Original Article

How specialized volatiles respond to chronic and short-term physiological and shock heat stress in *Brassica nigra*

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ABSTRACT

Brassicaceae species release volatile glucosinolate breakdown products upon tissue mechanical damage, but it is unclear how the release of glucosinolate volatiles responds to abiotic stresses such as heat stress. We used three different heat treatments, simulating different dynamic temperature conditions in the field to gain insight into stress-dependent changes in volatile blends and photosynthetic characteristics in the annual herb *Brassica nigra* (L.) Koch. Heat stress was applied by either heating leaves through temperature response curve measurements from 20 to 40 °C (mild stress), exposing plants for 4 h to temperatures 25–44 °C (long-term stress) or shock-heating leaves to 45–50 °C. Photosynthetic reduction through temperature response curves was associated with decreased stomatal conductance, while the reduction due to long-term stress and collapse of photosynthetic activity after heat shock stress were associated with non-stomatal processes. Mild stress decreased constitutive monoterpene emissions, while long-term stress and shock stress resulted in emissions of the lipoxygenase pathway and glucosinolate volatiles. Glucosinolate volatile release was more strongly elicited by long-term stress and lipoxygenase product released by heat shock. These results demonstrate that glucosinolate volatiles constitute a major part of emission blend in heat-stressed *B. nigra* plants, especially upon chronic stress that leads to induction responses.

Key-words: Brassicaceae; glucosinolate breakdown products; heat shock; high temperature; lipoxygenase pathway; terpenoid emission; volatile organic compounds.

INTRODUCTION

Among abiotic stresses, heat stress is one of the most deleterious factors resulting in major cellular damage once the heat stress threshold has been exceeded (Bidart-Bouzat & Imeh-Nathaniel 2008). Such deleterious heat effects are manifested in ubiquitous stress responses such as collapse of leaf photosynthetic activity and formation of reactive oxygen species in leaf tissues (Vacca et al. 2004; Hüve et al. 2011) and elicitation of release of lipoxygenase (LOX) pathway volatiles (Maccarrone et al. 1992; Copolovici et al. 2012). Nevertheless, even mild to moderate heat stress that does not result in visible lesions can result in significant reductions in leaf photosynthetic activities (Sharkey 2005; Zhang & Sharkey 2009; Zhang et al. 2009) and modifications in volatile emission profiles (Loreto et al. 1998; Kleist et al. 2012; Possell & Loreto 2013). In fact, release of several constitutive and induced volatiles can be extremely temperature sensitive and only moderate increases in temperature, even in the range of 30–38 °C can result in major changes in the emissions (Hartikainen et al. 2009; Kleist et al. 2012; Hu et al. 2013; Farré-Armengol et al. 2014).

Apart from ubiquitous stress responses elicited in a wide range of species in response to practically any severe stress, several plant taxonomic groups have specialized volatile defence pathways (Karban 2011). Glucosinolates constitute the unique secondary metabolites in the order Brassicaceae (Fahey et al. 2001; Redovniković et al. 2008; Ishida et al. 2014), and so far the occurrence of more than 130 natural glucosinolates has been documented (Agneta et al. 2014). Depending on their molecular structure, they can be divided among aliphatic-, aromatic- or indole glucosinolates (Hopkins et al. 1997; Ishida et al. 2014). Members of each group are biosynthesized from different precursors via slightly different pathways. Yet, in general, the biosynthesis starts with the chain elongation of an amino acid, continues with the creation of glucosinolate basic structure and ends up with the transformation of the core structure into the final glucosinolate molecule (Ishida et al. 2014). Glucosinolates are hydrolyzed to toxic volatile products by myrosinases that are released from specialized cells upon mechanical wounding, for example, upon insect herbivory (Barth & Jander 2006; Wittstock & Burow 2010; Najar-Rodriguez et al. 2015). These breakdown products can be isothiocyanates, thiocyanates, nitriles, epithionitriles and oxazolidines (Bones & Rossiter 2006; Kos et al. 2012), disulfides and thiols (Olivier et al. 1999; Agrawal & Kurashige 2003; Crespo et al. 2012) shown to significantly reduce herbivory by omnivorous insects (Hopkins et al. 2009), but also performance of specialist herbivores (Bruinsma et al. 2007; de Vos et al. 2008).

Apart from mechanical damage due to herbivory, multiple stress factors including heat stress can lead to cellular damage with potential release of myrosinases, but there is surprisingly little information about the relationship of abiotic stressors and volatile glucosinolate degradation products in brassicoid species (Wittstock & Burow 2010). Provided myrosinases are

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Indeed released as the permeability of cellular membranes increases upon developing heat stress, release of glucosinolate breakdown products is likely, and the release of these specialized volatiles might importantly contribute to the total heat-triggered volatile blend next to ubiquitous emissions of LOX products. Furthermore, there is recent evidence that exogenously applied glucosinolate volatiles, isothiocyanates, enhanced the development of heat-tolerance of *Arabidopsis thaliana* (Hara et al. 2013). Thus, heat-dependent induction of glucosinolate volatile emissions might contribute to development of induced abiotic stress resistance, but which temperature conditions lead to the release of glucosinolate volatiles and how these potential emissions are related to ubiquitous stress responses is not known.

During their lifetime, plants can be exposed to a wide variety of heat episodes differing in duration and temperature during the stress, including short-term to mid-term excursions of leaf temperature to high values upon light flecks and upon clearing up the sky when shaded leaves are suddenly exposed to strong beam irradiance (Singasas & Sharkey 1998; Sharkey 2005; Behnke et al. 2007; Way et al. 2011), as well as during heat waves that are predicted to become more common in the future (Ameye et al. 2012). Given this variety, it is relevant to consider that the heat stress threshold is determined by the heat dose (heat sum) that is dependent on both the actual temperature and the duration of the heat episode (Bilger et al. 1984; Niinemets 2010a) as well as on possible increases of heat stress resistance due to acclimation and priming responses occurring through the heat wave (Niinemets 2010b). Thus, modifications in the volatile blend triggered by heat stress can depend on the type of heat stress that ultimately determines whether the stress threshold for physiological damage is exceeded and whether acclimation or priming responses can occur.

The goal of the present study was to investigate how foliage photosynthetic characteristics and emissions of constitutive and ubiquitous and specialized stress-elicited volatiles respond to heat-stress of various types in black mustard (*B. nigra* (L.) Koch, Brassicaceae). To our knowledge, there is no information about the relationships between the volatile glucosinolate degradation products and volatiles of other biosynthetic pathways through abiotic stress treatments. Hence, next to the volatile glucosinolates, we also studied the heat responses of emissions of lipoxygenase, terpenoid and shikimate pathway products. We used three different heat treatments simulating dynamic temperature conditions in field environments that can occur during short-term heat episodes and longer-term heat waves to gain an insight how the share of different volatiles changes in dependence on reversible and irreversible stress conditions of different duration. We hypothesized that severe heat stress leads to elicitation of glucosinolate volatiles and that the emissions are quantitatively more significant upon long-term stress due to elicitation of induction responses.

*B. nigra* is a fast-growing 1- to 2-m-tall annual herb native to the southern Mediterranean region of Europe, growing over a broad temperature range and therefore classified as a stress-tolerant species (Duke 1983). It is occasionally cultivated for its seeds (Rajamurugan et al. 2012) as well as for leaves, extracts of which have allelopathic effects due to its secondary plant chemicals (Turk & Tawaha 2003). However, as a rapidly growing plant, *B. nigra* has become an aggressive weed in temperate Europe where it colonizes old fields (Gomaa et al. 2012). Because of extensive spread, more complex genome and greater stress tolerance, it has become next to *A. thaliana*, an additional brassicoid model system in studies on plant biology, ecology and plant-insect interactions (Dicke & van Loon 2000; Fatouros et al. 2012).

**MATERIALS AND METHODS**

**Plant material**

Plants of *B. nigra* were grown from the seeds provided by the Department of Entomology, University of Wageningen, the Netherlands. This standardized seed-lot corresponds to a wild-grown Dutch *B. nigra* population that has been used in multiple studies on plant-insect interactions (Bruinsma et al. 2008; Khaling et al. 2015; Pashalidou et al. 2015). The seeds were sown in 0.8 L plastic pots filled with a mixture of commercial garden soil with slow-release nutrients (Biolan Oy, Era, Finland) and quartz sand. Day length was 12 h, and light intensity at plant level of 400 μmol m⁻² s⁻¹ was provided by metal halide lamps (HPI-T Plus 400 W, Philips, Eindhoven, The Netherlands). Day/night temperatures were maintained at 24/20°C and relative humidity of 60%. The plants were watered every other day to soil field capacity. Five- to six-weeks-old non-bolted plants with at least three fully developed leaves were used in the experiments. Temperature response curves were measured, and two different heat stress treatments were conducted in three to seven replications with different plants (Fig. 1 for entire experimental protocol). New plants were used for individual temperatures within heat stress treatments, and each plant was stressed and analysed only once. Hence, emissions from 57 plants were analysed with GC-MS, and 65 plants were used in gas-exchange measurements (8 volatiles samples were lost due to malfunctioning of GC-MS cartridge autosampler, but nevertheless, the sample size was never below three for individual treatments).

**Long-term heat stress treatment**

For long-term stress application, the potted plants were placed in a Percival growth chamber (model E-36HO, Percival Scientific, Inc., Perry, IA, USA) under controlled conditions of light intensity at plant level of 400 μmol m⁻² s⁻¹ provided for 16 h day⁻¹ (6:00–22:00 h), 60% of humidity and day/night temperature of 25/21°C. Before the start of the heat stress treatment, the plants were acclimated for 24 h under these growth chamber conditions. After the acclimation, the heat stress was applied in two heat waves, one in the evening between 20:00–22:00 h and the second in the following morning between 06:00–08:00 h, providing a total treatment period of 4 h, but intervened with a night-time non-stressed period at 21°C to allow for a recovery and induction of volatile stress responses. Although the increase of temperature to preset conditions took ~0.5 h, the chamber cool down to 21°C after turning off the lights in the evening took ~1 h. Thus, the total stress period
The study was conducted using Brassica nigra plants grown under controlled conditions. Plants were divided into three groups based on stress treatments: moderate exposure through stepwise raising temperatures, shock stress, and heat shock stress, both by stepwise increase in temperature but differing in duration and treatment temperature. The protocol for the heat shock response was studied at temperatures of 25°C (control), 45, 48, and 50°C, with individual plants used for each treatment.

**Gas-exchange measurement system**

Foliage gas-exchange rates were measured within a custom-made open gas-exchange system, which is described in detail in Copolovici & Niinemets (2010). The system has a temperature-controlled 1.2 L chamber. The glass vessel is made of double-walled glass and stainless steel and has a bottom ring specially designed for volatile compound measurements. The chamber temperature is controlled by circulating thermostated water between the chamber walls (Copolovici & Niinemets 2010). An infra-red dual-channel gas analyzer operated in differential mode (CIRAS II, PP-Systems, Amesbury, MA, USA) was used to measure CO₂ and H₂O concentrations at the chamber inlet and outlet (Copolovici & Niinemets 2010). Ambient air was drawn from outside, passed through a 10 L buffer volume, and an HCl-activated copper tubing to scrub ozone and was humidified to ~60% humidity using a custom-made humidifier. After passing the ozone scrubber, ozone concentrations were less than 1 nmol mol⁻¹ (Sun et al. 2012). The chamber CO₂ concentration was 380–400 μmol mol⁻¹ in these experiments.

**Gas-exchange measurements and volatile sampling in heat-stressed leaves**

For plants subjected to long-term heat stress, at least three top leaves, and for heat shock treated leaves all treated leaves were inserted into the leaf chamber (approximately 80–100 cm² leaf area), and standard conditions of light intensity of 800 μmol m⁻² s⁻¹ and chamber temperature of 25°C (leaf temperature was within ± 1°C of chamber temperature) were established. Measurements of net assimilation and transpiration rates were taken immediately after the gas flows had stabilized in the system, typically in 10–20 min after plant enclosure.

After the gas flows had stabilized, volatiles were collected onto multi-bed stainless steel cartridges filled with three different carbon-based adsorbents for optimal adsorption of all volatiles. A portable 210-1003MTX air sampling pump (SKC Inc., Houston, TX, USA) was used for sampling the chamber outlet air with a constant flow rate of 200 mL min⁻¹. The sampling time was 20 min, and 4 L of air was sampled. Blank samples from the empty cuvette were taken before the plant measurements.

**Measurements of temperature response curves of net assimilation and volatile release**

Temperature response curve measurements started at 9:00 in the morning, 1 h after automatic turn-on of the light in the plant room. Three upper leaves were enclosed in the leaf chamber and stabilized at the reference conditions of light intensity at leaf level of 800 μmol m⁻² s⁻¹, chamber temperature of 20°C, CO₂

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**Figure 1.** A schematic representation of the study experimental design. *Brassica nigra* plants were subjected to three different heat treatments: moderate exposure through stepwise raising temperatures through temperature response curve measurements, long-term heat stress, and shock stress treatments both achieved by stepwise increase in temperature, but differing in duration and treatment temperature. Plants were placed in a gas exchange system to collect volatiles and measure foliage net assimilation and transpiration rates. Collected volatiles were analyzed with a GC-MS system followed by data analyses. In the case of temperature response curve measurements, the treatment and physiological measurements occurred simultaneously at the treatment temperature. In the case of the two other experimental protocols, the physiological measurements were performed after the heat stress treatment at 25°C.

**Heat shock stress**

Shock stress treatment started at 9:00 in the morning, approximately 1 h after the light regime was automatically turned on in the plant room. Heat shock treatments followed the protocol of Copolovici et al. (2012). A temperature-controlled glass vessel equipped with a magnetic stirrer (Heidolph MR Hei-Standard with an EXT Hei-Con temperature sensor, Heidolph, Schwabach, Germany) was used. In the glass vessel, distilled water was heated to the desired temperature, and the plant’s uppermost part with three fully developed leaves was inserted in the water with given temperature for 5 min. After the treatment, leaves were left to air-dry for approximately 5 min and then measured for gas-exchange and volatile emissions using the custom-made gas-exchange system at 25°C as described later (Fig. 1). The heat shock response was studied at temperatures of 25 (control), 45, 48 and 50°C, and individual plants were used for each treatment.
concentration of 380–400 μmol mol⁻¹ and air humidity of 60% until stomata opened and gas-exchange rates stabilized, typically in 20–30 min since leaf enclosure. After reaching the steady-state, net assimilation and transpiration rates were recorded and volatile organic compounds were collected for 20 min as described earlier (Fig. 1). The chamber temperature was raised to the next higher temperature, the plant was conditioned again at this temperature for 20–30 min, and gas-exchange rates were recorded and volatiles collected. Foliage gas-exchange rates were measured at temperatures 20, 25, 30, 35 and 40 °C, while volatile organic compounds were collected at 20, 25, 30, and 40 °C.

**GC-MS analyses**

The cartridges with adsorbed volatiles were analysed with a combined Shimadzu TD20 automated cartridge desorber connected to a Shimadzu 2010 Plus GC–MS system (Shimadzu Corporation, Kyoto, Japan). Adsorbent cartridges were back flushed with high purity He (99.9999% AGA, Linde Group, Tallinn, Estonia) during thermal desorption with the following TD20 parameters: He purge flow rate of 40 mL min⁻¹, primary desorption temperature of 250 °C, primary desorption time of 6 min, the second stage trap temperature during primary desorption of –20 °C, the second stage trap desorption temperature of 280 °C, hold time of 6 min. The compounds were separated on a Zebron ZB-624 fused silica capillary column (0.32 mm i.d., 60 m length, 1.8 μm film thickness, Phenomenex, Torrance, CA, USA) using He with a flow rate of 1.48 mL min⁻¹ as the carrier gas. The following GC oven programme was employed: 40 °C for 1 min, 9 °C min⁻¹ to 120 °C, 2 °C min⁻¹ to 190 °C, 20 °C min⁻¹ to 250 °C for 5 min. The Shimadzu QC2010 Plus mass spectrometer was operated in the electron impact mode. The transfer line temperature was 240 °C and ion-source temperature 150 °C. The GC-MS system was calibrated as explained in Kännaste et al. (2014) and the compound quantification follows Copolovici et al. (2009). The compounds were identified by comparing the mass-spectra of volatiles with the spectra of authentic standards of the highest purity purchased from Sigma-Aldrich (St. Louis, MO, USA, GC purity, most of the standards) and Fluka (Buchs, Switzerland, GC purity, 1-hexanol and methyl salicylate). For quantification of emissions, the GC-MS system was calibrated with standard compounds in hexane solution. Six concentrations of each compound (range 0.1–1 μL/mL) were prepared, and 1 μL of each sample was injected into the adsorbent cartridge. The cartridge was back flushed with a stream of N₂ at 200 mL min⁻¹ to simulate conditions during sampling of volatiles. Ultimately, the calibration factor for each compound was estimated as the slope of the GC chromatogram peak area versus compound mass concentration.

We grouped the volatile compounds released according to their formation pathways (Table 1) as fatty acid derived compounds (lipoxigenase volatiles, also called green leaf volatiles) (Matsui 2006), geranyl diphasphate (GDP) derived volatiles (GDP-pathway, various monoterpenoids synthesized from GDP) (Maffei 2010), geranylgeranyl diphasphate (GGDP) derived volatiles (homoterpenes such as DMNT and some carotenoid breakdown products such as geranyl acetone) (Arimura et al. 2009), shikimate pathway volatiles (different benzenoids such as methyl salicylate) (Wahid et al. 2007; Betz et al. 2009) and glucosinolate breakdown compounds (various sulphur- and nitrogen-containing compounds, often containing the CN functional group) (Sønderby et al. 2010; Ishida et al. 2014). It is primarily these latter compounds that give the plants from Brassicaceae the characteristic ‘cabbage smell’ (Buttery et al. 1976). No sesquiterpenes were observed in the emission blends in these experiments.

**Data analyses**

Net assimilation rate (A) and stomatal conductance (gs) per leaf area and intercellular CO₂ concentration (Ci) were calculated according to von Caemmerer & Farquhar (1981) and the volatile emission rates according to Niinemets et al. (2011).

For normalization of data and residuals, logarithmic data transformation was used, and average values of gas-exchange and volatile emission rates at different temperatures were compared with one-way ANOVA followed by Tukey’s post-hoc test. In addition, linear- and non-linear regression analyses were conducted to explore the relationships among gas-exchange and volatile emission rates and among the emission rates of different volatile compound classes. (StatSoft Inc., Tulsa, OK, USA) was used in these analyses. Heat stress effects on volatile bouquets and changes in the volatile bouquets through the temperature response curve were evaluated by principal component analysis (PCA) (Wold et al. 1987). Loading and score plots were derived after mean-centering and logarithmic data transformation. Redundancy data analysis was also used to test for the differences in bouquets among stress treatments. Monte-Carlo permutation tests were used to evaluate the statistical significance of the model. Multivariate data analyses were performed with CANOCO 5.0 software (ter Braak and Smilauer, Biometris Plant Research International, the Netherlands). All statistical tests were considered statistically significant at P < 0.05.

**RESULTS**

**Effects of heat stress on foliage photosynthetic characteristics**

Temperature response curve measurements indicated that leaf net assimilation rate (A) of *Brassica nigra* had a broad temperature optimum between 20 and 30 °C (Fig. 2a). Net assimilation rate declined over temperatures 35–40 °C, reaching ~4-fold lower values at the highest measurement temperature than at 20 °C (Fig. 2a). Stomatal conductance to water vapor (gs) decreased with increasing temperature through the entire temperature range from on average (±SE) 99 ± 13 mmol m⁻² s⁻¹ at 20 °C to 13 ± 4 mmol m⁻² s⁻¹ at 40 °C (Fig. 2b). Thus, the intercellular CO₂ concentration (Ci) decreased with increasing temperature to low values of 60–100 μmol mol⁻¹ at the highest measurement temperature (data not shown).

Exposure of plants to long-term heat stress (4 h exposure to given temperature, measurements of photosynthetic characteristics at 25 °C) was associated with minor modifications in A and gs over the treatment temperature range of 25–35 °C, but
further increases in temperature were associated with both reduced $A$ (4.5-fold reduction at 44 °C compared with the value at 25 °C) and $g_s$ (2.8-fold reduction), from 69 to 24 mmol m$^{-2}$ s$^{-1}$ (Fig. 2a, b, respectively). Intercellular CO2 concentration was similar through temperatures 25–40 °C (192 ± 16 μmol mol$^{-1}$), but there was a significant increase in $C_i$ at 44 °C (334 ± 30 μmol mol$^{-1}$, P < 0.01 for the difference among the means). Heat shock treatment (exposure for 5 min to given temperature, measurements of photosynthetic characteristics at 25 °C) was associated with major reductions in both $A$ and $g_s$, with barely positive rates of net assimilation observed after 45 °C treatment, and negative net assimilation rates observed at treatment temperatures 48 and 50 °C (Fig. 2a). Intercellular CO2 concentration was greater in heat shock treated than in control leaves (P < 0.005).

**Temperature response curves of volatile emission**

Total emission of fatty acid derived compounds was low and weakly affected by temperatures through the temperature response curves (Fig. 3a). Among C6-volatiles, only 1-hexanol and (Z)-3-hexen-1-ol were above the detection limit at 25 and 40 °C, and the rest of the emissions were due to aliphatic aldehydes (Table 1). Total emission of monoterpenoids (GDP-

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**Table 1.** Average ±SE emission rates (pmol m$^{-2}$ s$^{-1}$) of different volatiles released from leaves of *Brassica nigra* in response to three different heat treatments grouped according to the compound formation pathways

Different stress treatments as outlined in Fig. 1. Five primary compound groups were distinguished on the basis of compound synthesis pathways: fatty acid derived volatiles (products of lipoxygenase pathway, also called green leaf volatiles), geranyl diphosphate (GDP) derived volatiles (GDP-pathway, various monoterpenoids), geranylgeranyl diphosphate (GGDP) derived volatiles (homoterpenes such as DMNT and some carotenoid breakdown products including geranyl acetone), and shikimate pathway volatiles (different benzenoids such as methyl salicylate) and glucosinolate breakdown compounds (various sulphur- and nitrogen-containing volatiles). The compound number corresponds to the number in the PCA factor loading plot (Fig. 4). Number of replicates (individual plants) is shown in parenthesis below each stress temperature.

*for emissions of compounds, which were above the detection limit in only one of the replicate experiments, no SE values could be calculated.

*different letters indicate statistical significance at $P < 0.05$.
pathway compounds) decreased considerably from 20 to 40 °C (Fig. 3b). Among GDP-pathway compounds, α-pinene followed by 3-carene was the dominating volatiles at all temperatures (Table 1). Similarly to LOX-compounds, total emission of glucosinolate breakdown products was low and not affected by temperature (Fig. 3c). Allyl isothiocyanate and 2-propenenitrile were rare volatiles at only at 20 °C, while the emissions of cyclohexyl isothiocyanate were not affected by temperature (Table 1). Total emission of GGDP-pathway volatiles (carotenoid breakdown products) was overall low, and the emissions were dominated by geranyl acetone (Table 1). Similarly to GDP-pathway volatiles, GGDP-pathway volatiles decreased from $0.0309 \pm 0.0027 \text{nmol m}^{-2} \text{s}^{-1}$ at 20–25°C to $0.0043 \pm 0.0012 \text{nmol m}^{-2} \text{s}^{-1}$ at 30–40°C (Fig. 3d).

### Effects of long-term heat stress on volatile emissions

Long-term heat stress had no significant effect on LOX-compounds over the treatment temperatures 25–35°C, but the emissions were strongly enhanced upon exposure...
to 40 °C and 44 °C (Fig. 3a). Moreover, the emission composition significantly changed as at 40 °C treatment, the plants began to release 2-ethylfuran and 1-penten-3-ol and at 44 °C treatment, the emission of these volatiles increased even more (Table 1). At 44 °C treatment, the LOX bouquet was dominated by \((Z)-3\text{-hexen-1-ol}, 1\text{-penten-3-ol} \text{ and } 1\text{-penten-3-one} \text{ and aliphatic aldehydes hexanal, nonanal and octanal (Table 1). Heat stress effects on the release of glucosinolate breakdown products followed the same pattern as that observed for LOX-compounds (Fig. 3c). At treatment temperatures of 25\text{–}35 °C, total emission of glucosinolate breakdown products remained at a low level of 0.0087\text{–}0.0193 \text{nmol m}^{-2}\text{s}^{-1} \text{ (Fig. 3c) but increased somewhat already at 40 °C treatment, and a major emission burst of 1.10 ±0.43 \text{nmol m}^{-2}\text{s}^{-1} \text{ was observed at the highest applied temperature treatment (Fig. 3c). At this temperature treatment, allyl isothiocyanate was the dominating volatile followed by tetramethylthiourea, cyclohexyl isothiocyanate and 2-propenenitrile (Table 1). Emissions of GDP-pathway and GGDP-pathway compounds were not affected by treatment temperature (Figs. 3b, d), but emissions of the benzenoid and methyl salicylate were detected after higher temperature treatments (Table 1).}
Influences of heat shock stress on volatile release

Short-term exposure of leaves to severe heat stress increased total emissions of all volatiles at heat shock temperatures higher than 48 °C and for glucosinolate breakdown products already at 45 °C treatment (Fig. 3). A particularly strong enhancement was observed for LOX volatiles that increased from 0.036 ± 0.019 nmol m⁻² s⁻¹ at 25 °C treatment to 1.4 ± 1.0 nmol m⁻² s⁻¹ at 50 °C treatment (Fig. 3a). (E)-3-hexen-1-ol was released at temperature treatments of 45 and 48 °C as well, but at 50 °C treatment, the plants started to release additional C5-volatiles and C6-volatiles such as (E,E)-2,4-hexadienal, (Z)-3-hexenyl formate, 1-pentanol, 1-penten-3-ol and 1-penten-3-one (Table 1). The total emission of GDP-pathway volatiles rose from 0.013 ± 0.008 nmol m⁻² s⁻¹ at 25 °C to 0.048 ± 0.016 nmol m⁻² s⁻¹ at 50 °C treatment, mainly due to enhanced emissions of α-pinene and 3-carene (Table 1, Fig. 3b). Glucosinolate breakdown products were not detected at 25 °C treatment (Fig. 3c), but their emission increased from 0.113 ± 0.035 nmol m⁻² s⁻¹ at 45 °C treatment to 0.37 ± 0.09 nmol m⁻² s⁻¹ at 50 °C treatment (Fig. 3c). Methyl isothiocyanate was detected only at 50 °C treatment and tetramethylthiourea together with allyl isothiocyanate were the dominating volatiles at 50 °C treatment (Table 1). Total emission of GGDP-pathway volatiles remained similarly low as in the long-term stress (0.006 to 0.012 nmol m⁻² s⁻¹), and it was not significantly different among the heat shock treatments (Fig. 3d).

Changes in emission blends among different temperature treatments and among different temperatures within treatments

Principal component analysis demonstrated that the emission blends in three different treatments (temperature response curves) of Brassica nigra were distributed close to the control plants for long-term heat stress and heat shock stress (Fig. 4). Monte-Carlo permutation test, $P < 0.05$). A certain plant-to-plant variability was observed in the release of LOX volatiles, (E)-3-hexen-1-ol, (Z)-3-hexenyl formate and 1-penten-3-one (Table 1), upon heat stress. The plants emitting these volatiles, and experiencing a severe stress under the imposed conditions were grouped in the upper right corner of PCA score plot (Fig. 4b). In the case of other stressed plants, C5-volatiles such as 1-pentanol, 1-penten-3-ol and 2-ethylfuran and some glucosinolate degradation products such as methyl isothiocyanate constituted a stress signal of heat-stressed B. nigra. In the case of emissions higher than approximately 10 pmol m⁻² s⁻¹ (Z)-3-hexen-1-ol, tetramethylthiourea, isocyanides, methyl isothiocyanate and tetramethyleurea became stress signals (Table 1 and Fig. 4a, b). In the case of allyl isothiocyanate, the limit of emission for classification the plant as stressed in the PCA plot was about 200 pmol m⁻² s⁻¹ (Table 1 and Fig. 4a, b).

In the case of emissions observed at different temperatures through the temperature response curve, the blend of emissions at 25 °C did not differ from that at 20 °C (Fig. 4b). Analogously, the emission blends at higher temperatures (30 and 40 °C) did not differ from those at 20 and 25 °C (Fig. 4b). In contrast, in long-term stress, emissions after treatments at 40 and 44 °C differed significantly from the control treatment (Fig. 4b). Analogously, heat shock of 45–50 °C resulted in major changes in the emission blend compared with the controls (Fig. 4b).

As emissions of LOX products and glucosinolate breakdown products were low through the entire temperature response curve, 20–40 °C (Fig. 3a, c), all temperature response curve data were distributed close to the control plants for long-term – and heat shock stresses in the upper corner of the left side of PCA score plot (Fig. 4b). High emission of glucosinolate breakdown curves measurements constituting a mild-short stress, long-term heat stress and heat shock stress) differed significantly from each other (Fig. 4. Monte-Carlo permutation test, $P < 0.05$).

**Figure 4.** Loading-plot (A) and score-plot (B) of PCA analysis based on the emissions of volatiles released (Table 1 for the emission rates) from non-stressed and heat-stressed Brassica nigra plants. In the loading plot, the numbers represent different volatiles (Table 1 for the coding of individual compounds), while in the score plot, each symbol represents an individual non-stressed (empty symbols) or heat-stressed plant (filled symbols). Red symbols correspond to temperature response curve measurements, black symbols to long-term heat stress and green symbols to heat shock stress (Fig. 1 for the details of heat shock treatments and Fig. 3 for the heat stress effects on key volatile groups). In the loading plot, the impact of chemical compounds on PCA increases with the distance from the origin of the co-ordinate system.
volatiles (Fig. 4a), elicitation of methyl salicylate emissions and changes in the composition of GDP-pathway (e.g. induction of camphene emissions) were characteristic to plants exposed to long-term stress at 40 and 44 °C (Fig. 4b). Finally, heat shock treatments differed from long-term heat stress by greater elicitation of LOX-compounds and GDP-pathway volatiles and lower induction of glucosinolate breakdown products (Figs 3 & 4).

Correlations among emissions of different volatile compound classes and among emissions and photosynthetic characteristics

Through the temperature response curves, the emissions of GDP-pathway compounds (Fig. 3b) were positively correlated with $A$ ($r = 0.71$) and $g_s$ ($r = 0.83$, $P < 0.05$ for both), but low-level emissions of LOX-compounds (Fig. 3a) and glucosinolate breakdown products (Fig. 3c) were not correlated with foliage photosynthetic characteristics.

In long-term stress treatment, emissions of glucosinolate breakdown products and LOX-compounds were positively correlated through treatment temperatures of 25 to 40 °C, but the correlation was lost in leaves at 44 °C treatment where the increase in the emission of glucosinolate breakdown products was much stronger than that in LOX-compounds (Fig. 5). Emission of GDP-pathway compounds was also positively correlated with LOX-compound emission, but the slopes differed for treatment temperature range 25–35 °C and 40 and 44 °C, reflecting the stronger increase of LOX-compounds over this temperature range (Fig. 6a). Foliage photosynthetic characteristics were negatively correlated with emissions of glucosinolate breakdown products (Fig. 7a, b) and LOX-compounds (Fig. 7c, d), whereas the correlations were strongly non-linear (Figs 7 & 8).

In the heat shock treatments, glucosinolate breakdown products and LOX emission were not correlated ($P > 0.8$), although the slope was shallower than that observed for long-term heat treatments due to greater elicitation of emissions of LOX-compounds in heat shock treatments (cf. Figs 5 & 3). The emissions of GDP-pathway volatiles and LOX-compounds were positively correlated over the temperature range of 25 to 48 °C (Fig. 6b), but the treatment at 50 °C was characterized by much stronger elicitation of LOX-compounds (Fig. 3).

Analysis of relationships among photosynthetic characteristics and emissions of glucosinolate and LOX-compounds, indicated that photosynthetic activity was lost earlier than stress volatile emissions were elicited (cf. Figs 2 & 3).

DISCUSSION

How different types of heat stress affect leaf photosynthetic characteristics in B. nigra

In the current study high temperature resistance of *B. nigra* was studied by three sets of experiments with differing severity of heat stress, including measurements of temperature responses where temperature was raised up to 40 °C (mild stress), long-term moderate heat stress where plant temperature was raised up to 44 °C for 4 h and heat shock stress where leaves were exposed to sublethal to lethal temperatures of 45–50 °C for 5 min. Given that Brassicaceae have a specialized defense system constituting of high constitutive levels of glucosinolates and release of glucosinolate breakdown products, the key aim of the study was to gain insight into the relationships among ubiquitous stress responses and brassicoid-specific stress responses through the different heat stress treatments.

Among the ubiquitous stress responses, foliage net assimilation rate (\(A\)) and stomatal conductance (\(g_s\)) decreased in all heat stress treatments (Fig. 2), but the mechanism of photosynthetic decline differed among the different types of heat treatment (Figs 2 & 8a). In temperature response curve measurements, the temperature-dependent reduction in \(A\) resulted from reduced intercellular CO₂ concentration (\(C_i\)) due to a reduction in \(g_s\) (Fig. 2). Closure of stomata is often observed at higher temperatures (Cui *et al.* 2006; Hüve *et al.* 2006; Hüve *et al.* 2011; Copolovici *et al.* 2012), and this response reduces water loss in conditions of higher vapor pressure deficit typical to high temperature (Shinohara & Leskovar 2014). However, after long-term heat stress at 40 and 44 °C and heat shock stress at 45–50 °C, \(C_i\) actually increased, indicating that heat stress resulted in stronger reductions in leaf photosynthetic capacity than in stomatal conductance. This result is in agreement with previous studies indicating heat dose dependent reductions in foliage photosynthetic capacity after a certain threshold heat dose has been exceeded (Kadir *et al.* 2007; Hüve *et al.* 2011). Such decreases in photosynthetic capacity might reflect inactivation of foliage photosynthetic electron transport processes due to increased leakiness of membranes and enhanced non-photochemical quenching (Havaux 1993; Lu & Zhang 2000; Zhang & Sharkey 2009; Zhang *et al.* 2009), but they might also result from irreversible cellular damage (Hüve *et al.* 2011). As the result of sustained inhibition of photosynthetic activity or cellular damage, foliage photosynthetic activity does not recover upon return to lower temperatures as was also observed in our study after long-term heat stress at 44 °C and heat shock treatments between 45–50 °C (Figs 2 & 8a).

Different heat stresses have varying effects on lipoygenase pathway volatiles

The release of LOX volatiles in low amounts from flowers, leaves or fruits is a widespread phenomenon (Bengtsson *et al.* 2001; Ceuppens *et al.* 2015). In our study, characteristic C6 LOX volatiles such as 1-hexanol and (Z)-3-hexen-1-ol were emitted in small quantities, close to the analytical detection limit, at low temperatures (Table 1). In addition, aliphatic saturated aldehydes hexanal, heptanal, octanal and nonanal were consistently emitted at low level through all three sets of experiments (Table 1). Although in the literature, the LOX-pathway

is primarily associated with the emission of C6 aldehydes and their derivatives (Wildt et al. 2003), longer chain length aldehydes are often found in plant emissions, including emissions from *Brassica rapa var. rapa* (Taveira et al. 2009), tomato (*Solanum lycopersicum*) (Wang et al. 2001) and hybrid poplar (*Populus simonii* × *Populus pyramidalis*) (Hu et al. 2011), and there is evidence that activation of LOX-pathway is responsible for the emissions of all these aliphatic aldehydes (Hu et al. 2009; Hu et al. 2011).

In addition to the low-level emissions of LOX volatiles in non-stressed conditions, a major burst of LOX volatiles upon severe stress constitutes a key ubiquitous stress response (Matsui 2006; Copolovici et al. 2012). Multiple LOXs are constitutively active in leaves, and thus, the release of volatile LOX-compounds occurs rapidly as soon as the substrate, polyunsaturated fatty acids, becomes available because of membrane lesions (Feussner & Wasternack 2002; Liavonchanka & Feussner 2006; Andreou & Feussner 2009). Accordingly, elicitation of emissions of LOX-compounds constitutes a classic indicator of cellular damage (Matsui 2006; Jansen et al. 2009; Matsui et al. 2012). In our study, long-term heat stress at 40 and 44 °C and heat shock treatment at 48 and 50 °C resulted in a major increase in lipoxygenase pathway volatiles, while the lipoxygenase volatile emissions remained low through the temperature response curve measurements (Figs 3a & 8, Table 1). At these high temperatures in both heat stress treatments, the plants began to release next to C6-volatiles also various C5-volatiles such as 1-pentanal, 1-penten-3-ol, 1-penten-3-one, and C7-volatile (Z)-3-hexenyl formate (Table 1) that are also formed through the LOX-pathway (Shen et al. 2014) and are emitted upon several other stresses (de Gouw et al. 1999; Brilli et al. 2012).

Previous studies indicate that upon short-term heat pulses as those applied in the heat shock treatments, LOX product emissions are elicited between temperatures 46–49 °C according to a highly non-linear switch-type response (Loreto et al. 2006; Copolovici et al. 2012) as was also observed in our study (Fig. 3a). This temperature range corresponds to major increases in plasmalemma membrane permeability and time-dependent reductions in foliage photosynthetic activity upon return to lower temperature (Hüve et al. 2011). However, photosynthesis rate of *B. nigra* strongly decreased upon heat shock at 45 °C as well (Fig. 2), but no significant elicitation of LOX-compounds was observed (Fig. 3a). This discrepancy suggests that the reduction in photosynthetic activity at this temperature likely reflected enhanced engagement of non-photochemical processes or impaired photochemistry without direct membrane-level damage.

Similar to our study (Fig. 3a), long-term exposure, from several hours to days, to moderately high temperatures of 35–45 °C resulted in elicitation of LOX product emissions that was accompanied by reduced *A* (Fig. 7c) in several tree species (Kleist et al. 2012). This evidence together with our observations further underscores that heat stress impact on cellular processes is dose-dependent, and even moderately high sustained heat waves can result in major cellular lesions progressively leading to the cessation of photosynthetic activity.

**Constitutive terpenoid release upon heat stress**

Several plant species emit GDP-pathway compounds, mainly monoterpenes, constitutively. Constitutive monoterpene synthesis occurs in plastids where the terminal enzymes and monoterpene synthases are located (Tholl 2006; Chen et al. 2011; Rajabi Memari et al. 2013). Constitutive monoterpene emissions either come from specialized storage tissues or from immediate *de novo* synthesis (Grote et al. 2013). In the latter case, the emissions are strongly related to foliage photosynthetic characteristics, and thus, reduction in foliage photosynthetic rate upon heat stress typically also leads to reduction in constitutive monoterpene emissions (Loreto et al. 1998; Peñuelas & Llusia 2002; Kleist et al. 2012). In *B. nigra*, α-pinene and 3-carene followed by limonene were the main monoterpenes emitted under moderate temperatures in heat stress treatments and through the temperature response curve measurements (Table 1). Through the temperature response curve
measurements, the rates of total monoterpene emission and net assimilation were positively correlated, and the emissions decreased with increasing temperature parallel to photosynthesis (Figs 2a & 3b), suggesting that these emissions resulted from de novo synthesis.

However, sustained heat stress can result in the induction of monoterpene synthesis (Staudt & Bertin 1998), although not always (Kleist et al. 2012). These induced monoterpene emissions typically consist of different monoterpene than constitutive emissions, reflecting expression of new terpene synthases (Staudt & Bertin 1998; Niinemets et al. 2010a,b; Copolovici & Niinemets 2016). In B. nigra, the total emission rate of monoterpenes was not affected by long-term heat treatment, but the emission rates of GDP volatiles correlated with the emissions of LOX-compounds (Fig. 6a). These correlations differed for different treatment temperature ranges, suggesting that the constitutive plant defense was gradually replaced by induced plant defense as the treatment temperature raised.

Heat shock treatment was associated with a significant increase of monoterpene emissions (Figs 3b & 8b) as has been observed in tomato (S. lycopersicum) (Copolovici et al. 2012), but the mechanism of this increase is unclear. Enhanced substrate availability for monoterpene synthases due to disruption of other metabolic pathways consuming isopentenyl diphosphate and dimethylallyl diphosphate such as carotenoid synthesis could provide an explanation for the increase of monoterpene emission. It can also reflect a certain storage capacity of monoterpeneoids in idioblasts, also called the myrosin cells or ‘mustard oil bombs’ (Ahuja et al. 2009; Borgen et al. 2012), or non-specific storage of monoterpene in cellular membranes as is common in constitutive de novo monoterpene emitters (Niinemets & Reichstein 2002; Niinemets et al. 2010b). Thus, the release of these compounds, especially the release of α-pinene (Table 1) upon heat shock can occur due to cellular damage. A positive correlation between LOX-compounds and monoterpene emissions through the heat shock treatments (Fig. 6b) suggests that this is a plausible explanation, although the correlation collapsed at 50 °C where the increase in LOX emissions vastly exceeded that in monoterpene emission.

Release of specialized brassicoid volatiles as a major trait differentiating among heat stress treatments

Synthesis of glucosinolates and formation of their volatile toxic hydrolysis products by myrosinases constitute the characteristic defence system in Brassicaceae (Halkier & Du 1997; Raybould & Moyes 2001; Wang et al. 2011). Formation of glucosinolates primarily occurs in vascular tissues (Li et al. 2011), while myrosinases are stored in myrosin cells diffusely distributed through plant tissues (Kelly et al. 1998; Burow et al. 2007; Zhao et al. 2008; Misra et al. 2015). Thus, the release of myrosinases upon damage of myrosin cells is the first step required for the formation of glucosinolate volatiles (Winde & Wittstock 2011), whereas the blend of volatiles released depends on the mixture of structurally different glucosinolates, reaction conditions and protein cofactors (Ahuja et al. 2009; Borgen et al. 2012). In B. nigra, we observed eight different glucosinolate breakdown products (Table 1). As with the emissions of LOX volatiles, emissions of glucosinolate breakdown products was enhanced at 40–44 °C in long-term stress and at 45–50 °C in heat shock treatments (Figs 3c & 8b). Moreover, in long-term stress treatments, the emissions of LOX volatiles and glucosinolate breakdown products were correlated over temperatures of 25 to 40 °C (Fig. 5). Similar elicitation of LOX volatiles and volatile glucosinolate products suggests that both reflect the propagation of lesions with increasing the severity of heat stress.

However, long-term and heat shock stresses importantly differed in the quantitative relationship between LOX volatiles and glucosinolate breakdown products (Figs 3a, c & 8b). In particular, long-term heat stress led to a much stronger elicitation of glucosinolate volatiles than the heat shock stress and at the highest long-term heat treatment temperature of 44 °C, glucosinolate volatile production exceeded LOX volatile production (Figs 3a,c & 8b). This evidence suggests that while the release of glucosinolate volatiles upon heat shock reflects a release of myrosinases upon disruption of plant cells, a certain induction process is activated upon long-term heat stress. In fact, there is evidence that myrosinase expression can be enhanced by different biotic and abiotic stresses (Jost et al. 2005; Pan et al. 2014; del Carmen Martinez-Ballesta & Carvajal 2015). In addition, reactive oxygen species such as H2O2 can directly enhance myrosinase expression (Pan et al. 2014). As heat stress leads to a major burst of H2O2 (Hüve et al. 2011), heat stress dependent enhancement of myrosinase activity is likely. On the other hand, there is still limited information of tissue-specific expression of different isoforms of myrosinases as well as stress effects on the synthesis of glucosinolates. For example, methanethiol, that has been previously observed in Brassica upon tissue damage (Tulio et al. 2002; van Dam et al. 2012) was mostly detected in long-term stress experiment, suggesting a certain reprogramming of glucosinolate synthesis. Furthermore, the spatial separation of myrosinases and glucosinolates, any structural or physiological change that reduces the degree of separation, for example, expression of a different myrosinase closer to the site of synthesis of glucosinolates or vice versa is also expected to enhance the release of glucosinolate breakdown products.

Clearly, the release of glucosinolate volatiles constitutes a stress marker in Brassicaceae, but there is also evidence that glucosinolate volatiles may also play a key signalling role. In particular, exposure to allyl isothiocyanate has been shown to enhance thermotolerance of A. thaliana (Hara et al. 2013). Application of allyl isothiocyanate has also been shown to lead to reactive oxygen species formation and activation of a signalling cascade leading to the closure of stomata (Khokon et al. 2011; Hossain et al. 2013). In our study, stomatal conducance and glucosinolate release were correlated through the long-term heat treatment (Fig. 7b), however, given that stomatal closure also occurred through temperature response curves where glucosinolate volatile release was minimal (Fig. 3c), the correlation in Fig. 7b likely is not causal, but part of the heat stress syndrome.
Heat stress effects on benzenoids and geranylergynyl diphosphate pathway volatiles

In addition to LOX-pathway, GDP-pathway and glucosinolate volatiles, heat treatments were associated with differences in emissions of two other volatile compound classes. The only benzenoid, methyl salicylate (MeSA), was detected in long-term stress treatment at temperatures 30, 40 and 44°C (Table 1). MeSA is a common plant stress volatile, activating multiple biochemical pathways upon biotic and abiotic stresses (Arimura et al. 2005; Zhao et al. 2010), and its release has been observed in several cases upon long-term exposure to moderately high temperatures (Karl et al. 2008; Kleist et al. 2012). MeSA can be de novo synthesized upon stress, but it can also be released from a glycosidically bonded form (Blažević & Mastelić 2009). Given that no MeSA release was observed upon heat shock treatment, the release of MeSA upon long-term stress suggests that it was de novo synthesized.

In the case of geranylergynyl diphosphate (GGDP) pathway volatiles, we observed emissions of geranyl acetone and 6-methyl-5-hepten-2-one that are suggested from oxidative cleavage of carotenoids (Buttery et al. 1988; Goff & Klee 2006; Tieman et al. 2006). Both volatiles, geranyl acetone (Taveira et al. 2009; Truong et al. 2014) and 6-methyl-5-hepten-2-one (Geervliet et al. 1997) have been observed in emissions from Brassicaceae species. In B. nigra, only a reduction of emission with raising temperature was observed in temperature response curve measurements similarly to changes in GDP-pathway volatiles (Fig. 3d). Analogously, geranyl acetone emissions decreased in A. thaliana with increasing temperature (Truong et al. 2014). As both GDP-pathway and GGDP-pathway are confined to plastids (Rajabi Memari et al. 2013), the release of these volatiles might be associated with turnover of carotenoids that occurs as part of everyday plant metabolism (Beisel et al. 2010). If so, inhibition of the release of GGDP-volatiles with raising temperatures might indicate reversible inhibition of carotenoid synthesis.

CONCLUSIONS

Overall, the results indicated that different types of heat treatment are associated with major variation in photosynthetic and volatile responses in B. nigra (Figs 2–4 & 8). Temperature response curve measurements constituted a mild, physiological stress that led to reductions in constitutively synthesized volatiles associated with immediate photosynthetic metabolism. Both long-term and heat shock stress resulted in elicitation of lipoygenase and glucosinolate volatiles once the threshold heat dose was achieved. However, these two types of stresses primarily differed in the extent to which glucosinolate volatile emission was induced relative to LOX product release (Fig. 8). In particular, long-term heat stress was associated with much stronger elicitation of glucosinolate emissions than the heat shock response. In addition, methyl salicylate emissions were only induced by long-term heat stress. Although both long-term and short-term shock stress resulted in major releases of stress volatile emissions, sustained moderate heat stress resulted in the engagement of induced metabolic defense systems that did not occur upon short severe stress. As the result, different types of heat stress, mild, chronic and shock stress, are associated with different volatile fingerprints (Fig. 4). These different volatile blends could play important roles in heat-elicted signalling responses as well as in multitrophic interactions in natural stressful environments (Hopkins et al. 2009; Copolovici et al. 2014). Further work is needed to gain insight into the role of induction of glucosinolate volatiles in heat resistance and into how different types of heat stress affect plant-insect interactions in Brassicaceae.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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