se*.

Fumigation with MVK		
	TOTAL NET FLUX (μgC/m^2)	% of MVK uptake
MVK	-836 ± 257	
MEK	659 ± 242	73% ± 6%
3-buten-2-ol	38 ± 13	4% ± 0.4%
2-butanol	207 ± 32	19% ± 2%
MEK + 3-buten-2-ol + 2-butanol	839 ± 281	97% ± 4%
Fumigation with MEK		
	TOTAL NET FLUX (μgC/m^2)	% of MEK uptake
MVK	n.d.	
MEK	-270 ± 53	
3-buten-2-ol	n.d	
2-butanol	171 ± 34	87% ± 13%
Fumigation with 2-butanol		
	TOTAL NET FLUX (μgC/m^2)	% of 2-butanol uptake
MVK	n.d.	
MEK	47 ± 6	90% ± 29%
3-buten-2-ol	n.d	
2-butanol	-63 ± 10	
Fumigation with 3-buten-2-ol		
	TOTAL NET FLUX (μgC/m^2)	% of 3-buten-2-ol uptake
MVK	n.d.	
MEK	33 ± 7	33% ± 10%
3-buten-2-ol	-107 ± 10	
2-butanol	14 ± 2	14% ± 4%
MEK + 2-butanol	48 ± 9	47% ± 14%
Fumigation with MACR		
	TOTAL NET FLUX (μgC/m^2)	% of MACR uptake
MACR	-431 ± 110	
Isobutyraldehyde	33 ± 9	6.5% ± 0.8% (6.4%*)
2-Methallyl alcohol	4 ± 1	0.7% ± 0.3% (1.1%*)
Isobutanol	10 ± 1	2.1% ± 0.3% (1.8%*)
Isobutyraldehyde + 2-Methallyl alcohol + Isobutanol	48 ± 11	9.3% ± 0.8%

** Results in brackets represents values reported by Muramoto et al. (2015) in similar experiments using tomato plants.*

p. 2, lines 21-22: you disregard here that there is no possible direct conversion of 3-buten-2-ol to MEK. How sure are you about these data? I guess it is really tricky to properly distinguish 3-buten-2-ol from MEK using solely PTR-MS.
It is possible to distinguish 3-buten-2-ol from MEK using PTR-TOF. In fact, in H_3O^+ mode MEK undergoes proton transfer leading to $C_4H_9O^+$ at 73.0648 Th, while 3-buten-2-ol also undergoes dehydration leading to $C_4H_7^+$ at 55.0542 Th. We agree with the reviewer that the conversion of 3-buten-2-ol to MEK should be added.
In page 3, line 22, the following sentence was added:

"Conversion of 3-buten-2-ol to MEK is also possible (Figure 1d)."

p. 4, lines 13-15: The calculated assimilation rate is very low. I've never seen assimilation rates in a comparable range as the dark respiration values in a light (!) experiment.
What was the PAR you used in these experiments? Apparently, you used the PTRToF-MS to measure CO2 levels: have you considered different humidities in dark/light experiments when calibrating the PTR-ToF-MS for CO2? Can you comment on the accuracy of this method to measure CO2?
We reported the calibration curve and the correction factors used at different humidities in the Supplementary Material (Figure S2). The method is estimated to have an accuracy of about 5% using an integration time of 90 s and its applicability is limited to CO2 concentrations in the ppm range since the sensitivity is very much lower than typical PTR-MS sensitivities for VOCs in general. It has been used in this case since a CO2 detector was not available. As reported in the p.8 line 12, the PAR was 90 µmol m^{-2} s^{-1}. The results for both the assimilation rates and the isoprene emission are comparable with those of previous literature experiments on the same plant species (Loreto and Sharkey, 1990). The results for dark respiration are also comparable with the values reported by other studies on red oak (Turnbull et al., 2001).
In the Supplement, Figure S2 was added.

p. 5, lines 20-23: These reaction yield calculations require further explanations, either here or in the Methods section. Where do you "SHOW" that 73% of MVK is converted into MEK?
We added a table reporting all conversion percentages (including MVK to MEK).
In the Supplementary Information, we added Table S1.

p. 8, line 16: is there a reason why you heated your sample line to 110◦C? At such high temperatures you may encounter surface assisted reactions and thermal decomposition of larger compounds, possibly interfering with the ion signals of interest. The compounds you were interested in should all be fairly volatile, excessive line heating is therefore counter-productive in this case.
We clarified that only the last part (about 1 m) of the PEEK sampling line was heated. It avoids memory effects and condensation. The residence time is low (PEEK inner diameter 1.01 mm, 40 sccm flow) and no evidence of surface assisted reactions and thermal decomposition of larger compounds interfering with the ion signal of interest was found. Moreover, our conclusions are based on the simultaneous behavior of several compounds (e.g. MEK, 3-butenl-2-ol, 2-butanol). It is highly unlikely to have interference in all channels.
At page 8, line 16, the sentence now reads:
"(…) interfaced with the PTR-ToF-MS via polyetheretherketone (PEEK) capillary tubes (ca. 1.5 m length x 1.01 mm ID, temperature: 110°C (part outside the climatic chamber, ca. 1 m), flow: 40 sccm)".

p. 8, line 27: I guess this is 10ul/min of liquid standard. What is the actual volume

mixing ratio of the compound in the VOC-bag inlet air?
We clarified that the actual volume mixing ratios of the compounds in the VOC-bag inlets were the following: 290 ppbv for MVK, 330 ppbv for MEK, 90 ppbv for 2-butanol, 96 ppbv for 3-buten-2-ol, 140 ppbv for MACR.
At page 8, line 16, the following sentence was added:
"The volume mixing ratio of the compound used for the fumigation in the VOC-bag inlets was the following: 290 ppbv for MVK, 330 ppbv for MEK, 90 ppbv for 2-butanol, 96 ppbv for 3-buten-2-ol, 140 ppbv for MACR".

p. 9, line 3: what were the CO2 concentrations at the outlet of your VOC-bag? Depending on the enclosed leaf area, during light conditions at this modest flow rates you might have run into CO2 deficit conditions for your plant. This could have affected your measured VOC signals.
The CO_2 concentration (or $^{13}CO_2$ concentration) at the outlet of the VOC-bag was above 350 ppm in all conditions, indicating that the plants did not experience carbon dioxide deficit conditions.

Figure 1: are these data of a single experiment or the mean over several experiments? This should be indicated in the figure caption. Since you have a possible interconversion of the measured compounds as well as emission and re-uptake, the y-axes should be labeled "Net VOC flux".
This figure was revised as part of the previous authors' comment. Figure 1 reports the mean over experiments. This point was clarified in the updated figure caption.
In updated Figure 1:
The y-axes were relabeled "Net VOC flux (nmol m^{-2} s^{-1})".

Figure 2: The overall quality of this figure is very bad! The resolution is indisputably low. The error bars and asterisks are almost not visible. I assume the compound grouping here is based on the different ion signals when using H3O+ ions for chemical ionization in PTR-MS, yielding the same ion for the different groups. This should be stated somewhere. As you are focusing on endogenously formed compounds here, it would make sense to normalize the signals measured in the different conditions to the stomatal conductance of the leaves. This way you might get an idea on the actual concentration of these compounds within the leaves.
This figure was revised as part of the previous authors' comment. As the reviewer pointed out above, the quality of the updated figures is ok. Although we agree that in general it would be interesting to get an idea of the actual compound concentration within the leaves, we propose not to carry out a further normalization since in the rational of the paper the figure is reported in order to prove that the compounds of interest are emitted under heat stress conditions. Such conclusion can be based on the compound net flux as reported in the updated figure.
We clarified that the compound grouping was based on H3O+ ionization in the caption of the updated figure 2.
Figure 2 was revised to include six replicates and the values reported in page 3 line 29 – page 4 line 25 referring to Figure 2 were changed accordingly.

Figure 5: you completely neglect the conversion of 3-buten-2-ol to MEK here, although, considering Figure 1, this seems its major conversion pathway. Again, the resolution of the background image could be improved.

We added the arrow for conversion from 3-buten-2-ol to MEK, which was missing. We thank the reviewer for pointing this out. We improved the resolution of the background figure.

Technical corrections:

p. 2, line 3: remove "plenty of". How can you claim there is plenty of evidence for the heat dissipating and thylakoid membranes stabilizing properties of isoprene, when you cite only two publications? Btw: how large can the heat dissipating effect of isoprene be when you compare the isoprene emission fluxes (nmol/(m2 *s)) with leaf transpiration (mmol/(m2 *s))?

This point has been corrected.

At page 2, line 3:

We removed "plenty of".

p. 2, lines 19-20: remove this sentence. Why would the plant produce isoprene to scavenge ROS, if the isoprene oxidation products are similarly cytotoxic and in turn need to be scavenged themselves?

The sentence was removed.

p. 3, lines 2: "..., though the full mechanism was not described.": nor is it described here. What are the enzymes involved in the detoxification reactions? Just saying.

This point has been corrected.

At page 3, line 2, the sentence now reads:

"We suggested that MVK could be efficiently detoxified by reduction reactions which lead mostly to MEK and, to a minor extent to 2-butanol, though the full interconversion scheme was not studied."

p. 4, line 14: this is no proper sentence. You compare assimilation and isoprene emission values with a light intensity.

We clarified that we compared carbon assimilation and isoprene emission to literature values at analogous light intensity and the results were consistent.

At page 4, line 14, the sentence now reads:

"These values for carbon assimilation and isoprene emission are consistent literature findings, considering the light intensity to which plants were exposed (e.g. see (Loreto and Sharkey, 1990))."

p. 5, line 18: Fig. 5 is a possible pathway for the biogenic formation and emission of MEK, but does not really explain it. A proper explanation would require the investigation of the enzymatic pathways involved in the MEK production.

This point has been corrected.

At page 5, line 18, the sentence now reads:
"This mechanism is the first possible pathway for the biogenic production and emission of MEK."

p. 5, line 19: The results suggest that WITHIN PLANT isoprene oxidation is not the source of these VOCs IN YOUR EXPERIMENT! Atmospheric oxidation of isoprene is undoubtedly the main source of MVK in the atmosphere.
This point has been corrected.
At page 5, line 19, the sentence now reads:
"The results also suggest that within plant isoprene oxidation is not the source of these VOCs in our experiments."

p. 10, lines 4-5: the cited reference does not contain any information on the spectral peaks to monitor!
This point has been corrected.
At page 10, lines 4-5, the sentence now reads:
"Details on the spectral peaks used to monitor each compound in red oak are reported in the Table S2 in Supplementary Information".

We hope that the revised manuscript will be acceptable for publication in ACP. We thank you for your attention and look forward to hearing from you.

Sincerely,

Luca Cappellin

A mechanism for biogenic production and emission of MEK from MVK decoupled from isoprene biosynthesis

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Abstract.

Methyl ethyl ketone (MEK) is an important compound in atmospheric chemistry. While attention has been paid mostly to anthropogenic sources of MEK, recently it has been shown that biogenic sources are globally as important as anthropogenic ones. However, the origin of biogenic MEK has yet to be completely elucidated. We present the full mechanism by which within-plant transformation of methyl vinyl ketone (MVK) and, to a minor extent, of 2-butanol and 3-buten-2-ol, is a source of biogenic MEK. Such transformation is observed in red oak for both exogenous MVK, taken up from the atmosphere, and endogenous MVK generated within plant upon stress (e.g. heat stress). Endogenous MVK emitted by plants is typically explained by within-plant oxidation of isoprene caused by oxidative stress. In this study we show that MVK and MEK emissions caused by heat stress are~~is~~ not related to isoprene in isoprene-emitting plants, implying that the massive carbon investment that plants commit to isoprene production is not explained by a direct antioxidant role. The presented mechanism can be important for inclusion in plant emission and in plant-atmosphere interaction models.

Keywords

Isoprene; oxidative stress; heat stress; within-plant isoprene oxidation; methyl ethyl ketone, methyl vinyl ketone ~~ketone~~, ~~2-butenol~~2-butanol; 3-buten-2-ol; $^{13}CO_2$ labelling.

Introduction

Isoprene is the major biogenic volatile organic compound (VOC) emitted by the biosphere into the atmosphere. A wide breadth of research, and particularly the latest studies employing genetic engineering to produce transgenic plants with modified isoprene emission, have~~s~~ demonstrated that isoprene increases plant tolerance to oxidative stresses, caused by factors such as high temperature and ozone (Behnke et al., 2007; Loivamäki et al., 2007; Sasaki et al., 2007; Sharkey et al., 2005; Vickers et al., 2009, 2011). However, the mechanisms by which isoprene confers resistance against oxidative stresses remain unclear. A first hypothesis is that isoprene strengthens thylakoid membranes (Velikova et al., 2011). It has been shown that under physiological conditions there are less than a hundred isoprene molecules per million lipid molecules in the membranes

(Harvey et al., 2015), casting doubt on this mechanism. However, there is ~~plenty of~~ evidence that isoprene positively influences the photosynthetic electron flow, reducing heat dissipation mechanisms and stabilizing thylakoid membranes, especially under stressful environments (Pollastri et al., 2014; Velikova et al., 2011). Recently, it has been shown that isoprene may act as a signaling molecule, inducing up-regulation of phenylpropanoid biosynthetic genes, indirectly enhancing plant resistance to heat and light stress (Harvey and Sharkey, 2016). Alternatively, isoprene may simply act as a proxy of induced activation of secondary metabolism (Tattini et al., 2015). A last hypothesis is that isoprene scavenges reactive oxygen species (ROS) by direct or indirect reaction (Loreto and Velikova, 2001). ROS exert an important signaling role in plants (Mittler et al., 2011), but can be generated in high amounts upon abiotic stresses, leading to cell damage and programed cell death (Delledonne et al., 2001). Isoprene biosynthesis has been ascribed a ROS quenching effect, but, again, isoprene concentration in the leaf may be too low; abundant lipids and carotenoids can react with ROS much more rapidly than isoprene (Harvey et al., 2015). Nevertheless, emission of putative isoprene oxidation products that may be formed upon isoprene reaction with ROS has been reported, e.g. in a tropical rainforest mesocosm (Jardine et al., 2012), in mango trees (Jardine et al., 2012, 2013) and creosote bush (Jardine et al., 2010). The putative isoprene oxidation products, the carbonyls methyl vinyl ketone (MVK) and methacrolein (MACR), are cytotoxic and must be rapidly removed from leaves once formed (Oikawa and Lerdau, 2013; Vollenweider et al., 2000). Plants also rapidly take up MACR and MVK (Andreae et al., 2002; Karl et al., 2004, 2005, 2010), suggesting that these compounds may be metabolized. Detoxification mechanisms for MACR have been reported (Muramoto et al., 2015), whereas this is not the case for MVK. ~~The existence of in-plant detoxification mechanisms for these oxidation products supports the hypothesis that isoprene acts directly as an antioxidant.~~

Methyl ethyl ketone (MEK) is an important oxygenated volatile organic compound (OVOC) for the atmosphere. In several studies the MEK concentration in the free troposphere has been reported to be approximately 25% of the concentration of acetone (Moore et al., 2012; Singh, 2004). However, MEK has a much shorter lifetime than acetone due to a much higher reactivity (by about one order of magnitude) with hydroxyl radical (OH, $k_{OH} = 1.15 \cdot 10^{-12}$ cm^3 s^{-1} at 296 K (Chew and Atkinson, 1996)), which makes it important to total OH reactivity (Nölscher et al., 2016). Both biogenic and anthropogenic sources contribute to global atmospheric MEK (Yáñez-Serrano et al., 2016). In urban environments, MEK is typically the most abundant ketone after acetone (Feng et al., 2005; Grosjean et al., 1996), as it is widely produced in industrial processes (Legreid et al., 2007; Sin et al., 2001). The regional background of MEK was once attributed to anthropogenic sources only. More recent findings suggest that the biogenic source of MEK is comparable to the anthropogenic source (Yáñez-Serrano et al., 2015, 2016), but the origin of biogenic MEK has not been fully elucidated so far. Direct emission of MEK from vegetation seems to be the largest contribution since only a few minor biogenic VOCs (e.g, n-butane and 2-butanol) can lead to MEK via atmospheric oxidation (de Gouw et al., 2003; Jenkin et al., 1997; Singh, 2004; Sommariva et al., 2011). We recently found a possible relation between the detoxification by vegetation of MVK and the biogenic emission of MEK (Cappellin et al., 2017). We suggested that MVK could be efficiently detoxified by reduction reactions which lead mostly to MEK and, to a minor extent to 2-butanol, though the full ~~mechanism~~ interconversion scheme was not ~~described~~studied.

In this study we explore the links between production of isoprene from photosynthetic carbon, within-plant isoprene oxidation, and the biogenic emission of putative isoprene oxidation products MVK, MEK, and 2-butanol. We performed a series of fumigation experiments in which leaves were exposed to exogenous levels of a single carbonyl, and investigated the relationships between the uptake of each compound and the release of related products. We then studied emission of carbonyls by leaves under progressively higher temperature conditions that likely inhibit photosynthesis and increase isoprene biosynthesis and the oxidative environment within leaves. We finally explored whether MVK, MEK, and ~~2-butenol~~2-butanol produced under heat stress are products of in-plant isoprene oxidation by determining whether they share a labeling pattern with isoprene when leaves are fed $^{13}CO_2$. Different labeling patterns would indicate that emitted carbonyls are not isoprene oxidation products, and would cast doubt on the hypothesis that isoprene acts as an antioxidant by reacting with ROS inside leaves.

Results

Fumigation of leaves with OVOCs. Individual plants were fumigated with MVK, MEK, 2-butanol, 3-buten-e-ol, or MACR, as described in the Materials and Methods (Figure 1). Both MVK and MACR were efficiently taken up by the fumigated leaves but, while MVK was continuously transformed into other volatile products that were subsequently released (Figure 1a), in the case of MACR only a transient release of other volatiles was observed (Figure 1e). MVK reduction generated either MEK or 3-buten-2-ol, depending on whether the alkene moiety or the carbonyl moiety of MVK is reduced. Further reduction reactions converted MEK and 3-buten-2-ol into 2-butanol (see also Figures 1b and 1d). Conversion of 3-buten-2-ol to MEK is also possible (Figure 1d). The release of MEK, 3-buten-2-ol, and 2-butanol corresponded to 97 ± 6% (mean ± std, n=3) of the total MVK uptake (Table S1 in the Supplement). Compound identification is corroborated by corollary GC-MS experiments (Figure S1). In the MEK fumigation experiments (Figure 1b), the release of 2-butanol corresponded to 87 ± 22% of the total uptake of MEK. Remarkably, ~~2-butenol~~2-butanol and 3-buten-2-ol may also convert into MEK (Fig. 1c, d). In the 2-butanol fumigation experiments (Figure 1c), the release of MEK corresponded to 90 ± 50% of the total uptake of 2-butanol. In the 3-buten-2-ol fumigation experiments (Figure 1d), the release of MEK and 2-butanol corresponded to 56 ± 18% and~~-~~ 22 ± 6% of the total uptake of 3-buten-2-ol, respectively. Finally, in the MACR fumigation experiment (Figure 1e) we observed a small transient release of isobutyraldehyde, in an amount accounting for 6.5 ± 1.3% of the MACR taken up, and, to a lesser extent, of 2-methallyl alcohol (0.7 ± 0.5%) and isobutanol (2.1 ± 0.5%). The total emission of these three compounds corresponded to 9.3 ± 1.4% of the total MACR taken up.

Emission of isoprene and OVOCs under heat stress. Emissions from darkened, unstressed leaves were measured (Fig. 2a). As expected (Loreto and Sharkey, 1990), a very small basal emission of isoprene (0.0152~~2~~±0.0041~~1~~ nmol m^{-2} s^{-1}, mean ± se, n=6) was found. In these leaves the emissions of putative isoprene oxidation products (MVK+MACR+ISOPOOH, Fig. 2b) and their reduction products (MEK and 2-butanol. Fig. 2c and d, respectively) were near background level, and dark respiration

was low (0.495±0.072 μmol m^{-2} s^{-1}, Fig. 2e). When darkened red oak leaves were exposed to mild heat-stress (35°C for 2 h) a small but statistically significant increase in isoprene emission to 0.0345±0.0107 nmol m^{-2} s^{-1} was observed. Emission of MVK+MACR+ISOPOOH was detected at a level of 0.0021±0.0012 nmol m^{-2} s^{-1}, and emissions of further transformation products of MVK were also detected, namely MEK (0.0056±0.0014 nmol m^{-2} s^{-1}) and 2-butanol (0.010007±0.001 nmol m^{-2} s^{-1}). However, only the increase of 2-butanol was statistically significant compared to unstressed leaves. Dark respiration also increased to 0.8±0.24 μmol m^{-2} s^{-1}. More severe heat stress was then imposed, maintaining the darkened leaves at 45°C for 2 h. This led to a further significant increase in isoprene emission (0.0810±0.013 nmol m^{-2} s^{-1}) (Fig. 2a). Emission of MVK+MACR+ISOPOOH also increased to 0.006±0.0013 nmol m^{-2} s^{-1} (Fig. 2b) and emission of MEK and 2-butanol, increased to 0.0293±0.0071 nmol m^{-2} s^{-1} and 0.0334±0.0072 nmol m^{-2} s^{-1}, respectively (Fig. 2c, d). Dark respiration also increased to 1.1±0.12 μmol m^{-2} s^{-1} (Fig. 2e). All these changes were statistically significant with respect to the values measured at 25°C. After a recovery phase of 2 h at room temperature (25°C), pre-stress emissions were again found for all compounds (Fig. 2a-e).

The same experiment was repeated on illuminated red oak leaves (Fig. 2f-j). Non-stressed leaves (maintained at 25 °C) photosynthesized at a rate of 1.21±0.25 μmol CO_2 m^{-2} s^{-1} (Fig. 2j) and emitted isoprene at a rate of 3.74±0.52 nmol m^{-2} s^{-1} (Fig. 3f). These values for carbon assimilation and isoprene emission are consistent literature findings, considering~~with~~ the light intensity to which plants were exposed (e.g. see (Loreto and Sharkey, 1990)). In non-stressed leaves, the emission of MVK+MACR+ISOPOOH was below the detection limit (Fig. 2g) and the emissions of MEK and 2-butanol were also close to background (Fig. 2h, i). Exposure to 35°C for 2 h led to a non-significant decrease of photosynthesis (Fig. 2j) and to a large increase of isoprene emission, reaching 10.8±1.35 nmol m^{-2} s^{-1} (Fig. 2f). Detectable emission rates of MVK+MACR+ISOPOOH were present (Fig. 2g), and MEK and 2-butanol emissions were also detectable (Fig. 2h, i). However, the change in emissions compared to that observed at 25°C was statistically significant only for 2-butanol. Severe stress (45°C for 2 h) caused a reduction in the emission of isoprene as compared to the maximum emission observed at 35°C (Fig. 2f), and photosynthesis was also inhibited (Fig. 2j). However, the emission of MVK+MACR+ISOPOOH increased to 0.006±0.0602 nmol m^{-2} s^{-1} (Fig. 2g), and MEK and 2-butanol increased to 0.0263±0.0041 nmol m^{-2} s^{-1} and 0.0253±0.0051 nmol m^{-2} s^{-1}, respectively (Fig. 2h, i). After recovering for 2 h at room temperature (25°C), VOC emissions and photosynthetic rate were similar to those of pre-stressed leaves (Fig. 2f-j).

^{13}C labelling of isoprene and OVOCs. To provide better insights about isoprene production and possible within-leaf oxidation under severe heat stress, plants were exposed to isotopically labeled CO_2 at 45°C. ^{13}C labelling of isoprene was clearly visible. All ^{13}C-labeled isoprene isotopomers (m/z 70-74) appeared, and the unlabeled isoprene (m/z 69) declined rapidly (Fig. 3). After 20 min of $^{13}CO_2$ labelling, the percentage of labelled isoprene was above 80%, and a stable labeled fraction of 95±1 % of the emitted isoprene was reached ca. 40 min after starting the $^{13}CO_2$ fumigation (Fig. 4a). However,

MVK+MACR+ISOPOOH, MEK and 2-butanol were not labelled by ^{13}C. Even after 2 h of $^{13}CO_2$ labelling, the isotopic ratios of MVK+MACR+ISOPOOH (Fig. 4b) and those of MEK and 2-butanol (Fig. 4c, d) remained at their natural abundance levels.

Discussion

There are consistent indications that methyl ethyl ketone (MEK) is released by vegetation at the leaf-, canopy-, and ecosystem-scale and that biogenic sources influence global atmospheric concentrations of MEK ((Yáñez-Serrano et al., 2016) and references therein). However, no mechanism for biogenic MEK production has been demonstrated previously. It has been hypothesized that MEK could be generated following similar pathways to acetone, as a by-product of cyanogenesis in the transformation of a cyanohydrin lyase (Yáñez-Serrano et al., 2016). This idea has not been proven and emission would be limited to cyanogenic plants. Plant uptake of atmospheric methyl vinyl ketone (MVK) is a well-established phenomenon (Karl et al., 2010) and bidirectional exchange of MVK has been sometimes reported (Karl et al., 2005). Emissions of MVK and MEK haves ~~s~~ been rarely measured at leaf level and ~~is~~ are challenging to measure because i) emissions are ~~is~~ small, especially in the case of MVK; ii) separation of leaf and atmospheric sources in the presence of high isoprene emissions is difficult. Jardine and co-workers (Jardine et al., 2012, 2013) reported emissions of methacrolein (MACR) and ~~methyl vinyl ketone (~~MVK~~)~~ in isoprene emitting trees. They attributed carbonyl emissions to within-leaf isoprene oxidation, possibly by ROS induced under environmental stress conditions (e.g. heat stress).

Our results confirm emission of carbonyls and show that reduction of several carbonyls may occur in plants. In particular, we show that reduction reactions transform MVK into MEK, 3-buten-2-ol, and 2-butanol, and that the conversion of MEK into 2-butanol occurs in both directions (Fig. 5). This mechanism is the first ~~to explain~~possible pathway for the biogenic production and emission of MEK. The results also suggest that within plant isoprene oxidation is not the source of these VOCs in our experiments.

Our fumigation experiments establish a causal link between plant metabolism of MVK and MEK production. Reaction yield calculations were performed considering the net total amount of MVK taken up during the experiment and the net total amount of MEK released. We show that 73 ± 10% of the MVK taken up by leaves was converted into MEK. Most of the remaining MVK was converted into 3-buten-2-ol and 2-butanol. Thus 97 ± 6% of MVK uptake was re-emitted as other volatile products. These data confirm previous reports at Harvard Forest, corroborated by laboratory experiments, where a correlation between uptake of MVK and emission of MEK, 2-butanol and 3-buten2-ol was found (Cappellin et al., 2017). The implication is that metabolism of MVK is a likely source of MEK emissions.

Whether other mechanisms for MEK production by plants exist remains an open question. Processes on the leaf surface such as MVK adsorption or metabolization by leaf surface bacteria have been shown to be of minor importance in previous investigations of ketone and aldehyde leaf uptake (Omasa et al., 2000; Tani et al., 2010, 2013; Tani and Hewitt, 2009, p.20).

Future studies should investigate the fraction of global biogenic MEK emissions that could be accounted for by the proposed production mechanism.

Rapid transformation of MVK by secondary reactions can be interpreted as a necessary and efficient detoxification mechanism. MVK toxic effects include accumulation of hydrogen peroxide and the activation of some stress-responsive genes (Alméras et al., 2003; Vollenweider et al., 2000). MVK metabolism has been reported in several plants (Karl et al., 2010; Tani et al., 2010). It has also been demonstrated that various plant cells are able to reduce unsaturated ketones (Kergomard et al., 1988). Long-term fumigation experiments with houseplants (Tani and Hewitt, 2009) have established that the uptake of several aldehydes and ketones exceeded by orders of magnitude the amount dissolved in leaf water, thus implying their metabolization as well. Transformation of MVK into MEK may be especially important in stressed plants as oxidative stress increases and endogenous production of MVK occurs (Jardine et al., 2012). The fumigation experiments suggest that the process can occur for exogenous MVK as well. Hence, the reduction mechanism also provides an explanation for plant uptake of MVK, a cytotoxic compound, which has been observed in many studies (e.g. (Brilli et al., 2016; Karl et al., 2005, 2010)).

MACR was also efficiently metabolized, consistent with other literature reports on tomato plants (Muramoto et al., 2015). In this case, however, detected volatiles produced by MACR reduction represented only about 9% of the MACR uptake. By analogy with the case of MVK, the expected reduction products of MACR would be isobutyraldehyde, 2-methylprop-2-en-1-ol, and isobutyl alcohol. However, only a transient release of such products was detected. Isobutyraldehyde emission accounted for 6.5% of the MACR uptake within two hours, confirming results from a previous study on tomato plants (6.4% (Muramoto et al., 2015)). Muramoto *et al*. (Muramoto et al., 2015) reported that most MACR in the leaf undergoes glutathionylation. Therefore, no MACR volatile products are released. Our results are in line with this interpretation, as MACR uptake implies a sink within leaves, suggesting a continuous metabolization of the compound, but the metabolic products are not released as volatiles.

The foliar uptake of MVK and MACR was rapid and sustained throughout the entire fumigation period, suggesting a large and fast metabolic sink. On the other hand, the uptake of secondary transformation products (namely MEK, 3-buten-2-ol, and 2-butanol) was not sustained, indicating that the metabolic sink was smaller and insufficient to rapidly scavenge the metabolites, leading to their temporary accumulation within leaves. Upon ending the fumigation, accumulated MEK, 3-buten-2-ol, and 2-butanol were released from the leaves back to the atmosphere (Fig. 2b, 2c, 2d).

A further aim of this study was to verify whether carbonyls emitted by plants come from isoprene oxidation in plants, as elsewhere proposed (Jardine et al., 2012). The experiments were conducted on red oak, one of the strongest isoprene-emitting plant species (Loreto and Sharkey, 1990). As isoprene biosynthesis is light- and temperature-dependent (Loreto and Sharkey, 1990), an increase of isoprene transformation products would be expected when leaves are illuminated and exposed to

moderately high temperatures. This behavior was indeed represented in the present data (Fig. 2). Under heat stress, photosynthesis and isoprene emission generally decouple and carbon sources other than direct products of photosynthetic carbon fixation are used for isoprene biosynthesis (Brilli et al., 2007). When leaves were severely heat-stressed we did not observe the expected uncoupling between photosynthesis and isoprene emission, as both parameters decreased. However, MVK+MACR+ISOPOOH, MEK and 2-butanol increased. This increase would be predicted to occur under an increasingly oxidative environment where molecular O_2 is directly photoreduced instead of being used for photosynthetic electron transport. Interestingly, the amount of MVK+MACR+ISOPOOH, MEK and 2-butanol was similarly enhanced in illuminated and darkened leaves under the effect of high temperatures, despite the fact that no photosynthesis and minimal isoprene emission was present in darkness.

This unexpected observation was followed up with measurements of ^{13}C labeling to further characterize the carbon source of MVK, MEK, and 2-butanol. As photosynthetic metabolites and isoprene label very quickly and almost totally when exposed to a $^{13}CO_2$ atmosphere ((Delwiche and Sharkey, 1993), see also Figs 3, 4), we would expect that putative isoprene oxidation products and their further transformation products would be quickly and totally labeled as well. On the other hand, if carbon sources other than direct photosynthetic metabolites (including isoprene) are used for their biosynthesis these compounds would not be labeled. The observation that no ^{13}C labeling appeared in any of the emitted carbonyls suggests that isoprene is not involved in their biosynthesis. We are not aware of isoprene pools that label slowly in plants (Niinemets et al., 2004). However, sources independent of freshly assimilated carbon contribute to isoprene production when photosynthesis is stress-constrained (Brilli et al., 2007). Photorespiratory carbon may contribute to isoprene production especially during dynamic changes of environmental conditions (Jardine et al., 2014). However, photorespiration also labels quite rapidly with $^{13}CO_2$ (Delfine et al., 1999), and ^{13}C would appear in MVK and MEK skeletons, if contributed by photorespiratory carbon.

Isoprene labelling experiments using ^{13}C-enriched glucose under CO_2-free air conditions have provided a clear indication that isoprene may also incorporate carbon via the glycolytic pathway (Affek and Yakir, 2003), and possibly also by re-fixation of respiratory CO_2 (Loreto, 2004; Loreto et al., 2007). This alternative carbon source can become prevalent when photosynthesis is stress-inhibited (Brilli et al., 2007), and may sustain small yet measurable emissions of isoprene in CO_2-free air (Affek and Yakir, 2002) or in detached leaves having no photosynthetic activity (Brilli et al., 2011; Harrison et al., 2013; Loreto and Schnitzler, 2010). However, under the conditions used here photosynthesis was never dramatically impaired and isoprene emission was labelled completely, even under heat stress. We conclude that the emitted carbonyls are not isoprene oxidation products but derive from a different metabolism. It should be noted, however, that in nature, under high UV radiation, the emission of isoprene is generally far larger than that found in our experiment, and the oxidation potential of the atmosphere is also greater. Thus, we cannot completely rule out that isoprene also oxidizes forming carbonyls, namely MVK and MACR, in nature. If not isoprene, then what is the source of the emitted carbonyls in plants? MVK and MACR are peroxidation products of the trienoic fatty acid contained in chloroplast membranes (Alméras et al., 2003; Kai et al., 2012), and cause cytotoxicity

once formed in leaves. MVK also is a component of flower scent (Knudsen et al., 1993) and may be a lipid peroxidation product, activating the expression of stress-related genes in Arabidopsis (Vollenweider et al., 2000). As we have demonstrated that isoprene is not responsible for the production in plants of MVK and its transformation products, further research should concentrate on investigating the relationships between emitted carbonyls and lipid catabolism in cellular membranes.

2 Materials and Methods

2.1 Plant material and experimental design

Experiments were performed on two-year-old red oaks (*Quercus rubra*) obtained from a local nursery (Malleier, Lana, Italy). Forty plants were placed in 5 L polypropylene pots (Plastecnic, Perego, Italy), using TerraBrill® peat moss (Agrochimica, Bolzano, Italy). Prior to the experiments, plants were grown for 60 d in a greenhouse under a 14 h photoperiod with a light:dark temperature regime of 24.0:19.0 °C, 60 ± 10 % relative humidity, and ca. 90 µmol m^{-2} s^{-1} light intensity. Plants were watered every three days and developed fully expanded leaves after five weeks. Four days prior to the experiments, plants were transferred from the growth chamber into a climate cabinet (Climacell 707, BMT Medical Technology, Brno, Czech Republic). The climatic cabinet was employed to maintain constant climatic conditions (fumigation experiments) or to apply heat stress while maintain all other parameters constant (heat stress experiments). The~~is~~ early transfer allowed plant recovery from accidental mechanical injures and adaptation to the new environment. The climate cabinet was set with the same parameters of the greenhouse, but at a constant temperature of 25°C (except for heat stress experiments), and interfaced with the PTR-ToF-MS via polyetheretherketone (PEEK) capillary tubes (ca. 1.5 m length x 1.01 mm ID, temperature: 110°C (part outside the climatic chamber, ca. 1 m), flow: 40 sccm).

One day prior to the start of the experiments, a shoot portion of each plant was enclosed within a Teflon (perfluoroalkoxy; PFA) bag and three capillary tubes were attached to each shoot to be monitored. The three tubes included the following: a PFA tube providing a constant flow of humidified zero air (eventually including fumigation VOCs during fumigation experiments) to the VOC-bag, a second PFA tube removing the overflow air, and a PEEK capillary tube sampling the VOC-bag air into the PTR-ToF-MS. As negative control, volatiles were monitored in parallel on an empty VOC-bag connected to the PTR-ToF-MS with the same tubing system described above.

Fumigation experiments were performed on three independent plants and on the empty VOC-bag as a negative control. New plants were used for every fumigation. Pure liquid standards of each chemical were purchased from Sigma-Aldridge and diluted with Milli-Q water ~~in 1:25,000 ratio~~. A Liquid Calibration Unit (Ionicon Analytik GmbH, Innsbruck, Austria) was employed to continuously nebulize 10 µL min^{-1} of the diluted standard into 1 L min^{-1} of synthetic air generated from a cylinder (Aria Medicinale F.U., Rivoira) made by 80% N_2, 20% O_2, and 400 ppm CO_2. The volume mixing ratio of the compound used for the fumigation in the VOC-bag inlets was the following: 290 ppbv for MVK, 330 ppbv for MEK, 90 ppbv for 2-butanol, 96 ppbv for 3-buten-2-ol, 140 ppbv for MACR. Fluxes were controlled using mass flow controllers (MKS Instruments,

Deutschland GmbH). Water vapour was added to the air stream by constantly nebulizing water via a Liquid Calibration Unit (Ionicon Analytik GmbH, Innsbruck, Austria) to reach a RH of 60%.

Fumigation experiments were performed separately for each chemical using fresh plants from the greenhouse. Gas flow measurements in the PFA tubes providing the fumigation gas mixture were conducted using a flow meter at the beginning and at the end of each experiment (Defender 530, DryCal Technology, MesaLabs) and was ca. 250 sccm for each VOC-bag. Leaf area enclosed in the bag was measured using an open-source software (ImageJ, available at http://imagej.net/Welcome). VOC uptake or release by the plants was calculated as the difference between VOC concentrations in the plant VOC-bag and in the empty VOC-bag. Results were normalized by the total leaf area enclosed in the bag, and by the incoming air flows.

Heat stress experiments were conducted using a similar setup than in the fumigation experiments. After acclimation for four days, during the day of the experiment, basal emissions at 25°C were measured for two hours before raising the temperature to 35°C (mild stress) and subsequently to 45°C (severe stress). Each temperature was maintained for two hours. Measurements were continued during the recovery phase at 25°C for two more hours. Transitions between different temperatures required a 15 minute ramp.

Experiments using labelled $^{13}CO_2$ were performed a follows. A 1-L cylinder of ^{13}C-labelled CO_2 was purchased from Sigma-Aldrich (99 atom % ^{13}C purity). The heat stress experiment at 45°C described above was repeated by switching the CO_2 in the air stream from unlabelled CO_2 to ^{13}C-labelled CO_2. The labelling was triggered when the signals for isoprene and isoprene primary and secondary oxidation products became stable after the temperature change, i.e. about one hour after the onset of the stress. The temperature was kept constant at 45°C while feeding ^{13}C-labelled CO_2. Results were compared to analogous experiments employing unlabelled CO_2.

2.2 Gas analysis

Trace gas analysis performed via a PTR/SRI-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria). PEEK capillary tubes directly sampled the air mixture from each VOC-bag to the instrument inlet. A valve system allowed switching between the enclosures every two minutes. The PTR/SRI-ToF-MS was equipped with a switchable reagent ion system (Jordan et al., 2009), allowing to select either H_3O^+ or NO^+ as primary ion. Measurements were generally taken using H_3O^+ ion chemistry, while for the fumigation experiments measurements were taken in both modes in order to strengthen compound identification. Calibrations with pure standards (purchased from Sigma-Aldrich) were carried out for all measured compounds using a Liquid Calibration Unit (Ionicon Analytik GmbH, Innsbruck Austria). For isoprene, the calibration was performed using a standard gas cylinder (Scott Specialty Gases/Air Liquide) containing isoprene (80.0 ± 5% ppm). Isoprene signal was monitored on the ion peaks, corresponding to proton transfer reactions leading to $C_5H_9^+$ for H_3O^+ mode and $C_5H_8^+$, corresponding to charge transfer reactions, for NO^+ mode (Karl et al., 2012). The peaks corresponding to the isotopologues were used to monitor the isotopic labelling. Such peaks were $^{13}CC_4H_9^+$, $^{13}C_2C_3H_9^+$, $^{13}C_3C_2H_9^+$, $^{13}C_4CH_9^+$, $^{13}C_5H_9^+$ for H_3O^+ mode and $^{13}CC_4H_8^+$, $^{13}C_2C_3H_8^+$, $^{13}C_3C_2H_8^+$, $^{13}C_4CH_8^+$, $^{13}C_5H_8^+$ for NO^+ mode. Ketones undergo proton transfer reactions in H_3O^+ mode and three body association reactions in NO^+ mode. This was the case for MVK and MEK, leading to the ion signals $C_4H_7O^+$ and $C_4H_9O^+$,

respectively, in H_3O^+ mode and $C_4H_6O{\cdot}NO^+$ and $C_4H_8O{\cdot}NO^+$, respectively, in NO^+ mode. Aldehydes react at collision rates via proton transfer in H_3O^+ mode and mainly hydride ion transfer in NO^+. In NO^+ mode aldehydes also undergo three body association reactions at a much reduced rate (Liu et al., 2013). This is the case of MACR and isobutyraldehyde, mostly leading to the ion signals $C_4H_7O^+$ and $C_4H_9O^+$, respectively, in H_3O^+ mode and $C_4H_5O^+$ and $C_4H_7O^+$, respectively, in NO^+ mode. Alcohols typically undergo proton transfer followed by dehydration in H_3O^+ mode and hydride ion transfer in NO^+ mode. Details on the spectral peaks used to monitor each compound in red oak ~~and on the instrument sensitivities~~ are reported ~~elsewhere~~in the Table S2 in Supplementary Information ~~(Cappellin et al., 2017, p.2)~~. Isotopic labelling was monitored using the signal corresponding to isotopologues analogously to the case of isoprene. Photosynthesis and dark respiration were measured as CO_2 exchange. The exchange of CO_2 and $^{13}CO_2$ was estimated in H_3O^+ mode using the spectral peaks at m/z 44.9971 and 46.0005, respectively. Since CO_2 does not react with H_3O^+ at collision rate, extensive calibrations with CO_2 and $^{13}CO_2$ standards were made at the experimental conditions (Figure S2). The conditions in the instrument reaction cell were the following: 2.19 mbar drift pressure, 60 °C drift tube temperature, and 542 V drift voltage for the H_3O^+ mode, resulting in an *E/N* ratio of ca. 125 Townsend (Td) (*N* corresponding to the gas number density and *E* to the electric field strength; 1 Td=10^{-17} Vcm^2); 2.21 mbar drift pressure, 90 °C drift tube temperature, and 296 V drift voltage in NO^+ mode, resulting in *E/N* ratio of ca. 74 Townsend (Td). The purity of the primary ions was high. In H_3O^+ mode the fraction of spurious NO^+ and O_2^+ ions was <0.3% and <2.1%, respectively. The amount of primary ions was $3.1{\cdot}10^6$ cps and the signal of the first water cluster was about 11%. In NO^+ mode the fraction of spurious NO_2^+, O_2^+ and H_3O^+ were <3.5 %, <0.2 % and <0.1 % respectively, relative to the primary NO^+ ion signal. The amount of primary ions was $2.6{\cdot}10^6$ cps. The ion detector in PTR/SRI-ToF-MS was a time-of-fight (ToF) mass analyser having mass resolution of about 4000 (m/Δm, FWHM). The sampling time per bin of ToF acquisition was 0.1 ns, amounting to 350000 bins for a m/z spectrum ranging up to m/z = 400. The spectral ion signals used to derive VOC concentrations were calculated following the procedure described by (Cappellin et al., 2011b). The detector dead time was corrected for applying a procedure based on the Poisson statistics (Cappellin et al., 2011a; Titzmann et al., 2010). Internal mass calibration was applied achieving a mass accuracy better than 0.001 Th (Cappellin et al., 2010). Extraction of ion counts was performed according to (Cappellin et al., 2011b), employing an optimized peak shape from the sample spectra for fitting.

2.3 Statistics

Statistical analyses were performed using R routines (R Development Core Team, 2009) developed in-house. Statistical differences of emissions or uptakes in different conditions were assessed using Kruskal-Wallis test at a significance level of $p<0.05$.

Data availability

The dataset is available from http://doi.org/10.4121/uuid:63039e2e-03d6-455d-81de-e94c9671f21c.

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Competing interest. The authors declare that they have no conflict of interest.

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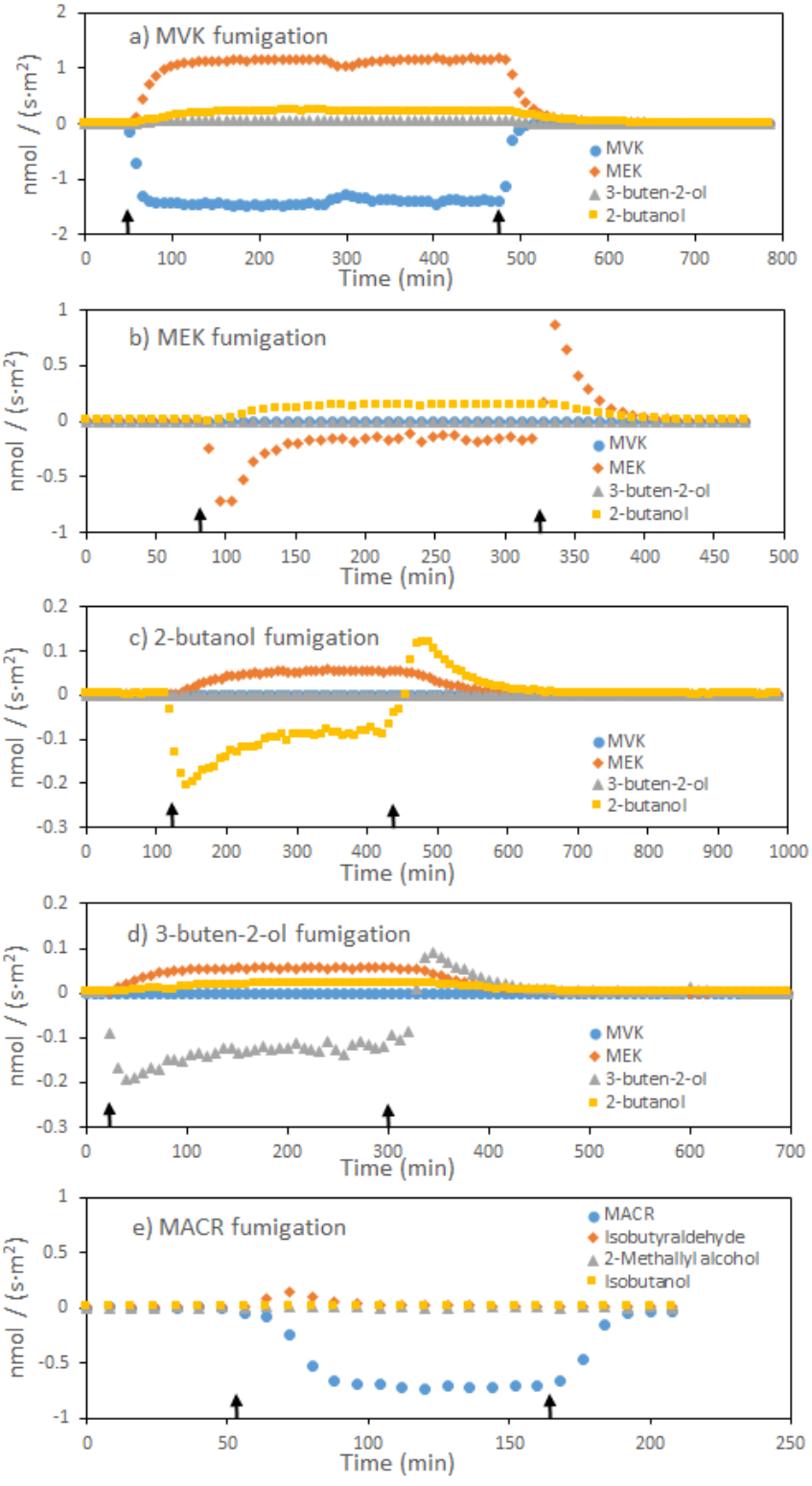
Figure captions


a) MVK fumigation
nmol / (s·m²)
Time (min)
MVK
MEK
3-buten-2-ol
2-butanol
b) MEK fumigation
nmol / (s·m²)
Time (min)
MVK
MEK
3-buten-2-ol
2-butanol
c) 2-butanol fumigation
nmol / (s·m²)
Time (min)
MVK
MEK
3-buten-2-ol
2-butanol
d) 3-buten-2-ol fumigation
nmol / (s·m²)
Time (min)
MVK
MEK
3-buten-2-ol
2-butanol
e) MACR fumigation
nmol / (s·m²)
Time (min)
MACR
Isobutyraldehyde
2-Methallyl alcohol
Isobutanol

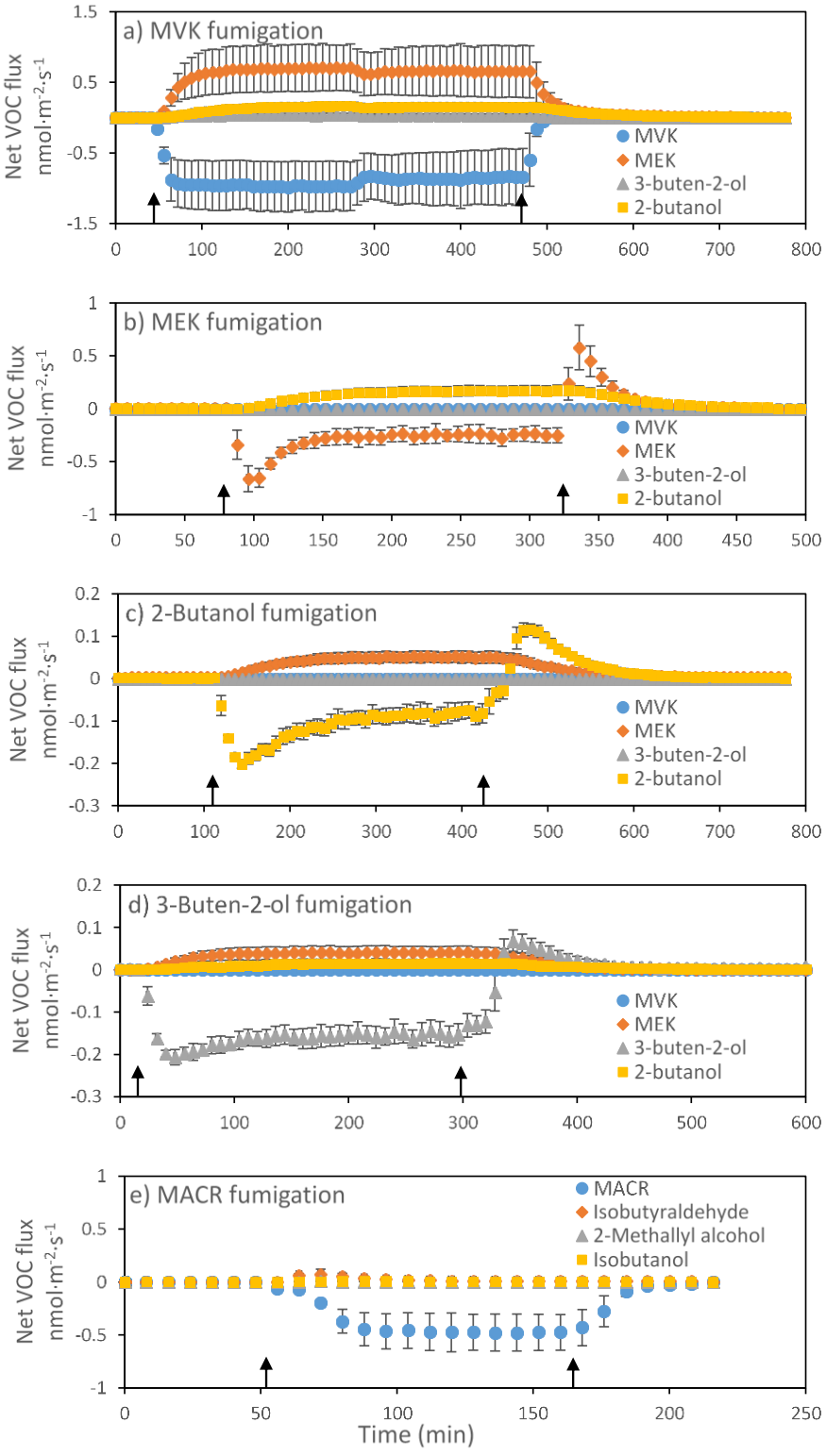
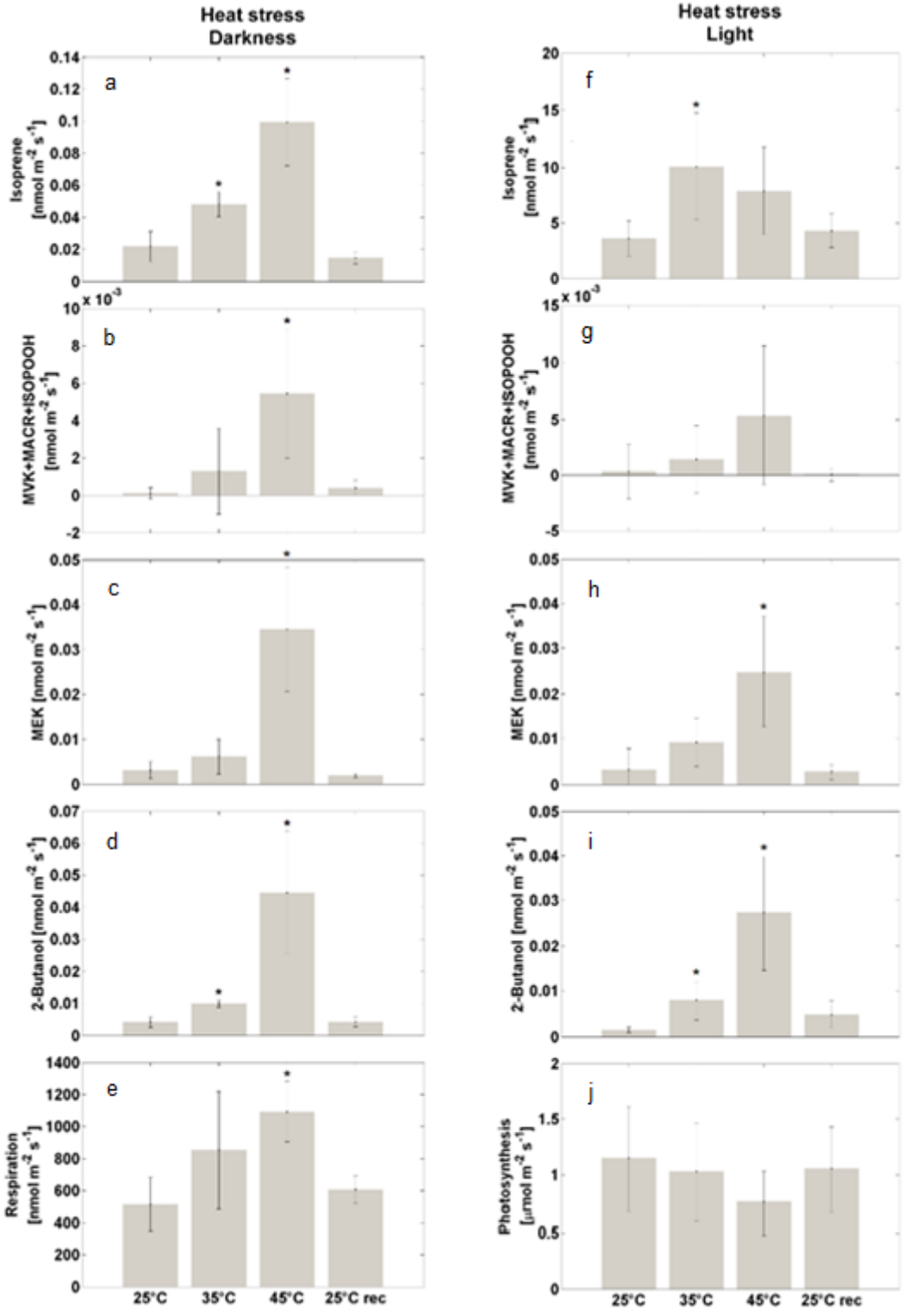

a) MVK fumigation
b) MEK fumigation
c) 2-Butanol fumigation
d) 3-Buten-2-ol fumigation
e) MACR fumigation
Net VOC flux
nmol·m-2·s-1
MVK
MEK
3-buten-2-ol
2-butanol
MACR
Isobutyraldehyde
2-Methallyl alcohol
Isobutanol
Time (min)

Figure 1.

Uptake of MVK (a), MEK (b), 2-butanol (c), 3-buten-e-ol (d) and MACR (e) and emission of their oxidized or reduced products in red oak leaves. Results are reported as mean ± standard deviation (n=3). Negative values denote the uptake - and positive values the emission of volatiles.~~Uptake and transformation of MVK (a) and MACR (e) and of MVK transformation products, MEK (b), 2 butanol (c), 3 buten e ol (d) by red oak leaves. Negative values denote uptake, while positive values indicate emission.~~ Black arrows indicate the beginning and the end of the fumigation. In panel (b), the pulses of MEK uptake at the beginning of the fumigation and of MEK release after the end of the fumigation correspond to formation and release, respectively, of a MEK pool dissolved into the leaf water. At equilibrium the uptake of MEK and subsequent release of 2-butanol is constant and indicates a constant rate of MEK transformation within leaves. Analogous considerations can be made for 2-butanol and 3-buten-2-ol in panels (c) and (d), respectively.


Heat stress
Darkness
Heat stress
Light
a
b
c
d
e
f
g
h
i
j
Isoprene [nmol m-2 s-1]
MVK+MACR+ISOPOOH [nmol m-2 s-1]
MEK [nmol m-2 s-1]
2-Butanol [nmol m-2 s-1]
Respiration [nmol m-2 s-1]
Photosynthesis [µmol m-2 s-1]
x 10-3
25°C
35°C
45°C
25°C rec

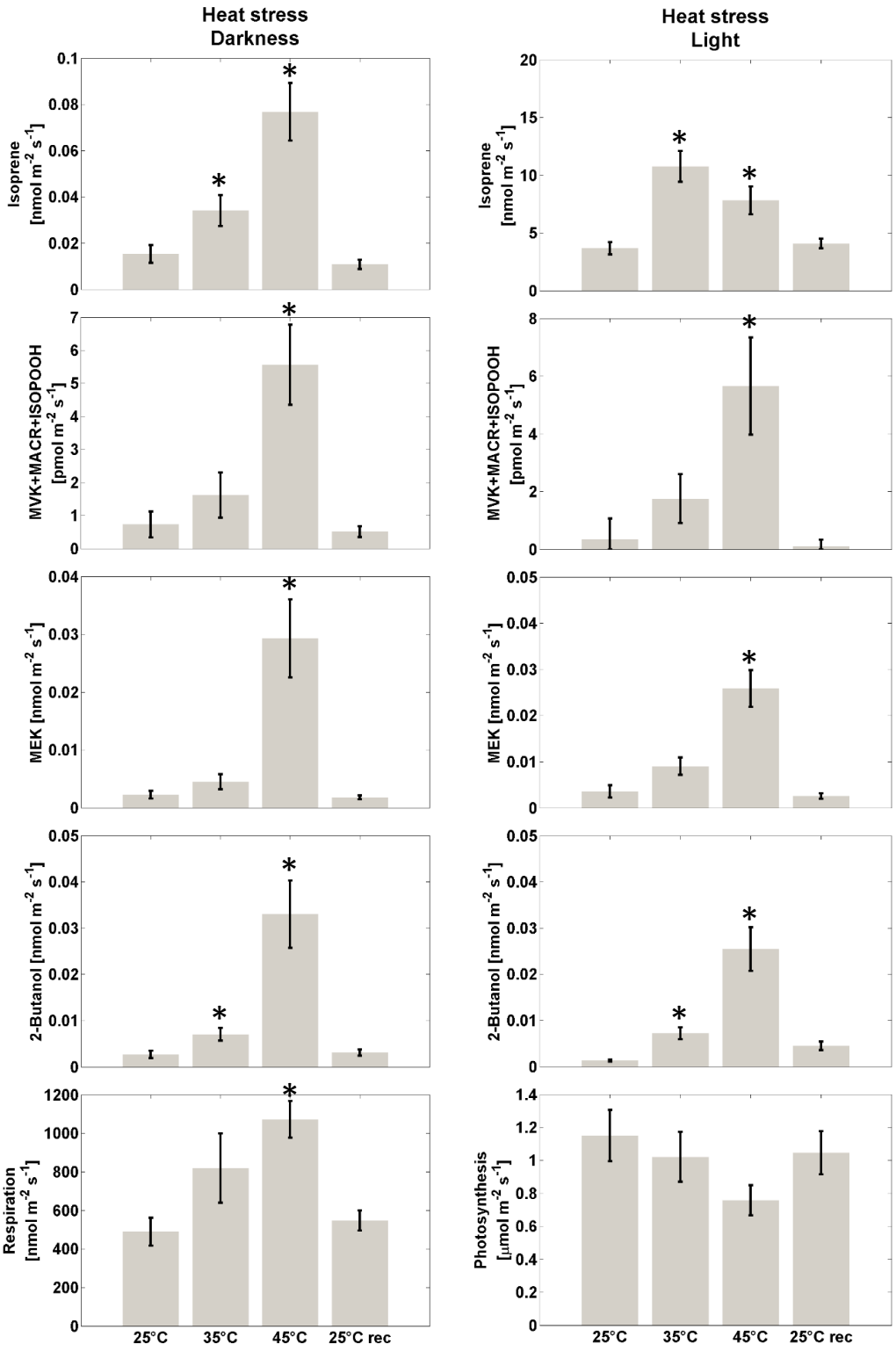

Heat stress
Darkness
Heat stress
Light
Isoprene [nmol m-2 s-1]
MVK+MACR+ISOPOOH [pmol m-2 s-1]
MEK [nmol m-2 s-1]
2-Butanol [nmol m-2 s-1]
Respiration [nmol m-2 s-1]
Photosynthesis [µmol m-2 s-1]
25°C
35°C
45°C
25°C rec

Figure 2. Left: ~~Emission~~ Net flux of isoprene (a), MVK+MACR+ISOPOOH (b), products from MVK reduction (MEK (c) and 2-butanol (d)) and respiration (e) upon exposure to moderate (35°C) or severe (45°C) heat stress, and recovery to 25°C by red oak plants in the dark. The compound grouping is based on PTR-TOF-MS in H_3O^+ mode. Results are reported as mean ± standard error (n=6). Asterisks indicate significant differences (Kruskal-Wallis, $p<0.05$) compared to the unstressed (25°C) case. Right: Emission of isoprene (f), MVK+MACR+ISOPOOH (g), products from MVK reduction (MEK (h) and 2-butanol (i)), and photosynthesis (j) upon exposure to moderate (35°C) or severe (45°C) heat stress, and recovery to 25°C in red oak plants in the light (ca. 90 µmol m^{-2} s^{-1} light intensity). The compound grouping is based on PTR-TOF-MS in H_3O^+ mode. Results are reported as mean ± standard error (n=6). Asterisks indicate significant differences (Kruskal-Wallis, $p<0.05$) compared to the unstressed (25°C) control.

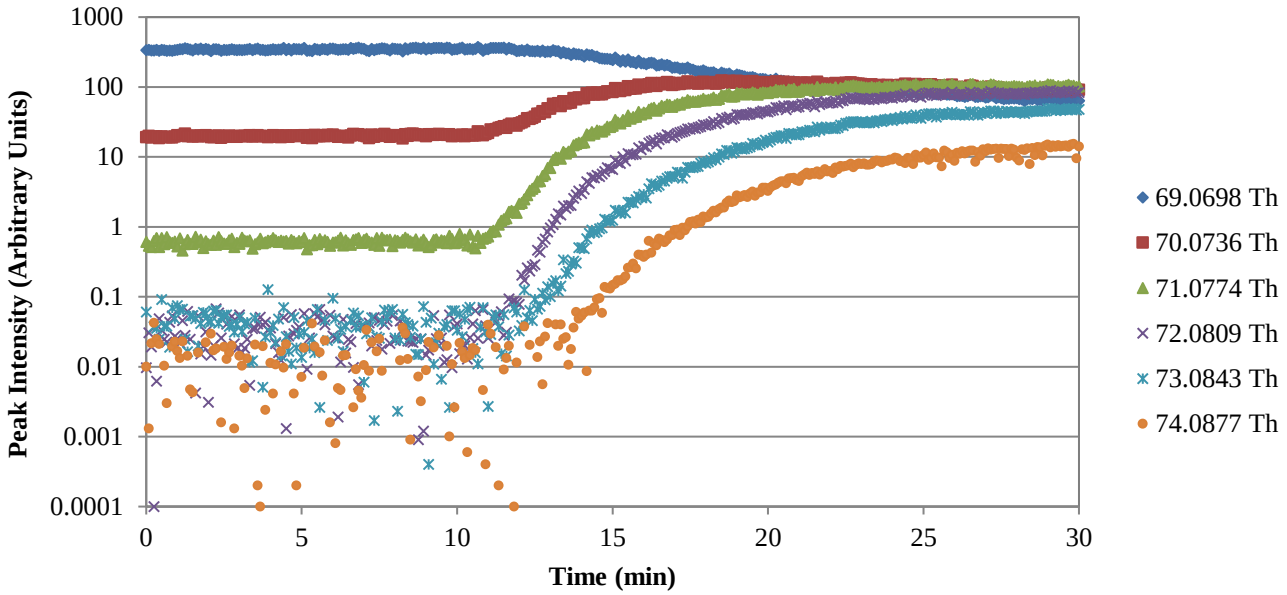
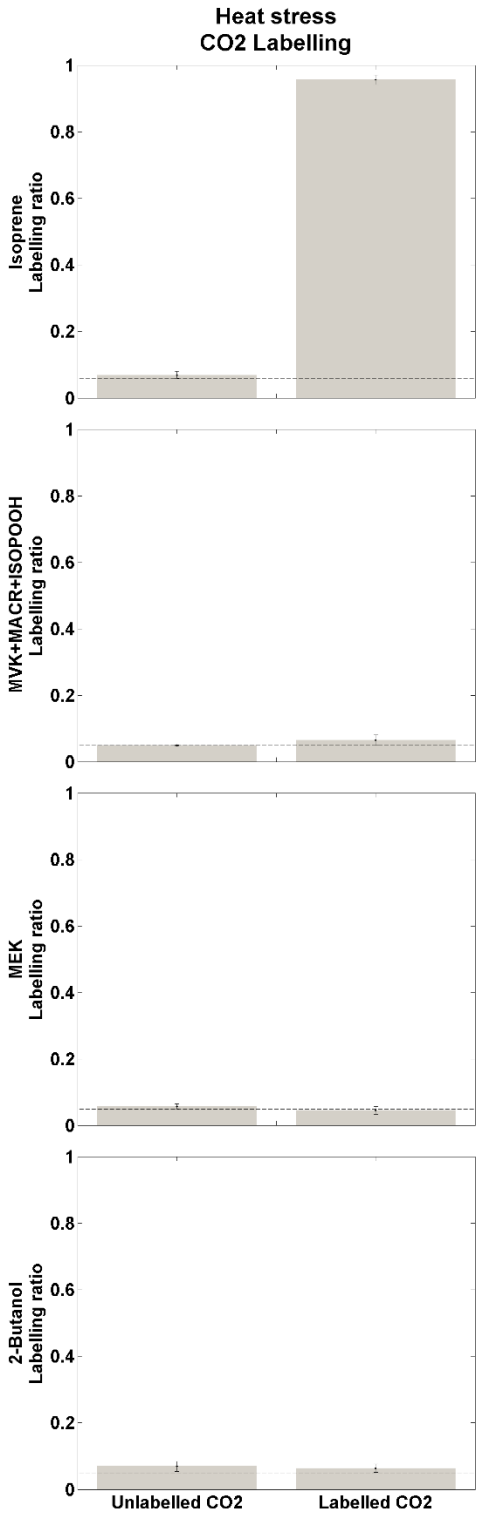


Figure 3. Labelling of isoprene emitted by red oak leaves upon $^{13}CO2$-feeding in the air. Fully labelled $^{13}CO_2$ is constantly supplied to the plants starting from time T = 10 min.


Heat stress
CO2 Labelling
a
b
c
d
Isoprene
Labelling ratio
MVK+MACR+ISOPOOH
Labelling ratio
MEK
Labelling ratio
2-Butanol
Labelling ratio
0
0.2
0.4
0.6
0.8
1
Unlabelled CO2
Labelled CO2

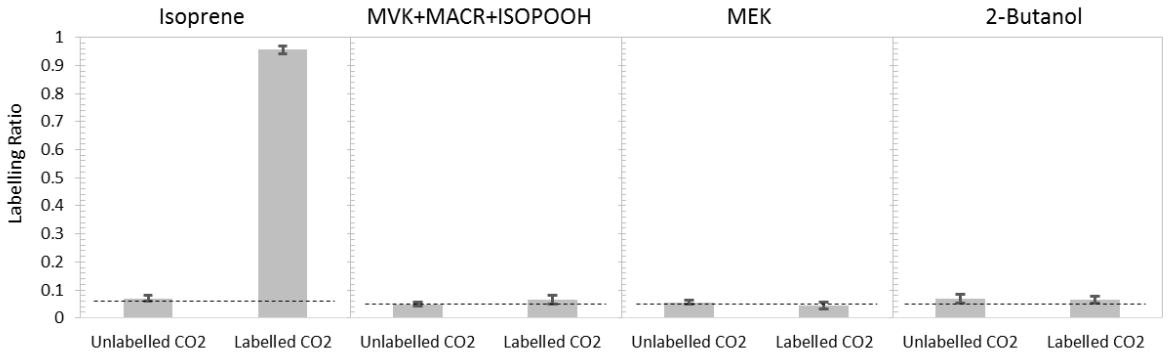
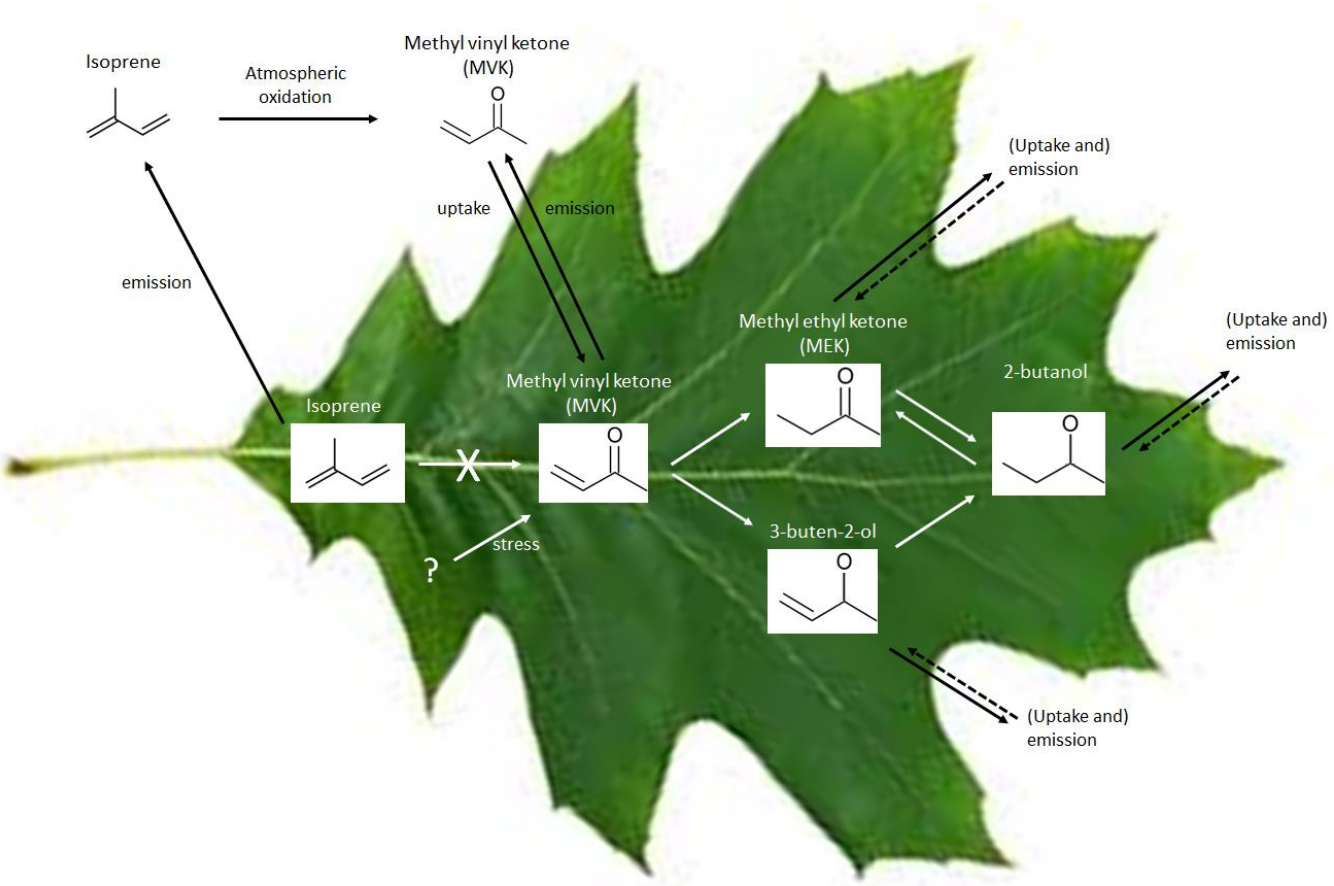


Figure 4. Emission of isoprene (a), MVK+MACR+ISOPOOH (b), MEK (c), and 2-butanol (d) in red oak plants heat stressed at 45°C for two hours in the light, under $^{12}CO_2$ or $^{13}CO_2$ atmosphere. Dashed horizontal lines represent natural abundances of isotopic compounds.


Isoprene
Atmospheric oxidation
Methyl vinyl ketone (MVK)
uptake
emission
emission
(Uptake and) emission
Methyl ethyl ketone (MEK)
(Uptake and) emission
Isoprene
Methyl vinyl ketone (MVK)
2-butanol
X
?
stress
3-buten-2-ol
(Uptake and) emission

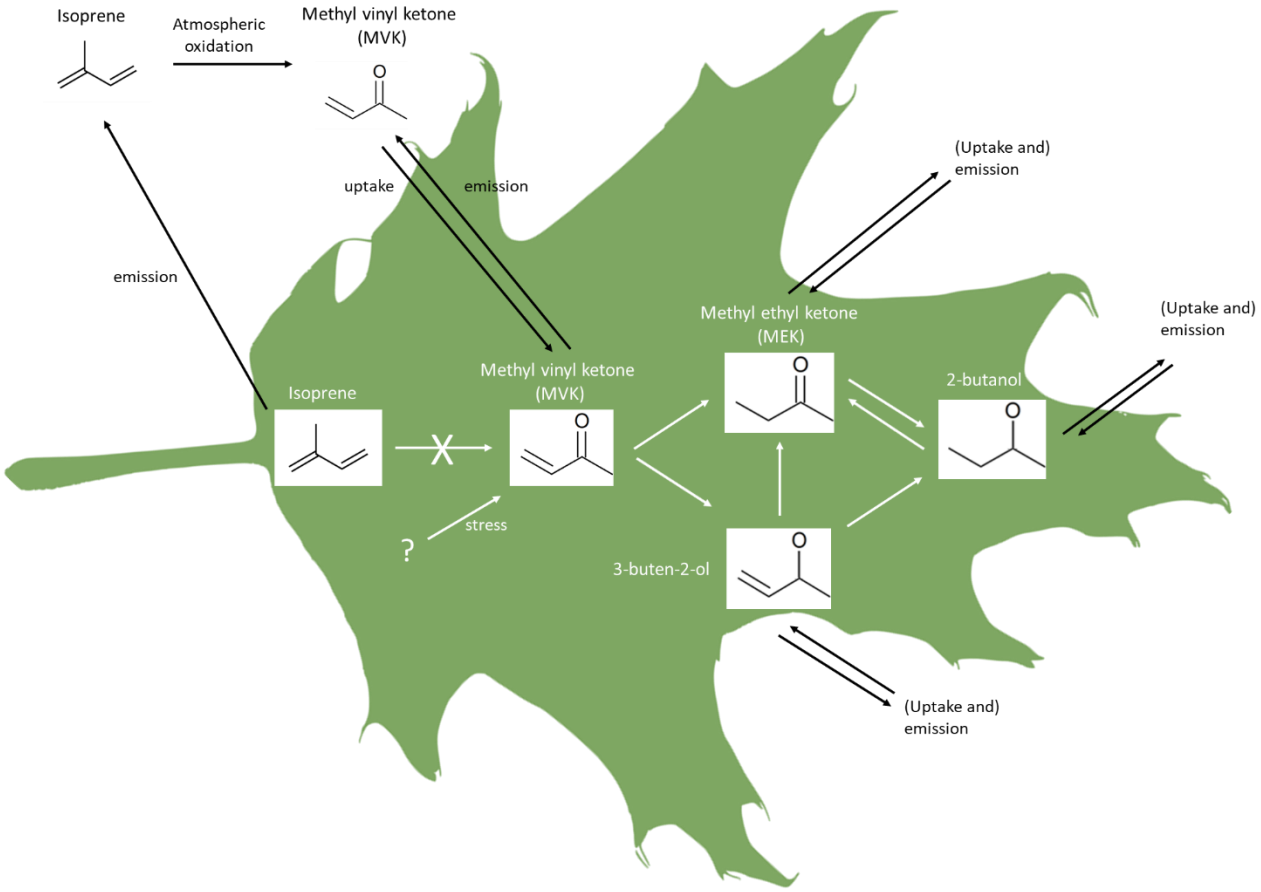


Figure 5. Schematics of MVK, MEK, 3-buten-2-ol, and 2 butanol origin and interconversion in leaves, as suggested by this study. The uptake of MEK, 3-buten-2-ol, and 2-butanol is indicated using dotted lines since it has not been reported so far but it is in principles possible given the present results.