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Lethal heat stress-dependent volatile emissions from tobacco leaves: what happens beyond the thermal edge?

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Abstract

Natural vegetation is predicted to suffer from extreme heat events as a result of global warming. In this study, we focused on the immediate response to heat stress. Photosynthesis and volatile emissions were measured in the leaves of tobacco (*Nicotiana tabacum* cv. Wisconsin 38) after exposure to heat shock treatments between 46–55 °C. Exposure to 46 °C decreased photosynthetic carbon assimilation rates (*A*) by more than three-fold. Complete inhibition of *A* was observed at 49 °C, together with a simultaneous decrease in the maximum quantum efficiency of photosystem II, measured as the F_v/F_m ratio. A large increase in volatile emissions was observed at 52 °C. Heat stress resulted in only minor effects on the emission of monoterpenes, but volatiles associated with membrane damage such as propanal and (*E*)-2-hexenal, (*Z*)-3-hexenol were greatly increased. Heat-induced changes in the levels of methanol, 2-ethylfuran that are indicative of modification of cell walls. In addition, the oxidation of metabolites in the volatile profiles was strongly enhanced, suggesting the acceleration of oxidative processes at high temperatures that are beyond the thermal tolerance limit.

Keywords

Chlorophyll fluorescence; heat stress; isoprene; methacrolein; methanol; monoterpenes; lipooxygenase pathway; photosynthesis; stress severity

1 Introduction

Biogenic volatile organic compounds (BVOC) are messengers in plant-to-plant, plant-to-herbivore, plant-to-pollinator and plant-to-seed-disperser communication (Arimura et al., 2005; Gershenzon and Dudareva, 2007; Dicke and Baldwin, 2010; Vickers et al., 2014; Vucetic et al., 2014; Cozzolino et al., 2015; Mishyna et al., 2015). Additionally, BVOC participate in atmospheric processes by contributing to the formation of tropospheric ozone,

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Conflicts of interest

The authors declare that they have no conflicts of interest.

cloud condensation nuclei and secondary aerosols (Guenther, 2000; Huff Hartz et al., 2005; Librando and Dipartimento, 2005; Fuentes et al., 2007; Dicke and Loreto, 2010; Kulmala et al., 2013).

The emission of BVOC is either constitutive or induced by different stress factors (Niinemets, 2010; Niinemets et al., 2013; Loreto et al., 2014; Copolovici and Niinemets, 2016). Wild tobacco plants are non-isoprene emitters, but for example, in non-stressed conditions several other plant species release constitutively isoprene and terpenoids (Guenther et al., 1994; Kesselmeier and Staudt, 1999; Vickers et al., 2009; Fineschi et al., 2013; Pollastri et al., 2014). While only a few species are strong constitutive emitters, induced volatile emissions caused by herbivores, pathogens, drought, low or high temperature, salinity, ozone and high light have been demonstrated both in strong and low constitutive emitters (Loreto and Delfine, 2000; Beauchamp et al., 2005; Loreto et al., 2006; Hartikainen et al., 2009; Lavoie et al., 2009; Dicke and Baldwin, 2010; Niinemets, 2010; Copolovici et al., 2011; Staudt and Lhoutellier, 2011; Copolovici et al., 2012; Jiang et al., 2016; Kask et al., 2016; Jiang et al., 2018; Kanagendran et al., 2018a; Kanagendran et al., 2018b).

Among different abiotic factors, high temperature is one of the most frequent and harmful stressors (Atkin et al., 2015; Heyduk et al., 2016; O'Sullivan et al., 2017; Niinemets, 2018; Zhu et al., 2018), and due to global warming, its impact on vegetation is expected to progressively increase worldwide (IPCC, 2013). In a natural setting, heat stress often occurs during heatwaves. In addition, sudden rises in leaf temperature to more than 50 °C can occur during lightflecks when shaded leaves are exposed to a bright light penetrating the canopy gaps (Singsaas et al., 1999; Singsaas and Sharkey, 2000; Valladares and Niinemets, 2007).

Plant physiological and molecular responses to heat stress depend on the severity of heat stress (Singsaas et al., 1999; Niinemets et al., 2009; Atkin et al., 2015; Ruiz-Vera et al., 2015; O'Sullivan et al., 2017; Niinemets 2018; Zhu et al., 2018). For example, mild heat stress inhibits photosystem II (PSII) activity, but upon return to a lower temperature, mild stress driven changes are typically fully reversible (Lu and Zhang, 2000; Zhang et al., 2009; Zhang and Sharkey, 2009; Hüve et al., 2011). Further increases in temperature lead to damaged PSII, membrane leakiness, production of various reactive oxygen species (ROS) and propagation of damage, ultimately leading to massive cellular death (Hüve et al., 2011; Grover et al., 2013; Pospíšil and Prasad, 2014; Kask et al., 2016; O'Sullivan et al., 2017; Zhu et al., 2018). Plants cope with heat stress by up-regulating heat-shock proteins and by scavenging ROS via enzymatic and/or non-enzymatic reactions involving antioxidative molecules (Vacca et al., 2004; Hasanuzzaman et al., 2013; Qu et al., 2013; Pospíšil and Prasad, 2014), but once the heat stress threshold is exceeded, ROS accumulation progressively increases and an apoptosis-like process is initiated (Bernardi et al., 1999; Jones, 2000; Dutilleul et al., 2003; Wang et al., 2015), which in turn leads to the time-dependent reductions in photosynthesis rate even after return to the lower temperature (Hüve et al., 2011).

Characteristic plant stress volatiles released rapidly upon different stresses are short-chained oxygenated compounds such as methanol, acetone, acetaldehyde and various lipooxygenase

(LOX) pathway derived compounds also known as green leaf volatiles (GLV) (Heiden et al., 2003; Loreto et al., 2006; Niinemets et al., 2009). Many of these volatiles are signal molecules, which activate regulatory genes involved in plant defense, stress tolerance and apoptosis (Kost and Heil, 2006; Scala et al., 2013; Ameye et al., 2017). Stress-dependent release of methanol occurs due to modifications in cell wall pectins (Koerner et al., 2009; Li et al., 2017). Lipoxygenase (LOX) pathway volatiles are synthesized from linolenic and linoleic acids in a cascade of reactions (Baur and Yang, 1969; Gigot et al., 2010). Their synthesis is initiated by cell membrane breakdown followed by the release of free linoleic and linolenic acids and further sequential action of lipoxygenases, hydroperoxide lyases, alcohol dehydrogenases and acetyltransferases (UI Hassan et al., 2015). Due to the constitutive activity of these enzymes, the emissions of LOX pathway derived volatiles occur rapidly as soon as their substrates become available (Feussner and Wasternack, 2002; Liavonchanka and Feussner, 2006; Rasulov et al. 2019). Next to GLV, plant stress may trigger or increase the emission of terpenoids (McConkey et al., 2000; Dudareva et al., 2006; Niinemets et al., 2013; Kanagendran et al., 2018b), but because their release requires expression of corresponding synthases, the stress-emissions typically occur with a time-delay (Pazouki et al., 2016). Heat stress alters the emission of monoterpenes and these emissions might increase or decrease depending on the duration and severity of stress and on whether terpenes are released from storage structures or whether they are de novo synthesized (Loreto et al., 1996; Loreto et al., 1998; Copolovici et al., 2012; Kleist et al., 2012).

The amount of different volatiles scales positively with the severity of heat stress (Copolovici et al., 2012; Kask et al., 2016). However, in previous studies, signaling and antioxidative controls on the heat stress response of transgenic tobacco (*Nicotiana tabacum*) have been studied only at temperatures below 50 °C (Wang et al., 2007; Xu et al., 2014; Liu et al., 2017). Yet, mitochondrial processes are functional even at higher temperatures (Hüve et al., 2011; Hüve et al., 2012; O'Sullivan et al., 2013; O'Sullivan et al., 2017) and such high temperatures do occur in nature. Thus, studies of higher temperatures are relevant, especially considering the vegetation responses to future heat waves that probably increase the BVOC emission from vegetation (Farré-Armengol et al., 2014; Jardine et al., 2015). Leaf temperature can be lower than the air temperature in windy conditions in actively transpiring plants, especially in small-leaved species, but leaf temperature can also be higher than air temperature in calm air and/or in water-stressed plants, especially in large-leaved species such as tobacco (Crawford et al., 2012; Yu et al., 2015; Urban et al., 2017; Wright et al. 2017; Blonder and Michaletz, 2018). Thus, we have used a water-bath methodology to apply highly controlled heat shock treatments to the leaves (Frolec et al., 2008; Copolovici et al., 2012; Kask et al., 2016; Pazouki et al., 2016).

The goal of the present study was to evaluate the effects of heat shock varying from moderate (46 °C) to extremely severe (55 °C) temperature. We hypothesized that once the critical heat stress threshold is reached, foliage photosynthetic characteristics are impaired, while key stress volatile emissions increase even above the temperatures that lead to the collapse of photosynthetic machinery. Additionally we show that upon the onset of an apoptosis-like process, plant leaves release a very high and surprisingly diverse blend of volatiles and that the release of stress volatiles also has a certain temperature optimum.

2 Material and Methods

2.1 Plant growth conditions and experimental material

Tobacco (*Nicotiana tabacum* cv. Wisconsin 38) seeds were germinated in Kekkilä garden soil (Kekkilä Group, Vantaa, Finland). After germination, individual seedlings were replanted in 4 L plastic pots and cultivated in the same substrate in a plant growth room at day/night temperatures of 27/23 °C and at a relative humidity of 60%. The day length was 14 h and illumination of 400-500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the plant level was achieved by Philips HPI-T Plus 400 W metal halide lamps (Eindhoven, The Netherlands). Plants were watered daily and fertilized with 0.5% fertilizer solution (Baltic Agro, Lithuania; NPK content ratio: 5:5:6; and micronutrients B (0.01%), Cu (0.03%), Fe (0.06%), Mn (0.028%), and Zn (0.007%) on a weekly basis.

In the experiments, fully-mature non-senescent leaves of nine- to ten-week old and 40 to 60 cm tall plants were used. All measurements were conducted with attached leaves. Due to short and winged leaf petioles of tobacco leaves, it was impossible to tighten the gas exchange chamber at the leaf to petiole junction (discussed below). Hence, two to three days prior to the stress treatments, part of the axial leaf tissue was removed so that the remaining portion of the leaf of 25-40 cm^2 could be air-tightly sealed in the gas-exchange chamber (Figure 1). The integrity of major veins was preserved such that the photosynthetic activity of the remaining portion of the leaf was not significantly different from the intact leaf. Leaf wounding elicited a significant release of LOX pathway derived volatiles, which was short-lived and lasted less than 30 min (Brilli et al., 2011; Brilli et al., 2012; Portillo-Estrada et al., 2015; Rasulov et al., 2019). At the time of the heat stress treatments, no LOX pathway derived or other stress volatiles were observed (Kanagendran et al., 2018a; Li et al., 2018) and there were also no quantitative or qualitative differences in the bouquets of base-level volatile emissions among mechanically wounded and intact leaves (data not shown).

2.2 Heat stress application

Heat shock was applied as characterized in earlier papers (Frolec et al., 2008; Copolovici et al., 2012). Briefly, a temperature-controlled water-bath (MB-5, Julabo GmbH, Germany) was heated to the desired temperature and the selected leaf was immersed in water for 10 min (Figure 1 for the experimental setup). Longer time that 10 min would have caused the lethal injuries to the leaf and thus, the emissions would have been dominated by unsaturated C6-aldehydes, alcohols and esters. Then, during 30 s the excess of water was gently removed with a soft tissue paper and the leaf was enclosed in the gas-exchange system for the measurements of photosynthetic characteristics and BVOC. Each experimental plant was measured twice. At first, the photosynthetic characteristics and BVOC of controls leaves were evaluated (immersed into 25 °C water), and then the same procedure was repeated at 46, 47, 48, 49, 50, 51, 52, 53, 54 and 55 °C. For every different heat shock temperature, separate leaves were used (Figure 1).

2.3 Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were conducted before heat stress and in 30 min after the given heat treatment using a Maxi-Imaging-PAM-fluorometer (Heinz Walz GmbH,

Effeltrich, Germany). Before the measurements the attached leaves were dark-adapted for 15 minutes. First, the minimum Chl *a* fluorescence (F_0) was measured, and then a saturating pulse of blue light of $2800 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 0.8 s was given to measure the maximum fluorescence yield (F_m). The maximum quantum yield of PSII in the dark-adapted state was calculated as F_v/F_m , where F_v is equal to $F_m - F_0$ (Schreiber, 2004).

In our study, F_v/F_m of the control leaves was on average ($\pm$ SE) 0.749 ± 0.005 (ca. 88% of theoretical maximum), which was somewhat lower than the theoretical maximum. The lower F_v/F_m reflects the circumstance that the maximum flash intensity of the imaging PAM was not fully saturating to yield the true F_m value and thus, the maximum F_v/F_m value was moderately underestimated (5-15%) (Masclaux-Daubresse et al., 2007; Ehlert and Hinch, 2008; Woo et al., 2008). Despite some underestimation, stress-dependent changes in F_v/F_m constitute an accurate measure of stress effects on leaf photochemistry and overall stress status (Ehlert and Hinch, 2008; Woo et al., 2008).

2.4 Gas exchange measurements

The treated leaves were enclosed in a custom-made gas-exchange system (Copolovici et al., 2010; Kask et al., 2016) with a modified leaf chamber. The leaf chamber consisted of a stainless steel cylindrical bottom part (4.4 cm height) and a double-layered glass upper part (50 cm² window area). The gas flow rate through the system was 1.26 L min^{-1} , and given the chamber volume of 0.22 L, the resulting chamber response half-time was 7.3 s (calculated for a first-order decay kinetics according to Niinemets, 2012). The chamber temperature was controlled by water that circulated between the double-glass layers of the chamber window. Thus, the temperature difference between the leaf surface and temperature-controlled chamber was less than 0.5 °C. The leaf temperature was measured with a thermocouple attached to the lower leaf surface. Four halogen lamps (50 W, Philips) provided the light intensity of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ at leaf surface. Ambient air drawn from outside by a pump passed through a 10 L buffer volume, a charcoal filter and a custom-made humidifier. CO₂ concentration was 380-400 $\mu\text{mol mol}^{-1}$ and humidity was ~60%. CO₂ and H₂O concentrations at the inlet and outlet of the chamber were measured with a LI-7000 infra-red CO₂/H₂O analyzer (Li-Cor, Inc., Lincoln, NE, USA). The analyzer was operated in the absolute mode and the readings were taken relative to the reference cell that was flushed with pure N₂. The measurement cell of the analyzer was switched between the chamber inlet (ingoing air) and outlet (outgoing air) with electronic valves.

Non-treated leaves were stabilized under standard measurement conditions for 10-20 min. When stomata fully opened and gas exchange rates stabilized, the gas exchange rates were recorded. In the case of heat stress treatments and control leaves immersed in 25 °C water, the measurements were always recorded in 30 min after the stress application. Net assimilation rate (A), transpiration rate (E), stomatal conductance to water vapor (g_s) and CO₂ concentration in sub-stomatal cavities (C_i) were calculated according to von Caemmerer and Farquhar (1981).

2.5 Volatile sampling and GC-MS analyses

Collection of volatiles was performed via the gas-exchange cuvette outlet with a flow rate of 200 ml min⁻¹ for 15 min after heat treatments. A constant flow air sample pump (1003-SKC, SKC Inc., Houston, TX, USA) was used to draw the cuvette air through the multi-bed stainless steel cartridges, which were filled with Carbotrap C (20-40 mesh), Carboxen B (40-60 mesh) and Carbotrap X (20-40 mesh) adsorbents (Supelco, Sigma-Aldrich) (Kännaste et al., 2014 for details). A blank (background) sample was taken from the empty cuvette prior to the measurement of each control leaf.

A Shimadzu 2010 Plus GC-MS system with Shimadzu TD20 automated cartridge desorber (Shimadzu Corporation, Kyoto, Japan) was used for the desorption and quantification of volatiles (Kännaste et al., 2014). The system was calibrated (Kask et al. 2016) with commercially available high-purity standards of LOX pathway products and terpenoids (Sigma-Aldrich). Volatiles were identified by comparing their retention times and mass spectra to the spectra of authentic standards and to the spectra in the NIST database (NIST05). The emission rates of volatiles were calculated according to Niinemets et al. (2011). In the current study, we focused mainly on the emissions of individual LOX pathway derived volatiles (2,4-hexadienal, hexanal, hexane, 1-hexanol, (*E*)-2-hexenal, (*Z*)-3-hexenal, pentane, 1-penten-3-one and propanal) and some 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway) volatiles. Additionally, we studied emissions of acetaldehyde, acetone, ethanol, 2-ethylfuran, 2-methyl-2-cyclopenten-1-one and methanol (Table 1). The detection limit of volatiles was better than 0.2 pmol m⁻² s⁻¹. Emission rates of sesquiterpenes, which are elicited several hours after the stress (Beauchamp et al., 2005; Pazouki et al., 2016b; Kanagendran et al., 2018a; Kanagendran et al., 2018b), remained below the detection limit in these experiments.

2.6 Methanol emission measurements

As methanol cannot be quantitatively trapped with the GC cartridges used, its emission was monitored with a proton transfer reaction mass spectrometer equipped with a quadrupole detector (high sensitivity version PTR-QMS, Ionicon GmbH, Innsbruck, Austria) by connecting the inlet of PTR-QMS to the gas exchange system. The flow drawn by PTR-QMS from the gas exchange system was 10 ml min⁻¹. Methanol was detected as the protonated parent ion at *m/z* of 33 (Copolovici and Niinemets, 2010). The PTR-QMS system was calibrated with a standard certified gas mixture (Ionimed Analytik, Innsbruck, Austria). Methanol emission rate was calculated from the difference between the measurements with a leaf and empty chamber according to Niinemets et al. (2011). Ultimately, the average methanol emission for 15 min after heat stress was calculated.

2.7 Statistical Analyses

Treatments of 46 to 47, 49 and 53 °C were repeated at least thrice and treatments of 48, 50 to 52, 54 and 55 °C were repeated four times. Hence, together with the blank and control measurements, 70 analyses were done. Foliage photosynthetic characteristics and emission rates of volatiles were expressed as average ± SE. Log-transformed data were used to study the impact of temperature on foliage physiological characteristics by ANOVA followed by a Tukey test (IBM SPSS Statistics 22, (IBM Corp, Armonk, NY). Additionally, the

relationships between the volatiles, or photosynthetic characteristics and volatiles were evaluated by Pearson correlation (Kännaste et al., 2013; Kask et al., 2016) and non-linear regression analyses (SigmaPlot ver. 12.5, Systat Software, Inc., San Jose California USA).

3 Results

3.1 Heat effects on Chl *a* fluorescence

In the temperature range of 46–48 °C, the maximum dark-adapted quantum efficiency of photosystem II (PSII) (F_v/F_m) diminished initially relatively slowly, but between temperatures 48–50 °C, it decreased rapidly to a value of 0.198 ± 0.019 (average $\pm$ SE), i.e. by more than 70% compared with the control leaves (Figure 2A). With further increases in heat stress severity, F_v/F_m gradually declined, reaching at 55 °C a value of about 3% of that in control leaves (Figure 2A).

Given that the minimum Chl *a* fluorescence F_0 did not change much until 49 °C, the first initial gradual change of F_v/F_m between 46–48 °C reflected primarily the reduction of F_m (e.g., 43% reduction from 0.661 ± 0.031 in control to 0.379 ± 0.014 in leaves exposed to 46 °C; Figure 2A). At 49 °C, F_0 started to increase (Figure 2A) and through all data, there was a negative correlation between F_v/F_m and F_0 (Figure 2B).

3.2 Changes in photosynthetic rate and stomatal conductance upon heat exposure

Already moderate heat stress at 46 °C that had a relatively minor effect on photochemistry (Figure 2A), reduced the net assimilation rate (A) by almost 80% from $12.5 \pm 0.7 \mu\text{mol m}^{-2} \text{s}^{-1}$ (control treatment) to $2.60 \pm 0.33 \mu\text{mol m}^{-2} \text{s}^{-1}$. A further declined with increasing heat stress severity reaching close to zero values after 48 °C treatment and negative values at higher treatment temperatures (Figure 3A). Differently from A , stomatal conductance to water vapor (g_s) also decreased in response to heat stress of 50 °C, but at higher temperatures g_s gradually increased (Figure 3B). Although g_s initially decreased together with A , the intercellular CO_2 concentration (C_i) actually slightly increased in high temperature treated leaves (Figure 3C) and the C_i to ambient CO_2 concentration (C_a) ratio (C_i/C_a) behaved analogously (data not shown).

3.3 Emission of volatiles

The emissions of hexane and pentane, acetaldehyde, acetone, ethanol, and terpenoids were unaffected by the heat stress (Table 1), but 2-ethylfuran, 2,4-hexadienal, hexanal, 1-hexanol, (*E*)-2-hexenal, (*Z*)-3-hexenol, methacrolein, 2-methyl-2-cyclopenten-1-one, and propanal increased with increasing treatment temperature after the heat stress threshold was exceeded (Table 1). Particularly massive emissions were observed at temperatures 52–54 °C, but at 55 °C the emissions declined again (Table 1).

In control plants and in plants exposed to 46–51 °C, the total emission of LOX pathway derived volatiles was low (Figure 4A). Starting from 52 °C LOX pathway derived volatiles began to increase and at 54 °C the total emission attained an average value of $14 \pm 5 \text{ nmol m}^{-2} \text{s}^{-1}$. After reaching the maximum, the LOX emission declined to $2.8 \pm 1.6 \text{ nmol m}^{-2} \text{s}^{-1}$ at 55 °C (Figure 4A). Heat stress enhanced also the emission of methanol that increased

from $2.9 \pm 0.6 \text{ nmol m}^{-2} \text{ s}^{-1}$ in control treatments to $270 \pm 50 \text{ nmol m}^{-2} \text{ s}^{-1}$ in plants exposed to 54°C (Figure 4B). Differently from LOX pathway derived volatiles emissions (Figure 4A), the elicited methanol emission stayed at a high level even at the hottest temperature of 55°C (Figure 4B).

Tobacco plants released also the hemiterpene isoprene and monoterpenes α -pinene, β -pinene, (*Z*)- β -ocimene and limonene (Table 1). In control and heat-stressed plants, the emission rates of individual volatile isoprenoids remained low (Table 1). Yet, the total emission of monoterpenes correlated positively with the treatment temperature through 46 to 55°C ($r = 0.41$, $P < 0.05$, data not shown).

3.4 Relationships of key BVOC with photosynthetic characteristics and among different BVOC

Relationships between all the volatiles derived via LOX pathway, photosynthetic characteristics (A , F_v/F_m) and methanol were evaluated via regression analysis. In the heat stress treatments, the total LOX pathway volatile emission rate showed a negative threshold-type non-linear relationship with F_v/F_m ($P < 0.0001$) (Figure 5A) and A ($P < 0.002$) (Figure 5B). A non-linear positive relationship was observed ($P < 0.0001$) between the total LOX pathway volatile and methanol emission rates (Figure 5C).

In control and heat-stressed plants, isoprene emission rate correlated positively with the emission rate of pentane ($r = 0.67$, $P < 0.001$ for controls plants and $r = 0.59$, $P < 0.01$ for stressed plants, data not shown). In addition, the emission rates of methacrolein and isoprene (Figure 6), and methacrolein and 2-ethylfuran were positively correlated, especially at 50 – 55°C (Figure 7A and 7B). Finally, significant positive relationships existed between two other LOX pathway derived volatiles pentane and propanal ($r > 0.48$ and $P < 0.01$ for controls or plants stressed at 50 to 55°C , data now shown). In most cases, the strength of these correlations and slopes were different for different treatment temperature ranges (Figure 6 and 7).

4 Discussion

Modifications of foliage photosynthetic characteristics due to moderate to extreme heat stress

Heat stress reduces leaf photosynthetic activity by inhibiting multiple processes and damaging key components of photosynthetic machinery (Law and Crafts-Brandner, 1999; Toth et al., 2005; Murata et al., 2007; Ashraf and Harris, 2013; Yan et al., 2013). Moderate heat stress inactivates photosystem II (PSII), ATP synthase, Rubisco, and Rubisco synthase activities, and typically also causes stomatal closure such that reduction in photosynthesis rate is both due to non-stomatal and stomatal factors, yet, upon return to lower temperatures, photosynthesis rate characteristically recovers (Law and Crafts-Brandner, 1999; Toth et al., 2005; Murata et al., 2007; Hüve et al., 2011; Ashraf and Harris, 2013; Kask et al., 2016). Onset of the rapid rise in F_0 during continuous heating indicates a critical temperature for the start of irreversible damage of PSII (O'Sullivan et al., 2017; Zhu et al., 2018). From a mechanistic point of view, the rise of F_0 level has been explained by dissociation of light-

harvesting chlorophyll a/b protein complexes from the reaction center complex of PSII, and an excessive reduction of plastoquinone pool, ultimately leading to damaged PSII reaction centers (Briantais et al., 1996). Although in our study F_0 level was assessed after the heat treatment at 25 °C (Figure 1), we argue that this reflects a sustained damage at PSII, analogously to a sustained photoinhibition (Demmig-Adams and Adams, 2006; Havaux, 1992). Continued decrease of F_v/F_m and F_0 at 50-55 °C (Figure 2) could be attributed to the propagation of lesions as the result of severe membrane damage, protein denaturation and complete impairment of repair processes due to ROS accumulation (Suzuki and Mittler, 2006; Allakhverdiev et al., 2008; Hüve et al., 2011; Ambastha et al., 2015).

Concomitant with reductions in F_v/F_m , net assimilation rate (A) also decreased (Figure 3A). A complete cessation of A observed at 49 °C was followed by negative values of A at even higher temperatures (Figure 3A), implying that the dark respiration rate was greater than the rate of gross photosynthesis. Although stomatal conductance (g_s) decreased under heat stress temperatures of up to 50 °C and then slightly increased (Figure 3B), intercellular CO₂ (C_i) concentration actually increased through the entire heat stress range (Figure 3C). This indicates that a decline in A was not immediately associated with the decrease in g_s similarly to other heat stress studies (Hüve et al., 2011; Jie et al., 2012). The reduction in A after exposure to temperatures 46-48 °C was stronger than in F_v/F_m , suggesting that dark reactions of photosynthesis were initially more strongly suppressed than the light reactions, possibly as the result of deactivation of Rubisco and activities of other Calvin cycle enzymes (Crafts-Brandner and Salvucci, 2000; Kurek et al., 2007). However, once the A was completely inhibited at 49 °C, the activity of light reactions was also rapidly lost (Figure 2).

Heat-triggered emissions of lipoxygenase pathway volatiles from tobacco leaves: a relationship with an optimum

A number of studies have demonstrated LOX pathway derived volatile emissions in response to wounding, herbivore attack, heat and other abiotic stresses at the immediate sites of stress/injury as well as in plant parts distant to the immediate site of damage (Major and Thomas, 1972; Kessler and Baldwin, 2002; Farag et al., 2006; Matsui, 2006; Wei et al., 2007; Adams et al., 2011; Blom et al., 2011; Wei and Kang, 2011; Copolovici et al., 2012; Rambla et al., 2016; Jiang et al., 2017; Rasulov et al. 2019). The release of LOX pathway derived volatiles plays an essential role in induced direct and indirect plant defenses including tri-trophic interactions and even in apoptosis, because the amount of LOX pathway derived volatiles and ROS produced by any stress determines the type of signaling or plant response they will induce (Geervliet et al., 1997; Engelberth, 2004; Ruther and Kleier, 2005; Kishimoto et al., 2008; Bruinsma et al., 2009; Wei and Kang, 2011; von Arx et al., 2012; Bhattacharjee, 2012; Tieman et al., 2012). There is evidence that the amount of LOX pathway derived volatiles scales quantitatively with the severity of stress (Jiang et al., 2017), but there is little information of whether this scaling is valid for extreme stress levels that can also inhibit LOX activity together with the cessation of entire plant metabolism. On the other hand, stress-generated ROS could directly oxidize plant volatiles and potentially also lead to the release of oxidized volatiles from other cell compartments including cell walls and membranes (Jardine et al., 2012; Jardine et al., 2013). That kind of a non-enzymatic

oxygenated volatile release might scale with stress severity without any signs of decline at extreme stress levels.

In the present study, the emission of LOX pathway derived volatiles was induced at higher temperatures according to a switch type response (Table 1) analogously to heat-stressed tomato (*Solanum lycopersicum*) (Copolovici et al., 2012) and black mustard (*Brassica nigra*) plants (Kask et al., 2016). Total LOX pathway derived volatile emission, F_v/F_m and A were related according to a threshold type response (Figure 5), which is in accordance with the circumstance that the rise of LOX volatile emission started not before the heat shock temperature raised at least to 53 °C (Figure 4A). At that temperature, F_v/F_m attained almost a minimum value and A was completely inhibited. This indicates that massive damage at the membrane level was absent until very high temperatures (Suzuki et al., 2011). However, the decline of LOX pathway derived volatile emissions at 55 °C suggests that the heat resistance of LOX enzymes or enzymes downstream LOX was compromised.

Constant emission of pentane and hexane from heat stressed plants (Table 1) is similar to the findings of Croft et al. (1993), where the release of pentane was not related to the heat stress of tobacco. On the other hand, the release of saturated aliphatic compounds including propanal (Table 1) under heat stress, has been attributed to by-products of lipid peroxidation (Anderson, 1994). Pentane is derived from linoleic acid (Kunert and Tappel, 1983), but propanal from linolenic acid (Lieberman and Kunishi, 1967) albeit other, yet unknown pathways such as ethylene pathway, can be responsible for the biosynthesis of saturated aldehydes (Anderson, 1994; Morgan and Drew, 1997; Wildt et al., 2003); however, ethylene could not be detected by our analytical setup.

The overall composition of emitted LOX pathway derived volatiles differed at different temperatures (Figure 4), and this might indicate differences in the activation of different LOX pathway enzymes, in the sites of LOX action and in heat resistance of different processes downstream of LOX and possible non-enzymatic reactions. Further studies are needed to gain an insight into processes governing the blend of LOX pathway derived emissions under heat stress.

Heat stress effects on volatiles from cell walls in tobacco

Severe abiotic stress, e.g. ozone stress leads to major destruction of cell walls and thus, to a complete loss of internal leaf structure (Matyssek et al., 1991; Günthardt-Goerg et al., 1997). So far, there are limited data of cell wall-associated volatile 2-methyl-2-cyclopentene-1-one. Yet, in our study, its enhanced emission at 53 to 54 °C provided a clear evidence that heat stress causes the decomposition of cell walls (Table 1). 2-Methyl-2-cyclopentene-1-one is suggested to originate from hemicellulose (Carrier et al., 2012), and methanol (Figure 4B) from cell wall pectins as the result of activation of pectin methylesterases and demethylation of pectins (Pelloux et al., 2007).

There is much more information of stress-dependent methanol emissions (Anderson, 1994; Graus et al., 2004; Beauchamp et al., 2005; Peñuelas et al., 2005; Cojocariu et al., 2006; Loreto et al., 2006; Copolovici and Niinemets, 2010; Brillì et al., 2011; Li et al., 2017; Niinemets et al., 2018). Pectin-released methanol is considered as a signaling compound,

which triggers systemic resistance processes in neighboring plants and also in non-stressed parts of a stressed plant (Dorokhov et al., 2014; Komarova, 2014). However, in our study, methanol emissions reached extremely high levels and differently from LOX pathway derived volatiles, methanol emissions were not inhibited in leaves exposed to 55 °C (Figure 4). This suggests that pectin methylesterases were likely more heat-resistant than all symplastic processes that became completely inhibited at the highest temperature.

Heat stress effects on the release of volatile terpenoids from tobacco leaves

Nicotiana tabacum is a low-level constitutive emitter of monoterpenes due to their presence in glandular trichomes on the leaf surface (Kanagendran et al., 2018a; Kanagendran et al., 2018b). Thus, severe stress can lead to either enhanced terpene emission due to the breakage of trichomes or due to the increased permeability of glandular trichome outer surfaces (Guenther et al., 1993; Jansen et al., 2009). Such an enhanced emission response has been observed in heat shock-treated tomato (*Solanum lycopersicum*) (Copolovici et al., 2012; Pazouki et al., 2016). In our study, the effect of heat stress on the emission of monoterpenes remained small through the entire temperature range (Table 1), but still, a significant positive relationship with heat shock temperature was observed according to regression analyses. Although this positive correlation suggests that heat stress might have changed the permeability of glandular trichomes, emission of terpenes changed much less compared to the changes in leaf photosynthetic apparatus and cellular oxidative status.

Different biotic and abiotic stresses lead to an induction of terpene synthesis and release from mesophyll cells (Loreto and Schnitzler, 2010; Niinemets, 2010). The stress-induced terpene emissions depend both on the induction of expression of corresponding terpene synthases and on substrate availability (Chern et al., 2013; Pazouki et al., 2016). In *N. tabacum*, enhancement of mono- and sesquiterpene release has been observed both in response to pathogen attacks (Huang et al., 2003) and in response to ozone stress (Kanagendran et al., 2018a; Kanagendran et al., 2018b). However, due to the delays associated with gene expression and synthesis of relevant terpene synthase proteins, there is generally a time-delay between the stress impact and the start of the release of terpenes (Pazouki et al., 2016). Nevertheless, we cannot rule out that elicitation of de novo terpene emissions did contribute to the positive relationship between heat shock temperature and terpene emission rate.

Isoprene, methacrolein and 2-ethylfuran release from heat-stressed tobacco leaves

Tobacco lacks the key terminal enzyme, isoprene synthase, but transgenic isoprene-emitting *N. tabacum* have been constructed and their oxidative stress resistance has been studied (Vickers et al., 2009; Jardine et al., 2012). Thus, it was surprising to find isoprene, albeit at a low level, in the emissions of control and heat-stressed wild-type tobacco plants (Table 1, Figure 6). Lack of isoprene synthase in tobacco arises the question what was the source of isoprene emissions in these plants? Non-enzymatic formation of isoprene from plastidial or cytosolic pools, especially under heat stress could lead to release of some isoprene. Tobacco smoke contains large amounts of isoprene (Sleiman et al., 2014), which suggests non-enzymatic isoprene formation from tobacco metabolites. The emission of isoprene may also be associated with the presence of polyisoprenoid solanesol (C45, i.e. 9 isoprene units) in

tobacco leaves (Yan et al., 2017) and its decomposition under heat stress. On the other hand, many plant species contain hemiterpene glycosides (Ward et al., 2011; Ono et al., 2015; Lihavainen et al., 2016), including the Solanaceae sp. (Ono et al., 2015), and thus isoprene release as the result of specific glycosidases is possible. Next to isoprene, also methacrolein is present in tobacco smoke (Sleiman et al., 2014). Differently from isoprene, significantly elevated emissions of methacrolein at high temperatures (Table 1, Figure 6 and 7) could be related to the within-plant isoprene oxidation by ROS (Jardine et al., 2012) or other/additional sources for methacrolein (Cappellin et al., 2019). In fact, also the co-existence of 2-ethylfuran and methacrolein refers the overall rise of leaf ROS status upon heat stress (Figure 7). The exact biosynthesis pathway of 2-ethylfuran is unclear, but according to the literature, the biosynthesis of furans may be related to the oxidation of phenolic compounds and even isoprene (Atkinson et al., 1989; Krause et al., 2014). Finally, 2-ethylfuran was recently detected in pathogen-infected leaves of grapevine (*Vitis vinifera*) (Lazazzara et al., 2018).

5 Conclusions

Tobacco is an important agricultural plant and a model in plant biology, and thus, the results obtained contribute to the understanding of the mechanisms of stress signaling and plant response to heat stress. This study indicates that in heat-stressed tobacco leaves, net assimilation rate (A) is inhibited first, followed by cessation of light reactions of photosynthesis, and ultimately by enhancement of stress volatile emissions, whereas elicitation of foliage stress volatiles occurs according to a switch-type response. The critical heat shock temperature leading to a rapid increase of stress volatile emissions was 52 °C. Differently from methanol, emissions of which increased even at the highest studied temperature, the emissions of most stress volatiles such as membrane-derived lipoxygenase pathway (LOX) derived volatiles, cell-wall derived methyl-2-cyclopentene-1-one, oxidation products of cellular volatiles and/or non-volatiles initially increased with increasing the severity of heat stress, but decreased at the most severe heat stress of 55 °C. This indicates that extreme heat stress resulted in the collapse of leaf metabolism, including the capacity to form ROS and respond to stress via volatiles. Although the total volatile emission rates decreased at the most severe heat stress treatment, the composition of volatiles was altered, providing a unique fingerprint of this extreme temperature. Regarding the emission and synthesis of terpenes, further studies are needed to understand how elicitation of terpenoid synthases scales with the severity of heat stress and how heat stress affects the accumulation and/or degradation of terpenoids such as diterpenoids and solanesol. Overall, monitoring emissions of stress volatiles through heat stress may be used as a non-invasive tool for quantifying heat stress resistance in phenotyping studies in other plant species.

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Highlight

Heat shock impairs photosynthesis in tobacco and results in massive increases in the emission of key stress volatiles including methanol and green leaf volatiles.

Figure 1.

Description of the workflow for tobacco (*Nicotiana tabacum* cv. Wisconsin 38) heat stress experiment. Two to three days prior to the experimental treatment, a part of the leaf tissue was removed such that the leaf could be fitted in the gas-exchange system. Heat treatments were applied for 10 min. by immersion of leaves in water at a given temperature (T_{stress} , control leaves were immersed in water at 25 °C and heat stressed leaves in water at temperatures between 46-55 °C). After treatments, the physiological leaf characteristics were measured at 25 °C ($T_{\text{leaf}} = 25$ °C). The treated leaf was inserted in the cuvette of the gas-exchange system and foliage photosynthetic characteristics were measured (net assimilation rate, stomatal conductance to water vapor and intercellular CO_2) and volatiles (BVOC) were collected. After these measurements, the leaf was dark-adapted for 15 min. for chlorophyll fluorescence measurements. Each individual leaf was treated only once with one of the heat shock temperatures.

Figure 2.

Minimum (F_0) and maximum (F_m) dark-adapted chlorophyll *a* fluorescence yield, and maximum dark-adapted quantum yield of PSII (F_v/F_m ; a), and correlation between F_0 and F_v/F_m (b) in control (25 °C) and heat-stressed tobacco (*Nicotiana tabacum* cv. Wisconsin 38) leaves (10 different heat stress treatments). In (a), data correspond to averages $\pm$ SE of 3-4 replicates, while values for individual leaves fitted by linear regression are plotted in (b). The heat stress treatment lasted for 10 min. and gas-exchange measurements and volatile collection took 15 min (Figure 1). Thus, chlorophyll fluorescence measurements were

conducted in ca. 30 min. after the heat treatment at the control conditions (25 °C). Different leaves were used for each heat treatment.

Figure 3.

Heat stress effects on foliage net assimilation rate (a), stomatal conductance to water vapor (b), and intercellular CO_2 concentration (c) in *N. tabacum* cv. Wisconsin 38. Photosynthetic characteristics were measured 30 min. after the heat treatment at 25 °C (Figure 2 for heat treatments). Data are means of 3-4 replicate measurement at each temperature. Different letters indicate statistical difference (ANOVA followed by Tukey test, $P < 0.05$).

Figure 4.

Influence of heat stress on the emissions of volatile products of the lipoxygenase pathway compounds (LOX, a) and methanol (b) in leaves of *N. tabacum* cv. Wisconsin 38. The emission rates correspond to the average values for a period of 15 min. after heat stress and were measured with GC-MS (a) and PTR-QMS (b). Data reporting and statistical analysis as in Figure 3.

Figure 5.

Relationship between the dark-adapted quantum yield of PSII (a), net assimilation rate (b) and methanol emission rate (c) and the emission rate of lipoxygenase (LOX) pathway volatiles through the heat stress treatments in *N. tabacum* cv. Wisconsin 38. Data correspond to individual heat-stressed leaves. Heat stress effects on individual characteristics are shown in Figure 2a (chlorophyll fluorescence), Figure 3a (net assimilation rate) and Figure 4 (LOX pathway compounds and methanol). Data were fitted by non-linear regressions in the form:

$y = a_1 + b_1 / (1 + \exp[-(x - c_1)/d_1])^{e_1}$ for (a); $y = a_2 + b_2 \exp[-\exp(-(x - c_2)/d_2)]$ for (b); $y = a_3 x / (b_3 + c_3 x)$ for (c).

Figure 6.

Linear regressions between the emissions of isoprene and methacrolein from control (white circles) and 50-55 °C treated (gray circles) leaves of *N. tabacum* cv. Wisconsin 38. Stressed plants, which lacked the high emission of methacrolein (within the ellipse) were excluded from the regression test of heat-stressed plants (50-55 °C). The relationships of control and stressed plants were statistically significant at $P < 0.001$ (**) and $P < 0.01$ (***), respectively.

Figure 7.

Linear regressions between the emissions of methacrolein and 2-ethylfuran in *N. tabacum* cv. Wisconsin 38 in control plants and in plants treated with 46-49 °C (a) and plants treated with 50-55 °C (b). The relationships of control and stressed plants were statistically significant at $P < 0.01$ (**) and $P < 0.001$ (***), respectively.

Table 1

Average (SE) emission rates ($\text{pmol m}^{-2} \text{s}^{-1}$) of detected volatiles in heat-stressed *Nicotiana tabacum* after exposure to different heat shock treatments.

No	Compounds	Treatment temperature (°C)										
		25	46	47	48	49	50	51	52	53	54	55
Lipoxygenase pathway volatiles												
1	2,4-Hexadienal	1.74 (0.25) *	0.85 (0.30) a	7.1 (3.8) ab	1.85 (0.68) a	0.59 (0.43) a	1.16 (0.57) a	2.5 (1.9) a	13 (9) ab	130 (70) bc	220 (90) b	24 (22) ac
2	Hexanal	63 (13) a	40 (20) a	138 (58) abc	58 (19) a	28 (17) a	78 (33) ab	110 (33) ab	230 (130) abc	1260 (550) bc	2170 (840) c	310 (200) abc
3	Hexane	440 (110) a	480 (360) a	1600 (1400) a	460 (200) a	110 (65) a	190 (130) a	260 (110) a	540 (220) a	620 (310) a	350 (150) a	400 (120) a
4	1-Hexanol	1.03 (0.19) a	1.05 (0.46) a	2.4 (1.6) ab	1.56 (0.77) ab	2.2 (1.8) ab	3.0 (2.5) ab	4.3 (1.5) abc	9 (6) abc	20 (6) bc	54 (21) c	5.7 (3.8) ab
5	(E)-2-Hexenal + (Z)-3-hexenol	5.68 (1.79) a	1.75 (0.69) ab	5.0 (3.6) ab	3.9 (1.8) ab	0.434 (0.091) a	15 (15) ab	14 (7) ab	80 (61) abc	600 (530) bc	1980 (1270) c	33 (22) ab
6	Pentane	70 (15) a	54 (18) a	54.0 (2.1) a	87 (45) a	33 (12) a	37 (6) a	43 (12) a	165 (103) a	130 (30) a	100 (15) a	106 (19) a
7	1-Penten-3-one	9.0 (1.8) a	3.3 (2.6) a	4.1 (1.8) ab	9 (8) ac	5.4 (2.9) ab	6.0 (4.1) ab	3.34 (0.80) ab	5.1 (2.7) ab	180 (68) b	130 (41) bc	66 (39) ab
8	Propanal	72 (7) a	54 (7) a	103 (42) a	96 (25) a	97 (53) a	107 (35) a	88 (22) a	200 (100) a	2830 (1180) b	3110 (1450) b	720 (480) ab
MEP/DOXP pathway volatiles												
9	Isoprene	55 (12) a	50 (18) a	47 (12) a	59 (24) a	26 (13) a	31 (13) a	36 (21) a	88 (43) a	100 (55) a	65 (25) a	57 (17) a
10	Limonene	87 (20) a	35 (23) a	17.2 (4.6) a	47 (33) a	60 (49) a	17.2 (4.5) a	23 (11) a	73 (41) a	140 (65) a	47 (13) a	117 (70) a
11	Methacrolein	41 (5) a	27 (6) ab	65 (38) ab	33 (15) ab	43 (22) ab	33 (9) ab	24 (5) ab	43 (15) ab	240 (100) b	140 (80) ab	93 (64) ab
12	cis-β-Ocimene	24.9 (3.0) a	18 (9) a	13.7 (4.4) a	22 (7) a	11 (5) a	26 (14) a	26 (9) a	29 (10) a	27 (11) a	23 (7) a	23 (5) a
13	α-Pinene	29.8 (3.6) a	21 (9) a	22 (5) a	58 (30) a	29 (18) a	34 (12) a	25 (6) a	36 (14) a	29 (9) a	26.7 (3.8) a	28.1 (3.7) a
14	β-Pinene	6.5 (2.7) a	2.1 (1.2) a	9 (5) a	1.7 (0.9) a	4.8 (4.3) a	1.49 (0.64) a	2.6 (0.6) a	9 (8) a	4.6 (1.3) a	3.07 (0.73) a	5.3 (3.7) a
Volatiles of other pathways												

No	Compounds	Treatment temperature (°C)										
		25	46	47	48	49	50	51	52	53	54	55
15	Acetaldehyde	1030 (260) a	1380 (1190) a	660 (340) a	860 (670) a	1020 (890) a	460 (155) a	1760 (1310) a	1880 (1110) a	3870 (1795) a	3080 (630) a	1930 (1310) a
16	Acetone	630 (120) a	310 (160) a	1050 (445) a	500 (260) a	470 (315) a	810 (360) a	400 (80) a	1050 (480) a	845 (200) a	350 (50) a	370 (200) a
17	Ethanol	1530 (540) a	970 (960) a	1620 (1610) a	1260 (1260) a	1600 (1600) a	230 (220) a	580 (520) a	1600 (1580) a	2880 (2770) a	760 (520) a	2340 (2330) a
18	2-Ethylfuran	14.8 (2.3) a	8.1 (2.7) a	8.5 (2.1) a	10.6 (1.8) a	10.8 (1.9) a	14 (9) a	45 (36) ac	57 (22) ac	2620 (1260) b	1840 (550) b	700 (440) bc
19	2-Methyl-2-cyclopenten-1-one	4.65 (0.74) a	5.6 (2.4) ab	5.3 (3.2) ab	18.1 (2.7) ab	6.3 (4.4) ab	26 (22) ab	100 (80) ab	130 (100) bc	2030 (880) cd	4430 (1450) d	440 (390) bc

Leaves were exposed to each different treatment temperature for 10 min., transferred to 25 °C and at this temperature, volatiles were collected on adsorbent cartridges 30 min after the treatment and analyzed by GC-MS (Figure 1). Independent leaves were used for every temperature.

* statistical difference between the log-transformed emissions of volatiles was tested with one-way ANOVA followed by Tukey test. Letters in bold font indicate statistically significant differences at *P*-level of 0.05.