Eesti Maaülikool
Estonian University of Life Sciences

DIFFERENTIAL REGULATION OF RELEASE OF LEAF STRESS VOLATILES: FROM TERPENE SYNTHASE GENE EXPRESSION TO EMISSION RESPONSES UNDER HEAT, OZONE AND WOUNDING STRESSES

BIOGEENSETE LENDUVÜHENDITE EMISSIOONI REGULATSIOON STRESSITINGIMUSTES: GEENIEKSPRESSIOONIST LENDUVÜHENDITE EMISSIOONIVASTUSTENI ERINEVATE ABIOOTILISTE STRESSIDE KORRAL

AROORAN KANAGENDRAN

A Thesis
for applying for the degree of Doctor of Philosophy
in Agriculture

Väitekiri
filosoofiadoktori kraadi taotlemiseks põllumajanduse erialal

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To my wife,
Vithyani Arooran

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LIST OF ORIGINAL PUBLICATIONS

This thesis was prepared based on the following research papers:

I. Pazouki, L., **Kanagendran, A.**, Li, S., Kännaste, A., Rajabi Memari, H., Bichele, R., Niinemets, Ü. (2016). Mono- and sesquiterpene release from tomato (*Solanum lycopersicum*) leaves upon mild and severe heat stress and through recovery: from gene expression to emission responses. *Environmental and Experimental Botany* 132, 1–15.

II. **Kanagendran, A.**, Pazouki, L., Niinemets, Ü. (2018). Differential regulation of volatile emission from Eucalyptus globulus leaves upon single and combined ozone and wounding treatments through recovery and relationships with ozone uptake. *Environmental and Experimental Botany* 145, 21-38.

III. **Kanagendran, A.**, Pazouki, L., Li, S., Liu, B., Kännaste, A, Niinemets, Ü. (2018). Ozone-triggered surface uptake and stress volatile emissions in *Nicotiana tabacum* 'Wisconsin'. *Journal of Experimental Botany* 69, 681-697.

ABBREVIATIONS

ATP	Adenosine triphosphate
CAC	Clathrin adaptor complex
DMADP	Dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose-phosphate
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
FDP	Farnesyl diphosphate
GC-MS	Gas-chromatography-mass spectrometry
GDP	Geranyl diphosphate
GGDP	Geranylgeranyl diphosphate
GLM	Generalized Linear Models
GLV	Green leaf volatiles
IDP	Isopentenyl diphosphate
LOX	Lipoxygenase pathway
MVA	Mevalonic acid
PCR	Polymerase chain reaction
SAND	Sp100, AIRE-1, NucP41/75, DEAF-1 (SAND) family protein
TPS	Terpenoid synthase
VOCs	Volatile organic compounds

1. INTRODUCTION

Emissions of plant volatile organic compounds (VOCs) are typically comprised of various terpenoids, such as isoprene, mono- and sesquiterpenes, lipoxygenase pathway (LOX, also called green leaf volatiles) volatiles, saturated aldehydes, and benzenoids which are emitted either constitutively or upon biotic or abiotic stresses (Copolovici et al., 2014; Jiang et al., 2016; Kask et al., 2016; Niinemets, 2010; Semiz et al., 2012). VOCs alter the chemical properties of atmosphere and thus, they play key roles in the interactions between the biosphere and atmosphere. In addition, they act as a signalling cue for communication among organisms (Dicke and Loreto, 2010).

Isoprenoids (terpenoids) are produced from the universal C5 building blocks of isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP). There are two distinct pathways responsible for the biosynthesis of isoprenoids: (1) the plastidial 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway, existing in bacteria and in plastids of plants, and (2) the cytosolic mevalonate (MVA) pathway, found in archaea, some bacteria, and in the cytosol of plants, animals and fungi (Nagegowda, 2010; Smit and Mushegian, 2000). The simplest isoprenoid is isoprene (2-methyl-1,3-butadiene) that is biosynthesized via MEP pathway in plants. 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS) and DXP reductoisomerase (DXR) are the first two key enzymes in the MEP pathway regulating the biosynthesis of isoprene (Rosenkranz and Schnitzler, 2013; Sharkey, 2005). In addition, IDP isomerase enzyme also acts as a key regulatory enzyme for isoprene biosynthesis by maintaining the balance between DMADP and IDP (Brüggemann and Schnitzler, 2002). These intermediates are further used by prenyltransferases to produce substrates of various lengths for specific terpene synthases. For example, monoterpene synthases convert geranyl diphosphate (GDP) to monoterpenes (C10), sesquiterpene synthases convert farnesyl diphosphate (FDP) to sesquiterpenes (C15), and diterpene synthases convert geranylgeranyl diphosphate (GGDP) to diterpenes (C20) (Bohlmann et al., 1998; Chen et al., 2011). Plants utilize isoprenoids for an array of key functions, including communication among plants, acting as hormones, protection of photosynthetic apparatus from heat, photosynthetic pigments, and defensive substances (Dicke & Baldwin

2010; Loreto et al., 2014; Velikova et al. 2011). In addition, plant-produced isoprenoids are commercially utilized for the production of food, essential oils and flavors, pharmaceuticals and biofuels (Tholl, 2015). Isoprenoids with C5-C15 such as isoprene, monoterpenes, and some sesquiterpenes are volatile, while isoprenoids with more than C15 molecules are much less volatile (Dudareva and Pichersky, 2008). Although volatile isoprenoids are emitted from almost all parts of plants such as leaves, flowers, buds, stems, and roots, the highest emissions are often detected from leaves (Kesselmeier and Staudt, 1999).

In addition to isoprenoids, plants emit several volatile compounds constitutively and upon stress, i.e. LOX volatiles, saturated aldehydes, benzenoids, and GGDP pathway volatiles. LOX volatiles are the primary stress-induced volatiles, consisting of various C6 aldehydes and alcohols (Beauchamp et al., 2005; Jiang et al., 2016; Li et al., 2017). The emission of LOX volatiles is considered as a distinctive early stress response of plants (Jansen et al., 2009; Matsui, 2006) and thus, they can act as a sensitive indicator of the severity of stress that plants experience (Harrison et al., 2013; Niinemets, 2010). Upon stress, LOX volatiles are usually emitted first, followed by the emissions of volatile isoprenoids, benzenoids, and GGDP pathway volatiles (Copolovici et al., 2014; Kask et al., 2016; Semiz et al., 2012). In fact, the emission of LOX volatiles usually scales with the severity of stress (Beauchamp et al., 2005; Brilli et al., 2011; Copolovici et al., 2014).

High temperature is one of the key abiotic stress factors negatively influencing plant growth and development (Copolovici et al., 2012). The global mean temperature in the 21th century has been suggested to have already increased by 0.76 °C as a result of global warming (IPCC 2007). In addition, the reduction of plant growth and productivity due to frequent heat waves is a serious issue in tropical and Mediterranean crop ecosystems (Copolovici et al., 2012; Hüve et al., 2011). Furthermore, higher temperatures often modify biosynthesis and emission of volatile organic compounds in plants (Copolovici et al., 2012).

Ozone is a phytotoxic gas and a key stress elicitor, and a frequent chronic abiotic stressor (Simpson et al., 2014). A major share of tropospheric ozone is formed in a reaction involving nitrogen oxides (NOX) and VOCs in the presence of sunlight (Ryerson et al., 2003). Elevated ozone exerts oxidative stress in plants and therefore, it alters

biochemical adjustments and metabolic shifts mainly through gene expression changes and hypersensitive reactions (Gerosa et al., 2009; Heath, 2008). The current tropospheric ozone concentration in most parts of the world is around 40 ppb, but it greatly varies with the geographic location (Sicard et al., 2017). However, tropospheric ozone concentration is expected to increase by 2-4 folds in next two decades due to enhanced rate of industrialization and burning of fossil fuels, suggesting that ozone stress will be a severe abiotic stress elicitor in the near future (Vingarzan, 2004). In particular, crop plants such as rice, wheat, and beans are more sensitive to present level of tropospheric ozone and thus, a greater loss of yield is expected as a result of curbed photosynthesis (Liu et al., 2015; Proietti et al., 2016).

In nature, plants can face many concomitant stresses. In particular, wounding is a common mechanical stress factor frequently caused by wind, falling debris, moving objects and precipitation (Benikhlef et al., 2013; Portillo-Estrada et al., 2015). Thus, wounding can often interact with chronic ozone stress in natural conditions. Combined ozone and wounding effects are predicted to result in synergistic effects on primary and secondary metabolic activities of plants such as photosynthesis, and biosynthesis and emission of VOCs (Alexieva et al., 2003; Niinemets et al., 2017; Niinemets, 2010).

In addition, rising temperate and atmospheric ozone have serious harmful effects on global agricultural sector and therefore, investigating how acute heat, ozone and wounding stresses alter plant photosynthetic and terpenoid biosynthetic pathways is of a key economic significance. In addition, there is a scarcity of information of how photosynthetic and volatile emission responses are altered by heat, ozone, and wounding stresses and how the elicited VOC emissions can lead to concomitant changes in physical and chemical properties of atmosphere. There is limited information on the molecular basis of terpenoid biosynthesis upon different abiotic stresses, particularly on regulation of terpenoid synthase (TPS) gene expression in response to heat stress. Therefore, closing the gap between the influence of stress and concomitant changes in primary and secondary metabolic processes of plants is highly relevant to predict plant responses to global change.

2. REVIEW OF THE LITERATURE

2.1. Impact of heat, ozone and wounding stress on foliage photosynthetic characteristics

Temperature is a key abiotic stress factor that severely affects photosynthetic characteristics (Hüve et al., 2011; Hüve et al., 2006; Kask et al., 2016). Both freezing and high temperatures significantly reduce the rates of growth and development of plants (Copolovici et al., 2012). Generally, the optimum temperatures for photosynthesis vary in the range of 25–35 °C for plants in most ecological habitats (Sage and Kubien, 2007). Berry and Bjorkman (1980) demonstrated that photosynthesis is very sensitive to both high and low temperatures and thus, it can be completely impaired by extreme high and low temperatures. Generally, elevated temperature often result in a decline in stomatal conductance to water vapour (g_s) (Hüve et al., 2011). Severe heat stress irreversibly impairs key photosynthetic processes; particularly, photosyntem II (PSII) and the oxygen-evolving complex (Copolovici et al., 2012). In fact, PSII is the most heat-liable component of the electron transport chain and thus, a heat-induced damage in PSII leads to a partial or complete inhibition of photosynthesis depending on the severity of stress (Hüve et al., 2011). Generally, a decline in PSII activity is often correlated with the reduction in chlorophyll fluorescence (Havaux and Tardy, 1996) and thus, chlorophyll fluorescence is a sensitive indicator for quantitative characterization of the responses of the activity of photosynthetic apparatus to heat stress (Krause and Weis, 1991; Strasser, 1997). High temperature negatively affects Calvin cycle; particularly, through inactivation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) because Rubisco enzyme is the ultimate rate-limiting enzyme in carbon fixation chain (Hüve et al., 2011).

Similar to heat stress, acute ozone exposures reduce leaf photosynthetic activity, including reductions in net assimilation rate (A), g_s and maximum quantum yield of PSII (F_v/F_m) (Fusaro et al., 2016; Li et al., 2017). A decline in foliage A upon elevated ozone exposure is often associated with concomitant changes in g_s (Fiscus et al., 2005; Uddling et al., 2010). In addition, there is a strong relationship between cumulative stomatal ozone uptake and a decline in photosynthetic characteristics (Calatayud et al., 2003; Li et al., 2017). An ozone-induced reduction of F_v/F_m

is often regarded as a sensitive indicator of impaired use of light in photosynthesis (Li et al., 2017). Individual and combined application of ozone and wounding treatments can possibly be associated with a reduction in electron transport capacity and irreversible damage in chlorophyll reaction centers in PSII due to oxidative stress ultimately curbing photosynthetic traits (Guidi and Degl'Innocenti, 2008). Similar to heat stress, acute ozone exposure reduces *A* through inactivation of Calvin cycle due to inhibition of Rubisco activity becasue Rubisco is highly prone to acute ozone exposures (Fusaro et al., 2016; Niu et al., 2014; Zhang et al., 2011).

2.2. Regulation of volatile emissions in response to heat stress

Generally, elevated temperatures enhance volatile emission mainly through enhanced activity of rate- limiting enzymes responsible for the biosynthesis of VOCs (Niinemets et al., 2002). In parallel with the changes in rate limiting enzyme activity, high temperatures alter the expression of terpene synthase genes, ultimately regulating the biosynthesis and emission of volatiles. Kask et al. (2016) demonstrated that there were enhanced emission responses of monoterpenes, LOX volatiles, GGDP pathway volatiles, and benzenoids due to heat stress on in *Brassica nigra* foliage. In addition, acute and chronic heat stresses differentially regulate constitutive and induced volatile emission responses (Kask et al., 2016). Generally, heat stress primarily elicits LOX volatile emissions, followed by volatile isoprenoids (Copolovici et al., 2012). LOX volatiles are formed in the reactions involving multiple lipoxygenases and fatty acid hydroperoxide lyases from polyunsaturated fatty acids (Feussner and Wasternack, 2002; Jiang et al., 2016). Lipoxygenases are constitutively active in cells and they are released upon cellular membrane damage caused by heat stress, ultimately leading to the formation of characteristic LOX volatiles such as (*Z*)-3-hexenol and (*E*)-2-hexenal (Copolovici et al., 2012; Jiang et al., 2016).

Given that isoprene is biosynthesized *de novo*, heat induced emission responses of isoprene are primarily due to enhanced rate limiting enzyme activity and/or upregulation of isoprene synthase gene (Loreto and Sharkey, 1993; Mayrhofer et al., 2005). In the case of foliage mono- and sesquiterpene emissions, constitutive release of mono- and sesquiterpenes comes from either the storage structures present in the leaves or from *de novo* synthesis or from both sources (Copolovici and

Niinemets, 2016; Grote and Niinemets, 2008). In addition, heat stress can rupture cell membranes of storage structures, where mono-and sesquiterpenes are stored, and result in a rapid emission burst of volatiles immediately after stress application and through recovery (Copolovici et al., 2012). However, the *de novo* synthesis of monoterpenes is dependent on foliage photosynthesis rate and therefore, heat-dependent changes in foliage photosynthetic characteristics may influence *de novo* monoterpene emission in some cases (Grote and Niinemets, 2008; Loreto et al., 1996).

2.3. Regulation of volatile emissions upon ozone and wounding treatments

Elevated ozone effects on leaf physiological characteristics depend on the type of plant species exposed, length and severity ozone exposures (Heiden et al., 2003). However, there is limited information on the elicitation of volatile emissions upon ozone exposures (Li et al., 2017). Given that stomata are the primary channel through which ozone enters the leaf intercellular air spaces, from where it can generate oxidative damage in mesophyll cells, the stomatal ozone uptake determines the elicitation of volatile emission regardless of the concentration of ozone around leaf surface (Beauchamp et al., 2005; Jud et al., 2016; Li et al., 2017). Generally, upon zone exposure, volatile emissions scale exponentially or sigmoidally to a maximum, followed by a decline until the emission reach pre-stress level or lower than pre-stress level (Beauchamp et al., 2005; Li et al., 2017). However, there is a complex kinetics of volatile release during ozone exposure and through recovery phase, where biphasic and multiphasic emission patterns can be observed (Beauchamp et al., 2005; Li et al., 2017).

In the case of LOX emissions, similar to heat stress, ozone caused hypersensitive responses associated with severe cellular rupture and necrotic lesions (Bussotti et al., 2007), resulting in induction of a major emission burst of LOX volatiles after stress application and followed by a sharp decline through recovery phase (Beauchamp et al., 2005).

Wounding treatments damage plant cell membranes and thus, free polyunsaturated fatty acids are rapidly available for the biosynthesis of LOX volatiles. Therefore, an emission burst of classical LOX compounds is often observed upon wounding; the magnitude of this emission burst usually scales with the extent of damage (Portillo-Estrada et al., 2015).

Acute ozone exposure enhances isoprene emission (Fares et al., 2006; Loreto et al., 2004), whereas chronic ozone exposure reduces isoprene emission (Calfapietra et al., 2008; Calfapietra et al., 2007). In the case of mono- and sesquiterpene emissions, the damage in epidermal and epithelial cells of terpene storage structures due to ozone-induced hypersensitive reactions can lead to immediate emission burst of mono- and sesquiterpenes, and thereafter the emissions decline throughout the recovery phase (Loreto et al., 2000; Schuh et al., 1996). Generally, the wounding reduces isoprene emissions and this is associated with a decline in photosynthesis rate (Loreto and Sharkey, 1993). In addition, wounding may initially increase the emission of mono- and sesquiterpenes due to direct damage of terpene storage structures in leaves (Portillo-Estrada et al., 2015).

2.4. Agricultural significance of volatile organic compounds

Agriculture sector can directly and indirectly benefit from VOCs (Zwenger and Basu, 2008). Usually, insect-pollinated crop plants utilize VOCs as a means to attract pollinators and seed dispersers (Metcalf and Kogan, 1987) and thus, they act as info-chemicals in agricultural ecosystem (Zwenger and Basu, 2008). Furthermore, VOCs are involved in tritrophic interactions and therefore, crops infested with herbivores release certain signaling volatiles that attract parasitoids to kill the herbivores. Therefore, once volatile release is induced, crop plants can be protected from subsequent herbivore attacks (Fink, 2007; Kessler and Baldwin, 2001; Turlings et al., 1990).

In recent years, huge attention has been paid to terpenes by agricultural sector (Zwenger and Basu, 2008). Batish et al. (2008) demonstrated that eucalyptus oil of *E. globulus*, mainly consisting of 1,8- cineole, can be widely used as a natural pesticide. In addition, the essential oils consisting of α-pinene, 1,8-cineole, and γ-terpinene from various eucalypt species also showed broad-spectrum pesticide activities (Batish et al., 2008; Lucia et al., 2007). In farm animals, terpenes are usually applied to control multiplication of antibiotic resistant pathogenic bacteria due to their broad-spectrum antimicrobial properties (Islam et al., 2003; Zwenger and Basu, 2008). Plant oils, such as cinnamon oil primarily consisting of (*E*)-cinnamaldehyde, α-guaiene, copaene, and α-muurolene act as broad-spectrum antibiotics, particularly against *Pseudomonas aeruginosa*, that cause severe chronic infections in farm animals (Prabuseenivasan et al., 2006).

In addition, a plant oil from *Neolitsea foliosa* consisting of isoprenoids, β-caryophyllene, caryophyllene oxide, elemol and β-elemene, exhibits strong antibacterial effects against certain pathogenic bacteria of farm animals (John et al., 2007)

3. HYPOTHESES AND AIMS OF THE STUDY

This thesis intended to study the impact of elevated temperatures in tomato (*Solanum lycopersicum* 'Pontica'), acute ozone and wounding treatments in eucalypt (*Eucalyptus globulus* Labil), and acute ozone exposures in tobacco (*Nicotiana tabacum* 'Wisconsin') on time-dependent modifications of foliage photosynthetic characteristics, TPS gene expression and regulation of stress volatile emissions, and the relationship between stomatal and non-stomatal ozone flux and stress volatile emission responses. In this study, we hypothesized that:

a. heat shock in *S. lycopersicum* leaves will modify the foliage photosynthetic characteristics and the emission of LOX volatiles, and mono- and sesquiterpenes through recovery phase; and the emission of mono- and sesquiterpenes will primarily be regulated at mono- and sesquiterpene synthase gene expression levels (**Paper I**).

b. acute ozone and wounding treatments applied independently and in combination in *E. globulus* leaves will greatly reduce foliage net assimilation rate, stomatal conductance to water vapour, and maximum quantum yield of photosystem II (F_v/F_m); independent ozone and wounding treatments will result in strong emission responses of LOX volatiles, and volatile isoprenoids and the emission responses will be greater for ozone treatments than for wounding treatment; the combined application of ozone and wounding treatments will lead to synergistic effects on photosynthetic characteristics and stress volatile emission responses. As *E. globulus* is a strong isoprenoid emitter, it was further expected that volatile isoprenoids and antioxidants will scavenge a major fraction of ozone in leaf intercellular spaces and mesophyll cells, ultimately leading to a weak relationship between stress dose and volatile emissions (**Paper II**).

c. acute ozone exposure of the leaves of *Nicotiana tabacum* ozone-resistant cultivar will result in moderate changes in foliage photosynthetic characteristics and the emission of LOX volatiles, mono- and sesquiterpenes through recovery; ozone mainly induces changes in trichome surface permeability, and there is a greater share of leaf ozone absorption due to non-stomatal ozone deposition than due to stomatal ozone uptake (**Paper III**).

Paper I investigated the impact of heat stress on foliage photosynthetic characteristics, stress volatile emission responses and terpene synthase gene expressions at different times of recovery after stress applications. **Paper II** summarized how single and combined ozone and wounding treatments altered foliage photosynthetic characteristics and stress volatile emission responses through recovery and how temporal emission responses are regulated by stomatal ozone uptake. **Paper III** studied the impact of acute ozone exposures on the modification of foliage photosynthetic characteristics and emission of LOX volatiles and mono- and sesquiterpenes from stress application through recovery and analyzed the share of foliage total ozone uptake between stomatal ozone uptake and non-stomatal ozone deposition, and related different ozone fluxes with the degree of elicitation of stress volatile emissions.

The general aim of this thesis was to develop tomato (*S. lycopersicum*), tobacco (*N. tabacum*), and eucalypt (*E. globulus*) model systems to study: (1) the impact of heat, ozone, ozone and wounding stresses on foliage photosynthetic characteristics; (2) the temporal regulation of expression of terpene synthase genes and subsequent changes in mono- and sesquiterpene emission upon acute heat stress in *S. lycopersicum*; (3) time-dependent modification of stress volatile emissions from *E. globulus* and *N. tabacum* upon acute ozone and wounding treatments and relate the share of stomatal ozone uptake and non-stomatal ozone deposition with the stress volatile emission rate.

The specific aims of the thesis were to:

1. a. study the impact of heat stress on time-dependent changes in photosynthetic characteristics.
   b. analyze the effects of heat stress on the expression and regulation of β-phellandrene and (*E*)-β-caryophyllene synthase genes and concomitant changes in mono- and sesquiteprene emission responses in *S. lycopersicum* 'Pontica'.
   c. assess the correspondence between time-dependent modifications in mono- and sesquiterpene gene expression and emissions throughout the recovery phase (**Paper I**).
2. a. study the influence of combined ozone and wounding treatments on photosynthetic characteristics and stress volatile emission responses through recovery.

b. estimate the relationship between stomatal ozone uptake rates and emission rates of stress volatiles (**Paper II**).

3. a. investigate the relationships between ozone dose and temporal modifications in foliage photosynthetic characteristics and emissions of LOX volatiles and mono- and sesquiterpenes.

b. estimate the share of leaf ozone uptake rates between stomatal ozone uptake rates and non-stomatal ozone deposition rates and relate the leaf ozone uptake rates with the stress volatile emission rates (**Paper III**).

4. MATERIALS AND METHODS

4.1. Plant materials

For the study of heat stress (**Paper I**), *S. lycopersicum* seeds (seed source: Starsem, Romania) were sown in a commercial potting mixture (Biolan Oy, Kauttua, Finland). After germination, seedlings were replanted into 1 L pots consisting of the same potting soil. Seedlings were kept under controlled environmental conditions as follows: the light intensity at leaf surface of 400 μmol m^{-2} s^{-1} for 12 h photoperiod, day/night temperatures of 24/18 °C, and relative air humidity of 60%.

For the study of ozone and wounding stress (**Paper II**), *E. globulus* seeds (Seed source: OMC seeds Ltd., Lithuania) were grown in 5 L pots filled with a commercial potting soil (Biolan Oy, Kauttua, Finland) under the controlled environmental conditions as follows: light intensity at leaf surface of 500 μmol m^{-2} s^{-1} for 12 h photoperiod, chamber temperature (day/night) of 28/25 °C, ambient CO_2 concentration of 380-400 ppm, and relative air humidity of 60-70%. After three weeks of germination, the seedlings were transplanted into 10 L pots and grown in a plant growth room under the same environmental conditions as in the growth chamber until the completion of the experiment.

For the study of ozone stress (**Paper III**), *N. tabacum* 'Wisconsin' seeds were sown in 1 L pots filled with a commercial potting soil (Biolan Oy, Kekkilä group, Finland). Four weeks after germination, the seedlings were transplanted into 3 L plastic pots consisting of the same potting soil. Plants were grown under the light intensity at leaf surface of of 400-500 μmol m^{-2} s^{-1} for 12 h photoperiod, and relative air humidity of 60%; and day/night temperature of 24/18 °C provided until the completion of the experiment.

In all experiments (**Papers I-III**), the plants were watered to soil field capacity and fertilised once in a week with a combined NPK (5:5:6) fertilizer with micronutrients (B (0.01%), Cu (0.03%), Fe (0.06%), Mn (0.028%), Zn (0.007%)). In all experimental treatments, fully mature similar-sized leaves from similar canopy positions and similar leaf order were used.

4.2. Application of stress

Heat shock (**Paper I**) was applied by gently immersing 3-4 fully mature tomato leaves in a temperature-controlled water bath (VWR International, West Chester, Pennsylvania, USA). The treatments included immersion in distilled water of 25 (control), 37, 41, 46 and 49 °C for 5 minutes. Then the leaves were left to air-dry for 5-10 minutes before taking gas exchange and volatile emission measurements.

For the study of combined ozone and wounding stress in eucalypt (**Paper II**), ozone was generated by Certizon C100 ozoniser (Erwin Sander Elektroapparatenbau GmbH, Germany). There were four different sets of stress applications: control (untreated), wounding, ozone exposure, and ozone exposure followed by wounding. For ozone exposure, an arbitrarily selected branch consisting of 6 fully mature leaves was exposed to 4, 5 and 6 ppm ozone for 3 hours. Wounding was applied by rapidly (within 6 sec) punching four holes in leaf lamina by a paper punch. The area of each disc removed was ca. 25 mm^2 and thus, in total a 7 cm-length of wound per leaf (wound edge) was generated. In the case of combined ozone and wounding treatments, the leaves were first exposed to ozone (the same ozone treatments), followed by wounding similar to the wounding treatment alone.

In the case of ozone treatments in tobacco leaves (**Paper III**), ozone was generated by Certizon C100 ozoniser (Erwin Sander Elektroapparatenbau GmbH, Germany). Fully mature and arbitrarily selected individual leaves were exposed to 0, 400, 600, 800, and 1000 ppb ozone for 30 minutes. In Papers **II and III**, ozone concentration in the in- and outlets of the glass chamber was monitored using a UV photometric ozone detector (Model 49i, Thermo Fisher Scientific, Franklin MA, USA).

4.3. Measurement of photosynthetic characteristics

In **paper I**, changes in net assimilation rate, stomatal conductance to water vapour, and dark-adapted chlorophyll fluorescence yield (F_v/F_m) of control and treated leaves were measured using a portable gas-exchange chlorophyll fluorescence system (Walz GSF-3000, Walz GmbH, Effeltrich, Germany) under standard baseline conditions as follows: light intensity at leaf surface of 700 μmol m^{-2} s^{-1}, leaf temperature of 25 °C, CO_2 concentration of 400 μmol mol^{-1}, and relative air humidity of 60%.

In **papers II and III**, gas exchange measurements were conducted in a temperature-controlled double-layered glass chamber (see Copolovici and Niinemets (2010) for detail description of the glass chamber set-up). CO_2 and H_2O concentrations at the chamber in- and outlets were measured with an infra-red dual channel gas analyser operated in differential mode (CIRAS II, PP-Systems, Amesbury, MA, USA) under the baseline conditions as follows: light intensity at leaf surface of 700 μmol m^{-2} s^{-1}; temperature of 25 °C; ambient CO_2 concentration of 380-400 μmol mol^{-1}; and relative air humidity of 50-60%. Net assimilation rate, stomatal conductance to water vapour, and intercellular CO_2 concentration (C_i) were estimated according to von Caemmerer and Farquhar (1981). Changes in maximum dark-adapted (10-min darkening) quantum yield of photosystem II (F_v/F_m) of control and treated leaves was measured with the PAM flourometer (Walz IMAG-MIN/B, Walz GmbH, Effeltrich, Germany) providing a 1-s saturating pulse (light intensity 7000 μmol m^{-2} s^{-1}) of blue light.

4.4. Volatile measurement protocol and GC-MS analysis

Volatile sampling was carried out in the same glass chamber system mentioned in section 4.3. Foliage volatiles were collected when leaves had reached the steady-state conditions in the glass chamber, in ca. 10-15 min. after leaf enclosure. Volatiles were collected into adsorbent cartridges for the quantitative estimation of LOX volatiles and volatile isoprenoids. The adorbent cartridges were analysed with a combined Shimadzu TD20 automated cartridge desorber and Shimadzu 2010 Plus GC–MS system (Shimadzu Corporation, Kyoto, Japan) according to Copolovici et al. (2009) and Kännaste et al. (2014). Individual volatile compounds were identified based on mass spectra of pure standards (Sigma-Aldrich, St. Louis, MO, USA) and NIST 05 library (National Institute of Standards and Technology). The volatile emission rates were calculated according to Niinemets et al. (2011).

4.5. Leaf sampling, RNA extraction and cDNA synthesis for qPCR analysis

In *S. lycopersicum*, leaf sampling for qPCR was carried out in separate plants with the same age and size, similar to those used for gas exchange and volatile collection. However, leaf samples were collected at 2, 6, 10, 24, 48 h after heat stress treatments, consistent with the time of volatile

sampling starting from 2 h. The collected leaf samples were immersed in liquid nitrogen and stored in -80 °C until RNA was extracted (**Paper I**).

Leaf tissues were homogenised in liquid nitrogen using a mortar and pestle and then the RNA extraction was carried out using the RNeasy Plant mini kit (Qiagen, Germany) according to manufacturer's protocol. The concentration of extracted RNA was quantitatively analysed using Biophotometer Plus (Eppendorf, Germany) and adjusted with nuclease-free water when needed. cDNA was synthesized from 1 μg of RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, California, USA).

4.6. Primer designing and qPCR analysis

For the study of TPS gene expression in tomato, two reference genes, CAC (clathrin adaptor complex) and SAND (SAND family protein), were selected for qPCR analysis based on their most stable expression profiles (Expósito-Rodríguez et al., 2008). The target genes used in the study were terpenoid synthase genes, a monoterpene synthase (β-phellandrene synthase) and a sesquiterpene synthase ((*E*)-β-caryophyllene/α-humulene synthase). The primers for reference genes and target genes were designed using Primer3Plus, http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi (**Paper I**).

qPCR was performed in ViiA™ 7 qPCR System (Applied Biosystems, Courtaboeuf, France). The reaction was carried out in 25 μL volume consisting of 10 μL 2× iQ SYBR Green Supermix (Bio-Rad Laboratories Inc.), 300 nM of each primer and 1 μL of 5-fold-diluted cDNA. The PCR was run in an ABI Sequence Detection System (Applied Biosystems) using the following program: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melt curve analysis was generated to detect any non-specific amplification. The dissociation program was configured as follows: 95 °C for 15 s, 60 °C for 15 s followed by 20 min of gradual temperature ramp from 60 °C to 95 °C. Relative changes in gene expression were quantified according to $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

4.7. Data analysis

In Paper I, Generalized Linear Models (GLM) based on maximum likelihood model fitting were used to estimate the individual and interactive effects of treatment temperature and recovery time on the emission of total LOX volatiles, total mono- and sesquiterpenes, β-phellandrene, and (*E*)-β-caryophyllene. In addition, paired sample t-tests were carried out to compare the averages of relative expression levels of β-phellandrene, and (*E*)-β-caryophyllene synthase genes at different times of recovery and in different temperature treatments. Correlation analysis between relative expression levels of (*E*)-β-caryophyllene and β-phellandrene synthase genes was conducted at different temperature treatments.

In **paper II**, GLM using maximum likelihood model fitting was applied to test the individual and interactive effects of ozone and wounding, and time of recovery on total LOX volatile and volatile isoprenoid emissions.

In **Paper III,** GLM based on maximum likelihood model fitting was applied to estimate the individual and interactive effects of ozone and time of recovery on the emissions of total LOX volatiles, and total mono- and sesquiterpenes.

In all cases **(Papers I-III),** control and treated samples were replicated at least thrice. Data were analysed for normality of distribution and homogeneity of variances and the data were log-transformed when necessary. Correlation analysis was carried out using SigmaPlot Version 12.5 (Systat Software Inc, San Jose, CA, USA). All other statistical analyses were conducted using SPSS Version 24 (IBM SPSS, Chicago, IL, WA). All statistical effects were considered significant at $P < 0.05$.

5. RESULTS

5.1. Changes in foliage photosynthetic characteristics upon heat, ozone and wounding treatments

Exposure of *S. lycopersicum* leaves (**Paper I**) to 37 °C and 41 °C temperatures did not cause any changes in F_v/F_m compared with control leaves throughout the recovery phase, but higher temperature treatments at 46 °C and 49 °C dramatically reduced F_v/F_m (Fig. 1A). However, F_v/F_m at 46 °C temperate treatment partly recovered at 25 h of recovery phase, but there was no recovery of F_v/F_m observed for the leaves treated with 49 °C. Similarly, A and g_s were not significantly affected by 37 °C and 41 °C treatments, but a considerable reduction in A and g_s was observed in the leaves treated with 46 °C and 49 °C, compared to control leaves (Fig. 1B and C).

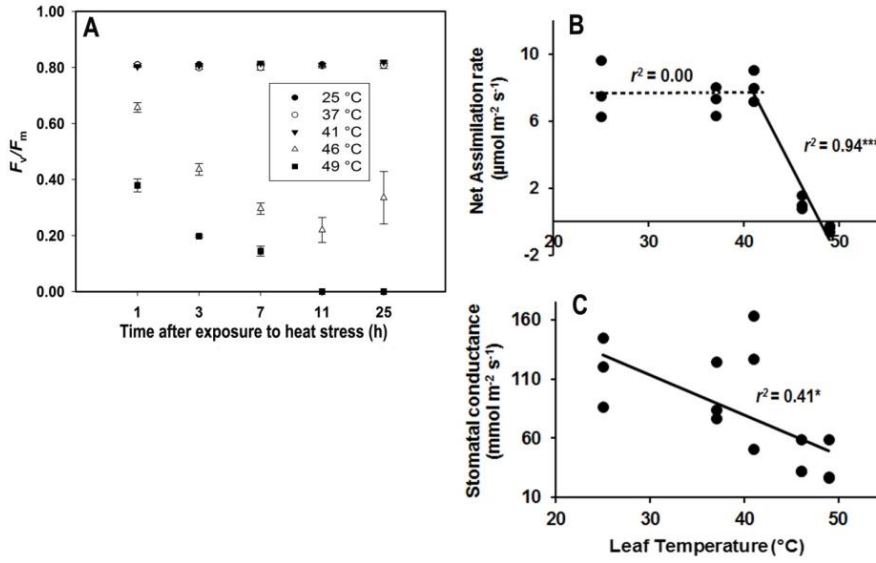

Figure 1. Changes in dark-adapted maximum quantum efficiency of PSII photochemistry estimated by chlorophyll fluorescence (Mean ±SE) (A) at different recovery times, and net assimilation rate (B) and stomatal conductance to water vapor (C) upon heat stress at 3.5 h of recovery phase at 25 °C in *Solanum lycopersicum* leaves. Each symbol represents an individual replicate. Data were fitted by linear regressions. Statistical significance is demonstrated as: * $P < 0.05$, *** $P < 0.001$ (reproduced from **Paper I**).

In *E. globulus* leaves (**Paper II**), single ozone and combined ozone and wounding treatments reduced F_v/F_m throughout the recovery phase (Fig. 2A, and Fig. 1A in **Paper II**). The decline was particularly severe for the leaves treated with 6 ppm ozone, and with combined 6 ppm ozone and wounding treatments at all recovery times. Wounding did not cause any substantial reduction in F_v/F_m. In addition, there was a partial recovery of F_v/F_m observed after 25 h of recovery time for all treatments. Single and combined ozone and wounding treatments reduced A at all recovery times, but the reduction was particularly pronounced until 25 h of recovery, and then A started recovering (Fig. 2B, and Fig. 1B in **Paper II**). However, there was no complete recovery of A observed in response to all stress applications at all recovery times. Generally, the reduction and recovery of A were associated with the corresponding changes in g_s (Fig. 2B and C, and Fig. 1B and C in **Paper II**).

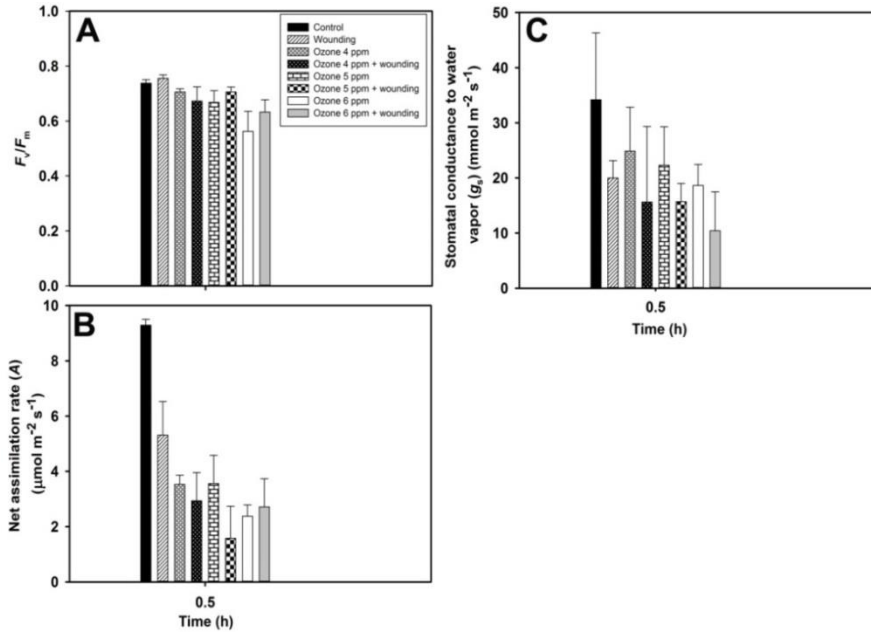

Figure 2. Changes (Mean +SE) in dark-adapted (10 min darkening) maximum quantum efficiency of PSII photochemistry estimated by chlorophyll fluorescence (A), and net assimilation rate (B) and stomatal conductance to water vapor (C) in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (first exposed to 4, 5, and 6 ppm ozone and then wounded) leaves of *Eucalyptus globulus* at 0.5h after stress applications (reproduced from **Paper II**).

Nicotiana tabacum leaves (**Paper III**) treated with 600-1000 ppb ozone demonstrated a progressive reduction in F_v/F_m at all recovery times, but the reduction was severe in response to 1000 ppb ozone treatment (Fig. 3, and Fig. 1 in **Paper III**). However, all ozone treatments led to a statistically significant reduction in A, g_s, and C_i immediately after ozone exposures (Fig. 4).

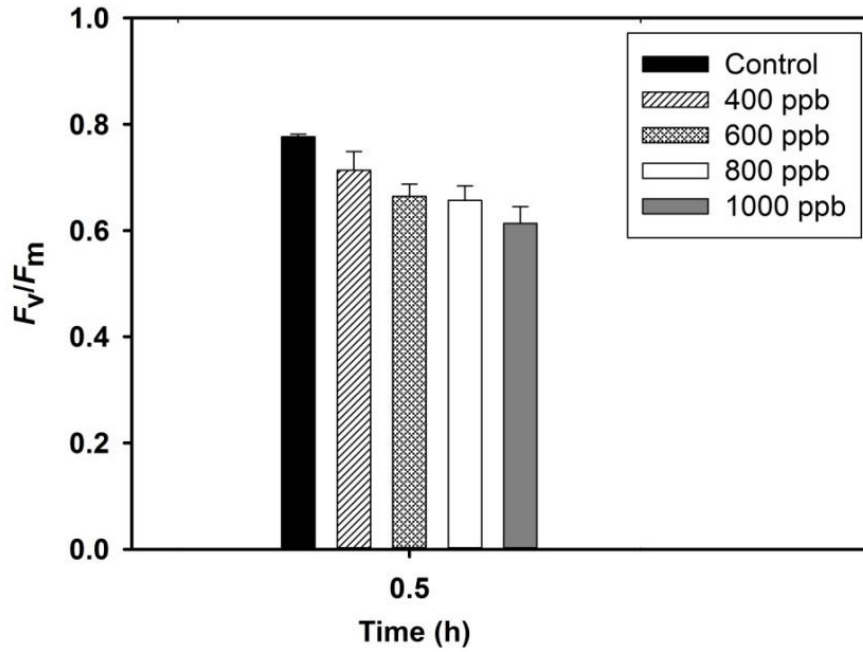

Figure 3. Dark-adapted (10 min darkening) average (+ SE) maximum fluorescence yield of photosystem II (PSII; F_v/F_m) of mature leaves of 10-12 weeks old *Nicotiana tabacum* 'Wisconsin' at 0.5 hr of recovery following the exposure with ozone concentrations of 0, 400, 600, 800, and 1000 ppb for 30 min (reproduced from **Paper III**).

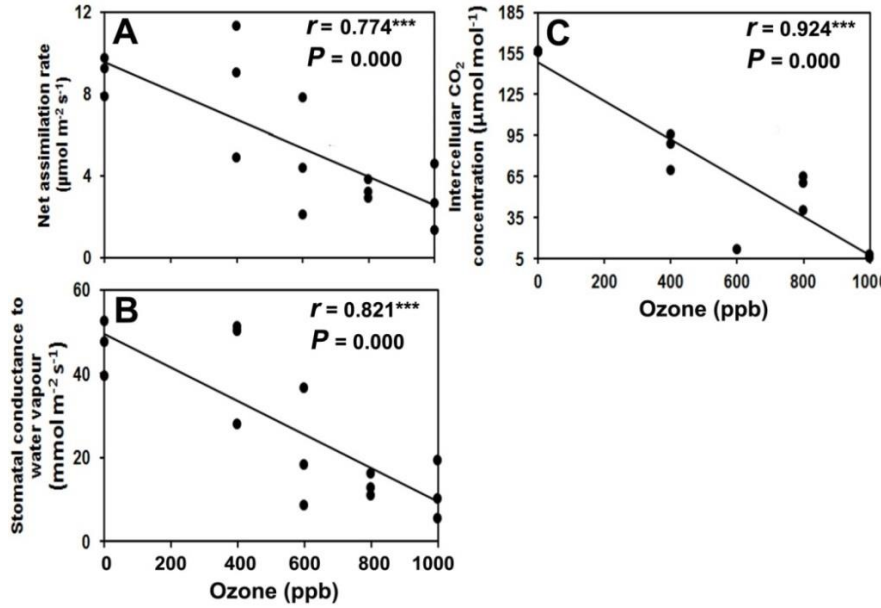

Figure 4. Changes in (A) leaf net assimilation rate (*A*), (B) stomatal conductance to water vapour (g_s), and (C) intercellular CO_2 concentration (C_i) in mature leaves of 10-12 weeks old *N. tabacum* 'Wisconsin' in relation to ozone concentration during exposure. The measurements were taken at 0.5 h after ozone exposure at 0, 400, 600, 800, and 1000 ppb for 30 min. Each data point corresponds to an individual replicate measurement. Data were fitted by linear regressions. Statistical significance of regressions is indicated as: *** -$P < 0.001$ (reproduced from **Paper III**).

5.2. Isoprene emissions in response to ozone and wounding treatments and through recovery

Eucalyptus globulus is a constitutive isoprenoid emitter (**Paper II**), and the control leaves emitted a relatively low level of isoprene, ca 1.4 ± 0.8 nmol m^{-2} s^{-1} (Fig. 5). However, single ozone and combined ozone and wounding treatments significantly enhanced isoprene emission throughout the recovery phase, but wounding alone reduced isoprene emission at all recovery times compared with the control leaves ($P < 0.05$ for impact of ozone, $P < 0.001$ for ozone and wounding, and $P < 0.001$ for wounding). In particular, the highest level of isoprene emission rate, ca. 17.6 ± 4.9 nmol m^{-2} s^{-1}, was achieved upon combined 5 ppm ozone and wounding treatment at 10 h of recovery, followed by 4 ppm ozone treatment throughout the entire recovery phase. There was no recovery of isoprene emission observed in any of the treatments.

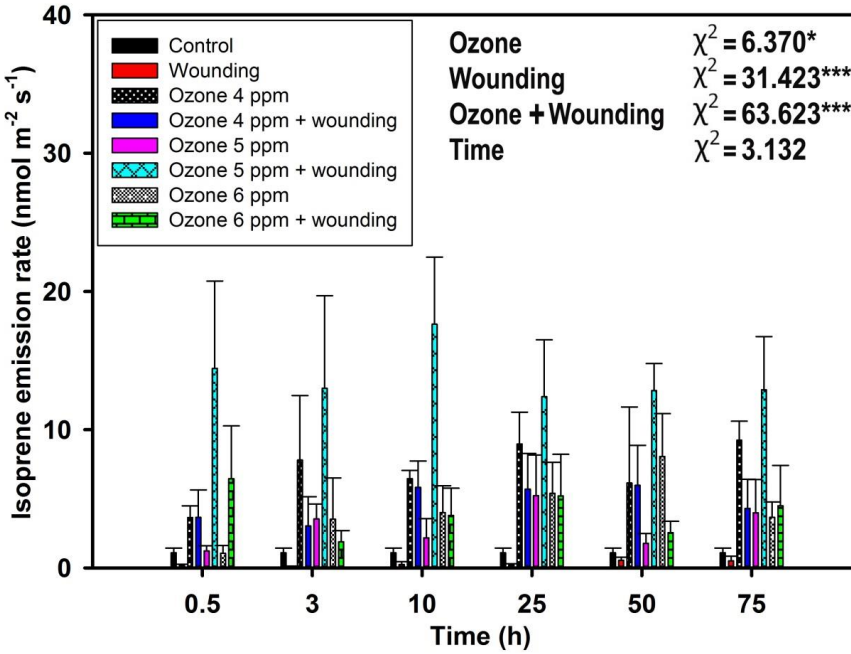

Figure 5. Emission (Mean + SE) rates of isoprene in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (4, 5, and 6 ppm ozone and wounding) leaves of *E. globulus* at different times of recovery (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Wald chi-squared test statistics (χ^2) is shown and its statistical significance is indicated as: * - $P < 0.05$, *** - $P < 0.001$ (reproduced from **Paper II**).

5.3. Time-dependent changes in foliage LOX volatile emissions upon heat, ozone and wounding treatments

Control leaves of *S. lycopersicum* did not emit significant quantities of total LOX volatiles, but heat stress substantially elicited total LOX volatile emissions at all recovery times (Fig. 4A in **Paper I**). The temperature effect was more pronounced immediately after stress applications and then the emission decayed throughout the recovery phase. In particular, total LOX emission was the highest for the leaves treated with 46 °C. The effect of temperature and recovery time had a statistically significant impact on total LOX volatile emission rates ($P < 0.001$ for temperature and $P < 0.05$ for recovery time). In this study, LOX emission blend was dominated by hexanal, followed by pentanal, 5-hexen-1-ol, and 1-hexanol (data not shown).

Untreated leaves of *E. globulus* (**Paper II**) emitted LOX volatiles at a very low level, ranging between ca. 0.001-0.002 nmol m^{-2} s^{-1} (Fig. 6A). Single and combined ozone and wounding treatments significantly enhanced total LOX emissions throughout the recovery phase ($P < 0.05$ for single ozone, and wounding treatments, $P < 0.001$ for combined ozone and wounding treatments, and recovery time). Furthermore, the LOX volatile blend comprised of hexanal, pentanal, (*E*)-2-hexenal, and (*Z*)-3-hexenol. In all treatments, LOX volatiles were dominated by (Z)-3-hexenol, followed by (*E*)-2-hexenal, hexanal, and pentanal immediately after stress applications (Fig. 4 in **Paper II**). In addition, the greatest emission rates of total LOX volatiles were observed for combined ozone and wounding treatments, followed by single ozone and wounding treatments. In this study, the emission of LOX volatiles scaled with the degree of stress applications (Fig. 6A, and Fig. 4 in **Paper II)**. The emission of LOX volatiles significantly declined throughout the recovery phase, but it did not achieve the pre-stress level until 75 h of recovery.

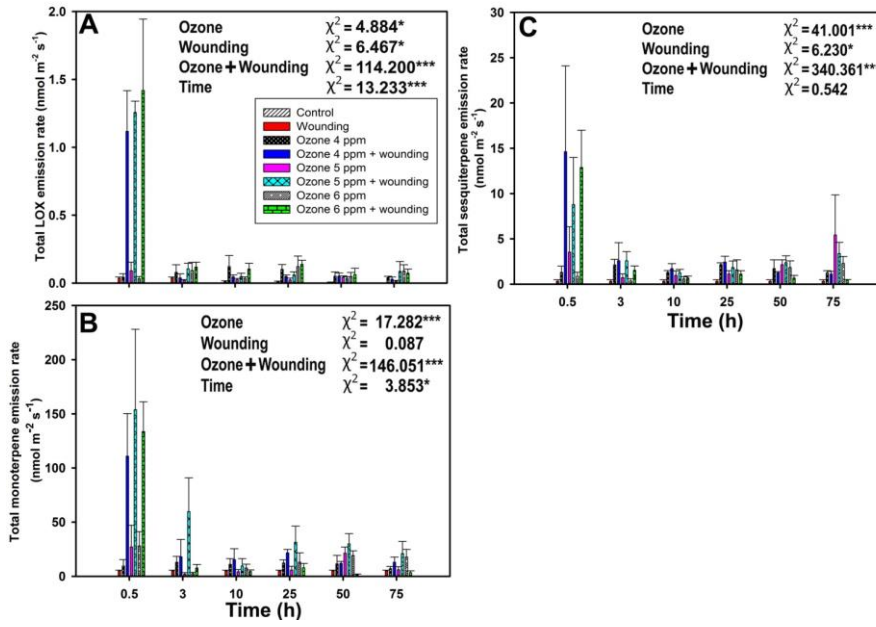

Figure 6. Emission rates (Mean + SE) of total LOX volatiles (A), total monoterpenes (B), and total sesquiterpenes (C) in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (4, 5, and 6 ppm ozone and wounding) leaves of *E. globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Shown are Wald chi-squared test statistics (χ^2) and its statistical significance as: * - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$(reproduced from **Paper II**).

Control leaves of *N. tabacum* (**Paper III**) emitted marginal levels of LOX volatiles, but ozone treatments significantly enhanced the emissions of LOX volatiles compared to control (Fig. 7A). The total emission of LOX was the highest immediately after ozone exposures and declined throughout the recovery phase, but the emissions achieved the pre-stress level at 48 h of recovery time ($P < 0.001$ for ozone treatments and $P < 0.01$ for the recovery time). In this study, ozone treatments elicited LOX volatile emissions in a dose-dependent manner. The ozone-induced LOX volatile blend consisted of hexanal and pentanal at all recovery times (Fig. 4A and B in **Paper III**). However, the emissions of the iconic LOX volatiles, (*Z*)-3-hexenol, and (*E*)-2-hexenal, were detected in trace levels only in response to 1000 ppb ozone exposure (data not shown).

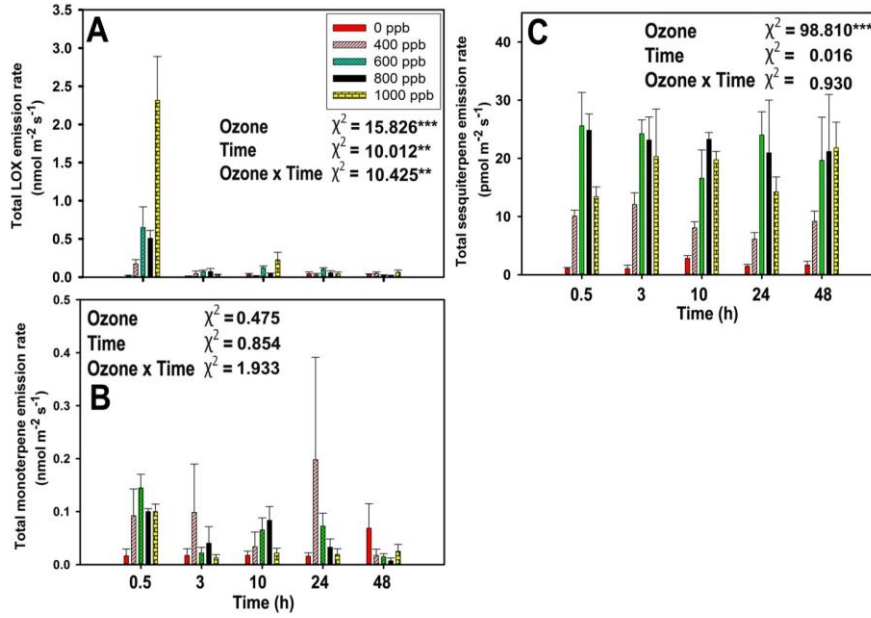

Figure 7. Emission rates (Mean + SE) of total LOX volatiles (A), total monoterpenes (B), and total sesquiterpenes (C) in mature leaves of 10-12 weeks old *N. tabacum* 'Wisconsin' at 0.5, 3, 10, 24, and 48 hours of recovery after ozone exposure at 0, 400, 600, 800, and 1000 ppb for 30 min. Individual effects of ozone, recovery time (Time), and their interactions on emission rates were tested by GLM with maximum likelihood model fitting. Wald chi-square (χ^2) test statistics is shown and statistical significance is indicated as: ** - $P < 0.01$, *** - $P < 0.001$ (reproduced from **Paper III**).

5.4. Temporal regulation of foliage monoterpene emissions upon heat, ozone and wounding treatments

The blend of monoterpenes emitted from *S. lycopersicum* leaves (**Paper I**) consisted of α-pinene, limonene, *p*-cymene, 2-carene, Δ^3-carene, β-phellandrene, (*E*)-β-ocimene and traces of α-terpinene and terpinolene, but β-phellandrene was the dominant monoterpene in all heat stress treatments (Fig. 8A). In this study, an iconic stress-induced monoterpene, (*E*)-β-ocimene, was observed and the emission rate (1-4 pmol $m^{-2} s^{-1}$) was increased with the severity of heat stress. Generally, all temperature treatments increased total monoterpene emission rate ($P < 0.001$ for temperature), but the effects were the highest immediately after stress applications and declined through the recovery phase (Fig. 4B in **Paper I**). The emission rate of β-phellandrene was increased immediately after all heat stress treatments and then it declined to a lower level at 6 and 10 h of recovery phase, until a slight enhancement of emission at 24 h after stress treatments (Fig. 8A).

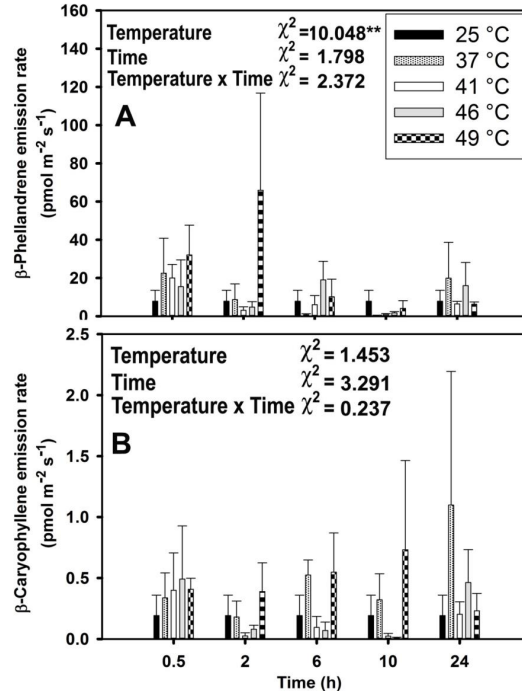

Figure 8. Emission rates (Mean + SE) of a monoterpene β-phellandrene (A), and a sesquiterpene (*E*)-β-caryophyllene (B) in heat-stressed (37-49 °C) and control (25 °C) leaves of *S. lycopersicum* at different recovery times (0.5-24 h) at 25 °C after heat stress applications. Individual and interactive effect of temperature and recovery times were tested by GLM. Wald chi-squared test statistics (χ^2) is shown and its statistical significance is indicated as: ** $P < 0.01$ (reproduced from **Paper I**).

Eucalyptus globulus is a constitutive monoterpene emitter (**Paper II**). The average total monoterpene emission by control leaves was ca. 0.20 ± 0.04 nmol m^{-2} s^{-1} (Fig. 6B). The blend of monoterpenes constituted of fifteen monoterpenes, dominated by 1,8-cineole (ca. 50% of total monoterpenes), α-pinene (ca. 20% of total monoterpenes) and limonene (ca. 10% of total monoterpenes) in controls and in all treatments (Fig. 5C, J, and F in **Paper II**). Total monoterpene emission rate was substantially enhanced upon single and combined ozone and wounding treatments, but the effect was the greatest for combined ozone and wounding treatments, and greater for single ozone and wounding treatments ($P < 0.001$ for ozone, and combined ozone and wounding treatments) (Fig. 6B). For example, total monoterpene emission was increased by ca. 750-fold for combined 5 ppm ozone and wounding treatment, and by 650-fold for combined 6 ppm ozone and wounding treatment at 0.5 h stress applications. Wounding treatment alone resulted in the lowest enhancement, ca. 25-times greater monoterpene emission rate (Fig. 6B). In addition, the total monoterpene emission peaked immediately after stress applications and decayed through the recovery phase for all stress treatments ($P < 0.05$ for recovery time). However, the total and individual monoterpene emission rates did not recover (Fig. 3B and 5 in **Paper II**). The emission rate of total monoterpenes increased non-linearly upon all stress applications. In this study, the emission of the iconic stress-induced monoterpene, (*Z*)-β-ocimene, was observed from all treated leaves, but the emission was the highest for combined ozone and wounding treatments and higher for single ozone, and wounding treatments (Fig. 5H in **Paper II**).

Nicotiana tabacum is a poor monoterpene emitter under non-stressed conditions. In this study (**Paper III**), the control leaves emitted total monoterpenes in the range of ca. 0.02 ± 0.01 nmol m^{-2} s^{-1}, but acute ozone treatments substantially increased total monoterpene emissions throughout the recovery phase (Fig. 7B). However, the total monoterpene emission by ozone-treated leaves was lower than that emitted by control leaves at 48 h of recovery phase. Ozone exposure did not have any statistically significant impact on total monoterpene emission rate. The blend of monoterpenes was dominated by limonene, followed by α-pinene, β-pinene, Δ^3-carene, and camphene both in control leaves and treated leaves (Fig. 5 in **Paper III**). In addition, the stress-induced monoterpene, (*E*)-β-ocimene, was emitted (1-10 pmol m^{-2} s^{-1}) upon higher ozone exposures (Fig. 5D in **Paper III**).

5.5. Time-dependent modifications in foliage sesquiterpene emissions in relation to heat, ozone and wounding treatments

Heat treatments of *S. lycopersicum* leaves (**Paper I**) had a minor impact on total sesquiterpene emission rates; sesquiterpene emissions were dominated by (*E*)-β-caryophyllene throughout the recovery phase (Fig. 4C in **Paper I** and Fig. 8B). The emission of (*E*)-β-caryophyllene upon heat stress was peaked immediately after stress applications and then it decreased at 2 h, followed by an increase for the leaves treated with 37 °C.

Total sesquiterpene emission in the control leaves of *E. globulus* (**Paper II**) was ca. 0.010 ± 0.002 nmol m^{-2} s^{-1} of (Fig. 6C). The total sesquiterpene emission was non-linearly enhanced in response to all stress applications with the highest emission responses observed for combined ozone and wounding treatments, followed by single ozone and wounding treatments at all recovery times ($P < 0.001$ for ozone, and combined ozone and wounding treatments) (Fig. 6C). There were eleven sesquiterpenes detected in this study and the emission blend was dominated by aromadendrene (ca. 40% of total sesquiterpenes), followed by α-gurjunene (ca. 20%) and alloaromadendrene (ca. 10 %) (Fig. 6A and B in **Paper III**). These three abundant sesquiterpenes made up ca. 70% of total sesquiterpene blend at 0.5 h of recovery.

Control leaves of *N. tabacum* (**Paper III**) were low-level emitters of total sesquiterpenes (1.66 ± 0.25 pmol m^{-2} s^{-1}). In fact, control leaves emitted α-caryophyllene as the only sesquiterpene, but ozone treatments elicited α-caryophyllene and γ-muurolene throughout the entire recovery phase (Fig. 6A and B **Paper III**). In addition, ozone treatments had a statistically significant impact on total sesquiterpene emission rates ($P < 0.001$ for ozone treatments) (Fig. 7C).

5.6. Expression analysis of mono- and sesquiterpene synthase genes in relation to heat stress in *S. lycopersicum*

Exposure of tomato leaves to high temperature treatments downregulated the expression of β-phellandrene and (*E*)-β-caryophyllene synthase genes (Fig. 9 and 10). In the case of β-phellandrene synthase gene, the expression level remained downregulated for 2–10 h after treatments with 37 °C and 41 °C, and then it reached the pre-stress level at 24 h of recovery (Fig. 9). In addition, the expression profile of (*E*)-β-

caryophyllene synthase gene was almost similar to the expression profile of β-phellandrene synthase gene at all recovery times (Fig. 10). Regression analyses indicated that the transcript abundance of β-phellandrene and (*E*)-β caryophyllene synthase genes was strongly correlated through the recovery phase except for 37 °C heat treatment (Fig. 8 in **Paper I**).

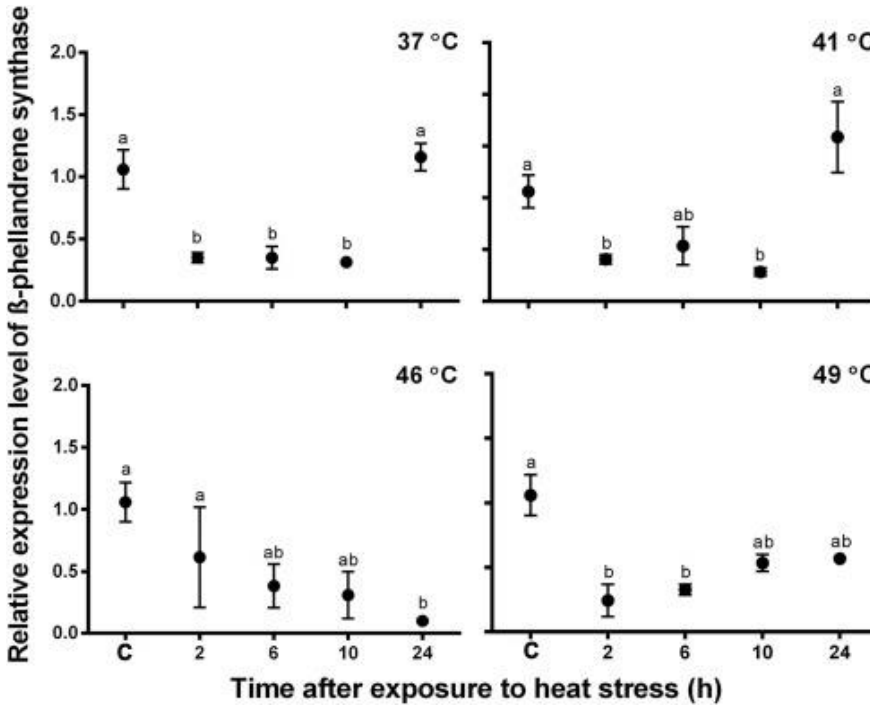

Figure 9. Relative expression levels of β-phellandrene synthase gene in *S. lycopersicum* leaves in response to heat treatments and at different times of recovery at 25 °C. Error bars indicate ±SE. Means with different letters are statistically different according to paired-sample t-tests (reproduced from **Paper I**).

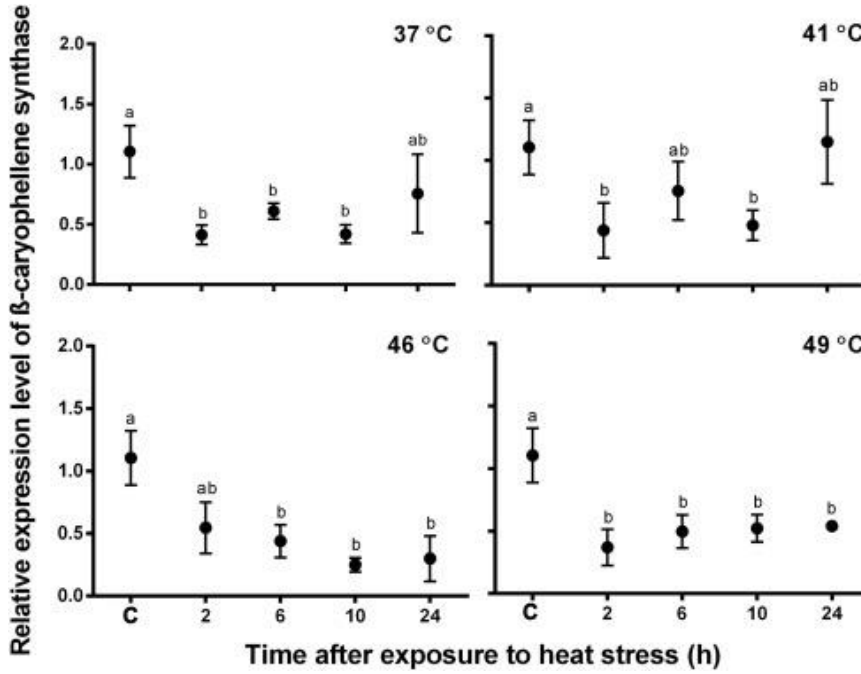

Figure 10. Relative expression levels of (*E*)-β-caryophyllene synthase gene in *S. lycopersicum* leaves in response to different treatment temperatures and at different times of recovery at 25 °C. Error bars indicate ±SE. Means with the different letters are statistically different according to paired-sample t-tests (reproduced from **Paper I**).

5.7. Emission of isoprene, total LOX, and total mono- and sesquiterpenes in response to stomatal ozone uptake

Overall, there were poor correlations between ozone uptake rate and emission rates of isoprene, total LOX, and total mono- and sesquiterpenes in *E. globulus* (Fig. 8 in **Paper II**). For total LOX emissions, the correlation with stomatal ozone uptake was negative at 0.5 h of recovery time, and statistically non-significant for other recovery times. In other cases, the correlations between total ozone uptake rates and isoprene emission rates were statistically non-significant, but there were marginally significant negative correlations observed between stomatal ozone uptake rates and total mono- and sesquiterpene emission rates at 0.5 h since ozone exposure. Similarly, the correlations between stomatal ozone uptake rates and emission rates of isoprene, total LOX, and mono- and sesquiterpenes were weak till 25 h since stress application for all combined ozone and wounding treatments (Fig. 9 in **Paper II**).

Furthermore, there were weak correlations between stomatal ozone uptake rates and the emission rates of total LOX, and mono- and sesquiterpenes in *N. tabacum*. However, the correlations were positive between non-stomatal ozone deposition rates and emission rates of total LOX, and mono- and sesquiterpenes (Fig. 8-10 in **Paper III**)

6. DISCUSSION

6.1. Impact of heat, ozone and wounding treatments on foliage photosynthetic characteristics

In the case of heat stress in *S. lycopersicum* leaves (**Paper I**), mild temperature treatments of 37 and 41 °C did not cause substantial changes in A and g_s, but severe heat stress at 46 and 49 °C significantly decreased both A and g_s (Fig. 1B and C). A sharp decline in net assimilation rate upon severe heat stress at 46 and 49 °C was likely associated with an irreversible damage of primary photosynthetic apparatus including PSII and oxygen-evolving complex (Camejo et al., 2005; Hüve et al., 2011). However, severe heat-stress often also increases membrane leakiness that could lead to reduced proton gradient and ATP synthesis, ultimately inhibiting overall photosynthetic rate (Zhang et al., 2009).

In the case of ozone and wounding stress in *E. globulus* leaves (**Paper II**), a sharp decline in F_v/F_m upon combined ozone and wounding treatments indicates that ozone induced damage in the reaction centers of PSII (Fig. 2A, and Fig. 1A in **Paper II**). This is in accordance with previous reports demonstrating that dissipation activity and efficiency of PSII are substantially reduced in ozone-exposed leaves (Guidi et al., 1999; Guidi and Degl'Innocenti, 2008) and the decline in PSII efficiency strongly scales with the stomatal ozone uptake rate (Gerosa et al., 2009). Furthermore, the reduction of PSII activity upon ozone treatments was also accompanied with sustained photoinhibition and in turn, decreased net assimilation rate (Guidi and Degl'Innocenti, 2008) consistent with our observations (Fig. 2B, and Fig. 1B in **Paper II**). In addition, elevated ozone reduces the activity of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) and thus, the overall activity of Calvin cycle was suppressed, leading to a decline in net assimilation rate (Guidi and Degl'Innocenti, 2008; Zhang et al., 2011). In this study, wounding treatment did not change F_v/F_m compared to control, suggesting that it did not led to the decline in the number of open PSII centers.

In the case of ozone treatments of *N. tabacum* leaves (**Paper III**), a sharp decline in A was due to the decline in g_s upon ozone exposure (Fig. 4A and B, and Fig. S1 in **Paper III**). This observation was further confirmed by corresponding changes in C_i and complete recovery of

A and g_s at later phases of recovery (Fig. 4C, and Fig. S1 in **Paper III**). In addition, the time-dependent reduction in stomatal conductance to water vapour in response to ozone and wounding (Fig. 2C) (**Paper II**) and ozone treatments (Fig. 4B) (**Paper III**) could be associated with an inhibitory effect on guard cell K^+ channels upon elevated ozone, leading to reduced stomatal conductance (Niu et al., 2014).

6.2. Effects ozone and wounding treatments on isoprene emission kinetics

Eucalyptus globulus is a strong isoprene emitter (**Paper II**). In our study, wounding reduced isoprene emission rate by ca. 85% at 0.5 h after stress application and by ca. 54% at 75 h of recovery and wounding reduced net assimilation rate by ca. 73% and by ca. 30% at 75 h of recovery (Fig. 2B and 5). This observation is consistent with the study of Loreto and Sharkey (1993) demonstrating that temporal emission of isoprene is more prone to wounding treatments and it responds faster than photosynthesis. However, *E. globulus* leaves treated with single ozone, and combined ozone and wounding stress showed increased isoprene emissions at all recovery times (Fig. 5). In particular, combined ozone and wounding treatments synergistically enhanced isoprene emissions at different times of recovery. As in our study, enhanced isoprene emissions upon ozone exposure have previously been reported by Fares et al. (2006) and Velikova et al. (2005). However, chronic ozone exposure reduced isoprene emission through downregulating isoprene synthase gene expression and isoprene synthase enzyme level in ozone-sensitive aspen (Calfapietra et al., 2008; Calfapietra et al., 2007), suggesting that different biochemical mechanisms are triggered upon acute and chronic ozone treatments.

6.3. Elicitation of foliage LOX pathway volatiles upon heat, ozone and wounding treatments

In general, lipoxygenase enzymes are constitutively active in plant cells and thus, LOX volatiles are rapidly biosynthesized and released from cell membranes when their substrates, polyunsaturated fatty acids become available (Feussner and Wasternack, 2002). Due to stress-induced damage in cell membranes, the release of classical LOX volatiles is considered as an initial stress response (Jiang et al., 2017; Jiang et al., 2016; Heiden et al., 2005; Copolovici et al., 2012; Toome et al., 2010).

Heat stress in *S. lycopersicum* leaves (**Paper I**) led to an emission burst of hexanal as the dominant LOX volatile, followed by pentanal. Similar to the responses to heat stress in *S. lycopersicum* leaves, hexanal and pentanal were the dominting LOX in *N. tabacum* leaves treated with acute ozone concentrations (**Paper III**). In fact, biosynthesis and emission of hexanal is partly due to the breakdown of membrane lipids by lipoxygenase and hydroperoxide lyase enzymes (Heiden et al., 2003) and therefore, enhanced emission of hexanal following heat and acute ozone stress could be caused by a direct effect of lipoxygenases in mesophyll cells that were activated as the result of the release of fatty acids from the leaf membranes upon stress. However, we cannot rule out the possibility of LOX volatile biosynthesis that involved the reaction of ozone with the secretory cells feeding leaf glandular trichomes of *N. tabacum* (Li et al., 2017).

Single and combined ozone and wounding treatments in *E. globulus* leaves (**Paper II**) led to an emission burst of classical LOX volatiles (*Z*)-3-hexenol, (*E*)-2-hexenal, along with hexanal and pentanal (Fig. 4 in **Paper II**). The emission of classical LOX volatiles is often associated with severe cellular damage, consistent with a partial recovery of photosynthesis (Fig. 2B, and Fig. 4 in **Paper II**). In addition, combined ozone and wounding treatments synergistically enhanced LOX emission responses. Similarly to the findings in our studies, there are several studies demonstrating emissions of classical LOX volatiles upon biotic (Arimura et al., 2005; Copolovici et al., 2014; Niinemets et al., 2013) and abiotic stresses (Copolovici and Niinemets, 2010; Kask et al., 2016; Wildt et al., 2003). Overall, this evidence collectively demonstrates that all the applied abiotic stresses did elicit LOX volatiles emissions, but also that the responses were complex and stress-specific.

6.4. Influence of heat, ozone and wounding treatments on mono- and sesquiterpene emissions

The blend of monoterpenes emitted by *S. lycopersicum* leaves upon heat stress (**Paper I**) were similar to the monoterpene blend emitted by tomato leaves infected with *Botrytis cinerea* (Jansen et al., 2009). Jansen et al. (2009) reported that there were 16 different monoterpenes emitted, but β-phellandrene, 2-carene, limonene, α-phellandrene and α-pinene comprised more than 95% of the emission blend. In our study, tomato leaves emitted (*E*)-β-caryophyllene, α-humulene and Δ-elemene at

a marginal level. Furthermore, these sesquiterpenes have also been observed in glandular trichomes of other tomato genotypes (Schilmiller et al., 2010). The emission kinetics of β-phellandrene resembled total monoterpene emission responses and (*E*)-β-caryophyllene resembled total sesquiterpene emission responses throughout the recovery phase (Fig. 4B in **Paper I**, and 8A).

Mono- and sesquiterpenes are generally stored in numerous sub-dermal secretory cavities and oil glands in *E. globulus* leaves (Goodger et al., 2010; Goodger et al., 2016) (**Paper II**). Therefore, there is a slow diffusion of these volatiles from the storage pools under non-stressed conditions, responsible for the constitutive emissions of mono- and sesquiterpenes in this species (Copolovici and Niinemets, 2016; Grote et al., 2013). In our study, application of single and combined ozone and wounding treatments activated MEP and MVA pathways for mono- and sesquiterpene biosynthesis, leading to enhanced *de novo* emission burst of mono- and sesquiterpenes. In parallel, all stress applications enhanced mono- and sesquiterpene emissions from terpene storage pools due to the damage of epidermal cells and epithelial cells of storage structures caused by ozone-induced hypersensitive reactions (Bussotti et al., 2007) and wounding treatments eventually leading to a greater terpene permeability of cell layers. Therefore, storage and *de novo* emissions contributed to the total emission of mono- and sesquiterpenes throughout the recovery phase in our study (Fig. 5 and 6 in **Paper II**, and Fig. 6B and C). However, our data suggest that at the later recovery phases, the emission responses of mono- and sesquiterpenes from storage pools are minor and thus, gene- and enzyme level controls primarily regulate enhanced *de novo* mono- and sesquiterpene emissions. In addition, combined ozone and wounding treatments synergistically upregulated mono- and sesquiterpene emission responses at all recovery times (Fig. 5 and 6 in **Paper II**, and Fig. 6B and C). In this study, single and combined ozone and wounding treatments did not elicit mono- and sesquiterpene emissions in a dose-dependent manner.

Slow diffusion of mono- and sesquterpenes from glandular trichomes and leaf lipid layers of tobacco leaves contributes to constitutive emission blend of mono- and sesquiterpenes under non-stressed conditions (**Paper III**) (Fig. 5, 7B and 7C, and 6A, B in **Paper III**). However, the terpenoid storage pool is considerably smaller in tobacco leaves, leading to a marginal-level constitutive emission of mono- and sesquiterpenes

(Cui et al., 2011; Ohara et al., 2003). However, exposure of acute ozone elicited constitutive and *de novo* emissions of mono- and sesquiterpenes, but their emissions were only partly exposure dose-dependent.

In all stress treatments (**Papers I**-**III**), the emission of a characteristic monoterpene, β-ocimene was observed, suggesting that defence pathways were activated upon oxidative stress. This finding is consistent with the previous studies, e.g., insect herbivore feeding of *Medicago truncatula* leaves (Navia-Giné et al., 2009), *Cabera pusaria* feeding of *Alnus glutinosa* leaves (Copolovici et al., 2014), and biotrophic fungus *Melampsora* spp. infection of *Salix* spp. leaves (Toome et al., 2010) induced β-ocimene emissions.

6.5. Analysis of expression profiles of two terpene synthase genes in response to heat stress

The expression profile of β-phellandrene synthase (TPS20) and (*E*)-β-caryophyllene/α-humulene synthase gene (TPS12) is representative to total mono- and sesquiterpene emissions through the recovery period due to the following reasons (**Paper I**); (1) temporal modifications in β-phellandrene emissions and (*E*)-β-caryophyllene emissions (Fig. 8A and B) were similar to the modifications in total mono- and sesquiterpene emissions upon all stress applications (Fig. 4B and Fig. 4C in **Paper I**); and (2) β-phellandrene emission contributes to the greatest share of total monoterpene emission and (*E*)-β-caryophyllene emission contributes to the greatest share of total sesquiterpene emission (Fig 4B and Fig 5A, and Fig 4C and Fig 5B in **Paper I)**. Expression of both genes recovered to the pre-stress level at 48 h of recovery phase for mild heat stress (37 and 41 °C), but there was no recovery observed in response to severe heat stress of 46 and 49 °C. Although β-phellandrene synthase is a plastidic enzyme and (*E*)-β-caryophyllene/α-humulene synthase is a cytosolic enzyme (Pazouki and Niinemets, 2016), the modifications in the transcript abundance of both genes through the recovery period were strongly coordinated (Fig. 8 in **Paper I**). This suggests that the same transcription factor might regulate their expression profile upon acute heat stress (Falara et al., 2011). Therefore, characterization of pertinent transcription factors and their regulation at different severities of temperature stress deserve further investigation to gain insight into the temporal kinetics of terpenoid gene expression as altered by heat stress.

6.6. A lag-time between TPS gene expression and emission: important factors to be considered

Mild temperature treatments of 37 and 41 °C of tomato leaves (**Paper I**) led to positive correlations between β-phellandrene and (*E*)-β-caryophyllene synthase gene expression and product emissions, whereas severe temperature treatments of 46 and 49 °C led to negative correlations. Under non-stressed conditions, there is a strong association between the expression of terpene synthase genes and terpenoid emissions in *S. lycopersicum* (Falara et al., 2011). However, a certain delay between gene expression and product emission occurred upon stressed conditions. Since the correlations between gene expression and product emission for the leaves treated with 37 and 41 °C were significant (Fig. 9 and Table 3 in **Paper I**), the corresponding time-delay could have been shorter than 2 h at mild heat stress.

There are several factors influencing the time-delay between the gene expression and emission responses. For example, all steps from the onset of stress application to terpene biosynthesis, including signal perception, signal transduction, activation of transcription factors, and gene expression are time consuming. In addition, there should be a continuous synthesis of corresponding proteins, such as terminal enzymes in MEP and MVA pathways to get a strong correlation between terpene synthase gene expression and terpene emission. In our study, the weak correlation between terpene synthase gene expression and phenotypic-level responses, particularly at severe heat stress (46 and 49 °C) could be due to the inactivation of certain enzymes at high temperatures; this finding is further confirmed by the observation that there was a complete cessation of photosynthetic characteristics at severe heat stress (Fig. 2A and 9 in **Paper I**). However, we cannot rule out the possibility of substrate-level controls acting simultaneously with gene expression-level controls. However, in-depth molecular studies are required to test possible changes in the lag-times of TPS gene expression and emission with the severity of heat shock treatments.

6.7. Emission of isoprene, and total mono- and sesquiterpenes in relation to stomatal ozone uptake

Stomata are the main channel through which ozone enters the leaf intercellular air spaces and generates oxidative stress (Fares et al., 2010).

Thus, stomatal ozone uptake plays a significant role in elicitation of emissions of stress volatiles. However, the ultimate physiological effect caused by ozone depends on the equilibrium between the quantity of stomatal ozone uptake and the rate with which ozone molecules are scavenged by the water-soluble antioxidants within the aqueous phase in cell membrane, and by volatile isoprenoids present in leaf gas and lipid phases (Fares et al., 2010).

In the case of ozone treatments of *E. globulus* and *N. tabacum* leaves (**Papers II and III**), generally, there were weak correlations between stomatal ozone uptake rates and emission rates of isoprene, total LOX, and total mono- and sesquiterpenes (Fig. 8 and 9 in Paper II and Fig. 8-10 in **Paper III**). This can be explained by three key factors: first, as *E. globulus* is a strong isoprenoid emitter (**Paper II**), the concentrations of volatile isoprenoids in intercellular spaces are usually much higher than those in and above the leaf boundary layer (Loreto and Fares, 2007). Therefore, as soon as ozone enters the leaf intercellular air spaces, it instantly reacts with volatile isoprenoids and thus, the quantity of ozone molecules entering the mesophyll cells is much lower than that taken up through stomata. However, *N. tabacum* is a poor isoprenoid emitter and therefore, scavenging of ozone by volatile isoprenoids was predicted to be small (**Paper III**); second, once ozone enters leaf mesophyll cells, it is also removed by antioxidants such as putrescine and apoplastic ascorbate in mesophyll cells (**Papers II and III**); and third, reactive oxygen species (ROS) generated by ozone are removed by the reactions involving ROS and volatile isoprenoids (Loreto and Fares, 2007) (**Papers II and III**). Therefore, the quantity of ozone molecules ultimately causing a physiological stress is much lower than that entering the leaf intercellular air space; in addition, due to reactions with ozone and ROS, the quantity of volatile isoprenoids emitted is also lower than those actually biosynthesized in *N. tabacum* and *E. globulus* leaves.

However, in the case of acute ozone treatments in *N. tabacum*, the positive correlations among non-stomatal ozone deposition rates and total LOX volatile, and mono- and sesquiterpene emission rates (Fig. 8E-H, Fig. 9E-H, and Fig. 10E-H in **Paper III**) are consistent with the hypothesis that the volatiles are released from glandular trichomes or from impacted secretory cells on leaf surface upon reacting with ozone. These results collectively imply that a significant share of stress volatile emission in response to ozone exposure in *N. tabacum* was released from leaf surface structures (**Paper III**).

7. CONCLUSION

There were three model systems developed in this thesis, tomato for heat stress, eucalypt for acute ozone and wounding stress, and tobacco for acute ozone stress, to understand the impact of abiotic stresses on primary and secondary metabolic processes of crop and economically important woody plants.

1. Impact of acute heat stress on temporal regulation of photosynthetic traits, stress volatile emissions, and mono- and sesquiterpene synthase gene expression profiles in *S. lycopersicum* leaves (**Paper I**). This line of work resulted in the following key outcomes:
   Mild heat stress did not have any significant impact on the changes in photosynthetic traits, but severe heat stress irreversibly reduced photosynthetic characteristics. Foliage mono- and sesquiterpene emissions were primarily regulated by β-phellandrene and (*E*)-β-caryophyllene synthase gene expression at mild heat stress, but the gene expression regulation for emissions was minor at severe heat stress. Based on time-delay analysis of mono- and sesquiterpene synthase gene expression and emission of corresponding compounds, the best correspondence between terpene synthase gene expression and emission was shifted by ca. 2 h for mild heat stress, but the time-shift relationship between gene expression and emission was not clear-cut for severe heat stress, implying that severe heat stress significantly impaired terpene metabolism.
2. Impact of combined ozone and wounding treatments on photosynthetic characteristics and stress volatile emission rates in *E. globulus* through recovery and the relationship between emission responses and stress severity, including ozone dose and stomatal ozone uptake rate (**Paper II**). This line of study resulted in the following key conclusions:
   The physiological and biochemical responses for combined ozone and wounding stress were different from the responses in plants treated with single ozone and wounding stresses. Particularly, the reduction of photosynthetic characteristics such as A, g_s and F_v/F_m, and enhancement of stress volatile emissions were greater for combined ozone and wounding treatments followed by single ozone and wounding treatments. Taken together, these key outcomes demonstrated that combined acute ozone and wounding treatments

led to synergistic effects on leaf photosynthetic traits and stress volatile emissions. LOX emissions scaled with the severity of stress, but there were no quantitative relationships observed between the severity of stress and emissions of other volatiles. The absence of stress dose vs. emission relationship was possibly due to scavenging of ozone by volatile and non-volatile antioxidants, and removal of ROS in reactions involving volatile isoprenoids in mesophyll cells through recovery.

3. Temporal modifications in foliage photosynthetic characteristics and emissions of LOX volatiles and mono- and sesquiterpenes upon acute ozone exposure in *N. tabacum*, and the share of stomatal ozone flux and non-stomatal ozone deposition to the degree of elicitation of different volatiles (**Paper III**). This study resulted in the following key outcomes:
   Acute ozone exposure resulted in a sharp decline in foliage photosynthetic characteristics, but the reductions in photosynthetic characteristics were completely reversible even at the highest ozone concentrations, underscoring high ozone resistance of the used variety, Wisconsin. The emission rate of LOX volatiles was dose-dependent, but the dose-dependency was weaker for mono- and sesquiterpene emissions. In addition, non-stomatal ozone deposition contributed to the greater share of ozone flux into leaf, associated with the greater share of terpene emission. This outcome collectively implies that a greater share of terpenoid emission upon ozone exposures mostly reflected the modification in terpene release from glandular trichomes.

Collectively these data (**Papers I-III**) indicated that multiple physiological and biochemical factors regulate the emission of volatile compounds upon abiotic stresses in a complex manner. In addition, the physiological and biochemical responses of plants to combined stresses were different from the responses in plants treated with the single stress factors separately. Therefore, future studies are required to understand how genetic, physiological, and physiochemical factors influence plant phenotypic responses upon different abiotic stresses and factors affecting the regulation of transcription activity of terpenoid synthases, and the complex linkages between gene expression and phenotypic responses scaling with the severity of abiotic stresses.

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SUMMARY IN ESTONIAN

BIOGEENSETE LENDUVÜHENDITE EMISSIOONI REGULATSIOON STRESSITINGIMUSTES: GEENIEKSPRESSIOONIST LENDUVÜHENDITE EMISSIOONIVASTUSTENI ERINEVATE ABIOOTILISTE STRESSIDE KORRAL

Taimed eritavad mitmeid erinevaid biogeenseid orgaanilisi lenduvühendeid nii konstitutiivselt kui vastusena stressitingimustele, mistõttu on lenduvühenditel oluline bioloogiline ja ökoloogiline tähtsus. Lisaks sellele võimaldab stressivastusena taimes sünteesitavate biogeensete orgaaniliste lenduvühendite kogus määrata taime mõjutava stressi ulatust. Lenduvühendite emissiooni kõrval mõjutab stress ka teisi olulisi parameetreid: fotosünteesi, õhulõhede juhtivust ja fotosüsteem II maksimaalset kvantsaagist.

Üks peamistest taime kasvu ja produktiivsust vähendavatest abiootilistest stressifaktoritest on kõrge temperatuur, mis mõjutab tugevalt taimede füsioloogilisi ja biokeemilisi protsesse, eriti fotosünteesi ja biogeensete orgaaniliste lenduvühendite sünteesi ning emissiooni. Kuumalained on tõsiseks probleemiks troopilistes ja vahemerelistes põllumajanduspiirkondades tuues kaasa suuri saagikadusid. Samuti on väga oluline abiootiline stressifaktor fütotoksiline gaas – osoon. Troposfääri osoon moodustub peamiselt lämmastiku oksiidide (NO ja NO_2) ja biogeensete orgaaniliste lenduvühendite omavahelise reaktsiooni käigus päikesevalguse käes. Kõrge troposfääri osooni tase tekitab taimedes oksüdatiivset stressi, mistõttu toimuvad muutused biokeemilistes reaktsioonides ning metaboolsetes radades. Praegune troposfääri osooni tase maailmas on umbes 40 osakest miljardist, juba selline kontsentratsioon võib põhjustada oksüdatiivset stressi paljudel taimedel, eelkõige põllukultuuridel. Lisaks eelnevatele on tavaliseks stressifaktoriks ka taimeosade mehhaaniline vigastus, mille peamisteks looduslikeks põhjustajateks on tuul, langev varis ja tugevad sademed. Arvatakse, et kombineerituna on osoonil ja mehhaanilistel vigastustel vastastikune koosmõju taime füsioloogiale, fotosünteesile ja lenduvühendite emissioonile.

Globaalsel skaalal on vähe informatsiooni kõrge temperatuuri, osooni ja mehhaaniliste vigastuste tagajärjel tekkinud oksüdatiivse stressi mõjudest fotosünteesile ning lenduvühendite emissioonile. Lisaks on vähe teada mil määral mõjutavad erinevad abiootilised stressifaktroid terpenoidide biosünteesi molekulaarsel tasandil. Seetõttu on äärmiselt oluline uurida täpsemalt stressi mõju taimede ökofüsioloogiale.

Töö eesmärgid

Antud töö täpsemad eesmärgid olid:

1. uurida ajast sõltuvat kuumastressi mõju nii fotosünteesile kui mono - ja seskviterpeenide emissioonile; analüüsida kuumastressi mõju β-fellandreeni ja (*E*)-β-karofülleni süntetaasi geenide regulatsioonile tomatil *(Solanum lycopersicum* 'Pontica'); (**I artikkel**).
2. uurida osooni ja mehhaaniliste vigastuste koosmõju fotosünteesile ja lenduvühendite emissioonile stressist taastumise perioodi vältel ning hinnata stressi tugevuse ja emissiooni vastuse kvantitatiivset suhet, võttes arvesse osooni doosi ja õhulõhede kaudu lehte sisenemise kiirust, liigil *Eucalyptus globulus* Labil (**II artikkel**)
3. hinnata seoseid osooni kontsentratsiooni ja lehestiku fotosünteesi, LOX-ühendite ning mono- ja seskivterpeenide emissiooni vahel; hinnata osooni jõudmist lehtedesse õhulõhede kaudu ja õhulõhedest sõltumatult; ning siduda seda erinevate volatiilsete ühendite sünteesi indutseerimisega tubakal (*Nicotiana tabacum* 'Wisconsin') (**III artikkel**).

Peamised tulemused

Antud töö käigus oleme välja töötanud kolm mudelsüsteemi mõistmaks abiootilise stressi mõju nii primaarse kui sekundaarse ainevahetuse protsessidele põllukultuuridel ja majanduslikult olulistel puittaimedel.

- Kerge kuumastress tomati (*S. lycopersicum*) fotosünteesile olulist mõju ei avaldanud, küll aga kahjustas tugev kuumastress pöördumatult fotosünteesiaparaati. Kerge kuumastressi puhul aset leidnud

muutused lehestiku mono - ja seskviterpeenide emissioonis olid seotud β-fellandreeni ja (*E*)-β-karofülleeni süntaasi geeni avaldumisega. Mono - ja seskviterpeenide süntetaasi geenide analüüsil eri ajahetkedel stressitöötluse järgselt ilmnes, et terpeeni süntaasi geenieksрessiooni ja emisiooni nihe kerge kuumastressi järgselt oli vaid 2 tundi, kuid tugeva kuumastressi puhul selget nihet geeni ekspressiooni ja lenduvühendi emissiooni vahel ei olnud, millest saab järeldada, et tugev kuumastress kahjustab terpeeni ainevahetust (**I artikkel**).

- Füsioloogilised ja biokeemilised muutused ning taastumisprotsess eukalüptil (*E. globulus*) erinesid olenevalt sellest, kas lehti vigastati mehhaaniliselt ning töödeldi osooniga samaaegselt või rakendati stressitöötusi eraldi. Kombineeritud stressitingimuste korral vähenesid fotosünteesi karakteristikud näiteks A, g_s ja F_v/F_m ning suurenes stressist tingitud lenduvühendite emissioon suuremal määral kui osooni ja mehhaanilise stressi puhul eraldi. Kokkuvõttes näitasid need tulemused, et kombineerituna on kõrgel osooni tasemel ja mehhaanilisel vigastusel sünergiline efekt fotosünteesile ning stressi poolt indutseeritud lenduvühendite emissioonile. LOX-ühendite emissioon oli vastavuses stressi tugevusega, kuid teiste stressitingimustes tekkivate lenduvühendite emissiooni ja stressi tugevuse vahel kvantitatiivset seost ei leitud. Stressi ulatuse ja lenduvühendite emissiooni vahelise kvantitatiivse seose puudumist saab seletada volatiilsete isopenoidide toimega, mis elimineerivad osooni rakuvaheruumidest ning reaktiivsed hapnikuühendeid (ROS) mesofülli rakkudest (**II artikkel**).

Osoon vähendas oluliselt tubaka (*N. tabacum* 'Wisconsin') lehtede gaasivahetust, kuid isegi kõige kõrgemate kontsentratsioonide korral oli see efekt pöörduv, mis viitab selle liigi tugevale osoonitaluvusele. Tubakal sõltus LOX-ühendite emissioon tugevalt osooni annusest, kuid mono - ja seskviterpeenide puhul oli kontsentratsiooni ja emissiooni vaheline kvantitatiivne suhe nõrgem. Lisaks sellele oli olulisem roll õhulõhedest sõltumatul osooni sisenemisel lehtedesse ning see kutsus esile terpeenide emissiooni, mis kokkuvõttes viitab sellele, et suurem osa osooni vastusena eritatavatest terpeenidest pärineb lehe pinnal asuvatest trihhoomidest (**III artikkel**).

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I

ORIGINAL PUBLICATIONS

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Mono- and sesquiterpene release from tomato (*Solanum lycopersicum*) leaves upon mild and severe heat stress and through recovery: From gene expression to emission responses

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ABSTRACT

Plants frequently experience heat ramps of various severities, but how and to what degree plant metabolic activity recovers from mild and severe heat stress is poorly understood. In this study, we exposed the constitutive terpene emitter, *Solanum. lycopersicum* leaves to mild (37 and 41 °C), moderate (46 °C) and severe (49 °C) heat ramps of 5 min and monitored foliage photosynthetic activity, lipoxygenase pathway volatile (LOX), and mono- and sesquiterpene emissions and expression of two terpene synthase genes, β-phellandrene synthase and (*E*)-β-caryophyllene/α-humulene synthase, through a 24 h recovery period upon return to pre-stress conditions. Leaf monoterpene emissions were dominated by β-phellandrene and sesquiterpene emissions by (*E*)-β-caryophyllene, and thus, these two terpene synthase genes were representative for the two volatile terpene classes. Photosynthetic characteristics partly recovered under moderate heat stress, and very limited recovery was observed under severe stress. All stress treatments resulted in elicitation of LOX emissions that declined during recovery. Enhanced mono- and sesquiterpene emissions were observed immediately after the heat treatment, but the emissions decreased even to below the control treatment during recovery between 2 and 10 h, and raised again by 24 h. The expression of β-phellandrene and (*E*)-β-caryophyllene synthase genes decreased between 2 and 10 h after heat stress, and recovered to pre-stress level in mild heat stress treatment by 24 h. Overall, this study demonstrates a highly sensitive heat response of terpenoid synthesis that is mainly controlled by gene level responses under mild stress, while severe stress leads to non-recoverable declines in foliage physiological and gene expression activities.

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1. Introduction

Average global temperature has been estimated to have increased by 0.76 °C during the 20th century and it is further expected to increase by 1.8 – 4.0 °C by the end of this century (Hansen et al., 2006; IPCC, 2007). Elevated temperatures constitute a major stress for plants restricting their growth and survival, altering the distribution of species and affecting the productivity of natural ecosystems and crops worldwide (Allen et al., 2010; Ainsworth and Ort, 2010). The effects of heat stress depend on the severity of heat stress with mild stress resulting in reversible inhibition of foliage photosynthetic characteristics, while severe stress leading to sustained reduction in photosynthesis or even to time-dependent decline after return to lower temperatures (Sharkey, 2005; Schrader et al., 2007; Hüve et al., 2011; Copolovici et al., 2012; Niinemets, 2010a). Irreversible and progressive reduction in photosynthetic activity is also associated with the release of volatile ubiquitous stress-induced volatiles, the products of lipoxygenase pathway (LOX products, also called green leaf

Abbreviations: CAC, clathrin adaptor complex; DNAJ, DnaJ-like protein; GLM, generalized linear model; HSP, heat shock protein; LOX, lipoxygenase pathway; M_A, leaf dry mass per area; MEP/DOXP pathway, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway; MVA pathway, mevalonate pathway; qPCR, quantitative PCR; RPL8, ribosomal protein L8; SAND, SAND family protein; TBP, TATA-box binding protein; TPSs, terpene synthase; VOCs, volatile organic compounds.

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volatiles), reflecting damage at membrane level (Copolovici et al., 2012; Loreto et al., 2006).

Apart from stress-induced LOX-emissions, several plant species are characterized by constitutive emissions of volatile organic compounds including isoprene, mono- and sesquiterpenes (Sharkey et al., 2013; Fuentes and Wang, 1999; Harrison et al., 2013; Fineschi et al., 2013). Under non-stressed conditions, these emissions typically consume 1–2% of photosynthetic carbon, but these emissions can consume even more than 10% of photosynthetic under stress conditions, in particular under high temperature stress (Fuentes and Wang, 1999; Sharkey et al., 2008; Guenther et al., 1993; Niinemets, 2010b; Fatouros et al., 2012; Copolovici et al., 2014). In fact, these constitutive emissions have been associated with enhanced tolerance of heat stress, either operating directly by improving membrane heat resistance (Sharkey et al., 2008; Singsaas et al., 1997; Loreto et al., 1998; Copolovici et al., 2005; Sharkey et al., 2001; Llusia et al., 2005) or indirectly by serving as membrane-dissolved antioxidants that can quench free-radicals formed upon heat stress (Possell and Loreto, 2013; Vickers et al., 2009). In addition, heat stress can not only enhance constitutive emissions, but can itself result in induction of volatile isoprenoid release (Staudt and Bertin, 1998; Kleist et al., 2012; Karl et al., 2008). The blend of these induced emissions can significantly differ from constitutive emissions, although not always (Staudt and Bertin, 1998; Kleist et al., 2012; Niinemets, 2010a). Furthermore, short- and long-term heat effects are often different with the emissions enhanced over the short term and reduced over the long term (Kleist et al., 2012; Loreto et al., 1998) due to reasons not fully understood. In fact, to our knowledge, heat-dependent regulation of expression of terpenoid synthases, the enzymes responsible for formation of terpenoids, has not been studied.

Even though temperature is a primary environmental factor for tree growth and development (Way and Oren, 2010) and for CO_2 assimilation in woody plants (Jia et al., 2016), similar to herbaceous plants, high temperature stress also has a considerable impact on gas exchange, and emission of volatile isoprenoid, and GLVs. Usually, in natural habitats, high temperature stress is accompanied by draught stress causing detrimental physiological effects on woody plants where different degrees of acclamatory responses of isoprene emission and photosynthesis, and light and dark respiration were especially observed due to high ratio of respiration to photosynthesis and also to higher quantity of carbon loss upon isoprene emission (Centritto et al., 2011).

Previous studies have revealed the distribution of terpene synthase (TPS) genes and their evolutionary relationships (Sharkey et al., 2013) in several plant species including *Arabidopsis* (Aubourg et al., 2002), poplar (*Populus trichocarpa*) (Tuskan et al., 2006), apple (*Malus domestica*) (Velasco et al., 2010), papaya (*Carica papaya*) (Ming et al., 2012), date palm (*Phoenix dactylifera*) (Al-Dous et al., 2011), pear (*Pyrus bretschneideri*) (Wu et al., 2013), maize (*Zea mays*) (Schnee et al., 2002), rice (*Oryza sativa*) (Wilderman et al., 2004), and tomato (*Solanum lycopersicum*) (Falara et al., 2011). The TPS gene family in *S. lycopersicum* consists of at least 44 TPS genes, of which 29 have been shown to be functional genes of corresponding TPSs (Falara et al., 2011) involved in the synthesis of mono-, sesqui- and diterpenes (Colby et al., 1998; Schilmiller et al., 2009; Schilmiller et al., 2010a; Falara et al., 2011). *Solanum lycopersicum* TPSs have a large sequence diversity and are distributed among all known angiosperm TPS clades, from TPS-a to TPS-g (Falara et al., 2011). These genes are expressed in different plant tissues (Falara et al., 2011), but not all of the synthases have been functionally characterized and the expression response of most genes to environmental stimuli is unknown.

Solanum lycopersicum is a constitutive mono- and sesquiterpene emitter under natural conditions (Jansen et al., 2009; Maes and Debergh, 2003), and these emissions are highly enhanced by both biotic and abiotic stresses (Copolovici et al., 2012; Maes and Debergh, 2003; Jansen et al., 2009). In the case of leaf terpenoid emissions, two genes are likely of special significance, β-phellandrene synthase gene (TPS20, located in chromosome 8 and belonging to the TPS-e/f clade) and (*E*)-β-caryophyllene/α-humulene synthase gene (TPS12, located in chromosome 6, and belonging to the TPS-a clade (Falara et al., 2011)). Both of these genes are expressed in leaves with the activity concentrated in glandular trichomes, especially for TPS20 (Falara et al., 2011; Schilmiller et al., 2009). In a previous study, both constitutive and heat-enhanced monoterpene emissions in *S. lycopersicum* were dominated by β-phellandrene, while sesquiterpene emissions were dominated by α-humulene (Copolovici et al., 2012). However, it remained unclear how the heat-dependent changes in emissions are associated with gene expression profiles. Furthermore, time-dependent modifications in terpene emissions after heat stress were not monitored in this study. In fact, there is overall limited information of the timing of gene expression response and resulting changes in trait expression. Cleary, there is a certain delay between elicitation of gene-level response and modifications in phenotype (Li et al., 2006; Mohyedinbonab et al., 2013; Straube et al., 2015), e.g. time-delays between biochemical elicitor application and onset of terpenoid synthesis have been demonstrated (Martin et al., 2003; Miller et al., 2005), but how rapidly the heat-elicited expression response is converted to modified terpenoid emission response has not been studied.

The aim of the present study was to investigate the effects of heat stress on leaf photosynthesis, LOX volatile and terpenoid emissions, and expression of β-phellandrene and (*E*)-β-caryophyllene synthase genes in *S. lycopersicum*. Both the responses to the immediate heat stress and recovery in these traits upon return to lower temperature were monitored to gain insight into the recovering capacity of leaves in dependence on the severity of heat stress and time of recovery, and estimate the correspondence between changes in gene expression and terpenoid emission through the recovery period. The results of the study demonstrate that short-term heat stress enhanced terpenoid emissions, but the expression of terpenoid synthases was reduced, leading to reduction in emissions in long-term with a certain recovery depending on the severity of heat stress.

2. Material and methods

2.1. Plant material and growth system

Tomato (*S. lycopersicum* L. cv. Pontica, seed source Starsem, Romania) seeds were sown in a commercial potting soil (Biolan Oy, Kauttua, Finland). After germination, the seedlings were transplanted and grown in 1 L clay pots in a plant growth room under controlled conditions of light intensity of 400 $\mu mol\ m^{-2}\ s^{-1}$ (HPI-T Plus 400 W metal halide lamps, Philips) for 12 h light period, day/night temperatures of 24/18 °C, and relative air humidity of 60%. The plants were watered daily to soil field capacity and fertilized once a week till completion of the experiment with a combined NPK (5:5:6) fertilizer with micronutrients (B (0.01%), Cu (0.03%), Fe (0.06%), Mn (0.028%), Zn (0.007%)). Each time, 70 mL of fertilizer solution (0.5% solution of the concentrated liquid fertilizer) was applied to every plant. The experimental treatments started when the plants were 25 days old. In all experiments, we used 25–30 cm tall plants, with similar biomass, stem thickness and number of fully mature leaves.

2.2. Experimental protocol

Two identical experiments were run in parallel, one to monitor time-dependent changes in foliage gas-exchange rates, chlorophyll fluorescence and emission rates of volatile organic compounds (VOCs), and the other to estimate time-dependent changes in gene expression. Because for gene expression, leaves had to be destructively harvested, separate plants had to be used for the two types of measurements.

For the experimental treatments, 3–4 fully mature leaves were selected on each individual plant. Before the treatment, emission rates of volatiles, dark-adapted chlorophyll fluorescence yield (F_v/F_m), net assimilation rate (A) and stomatal conductance to water vapor (g_s) were measured (section *Chlorophyll fluorescence and gas exchange measurements*). First, the measurement for volatile emission were conducted by a gas-exchange system using all the leaves selected, and thereafter measurement for F_v/F_m, A, and g_s were carried out on a single leaf selected from all the experimental leaves.

After these measurements in non-treated plants, the leaves were subject to the heat shock treatments of 25 (control treatment), 37, 41, 46 and 49 °C for 5 min by immersing them in distilled water at the target temperature according to the procedure of Frolec et al. (2008) and Copolovici et al. (2012). Highly stable temperature of the immersion medium was achieved with a thermostat (VWR International, West Chester, Pennsylvania, USA).

After the heat treatment, leaves were left to air-dry for ca. 5 min, and all physiological measurements were repeated. The sampling of volatiles together with stabilization of gas flows took ca. 25–30 min (see the next section), F_v/F_m measurements ca. 30 min. (first measurements taken in ca. 1 h since heat treatment), and A and g_s measurements 20–30 min. (first measurement taken in ca. 1.5 after the heat treatment). The physiological measurements were repeated in 2 h, 6 h, 10 h and 24 h after the completion of the first set of measurements. All the treated leaves were used for volatile measurements, while the same leaf initially selected for measurement of photosynthetic characteristics was used for F_v/F_m, A and g_s measurements.

2.3. Volatile sampling and GC–MS analyses

For volatile sampling, we used a custom-made temperature-controlled gas-exchange system with a temperature-controlled glass chamber described in detail by Copolovici and Niinemets (2010). Before enclosure of leaves in the chamber, the background volatile samples were collected from the empty chamber. After the leaves were enclosed in the chamber, the following conditions were established: light intensity at leaf surface of 700 μmol m^{-2} s^{-1}, temperature of 25 °C, and ambient CO_2 concentration of 380–400 μmol mol^{-1}. We used ambient air purified by passage through an activated carbon filter and custom-made ozone trap as in Copolovici and Niinemets (2010). Volatile sampling was started after stabilization of gas flows and leaf gas-exchange rates, typically in 5–10 min after leaf enclosure. Volatiles were collected onto a stainless steel cartridge using a constant flow air sample pump (210-1003MTX, SKC Inc., Houston, TX, USA) with a flow rate of 200 mL min^{-1} for 20 min. The cartridges were filled with carbon-based adsorbents Carbotrap C 20/40 mesh, Carbopack B 40/60 mesh and Carbotrap X 20/40 adsorbents (Supelco, Bellefonte, USA) for quantitative adsorption of volatiles in C_5–C_{15} range (for detail see Kännaste et al., 2014).

A Shimadzu 2010 Plus GC–MS system with Shimadzu TD20 automated cartridge desorber (Shimadzu Corporation, Kyoto, Japan) was used for volatile analysis as described in detail in previous papers of the team (Copolovici et al., 2009; Toome et al., 2010; Kännaste et al., 2014). Lipoxygenase pathway volatiles (LOX), also called green leaf volatiles, including *n*-pentanal, *n*-hexanal, (*E*)-2-hexenal, and 2,4-hexadienal, *n*-hexanol, (*Z*)-3-hexenol, 5-hexen-1-ol and mono- and sesquiterpenes were quantified. Pure standards (Sigma-Aldrich, St. Louis, MO, USA) were used for compound identification and GC–MS calibration.

2.4. Chlorophyll fluorescence and gas exchange measurements

A portable gas-exchange chlorophyll fluorescence system (Walz GSF-3000, Walz GmbH, Effeltrich, Germany) was used for the measurements of chlorophyll fluorescence (F_v/F_m), the rate of net CO_2 assimilation (A) and stomatal conductance to water vapor (g_s) immediately after volatile measurements. The baseline conditions used for these measurements were: leaf temperature of 25 °C, ambient CO_2 concentration of 400 μmol mol^{-1}, air humidity of 60% and light intensity of illuminated leaves of 700 μmol m^{-2} s^{-1}. The leaves were enclosed in the Walz 3055-FL leaf chamber fluorimeter and dark-adapted for 30 min. Thereafter the minimum dark-adapted quantum yield, F_0, was measured, and an 1 s saturating light pulse of 7000 μmol m^{-2} s^{-1} was given to measure the maximum fluorescence yield, F_m, and obtain the dark-adapted quantum yield, F_v/F_m. Then light was switched on and net CO_2 assimilation rate and stomatal conductance to water vapor were recorded after these characteristics stabilized, in 20–30 min after switching on the light.

2.5. Estimation of leaf dry mass per unit area

After the measurements, all leaves were harvested and scanned at 300 dpi to determine leaf area with a custom-made software. Leaves were further oven-dried for 48 h at 70 °C and their dry mass was estimated, and leaf dry mass per area (M_A) was calculated. For comparison with other studies, the estimates of M_A were used to calculate the volatile emission rates per unit dry mass that are sometimes the only values reported (Niinemets et al., 2011)

2.6. Leaf sampling for qPCR

Sampling of leaf material for qPCR occurred in parallel with physiological measurements using separate plants of the same age and size. In each case, all selected leaves were treated, and different plants were used for each temperature treatment. The timing of leaf sampling for qPCR followed that of volatile measurements, only that the first qPCR sample after heat stress exposure was taken in 2 h rather than immediately after heat stress, given that the elicitation of gene expression is typically time-consuming (Byun-McKay et al., 2006). The collected leaf samples were immersed in liquid nitrogen and stored in −80 °C until RNA extraction.

2.7. RNA extraction and cDNA synthesis

Plant RNA was extracted from leaf tissues using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol after homogenization of foliage with mortar and pestle in liquid nitrogen. The concentration of extracted RNA was measured by Biophotometer Plus (Eppendorf; Germany) and adjusted as needed with nuclease-free or diethyl pyrocarbonate (DEPC)-treated water. One microgram of RNA was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, California, USA).

2.8. qPCR primer design

The genes included in this study are expressed in vivo in tomato leaves (Schilmiller et al., 2010a; Schilmiller et al., 2009; Falara et al.,

Table 1
Sequences of primers for reference genes and monoterpene β-phellandrene and sesquiterpene (*E*)-β-caryophyllene/α-humulene synthase genes used in this study.

Gene	Primer sequences (5′ → 3′)	Amplicon length (bp)
Reference genes		
TATA-box binding protein (TBP)	GCTAAGAACGCTGGACCTAATG/TGGGTGTGCCTTTCTGAATG	184
Ribosomal protein L8 (RPL8)	CCGAAGGAGCTGTTGTTTGTA/ACCTGACCAATCATAGCACGA	184
DnaJ-like protein (DNAJ)	GAGCACACATTGAGCCTTGAC/CTTTGGTACATCGGCATTCC	158
SAND family protein (SAND)	TTGCTTGGAGGAACAGACG/GCAAACAGAACCCCTGAATC	164
Clathrin adaptor complex (CAC)	CCTCCGTTGTGATGTAACTGG/ATTGGTGGAAAGTAACATCATCG	173
Target genes		
β-Phellandrene synthase	GTTGATGTTGATGGAGCGAG/GGCCACATATTTCATCACTTTCTAG	176
(*E*)-β-Caryophyllene synthase	CTTCTGTCCAGACGTAGAAGTACC/CGACAGACTCAACCAGTAACG	155

Reference gene primers used are based on Expósito-Rodríguez et al. (2008). β-Phellandrene synthase gene ((FJ797957, Schilmiller et al., 2009); TPS20 according to (Falara et al., 2011)) primer was designed on the basis of Schilmiller et al. (2009) and (*E*)-β-caryophyllene/α-humulene synthase gene (GU647162, Schilmiller et al., 2010a); TPS12 according to (Falara et al., 2011) revised nomenclature) primer on the basis of Schilmiller et al. (2010a).

2011). Five reference tomato genes – DNAJ (DnaJ-like protein), TBP (TATA-box binding protein), RPL8 (ribosomal protein L8), CAC (clathrin adaptor complex) and SAND (SAND family protein) (Table 1) were used as candidate reference genes based on the study of Expósito-Rodríguez et al. (2008), See Table 1. Finally CAC and SAND were used in the qPCR experiment as recommended by Expósito-Rodríguez et al. (2008) due to most stable expression profile. Gene-specific qPCR primers were designed for two terpenoid synthase genes, (*E*)-β-caryophyllene/α-humulene synthase (EC No. 4.2.3.104) (GU647162, Schilmiller et al., 2010a), TPS12 according to Falara et al. (2011) revised nomenclature, and β-phellandrene synthase (EC No. 4.2.3.51) (FJ797957, Schilmiller et al., 2009), TPS20 according to Falara et al. (2011) using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), with the melting temperature between 60 and 62 °C and a primer length of 20–26 bp. The oligonucleotide primer sequences are shown in Table 1.

2.9. qPCR conditions

To assay the modifications in tomato gene expression under heat stress, qPCR was conducted with the ViiA™ 7 qPCR System (Applied Biosystems, Courtaboeuf, France). The reaction was performed in 25 μL volume containing 10 μL 2 × iQ SYBR Green Supermix (Bio-Rad Laboratories Inc.), 300 nM of each primer and 1 μL of 5-fold-diluted cDNA. The PCR reactions were run in an ABI Sequence Detection System (Applied Biosystems) using the following program: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melt curve analysis was carried out to recognize possible non-specific amplifications. The dissociation program was set up as follows; 95 °C for 15 s, 60 °C for 15 s followed by 20 min of slow temperature ramp from 60 °C to 95 °C. Three replicates of each reaction were performed and ultimately two different reference genes that showed the most stable expression profile (SAND and CAC, Table 1) were used as the internal controls to normalize gene expression levels. Relative changes in gene expression were quantified according to the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001). The qPCR assay efficiency for (*E*)-β-caryophyllene synthase gene was between 83.6-115.5% and for β-phellandrene gene was between 83.8-116.8%.

2.10. Data analyses

All temperature treatments were replicated with at least three different plants. Generalized Linear Model (GLM) analyses based on maximum likelihood model fitting were used to test for individual and interactive effects of treatment temperature and recovery time on the emission of total LOX products, total monoterpenes, total sesquiterpenes, and separately for β-phellandrene and β-caryophyllene. We have log transformed the data for GLM, whenever appropriate, to reduce the effects of inequality of variance.

The emissions of the last two compounds were separately analyzed to allow for comparison with their gene expression profiles. In addition, paired samples *t*-tests were conducted within temperature stress treatments to compare the averages at different times through recovery. Correlations of net assimilation rate, stomatal conductance, emission rates of volatiles and gene expression vs. treatment temperature and recovery time, and correlations among emissions and gene expression levels of β-caryophyllene and β-phellandrene synthases were also analyzed. As a certain delay is expected between gene expression and corresponding change in trait expression, time-delay analysis was conducted to find the time-shift(s) at which the gene expression and volatile release are best aligned (i.e. most strongly cross-correlated according to the value of the correlation coefficient). Time-delays in the emission response of 2–8 h were tested.

The rate of change in gene expression was determined using first order exponential decay model for downregulation of β-caryophyllene and β-phellandrene synthase genes using non-linear regression analyses. All statistical analyses were conducted with SPSS Version 23 (IBM SPSS, Chicago, IL, WA), except for the non-linear regressions used for estimation of the rate of change in gene expression that were conducted with SigmaPlot Version 12.5 (Systat Software Inc, San Jose, CA, USA). All statistical effects are considered significant at $P < 0.05$.

3. Results

3.1. Changes in photosynthetic characteristics upon heat stress

The maximum dark-adapted photosystem II (PSII) quantum yield estimated by chlorophyll fluorescence (F_v/F_m) was not affected by 37 °C and 41 °C temperature treatments compared with controls, but higher temperature treatments, 46 °C and 49 °C, resulted in significant reductions in F_v/F_m (Fig. 1). After treatment at these higher temperatures, there was a progressive time-dependent reduction in F_v/F_m, with some recovery observed at 46 °C in 25 h after heat stress (Fig. 1).

Similarly to F_v/F_m, net CO_2 assimilation (*A*) was little affected by mild heat stress of 37 °C and 41 °C, but higher temperature treatments resulted in major reductions in *A* (Fig. 2A). However, stomatal conductance to water vapor (g_s) started to decline already at less severe heat treatments, although the variability at 37 °C and 41 °C was large (Fig. 2B). Net assimilation rate was invariable

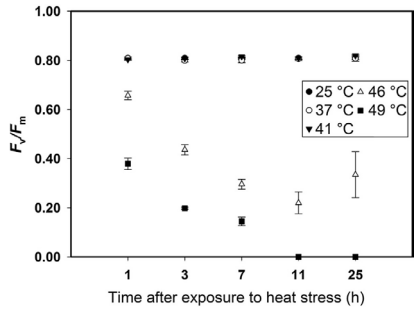

Fig. 1. Maximum dark-adapted quantum yield of photosystem II (PSII) estimated from chlorophyll fluorescence (F_v/F_m) in control (25 °C) and heat-treated (37–49 °C) leaves of *Solanum lycopersicum* L. cv. Pontica after recovery for 1, 3, 7, 11, and 25 h at 25 °C. In these experiments, 25-day-old plants were used and the heat treatment consisted of 10 min heating at given temperature.

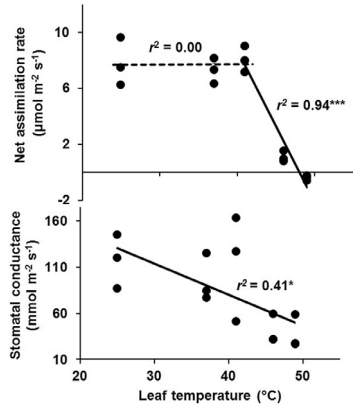

Fig. 2. Net assimilation rate (A) and stomatal conductance to water vapor (B) in relation to heat-treated (37–49 °C) and control (25 °C) leaves of *Solanum lycopersicum* L. cv. Pontica after 3.5 h recovery at 25 °C. Each symbol corresponds to an individual replicate. Data were fitted by linear regressions, whereas a segmented regression was used for (A) that apparently had a breaking point at 41 °C. Statistical significance of regressions as: * – $P < 0.05$, *** – $P < 0.001$.

through the recovery period in leaves exposed to the mild heat stress (Fig. 3A, B), but g_s slightly increased through the recovery period in leaves exposed to 37 °C (Fig. 3E). In the case of severe heat stress, there was a limited recovery in A and g_s in leaves exposed to 46 °C (Fig. 3C, G). A certain recovery in A was observed in leaves exposed to 49 °C, but by the end of the recovery period, A remained much lower than that in leaves exposed to 46 °C. These low levels of A in leaves exposed to 49 °C were accompanied by very low values of g_s (Fig. 3H).

3.2. Elicitation of lipoxygenase pathway volatiles (LOX products) after exposure to heat stress

No significant emission of LOX volatiles was observed from leaves in the control treatment, but heat stress induced emissions of LOX volatiles, albeit at a low level, in all heat stress treatments (Fig. 4A). Although the temperature effect was statistically significant and increased LOX emissions initially after heat stress compared to control, no clear patterns in total LOX emission were evident among the heat stress treatments (Fig. 4A). However, while in all temperature treatments LOX emissions were dominated by hexanal emissions followed by pentanal, 5-hexen-1-ol, and 1-hexanol, the emissions of (*E*)-2-hexenal, (*Z*)-3-hexenol, and 2,4-hexadienal were observed only at higher temperatures of 46 °C and 49 °C (data not shown). LOX emissions decreased significantly with the time of recovery (Fig. 4A).

3.3. Responses of mono- and sesquiterpene emissions to heat stress

Foliar monoterpene emissions were dominated by β-phellandrene, but α-pinene, limonene, *p*-cymene, 2-carene, Δ-3-carene, α-phellandrene, (*E*)-β-ocimene and traces of γ-terpinene and terpinolene were also detected in the emission blends. The average (±) total monoterpene emission rate of control leaves at 25 °C was 27.96 ± 17.37 pmol m^{-2} s^{-1}. The only stress-induced monoterpene was (*E*)-β-ocimene that was not emitted in control plants and increased with increasing treatment temperature reaching values of 1–4 pmol m^{-2} s^{-1} ($P < 0.001$ for the comparison of heat-stressed and control leaves). The dominant sesquiterpene emitted was (*E*)-β-caryophyllene (on average 82 ± 83% of total sesquiterpene emission) and the average total sesquiterpene emission of control leaves per area was 0.23 ± 0.20 pmol m^{-2} s^{-1}). Monoterpene emissions were initially enhanced by heat stress treatment compared to control and the effect was statistically significant, but the emissions gradually decreased with increasing recovery time, especially for milder heat treatments (Fig. 4B). In fact, emissions in heat-stressed plants even decreased to below the values in control plants at 6 and 10 h after the heat stress. Sesquiterpene emissions were also enhanced immediately after the heat treatment and the emissions decreased during recovery, except for 37 °C treatment and 49 °C treatment (Fig. 4C). Recovery time did not have any statistically significant impact on total mono-and sesquiterpene emissions (Fig. 4B and C).

Emissions of individual terpenes, the monoterpene β-phellandrene, and the sesquiterpene (*E*)-β-caryophyllene, were broadly consistent with the total emissions of given terpene classes (cf. Fig. 4B and Fig. 5A and B). In particular, emissions of β-phellandrene were enhanced immediately after the heat treatment compared to control and the emissions decreased to a low value in 6 and 10 h after heat treatment and slightly increased again in 24 h after heat treatment (Fig. 5A). Furthermore, the treatment effect on β-phellandrene emission was statistically significant. In the case of (*E*)-β-caryophyllene, after the initial enhancement, the emissions from treated leaves were decreased in 2 h, followed by an increase for leaves treated with 37 °C and 49 °C (Fig. 5B).

Recovery time did not have any statistically significant impact on β-phellandrene and (*E*)-β-caryophyllene emission (Fig. 5A and B).

3.4. Expression profiles of studied terpenoid synthase genes in response to heat stress

Heat treatment resulted in downregulation of expression of both β-phellandrene (Fig. 6) and (*E*)-β-caryophyllene (Fig. 7) synthase genes in 2 h after the stress treatment compared to the control plants. In the case of β-phellandrene synthase gene

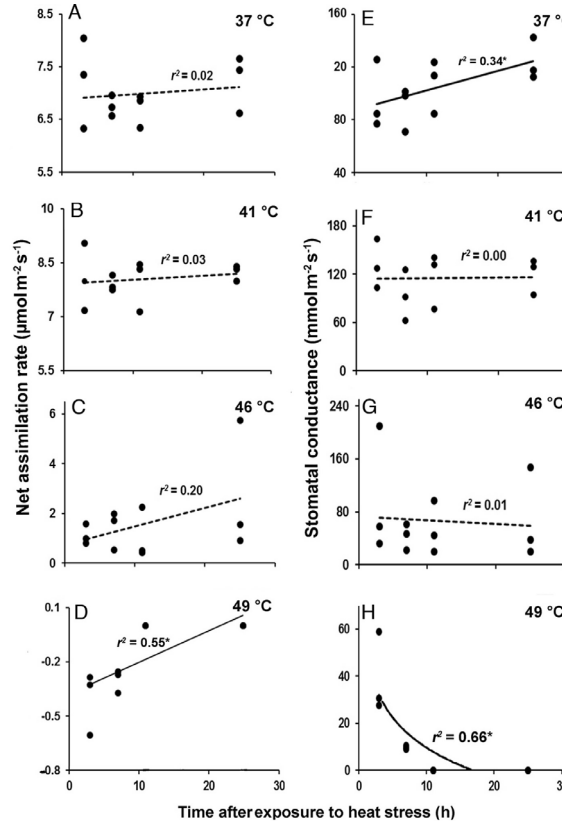

Fig. 3. Correlations of net assimilation rate (A-D) and stomatal conductance to water vapor (E-H) with the time of recovery (0.5–24 h) after heat treatment (37–49 °C) in *S. lycopersicum* L. cv. Pontica leaves. The treatment temperature is shown in each panel (experimental treatments as in Fig. 1). Each symbol corresponds to an individual replicate experiment. Data were fitted by linear regressions, except for (H) where the data were fitted by a non-linear regression. Statistically significant regressions are shown by solid lines and asterisks ($P < 0.05$).

expression in leaves treated with 37 °C and 41 °C, this suppression of gene expression continued for 2–10 h after the heat treatment, and the expression level reached the pre-stress level in 24 h (Fig. 6). However, no recovery was observed for leaves treated with 46 °C and only partial recovery was observed for leaves treated with 49 °C (Fig. 6). The expression response was similar for (*E*)-β-caryophyllene synthase gene expression, except that the recovery at 24 h was only partial for leaves treated with 37 °C and 41 °C. For both terpene synthase genes, the initial rate of gene expression was in most cases greater immediately after the heat stress treatment at 2 h than at 6 and 10 h (assuming the first order constant decay kinetics through the declining phase of gene expression), reflecting leveling off the decrease and onset of recovery of the gene expression response (Table 2). According to regression analyses, expression of β-phellandrene and (*E*)-β-caryophyllene synthase genes in leaves treated with different temperatures was correlated through the recovery phase except for the treatment with 37 °C (Fig. 8).

3.5. Expression of terpene synthase genes versus terpene emission through heat treatments

Positive correlations among the emission rates of β-phellandrene and (*E*)-β-caryophyllene gene expression levels through

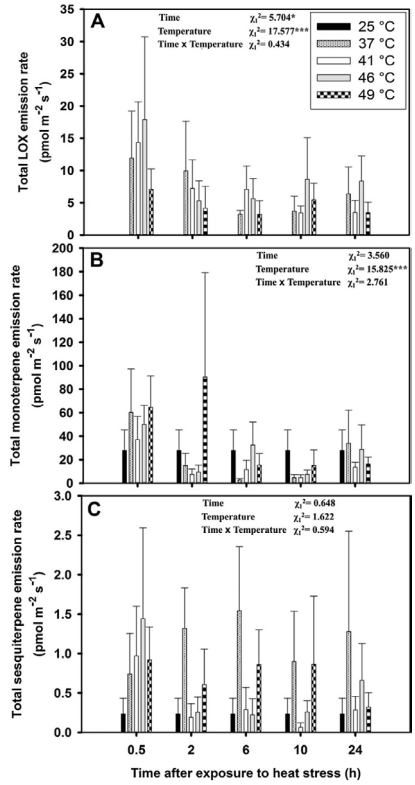

Fig. 4. Average total emissions of lipoxygenase pathway volatiles (LOX, also called green leaf volatiles, A), monoterpenes (B), and sesquiterpenes (C) in leaves of *S. lycopersicum* L. cv. Pontica upon heat-treatment (37–49 °C) and control (25 °C) (Fig. 1 for experimental treatments), and recovery time (0.5–24 h). Error bars show +SE. Effects of temperature treatment, recovery time and their interaction were tested by GLM, and Wald Chi-Square (χ^2_1) statistics along with their statistical significance are also demonstrated (* $-P < 0.05$, *** $-P < 0.001$).

recovery period were observed for the treatments with 37 °C (Fig. 9A, E) and 41 °C (Fig. 9B, F), while no correlation was observed for the treatment with 46 °C (Fig. 9C, G) and even negative correlations were observed for the treatment with 49 °C (Fig. 9D, H). According to the time-delay analysis, the cross-correlations were in most cases improved by introducing shifts of 2–4 h in emission responses with 2 h shift generally providing the strongest correlation (Table 3). In particular, introducing a lag-time between expression and emission resulted in positive correlations among expression and emission of both β-phellandrene and (*E*)-β-caryophyllene in leaves treated with 49 °C (Table 3). Nevertheless, the time-delay analysis did not identify any correlations for the 46 °C treatment (Table 3).

4. Discussion

4.1. Application of heat stress on Lycopersicum *L. cv. pontica leaves*

We note that heat stress affects the plants on heat dose (heat sum) dependent manner that is dependent on both the actual temperature and the duration of the heat episode (temperature X exposure time above the heat stress threshold) (Bilger et al., 1984; Kask et al., 2016). Although chronic heat stress can affect plants quite differently due to secondary acclimation responses (Kask et al., 2016), we consider the heat shock treatments as representative for quantitative characterization of plant response to different stress levels.

4.2. Response of tomato leaf photosynthetic characteristics to heat stress

Leaf net assimilation rates typically decline at supra-optimal temperatures, even under sustained moderate heat stress conditions of 35–40 °C (Zhang and Sharkey, 2009; Zhang et al., 2009; Hüve et al., 2012, 2006; Rasulov et al., 2015a). Such a moderate heat stress response is reversible and characteristically results from reductions in stomatal conductance to water vapor (g_s) (Hüve et al., 2012; Hüve et al., 2006; Copolovici et al., 2012) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity caused by impaired Rubisco activase activity at high temperatures (Haldimann and Feller, 2004; Hüve et al., 2011; Salvucci and Crafts-Brandner, 2004; Kim and Portis, 2005) as well as from partly impaired photosynthetic electron transport activity, mainly due to reduced activity of photosystem (II) (Zhang and Sharkey, 2009; Zhang et al., 2009; Rasulov et al., 2015a; Hüve et al., 2012, 2006). Exposure of plants to high temperature stress impairs photosynthetic apparatus associated with increased membrane fluidity (Copolovici et al., 2005). In addition, exposure to higher temperatures can cause irreversible damage of photosystem II and the oxygen-evolving complex ATPase (Camejo et al., 2006; Hüve et al., 2011, 2006), leading to time-dependent reductions in photosynthetic characteristics (Hüve et al., 2012). Analysis of foliage photosynthetic characteristics indicated that in our study, 37 °C and 41 °C treatments had a minor impact on foliage photosynthetic characteristics with some changes in only stomatal conductance in leaves treated with 37 °C (Figs. 1–3A, B, E, F). However, the treatment with 46 °C was a moderately severe stress that resulted in only a partial recovery of the maximum dark-adapted quantum yield of PSII (Fig. 1), while the treatment with 49 °C constituted a severe heat stress that was associated with very limited recovery (Fig. 3D). As both F_v/F_m (Fig. 1) and g_s (Fig. 3H) remained low at this latter treatment, both biochemical and stomatal factors likely limited net assimilation at this acute stress treatment. Overall, these results are consistent with the previous observations that tomato leaf resistance to shock-heating is between 49 and 51 °C (Copolovici et al., 2012).

4.3. Emission of LOX products under elevated temperatures

LOX volatiles (green leaf volatiles) are synthesized through lipoxygenase pathway from free fatty acids released by phospholipases in response to various stresses that lead to physical membrane damage (Matsui et al., 2012; Scala et al., 2013; Copolovici et al., 2012; Dicke et al., 1999; Loreto et al., 2006), including severe heat stress (Copolovici et al., 2012; Porta and Rocha-Sosa, 2002). In *S. lycopersicum*, Jansen et al. (2009) reported that biotic stress caused by necrotrophic fungus *Botrytis cinerea* infection led to a rich blend of LOX product consisting of (*E*)-3-hexenal, (*Z*)-3-hexenol, (*E*)-2-hexenal, 1-penten-3-ol and (*Z*)-3-hexenyl acetate. However, Copolovici et al. (2012) showed that

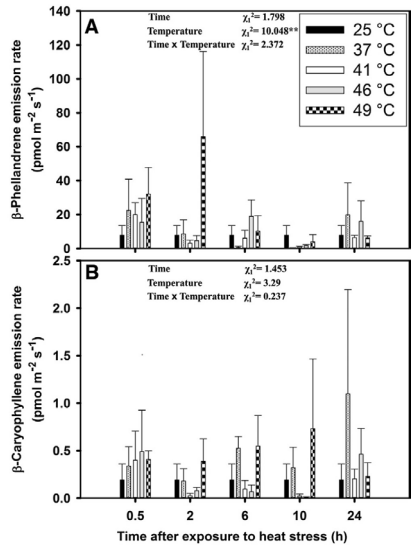

Fig. 5. Emission rates of β-phellandrene (A), and (*E*)-β-caryophyllene (B) in relation to heat-treated (37–49 °C) and control (25 °C) leaves of *Solanum lycopersicum* L. cv. Pontica and recovery time (0.5–24 h) (Fig. 1 specifics of heat treatments). Error bars show +SE. Effects of temperature treatment, recovery time and their interaction were tested by GLM, and Wald Chi-Square (χ^2_1) statistics along with their statistical significance are also demonstrated (** – $P < 0.01$).

heat-stressed tomato emissions were dominated by three LOX products (*Z*)-3-hexenol and (*E*)-2-hexenal, and 1-hexanol similar to our study. However, slightly higher LOX emissions were observed in Copolovici et al. (2012) with particularly strong emission bursts observed between 49 and 51 °C. In our study, such strong emission bursts were absent and the release of LOX products was similar across the treatments (Fig. 4A), reflecting slightly stronger heat resistance than in the previous study, perhaps associated with the use of more southern cultivar (an Estonian cultivar cv. Mato used in the study of Copolovici et al. (2012) vs. a Romanian cultivar cv. Pontica used in the current study).

4.4. Emission of monoterpenes and sesquiterpenes under elevated temperatures

Solanum lycopersicum is a constitutive terpene emitter (Copolovici et al., 2012; Jansen et al., 2009; Schilmiller et al., 2009, 2010a). These terpene emissions mainly rely on its specialized storage tissues for terpenes, glandular trichomes, where the de novo terpene synthesis is most active (Schilmiller et al., 2009, 2010b; Falara et al., 2011). Under non-stressed conditions, the release of terpenoids occurs at a very low level (Fig. 4B in this study; Copolovici et al., 2012; Jansen et al., 2009). As our study and the study of Copolovici et al. (2012) demonstrate, heat stress result in a major enhancement of terpenoid emissions (Fig. 4B, C). However, while constitutive and heat-enhanced monoterpene emissions were dominated by β-phellandrene in Copolovici et al. (2012) and in our study, sesquiterpene emissions were dominated by α-humulene in Copolovici et al. (2012) and by (*E*)-β-caryophyllene in our study. Such difference might reflect different cultivars used in these two studies as well as more severe heat stress applied in Copolovici et al. (2012).

While Copolovici et al. (2012) looked only at the initial enhancement of terpenoid emissions immediately after heat stress, analysis of the recovery after heat stress demonstrated that the immediate enhancement is transient and the emissions

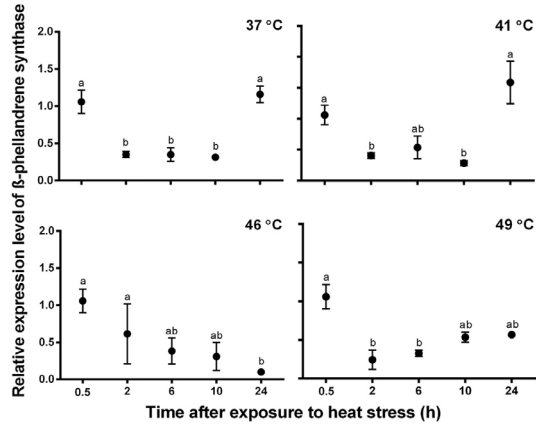

Fig. 6. Relative expression of (*E*)-β-phellandrene synthase gene in *S. lycopersicum* L. cv. Pontica leaves as affected by treatment temperature (37–49 °C) and time of recovery (0.5–24 h) at 25 °C (Fig. 1 for heat treatments). Data are means ± SE. Means with the same letter are not statistically different ($P < 0.05$) according to paired-samples *t*-tests.

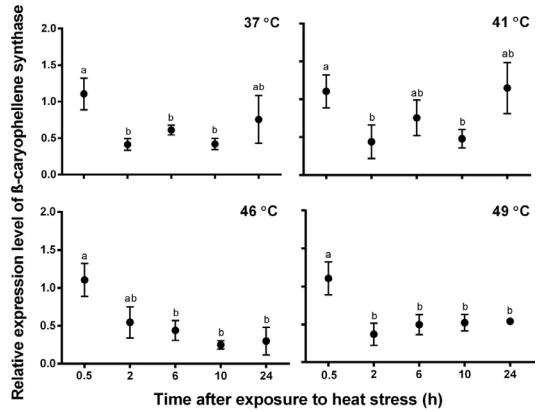

Fig. 7. Relative expression of (*E*)-β-caryophyllene synthase gene in *S. lycopersicum* leaves in relation to the treatment temperature (37–49 °C) and time of recovery (0.5–24 h) at 25 °C (heat treatments as in Fig. 1). Data presentation and statistical analysis as for Fig. 6.

Table 2
Rate of change in relative gene expression of *β*-phellandrene and (*E*)-β-caryophyllene/α-humulene synthase genes at different temperatures and different times of recovery.

Temperature	Time after exposure to heat stress (h)	Mean ± SE rate of change
β-Phellandrene		
37 °C	2	-0.85 ± 0.17^{a}
	6	-0.24 ± 0.08^{b}
	10	-0.144 ± 0.022^{b}
41 °C	2	-0.75 ± 0.14^{a}
	6	-0.16 ± 0.06^{b}
	10	-0.157 ± 0.021^{b}
46 °C	2	-0.63 ± 0.20^{a}
	6	-0.23 ± 0.07^{a}
	10	-0.163 ± 0.026^{a}
49 °C	2	-1.15 ± 0.11^{a}
	6	-0.242 ± 0.025^{b}
	10	-0.089 ± 0.027^{b}
(*E*)-β-Caryophyllene/α-humulene		
37 °C	2	-0.70 ± 0.13^{a}
	6	-0.115 ± 0.049^{b}
	10	-0.108 ± 0.032^{b}
41 °C	2	-0.84 ± 0.33^{a}
	6	-0.122 ± 0.055^{a}
	10	-0.097 ± 0.017^{a}
46 °C	2	-0.65 ± 0.36^{a}
	6	-0.20 ± 0.07^{a}
	10	-0.164 ± 0.040^{a}
49 °C	2	-0.84 ± 0.13^{a}
	6	-0.162 ± 0.014^{b}
	10	-0.086 ± 0.002^{b}

Figures 6 and 7 show time-dependent changes in gene expression. The rate of change in gene expression was calculated according to a first-order decay model using in each case the measurements in the control treatment at $t = 0$ as the reference gene expression level. Means within treatment temperatures were compared with one-way ANOVA followed by Tukey's test and different letters indicate means that are statistically different at $P < 0.05$.

decay over time (Fig. 4B, C, Fig. 5). In principle, changes in emissions through heat treatments and the following recovery can occur by four different mechanisms that are not mutually exclusive. First, heat stress can either damage or increase the permeability of epithelial and epidermal layers of glandular trichomes, thereby resulting in enhanced emissions until the terpenoid pools are either exhausted or the damage locations become sealed as the result of terpenoid oxidation and

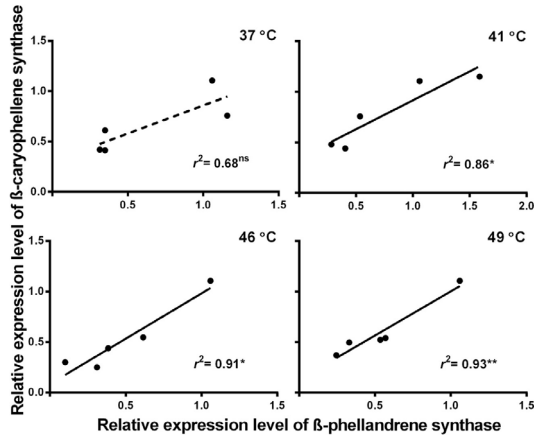

Fig. 8. Correlations among the relative expression levels of β-phellandrene and (*E*)-β-caryophyllene synthase genes at different treatment temperatures (37–49 °C) through recovery times (0.5–24 h). Each data point corresponds to an average estimate at different recovery time (Fig. 1 for heat treatments, and Figs. 6 and 7 for variation in expression levels of both synthase genes with treatment temperature and time of recovery). Statistical significance as: $^*P < 0.05$, $^{**}P < 0.01$.

polymerization (e.g., Loreto et al., 2000). Although plausible, such an explanation is not consistent with the correlations observed between gene expression and emission rate (see below, Fig. 9 and Table 3). Moreover, a physical damage exposing the trichome contents to ambient air is expected to result in a much stronger and sustained emission response.

Second, synthesis of monoterpenes scales positively with temperature due to the positive effects of temperature on the enzymatic activity of monoterpene synthases (Fischbach et al., 2000; Grote et al., 2006; Niinemets 2010a). Thus, faster synthesis of monoterpenes during the heat period could explain the higher emission rate immediately after the heat stress. However, monoterpene synthases typically have a relatively low optimum temperature, at around 40 °C (Niinemets et al., 2002b for a review), and given the short duration of the heat treatment, it seems unlikely that temperature-dependent transient speeding-up of synthase activities alone was responsible for the heat enhancement of the emissions right after the heat treatment.

Third, heat treatments could have altered the substrate availability for terpenoid synthesis. Given that monoterpenes are synthesized in plastids, including the monoterpenoids in tomato glandular trichomes (Schilmiller et al., 2009, 2010b; Falara et al., 2011), via the plastidial 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway, and sesquiterpenes in cytosol via mevalonate (MVA) pathway, substrate-level regulation can differ among the different terpenoid classes. In fact, monoterpene emission was more variable during the recovery phase than sesquiterpene emission (cf. Fig. 4B and C), possibly reflecting this circumstance. As the plastidial monoterpene synthesis typically relies on substrates provided by photosynthetic metabolism (Rajabi Memari et al., 2013; Rosenkranz and Schnitzler, 2013), the rates of monoterpene emission and photosynthesis are often correlated (Kesselmeier et al., 1996; Loreto et al., 1996; Staudt and Lhoutellier, 2011; Niinemets et al., 2002a), possibly reflecting the control of MEP/DOXP by the availability of plastidic NADPH and ATP (Niinemets et al., 2002a; Rasulov et al., 2009, 2015b). However, because photosynthesis was not affected, a reduction in substrate supply due to reduced foliage photosynthesis clearly cannot explain reduced monoterpene emissions during the recovery in leaves treated with 37 °C and 41 °C (cf. Figs. 1–3 and Fig. 4B). Furthermore, given that the photosynthetic potential of glandular trichomes is likely very low due to low expression of key photosynthetic enzymes (Schilmiller et al., 2010a), it is even not clear to what extent monoterpene synthesis in glandular trichomes relies on immediate photosynthetic metabolites. On the other hand, MEP/DOXP pathway products are also used for larger isoprenoids such as carotenoids and phytol residue of chlorophyll, and cytosolic MVA pathway products for phytosterols, inhibition of synthesis of these larger isoprenoids could have made the substrates available immediately after the heat stress in both plastids and cytosol.

Fourth, modified gene expression patterns could have led to changes in enzyme amounts. Indeed, heat stress was associated with major changes in gene expression patterns (Figs. 6 and 7), suggesting that such a control is plausible (see next section). In fact, similar modifications in mono- and sesquiterpene emissions upon treatments and recovery suggest that the same mechanism may be responsible for the alterations in the emissions of mono- and sesquiterpenes in tomato. A gene-expression level control can provide such a common regulatory mechanism.

Apart from herbaceous plants, thermal stress affects woody plants almost similarly. Application of heat stress on some woody species showed that, above certain threshold temperatures, constitutive *de novo* monoterpene emissions decrease largely when enhanced temperatures act as thermal stress. In the case of monoterpenes emitted from storage pools, higher rate of monoterpene emission was observed that was due to the damage of membranes surrounding the resin ducts and such monoterpene emission was associated with a pulse of LOX emissions. Moreover, application of thermal stress above heat stress threshold declined

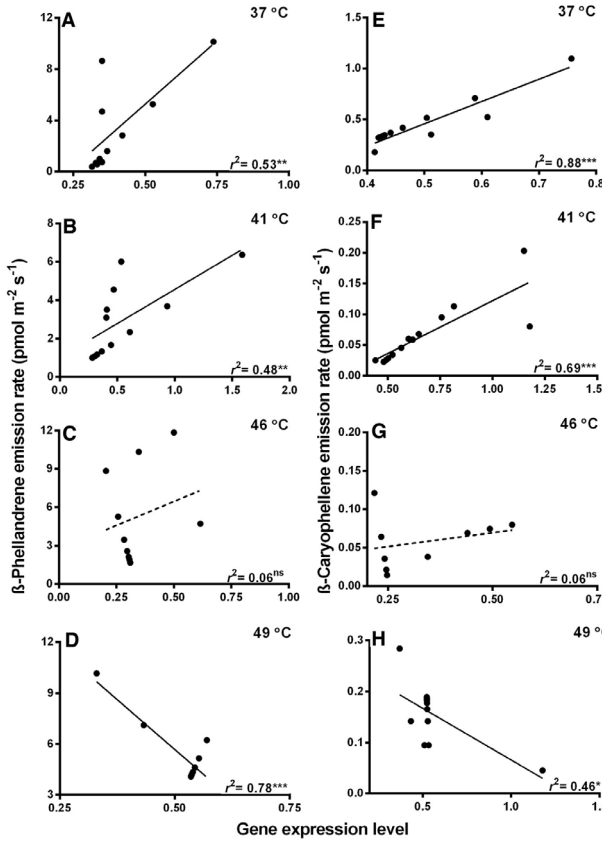

Fig. 9. Relationships among the relative gene expression and product emission for β-phellandrene (A-D) and (*E*)-β-caryophyllene (E-H) in *S. lycopersicum* L. cv. Pontica leaves at different treatment temperatures (37–49 °C) through recovery times (0.5–24 h) (Fig. 1 for treatments, Fig. 5A and C for emissions and Fig. 6 and 7 for gene expressions). Each symbol corresponds to a different replicate. Data were fitted by linear regressions and the statistical significance as: * $-P < 0.05$; ** $-P < 0.01$; *** $-P < 0.001$.

de novo sesquiterpene emission suggesting that heat induces irreversible effects on *de novo* emissions of mono-and sesquiterpenes (Kleist et al., 2012).

Volatile isoprenoids such as isoprene (Sharkey and Singsaas, 1995; Singsaas et al., 1997) and monoterpenes (Loreto et al., 1998; Delfine et al., 2000) improves thermotolerance of plants such that they protect membranes during heat stress by enhancing stability of membrane liquid-crystalline phase (Copolovici et al., 2005; Sharkey and Singsaas, 1995; Sharkey, 1996) Furthermore, heat stress also acts as an oxidative stress and in this case, isoprene and monoterpenes acting as antioxidents protect plants from heat associated with oxidative stress (Copolovici et al., 2005; Loreto and Velikova, 2001).

4.5. Expression profiles of key mono- and sesquiterpene synthase genes in response to heat stress

Heat stress treatment and time-dependent changes in β-phellandrene emission (Fig. 5A) were similar to changes in total monoterpene emission (Fig. 4B), and changes in (*E*)-β-caryophyllene emission (Fig. 5B) were similar to modifications in total sesquiterpene emissions and in particular, comparing Fig. 4B and Fig. 5A, and Fig. 4C and Fig. 5B, we found that β-phellandrene

Table 3
Time-delay analysis of the relationship between the emission and gene expression of (*E*)-β-caryophyllene and *β*-phellandrene.

Treatment temperature	Time shift in gene ex-pression (h)	Regression statistics			
		β-Phellandrene		(*E*)-β-Caryophyllene	
		r	*P*	*r*	*P*
37 °C					
	2	0.98	<0.001	0.56	ns
	4	0.97	<0.001	0.23	ns
	6	0.87	<0.01	0.08	ns
	8	0.47	ns	0.36	ns
41 °C					
	2	0.75	<0.01	0.71	<0.05
	4	0.54	ns	0.08	ns
	6	0.05	ns	0.23	ns
	8	0.38	ns	0.27	ns
46 °C					
	2	0.38	ns	0.40	ns
	4	0.50	ns	0.44	ns
	6	0.03	ns	0.54	ns
	8	0.55	ns	0.64	ns
49 °C					
	2	0.72	<0.05	0.82	<0.01
	4	0.66	<0.05	0.82	<0.01
	6	0.2	ns	0.02	ns
	8	0.64	ns	0.50	ns

Both terpenoid emission and gene expression were measured at the same time after the heat stress, and in this analysis, time-shifts of 2–8 h were introduced in the gene expression data to consider the circumstance that a change in gene expression comes first and emission response follows with a certain time delay. After each time-shift, data were fitted by linear regressions. *r* is the correlation coefficient for the cross-correlation and *P* the statistical probability.

emission contributes to a larger portion of total monoterpene emission and (*E*)-β-caryophyllene emission contributes to a larger portion of total sesquiterpene emission indicating that these two terpenoids were representative to the corresponding terpene classes. Thus, we analyzed the transcript abundance of *S. lycopersicum* β-phellandrene synthase gene (TPS20) and (*E*)-β-caryophyllene/α-humulene synthase gene (TPS12) to test whether the expression of these two genes is altered by heat stress treatment through the recovery period. We observed a rapid reduction in the expression of both of these genes after heat stress treatments (Table 2), and low expression level between 2 and 10 h in all cases (Figs. 6 and 7). In the case of lower treatment temperatures of 37 and 41 °C, there was almost a full recovery of gene expression levels by 24 h after heat treatment (Figs. 6 and 7). In the case of more severe heat stress, the recovery was absent, except for partial recovery of (*E*)-β-caryophyllene/α-humulene synthase gene expression at 49 °C (Fig. 7). Although the β-phellandrene synthase is a plastidic enzyme and the (*E*)-β-caryophyllene/α-humulene synthase is a cytosolic enzyme, expression of both genes through the recovery period was highly coordinated (Fig. 8). This suggests that the same transcription factor is regulating their activity, which is not surprising given the co-localization of their expression activity in *S. lycopersicum* leaves, in particular in leaf Type VI glandular trichomes (Falara et al., 2011). Further work is needed to characterize the pertinent transcription factor and its regulation by different temperatures to gain insight into the temporal kinetics of terpenoid gene expression elicited by heat stress.

Heat stress induces the transcriptional regulation of some important genes involved in heat stress responses. Thermal stress triggered heat stress signalling (Schoffl et al., 1998) and upregulated protective heat shock protein (HSPs) genes, *HSP18* and *HSP21*, in the roots and leaves of *Populus alba* × *Populus tremula* var. *glandulosa* saplings (Jia et al., 2016). However, certain types of HSP genes such as HSP40, HSP60, HSP70 and HSP80 were downregulated in response to heat stress suggesting that HSPs act differently (Song et al., 2014). In addition, heat stress downregulated some primary metabolism genes involved in light reaction, calvin cycle, and photorespiration (see Song et al. (2014) for further details). Overall, these findings suggest that the downregulation of secondary metabolism genes such as (*E*)-β-caryophyllene synthase and β-phellandrene genes studied here seem to follow the response for the downregulation HSP genes and primary metabolism genes in response to heat stress.

4.6. From synthase genes to emission: importance of consideration of time-delays

In a steady-state, there is evidence of a strong relationship between the product abundance in the blend of volatile terpenoids of *S. lycopersicum* and the level of expression of respective genes, e.g., a correspondence between the transcript abundance and the abundance of their products has been observed for TPS3 (camphene synthase) and TPS12 (Falara et al., 2011). In our study, in transient conditions after the heat stress when both the gene expression and the emission rates varied, the overall correlation between gene expression and emission rate was poor, especially at higher temperatures where the gene expression and product emission were even negatively correlated (Fig. 9). However, the time-delay analysis indicated that these non-correlations reflected delays between gene responses and emission with the delay of 2 h providing the best correspondence between gene expression and emission (Table 3). In fact, given the significant correlations between the emission and gene expression for leaves treated with 37 and 41 °C (cf. Fig. 9 and Table 3), the pertinent time-delay might have been even shorter in the case of mild heat stress. Higher resolution gene expression data are needed to test for possible changes in the delay-times with the severity of heat stress.

What factors can control the delay time between gene-level and phenotype-level responses? First, all steps from gene to protein, signal transduction, gene expression and protein synthesis, are time-consuming. Second, for a correlation between the gene product and gene expression to occur, there should be a continuous turnover of the corresponding target protein. In the case of terpene

synthases, there is limited information of the rate of turnover, but herbivory studies have shown that elicitation of synthesis of terpenes occurs within hours after start of herbivory feeding, and the terpenoid emissions reach to background level also within hours after removal of herbivores (Copolovici et al., 2011, 2014), supporting the continuous turnover of terpene synthase enzymes. Thus, the way heat stress affects the scaling between the expression of terpenoid synthases and the content of given terpene synthase enzymes depends on heat effects on time-delays between gene expression and protein synthesis and heat effects on protein turnover. A certain inhibition of protein turnover at severe heat stress might explain the weaker relationships of gene expression and emission observed at higher treatment temperatures, especially at 46 °C (Table 3, Fig. 9). However, we cannot also rule out substrate-level controls that can be operative simultaneously with gene-level controls. In fact, simultaneous reduction in emission and gene expression in leaves treated with the highest temperature of 49 °C (Table 3) might simply result from the overall reduction in foliage physiological activity, including RNA and protein synthesis and simultaneous reduction in substrate availability due to curbed photosynthesis rather than reflect a control by gene expression on emission.

5. Conclusion

This study demonstrated that both mild heat stress that did not affect foliage photosynthetic characteristics and severe heat stress that lead to irreversible photosynthetic reductions resulted in changes in emissions of mono- and sesquiterpenes in *S. lycopersicum* leaves immediately after heat stress and during recovery. Heat stress also lead to a sharp decline in the expression of β-phellandrene and (*E*)-β-caryophyllene/α-humulene synthase activity followed by a full recovery in leaves treated by mild heat stress. A time-delay of ca. 2 h was identified between gene expression and emission responses, but the relationships between gene expression and emission through recovery became increasingly weaker with increasing treatment temperature. This study overall underscores the complexity of linkages between gene-level and emission-level responses, especially in the case of severe heat stress that results in non-recoverable perturbation of leaf physiological activity. Such complexity can be partly considered in process-based emission models simulating terpenoid emissions in dependence on gene expression (e.g., Grote et al., 2013) by introducing a "delay factor". However, further studies are needed to gain insight into what controls heat-dependent regulation of transcription activity of terpenoid synthases, and how the delay between gene expression and phenotypic response scales with the severity of heat stress.

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Research paper

Differential regulation of volatile emission from *Eucalyptus globulus* leaves upon single and combined ozone and wounding treatments through recovery and relationships with ozone uptake

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ABSTRACT

Both ozone and wounding constitute two key abiotic stress factors, but their interactive effects on plant constitutive and stress-elicited volatile (VOC) emissions are poorly understood. Furthermore, the information on time-dependent modifications in VOC release during recovery from a combined stress is very limited. We studied the modifications in photosynthetic characteristics and constitutive and stress-induced volatile emissions in response to single and combined applications of acute ozone (4, 5, and 6 ppm) and wounding treatments through recovery (0.5–75 h) in a constitutive isoprene and mono- and sesquiterpene emitter *Eucalyptus globulus*. Overall, the photosynthetic characteristics were surprisingly resistant to all ozone and wounding treatments. Constitutive isoprene emissions were strongly upregulated by ozone and combined ozone and wounding treatments and remained high through recovery phase, but wounding applied alone reduced isoprene emission. All stress treatments enhanced emissions of lipoxygenase pathway volatiles (LOX), mono- and sesquiterpenes, saturated aldehydes (C7-C10), benzenoids, and geranylgeranyl diphosphate (GGDP) pathway volatiles. Once elicited, GGDP volatile, saturated aldehyde and benzenoid emissions remained high through the recovery period. In contrast, LOX emissions, and total mono- and sesquiterpene emissions decreased through recovery period. However, secondary rises in total sesquiterpene emissions at 75 h and in total monoterpenes at 25–50 h were observed. Overall, acute ozone and wounding treatments synergistically altered gas exchange characteristics and stress volatile emissions. Through the treatments and recovery period, stomatal ozone uptake rate and volatile emission rates were poorly correlated, reflecting possible ozone-scavenging effect of volatiles and thus, reduction of effective ozone dose and elicitation of induced defense by the acute ozone concentrations applied. These results underscore the important role of interactive stresses on both constitutive and induced volatile emission responses.

1. Introduction

Tropospheric ozone (O_3) is a major oxidative pollutant and a key plant stress elicitor (Karnosky et al., 2007). The bulk of tropospheric ozone is formed in the reactions involving nitrogen oxides NO and NO_2 (NO_X) and reactive volatile organic compounds (VOCs) in the presence of sunlight (Ryerson et al., 2003). Elevated ozone generates oxidative stress in plants that leads to biochemical adjustments and metabolic shifts as a result of ozone-induced gene expression changes and acclimation responses or hypersensitive or necrotic responses associated with foliar injury, impairment of shoot and root growth, and accelerated organ senescence (Calfapietra et al., 2008; Gerosa et al., 2009; Heath, 2008; Peñuelas and Staudt, 2010).

The current surface ozone level is around 40 ppb in most parts of the world (Sicard et al., 2017). Although it varies with the geographical location, the present tropospheric ozone levels are capable of causing physiological damage in plants (Ashworth et al., 2013; Proietti et al., 2016). Furthermore, it is anticipated that tropospheric ozone concentrations will increase by 2–4 folds in the next two decades, primarily due to industrialization and burning of fossil fuels, implying that ozone stress is expected to become more severe in the future (Vingarzan, 2004).

Abbreviations: BVOC, biogenic volatile organic compound; GGDP, geranylgeranyl diphosphate; GLM, generalized linear model; GLV, green leaf volatiles; LOX, lipoxygenase pathway; MEP/DOXP pathway, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway; MVA pathway, mevalonate pathway; PSII, photosystem II; ROS, reactive oxygen species

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Plants are a significant sink for atmospheric ozone both due to stomatal ozone uptake and non-stomatal ozone deposition (Fares et al., 2008). Stomatal uptake is the primary passage through which ozone enters the leaf intercellular spaces and generates physiological and oxidative damage in plant cells (Beauchamp et al., 2005; Gerosa et al., 2007). Apart from alteration of basic metabolic processes such as photosynthesis and plant growth, ozone exposure leads to major changes in plant volatile emission rates and emission profiles during the initial stress impact and through recovery. Upon acute ozone exposures, damaged plant cells release free fatty acids from their membranes, leading to an activation of lipoxygenase pathway (LOX) and rapid emission burst of LOX volatiles (also called green leaf volatiles, GLV) (Beauchamp et al., 2005; Copolovici et al., 2014; Portillo-Estrada et al., 2015). These early stress responses ultimately trigger the signal transduction pathways that activate defense reactions primarily through jasmonate (JA) and ethylene regulated transcription factors (Bailey et al., 2005). Typically, ozone-caused longer-term responses include elicitation of terpenoid and benzenoid emissions for hours to days following the initial stress impact (Beauchamp et al., 2005).

In nature, plants often encounter multiple stress factors simultaneously or in sequence. Interacting stresses can strengthen or weaken the effect of individual stress factors due to modification of overall stress severity or complex stress-priming responses (Copolovici et al., 2014; Ibrahim et al., 2008; Niinemets, 2010a,b). Leaf wounding is a key mechanical stress in the field that primarily results from herbivore feeding, but also from mechanical damage due to wind, falling debris and heavy precipitation (Benikhlef et al., 2013; Portillo-Estrada et al., 2015). Similarly to ozone, wounding leads to emissions of LOX volatiles that are quantitatively related to the degree of damage (Copolovici and Niinemets, 2015; Copolovici et al., 2017; Portillo-Estrada et al., 2015).

In fact, LOX emission is a very characteristic early stress response, followed by volatile isoprenoids, emissions of which have been detected upon many abiotic stresses such as heat shock in *Solanum lycopersicum* (Pazouki et al., 2016), drought and herbivory in *Alnus glutinosa* (Copolovici et al., 2014) and ozone in *Nicotiana tabacum* (Beauchamp et al., 2005), and biotic stresses such as feeding by larvae of common white wave (*Cabera pusaria*) in leaves of *Alnus glutinosa* (Copolovici et al., 2011) and leaf rust infection in *Salix* spp. (Toome et al., 2010) and *Populus* (Jiang et al., 2016).

Previous studies have revealed that the impacts of elevated ozone (Beauchamp et al., 2005; Llusià et al., 2002; Loreto et al., 2001, 2004; Peñuelas et al., 1999; Velikova et al., 2005), and wounding (Brilli et al., 2011; Loreto and Sharkey, 1993; Portillo-Estrada et al., 2015) alter primary and secondary metabolic process of plants and especially, LOX, MEP/DOXP (2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate) and MVA (mevalonate) pathways responsible for volatile emission. In all these studies, volatile emissions were quantitatively associated with the stress severity, characterized as the spread of damage or degree of biological infection, but it is unclear how combined application of two different stresses capable of causing cell-level injury can simultaneously affect plant volatile emission. Although both ozone and wounding stresses are known to induce LOX emissions, and ozone exposure further elicits isoprenoid emissions during recovery, it is also unclear whether wounding alone can also elicit volatile isoprenoid emission responses and whether wounding following ozone exposure amplifies the volatile emission responses during recovery. Furthermore, not only do the past studies lack the interaction effects, but they have typically analyzed immediate plant responses to stress rather than plant responses through recovery phase. Interaction studies are pertinent because in natural environments, occurrence of multiple stress factors with varying strength and duration is common and multiple stresses can significantly influence tree physiological responses through additive, synergistic, and antagonistic effects. In old-growth forest ecosystems, for example, low understory light levels combined with low soil nutrient and water availabilities can frequently limit understory regeneration (Bergh et al., 1999; Niinemets, 2010b). In addition to interacting stresses, an already existing stress can be superimposed by another stress factor, further complicating the stress situation. Due to stress priming and acclimation responses, the effects of superimposed and successive environmental stresses are often not additive, but different stresses can interact, leading to synergistic or antagonistic responses. The interactive effects on plant performance can either be negative, indicating an enhanced plant response to the given stressor due to an additional stress, or positive, implying a reduced plant response due to an additional stress (Niinemets, 2010b). Thus, the plant responsiveness to the given stress varies with the type and duration of the combined stresses imposed. In this study, wounding is a disturbance stress defined as a single episodic event or chronic impact that is associated with full or partial destruction of plant biomass, impairing plant physiological and functional activities such as photosynthesis and growth, whereas ozone is an oxidative stress that leads to a sustained deviation from optimal environmental conditions, causing reduced plant productivity and growth rates (Bansal et al., 2013). Therefore, ozone and wounding treatments are expected to lead to synergistic effects on plant functional activity. Especially for ozone, this is relevant as early and late stress responses can be qualitatively different (Calfapietra et al., 2013; Niinemets, 2010a).

Eucalyptus spp. are the dominant plant species in Australian forests and woodlands. Due to their high growth rates, they are considered economically highly valuable hardwoods for pulp industry (Külheim et al., 2015), and are therefore, widely grown in forest plantations around the world especially in tropical, sub-tropical and warm temperate regions (Loreto et al., 2000). Eucalypts are significant isoprene and monoterpene emitters under non-stressed conditions (Funk et al., 2006; Guenther et al., 1991; He et al., 2000; Loreto et al., 2000; Street et al., 1997; Winters et al., 2009). In this study, we used Tasmanian bluegum (*Eucalyptus globulus* Labill.) that is a classic species used in constitutive isoprenoid emission studies (Guenther et al., 1991; Loreto et al., 2000). However, much less is known of stress responses of volatile emissions in eucalypt species. We investigated how acute ozone and wounding stresses alone and in interaction change foliage photosynthetic characteristics, and volatile emissions through different recovery times in *E. globulus* leaves. Since plant-produced volatiles can quench ozone in ambient air and in leaf intercellular air spaces due to direct reaction with ozone (Fares et al., 2010), eucalypts are expected to be highly resistant to ozone stress, and we used acute ozone exposures in this study.

We addressed the following questions: (1) how do individual and interactive effects of acute ozone and wounding influence foliage photosynthetic characteristics, overall emission amounts and blend of emissions through recovery? (2) how are the quantitative emission responses through recovery phase associated with stress severity, including ozone dose and stomatal ozone uptake rate? Both the plant responses to immediate ozone and wounding application, and recovery of those responses upon returning the plants to ambient environment after stress were analyzed to gain conclusive insight into the correspondence among photosynthetic characteristics, ozone and wounding treatments, stomatal ozone uptake rates and volatile emissions. We hypothesized that (1) single and combined applications of acute ozone and wounding stresses would result in a major reduction in foliage photosynthesis rate, stomatal conductance to water vapor, and reduction in maximum quantum yield of photosystem II (PSII, F_v/F_m); (2) application of ozone and wounding treatments alone would lead to a strong emission response of LOX and volatile isoprenoids, saturated aldehydes, benzenoids, and GGDP pathway volatiles and that these responses scale with ozone dose and are greater than the responses due to wounding; (3) the combined application of ozone and wounding leads to synergistic effects on gas exchange and VOC emission responses; and (4) as *E. globulus* is a strong isoprenoid emitter, a major proportion of volatile isoprenoids and antioxidants scavenges ozone in leaf intercellular airspaces and ROS in plant cells, eventually resulting in a poor relationships between stomatal ozone uptake vs. volatile

emissions. To our knowledge, the sequence of events from elicitation to release of different volatiles upon application of individual ozone and wounding stresses through recovery and the interactive effects of combined ozone and wounding stresses on volatile emissions have not been studied yet, and it is also unclear how these responses are mediated by stomatal ozone uptake.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *E. globulus* were obtained from OMC seeds Ltd. (Lithuania) and sown in 5 L pots filled with a soil consisting of 1:1 mixture of quartz sand and commercial potting soil (Biolan Oy, Kekkilä group, Finland) that included essential macronutrients N (100 mg L^{-1}), P (30 mg L^{-1}), and K (200 mg L^{-1}) and micronutrients. The pH of the soil water was 6.2. Seedlings were grown for 3 weeks in a growth chamber (Percival AR-95 HIL, CLF Plant Climatics GmbH, Wertingen, Germany) under controlled environmental conditions of light intensity at leaf surface of 500 µmol m^{-2} s^{-1} provided for 12 h photoperiod, chamber temperature (day/night) of 28/25 °C, ambient CO_2 concentration of 380–400 ppm, and relative air humidity of 60–70%. The 3-week-old seedlings were transplanted into 10 L pots filled with the same potting mixture and the plants were further grown in controlled-conditions in a plant growth room under the light intensity of 400–500 µmol m^{-2} s^{-1} provided by HPI-T Plus 400 W metal halide lamps (Philips) for 12 h photoperiod, day/night temperature of 28/25 °C, ambient CO_2 concentration of 380–400 ppm, and relative air humidity of 60–70% until the completion of the experiment. Plants were watered every two days to soil field capacity and fertilized once a week with a liquid fertilizer (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%)). Each plant was supplied with 80 mL diluted liquid fertilizer (ca. 0.4% solution) to ensure optimum growth. One-year-old eucalypt plants were used in this experiment. In all experimental treatments, ca. 1 m tall plants with similar biomass, stem thickness, and fully mature leaves were used.

2.2. Experimental system for gas-exchange measurements, volatile collection and ozone fumigation

A temperature controlled, custom-made double-layered cylindrical glass chamber (1.2 L) with glass bottom was used for ozone fumigation, gas-exchange measurements, and volatile samplings (Copolovici and Niinemets, 2010, for details). The chamber temperature was maintained at 25 °C by circulating water between the double layers of the glass chamber; the temperature of circulating water was controlled by a thermostat. Four 50 W halogen lamps were used for chamber illumination, and the light intensity at the leaf level was controlled by a regulatory unit. Air temperature inside the chamber was monitored by a thermistor (NTC thermistor, model ACC-001, RTI Electronics, Inc., St. Anaheim, CA, USA) and leaf temperature was measured by a thermocouple attached to the lower leaf surface. Ambient air was purified by passing through a charcoal-filled filter and a custom-made ozone trap. The chamber was equipped with a fan (Sunon Group, Beijing, China), yielding a high air turbulence at relatively low wind speeds, ensuring high leaf boundary layer conductance and no pockets of still air in the chamber. Chamber glass bottom had openings for plant stem or leaf petiole and for sensors. Upon plant enclosure, the site of leaf insertion was sealed with low-emitting modeling clay to prevent any gas leakage from the chamber. All chamber connections were made of stainless steel and Teflon® was used for all the tubing.

The chamber inlet and outlet ports can be switched between the reference and measurement modes for gas exchange measurements and for volatile sampling. CO_2 and H_2O concentrations at chamber in- and outlets were measured by an infrared dual-channel gas analyzer operated in differential mode (CIRAS II, PP-systems, Amesbury, MA, USA). Upon branch enclosure, steady-state foliage net assimilation and transpiration rates were usually achieved in ca. 10–15 min after leaf enclosure in the chamber. When required, ozone was produced by a Certizon C100 ozonizer (Erwin Sander Elektroapparatenbau GmbH, Germany), and ozone concentration in the chamber in- and outlets was monitored with a UV photometric ozone detector (Model-49i, Thermo Fisher Scientific, Franklin MA, USA). A detailed description of the gas-exchange system setup is provided by Copolovici and Niinemets (2010), see also Niinemets et al. (2011, for a comparison of different gas-exchange systems).

Stainless steel adsorbent cartridges were used to collect volatiles from the leaf chamber using a portable pump 210–1003MTX (SKC Inc., Houston, TX, USA) operated with a constant suction flow rate of 200 mL min^{-1}. A detailed description of adsorbent cartridges and the method of volatile collection can be found in Kännaste et al. (2014) and Niinemets et al. (2010a). Air samples were also taken without the leaf to estimate chamber volatile background.

2.3. Experimental treatments

Mature leaves of *E. globulus* are comparatively thick, and this species is a very high isoprenoid emitter under control conditions. Preliminary experiments demonstrated that ozone was substantially scavenged and eucalypt leaves showed high resistance to acute ozone exposures. In fact, exposure to moderate to relatively high ozone concentrations of 0.3–2 ppm did not result in elicitation of LOX volatiles, and did not cause significant differences in photosynthetic characteristics (F_v/F_m, net assimilation rate and stomatal conductance to water vapor). Therefore, we decided to use higher acute ozone exposures. Four different sets of experiments were made: control (no treatment), wounding, ozone exposure (three different fumigations of 4, 5 and 6 ppm) and ozone exposure followed by wounding (the same three different ozone exposures). In all cases, a randomly selected branch consisting of six fully mature leaves was used, and standard environmental conditions maintained in the glass chamber were: light intensity at the leaf surface of 700–750 µmol m^{-2} s^{-1}, chamber temperature of 25 °C (leaf temperature of 25–27 °C), ambient CO_2 concentration of 380–400 µmol mol^{-1}, and relative air humidity of 60–70%. In the case of wounding treatment, prior to leaf enclosure, four holes were rapidly (within 6 s) punched in the leaf lamina by a paper punch. The area of each disc removed was ca. 25 mm^2 and the total perimeter length of four discs (wound edge) was 7 cm per leaf (Portillo-Estrada et al., 2015, for detailed methodology). For ozone treatments alone, the branch was exposed to either 4, 5, or 6 ppm ozone for 3 h. In the case of combined ozone and wounding treatment, the branch was taken out after ozone fumigation, and four punch holes were created in each leaf as for the treatment with wounding alone.

2.4. Gas-exchange and volatile measurement protocol and GC–MS analysis

Once the net assimilation and transpiration rates reached a steady-state, they were recorded and volatile sampling was carried out. After the initial measurement, the measurements for treated samples were repeated in 0.5, 3, 10, 25, 50, and 75 h after stress application. Separate measurements indicated that foliage gas-exchange and volatile emission rates of control samples were constant (variation less than 5%) during the measurement period.

The adsorbent cartridges were analyzed with a combined Shimadzu TD20 automated cartridge desorber and Shimadzu 2010 Plus GC–MS system (Shimadzu Corporation, Kyoto, Japan) for quantitative analysis of lipoxygenase pathway (LOX) volatiles, saturated aldehydes, volatile isoprenoids, benzenoids and geranylgeranyl diphosphate pathway (GGDP) volatiles (Kask et al., 2016 for definition of the compound classes). As lower molecular mass saturated aldehydes, pentanal (C5) and hexanal (C6) might partly reflect the LOX pathway activity (Heiden

Table 1
Average (± SE) emission rates (nmol $m^{-2} s^{-1}$) of stress volatiles released from the leaves of *E. globulus* in control, wounding, 5 ppm ozone, and 5 ppm ozone and wounding treatments at 0.5 h of recovery since stress applications. Compounds were grouped according to compound biosynthesis pathways.

Emission rate (nmol $m^{-2} s^{-1}$) of volatiles at 0.5 h since stress applications					
No	Compound	Control	Ozone 5 ppm	Wounding	Ozone 5 ppm and wounding
1	Isoprene	1.4 ± 0.8	1.2 ± 0.4	0.2 ± 0.1	14 ± 6
LOX volatiles					
2	Hexanal	0.004 ± 0.001	0.06 ± 0.04	0.010 ± 0.001	0.200 ± 0.014
3	Pentanal	0.005 ± 0.004	0.02 ± 0.01	0.010 ± 0.002	0.030 ± 0.001
4	(E)-2-Hexenal	nd	0.010 ± 0.004	0.010 ± 0.005	0.400 ± 0.030
5	(Z)-3-Hexenol	nd	0.010 ± 0.004	0.020 ± 0.003	0.45 ± 0.03
Monoterpenes					
6	Camphene	0.003 ± 0.002	0.13 ± 0.10	0.10 ± 0.02	1.6 ± 0.7
7	Δ^3-Carene	0.004 ± 0.001	0.03 ± 0.02	0.06 ± 0.03	0.20 ± 0.05
8	1,8-Cineole	0.130 ± 0.001	15 ± 12	3.0 ± 0.4	65 ± 30
9	p-Cymene	0.003 ± 0.001	0.4 ± 0.2	0.02 ± 0.01	4.0 ± 2.4
10	α-Fenchene	0.010 ± 0.001	0.03 ± 0.02	0.010 ± 0.004	0.4 ± 0.2
11	Limonene	0.02 ± 0.01	3 ± 2	0.80 ± 0.04	25 ± 11
12	β-Myrcene	0.005 ± 0.001	0.5 ± 0.4	0.2 ± 0.1	4.4 ± 3.5
13	(Z)-β-Ocimene	nd	0.2 ± 0.2	0.006 ± 0.004	1 ± 1
14	α-Phellandrene	0.001 ± 0.001	0.2 ± 0.2	0.003 ± 0.002	3 ± 2
15	α-Pinene	0.03 ± 0.02	6 ± 4	1.0 ± 0.2	37 ± 15
16	β-Pinene	0.006 ± 0.002	0.2 ± 0.2	0.02 ± 0.01	4 ± 2
17	α-Terpinene	0.005 ± 0.003	0.01 ± 0.01	0.10 ± 0.02	0.5 ± 0.2
18	γ-Terpinene	0.004 ± 0.002	0.3 ± 0.2	0.10 ± 0.01	1.3 ± 0.7
19	α-Terpinolene	0.004 ± 0.004	0.010 ± 0.006	0.10 ± 0.02	2.0 ± 0.1
20	α-Thujene	0.001 ± 0.001	0.10 ± 0.05	0.020 ± 0.006	1.0 ± 0.6
Sesquiterpenes					
21	Alloaromadendrene	0.001 ± 0.001	0.2 ± 0.2	0.01 ± 0.01	0.7 ± 0.4
22	Aromadendrene	0.003 ± 0.001	2.0 ± 1.3	0.103 ± 0.080	4 ± 2
23	(E)-β-Caryophyllene	nd	0.2 ± 0.2	0.010 ± 0.006	0.1 ± 0.1
24	α-Copaene	0.001 ± 0.001	0.10 ± 0.04	0.010 ± 0.003	0.3 ± 0.2
25	Epiglobulol	0.001 ± 0.001	0.10 ± 0.05	0.001 ± 0.001	0.24 ± 0.20
26	α-Gurjunene	0.001 ± 0.001	0.4 ± 0.4	0.04 ± 0.02	1.6 ± 1.3
27	β-Gurjunene	0.0003 ± 0.0002	0.020 ± 0.001	0.001 ± 0.001	0.13 ± 0.03
30	γ-Gurjunene	0.0002 ± 0.0001	0.10 ± 0.06	0.010 ± 0.001	0.13 ± 0.05
31	Isoledene	0.0004 ± 0.0001	0.1 ± 0.1	0.010 ± 0.003	0.4 ± 0.3
32	γ-Muurolene	0.0010 ± 0.0001	0.1 ± 0.1	0.010 ± 0.001	0.2 ± 0.1
33	Viridiflorene	0.002 ± 0.001	0.6 ± 0.5	0.10 ± 0.04	1.30 ± 0.70
Saturated aldehydes					
34	Decanal	0.003 ± 0.001	0.3 ± 0.1	0.002 ± 0.001	0.3 ± 0.1
35	Heptanal	0.0010 ± 0.0001	0.05 ± 0.03	0.0010 ± 0.0004	0.04 ± 0.02
36	Nonanal	0.003 ± 0.002	0.3 ± 0.2	0.004 ± 0.002	0.30 ± 0.02
37	Octanal	0.002 ± 0.001	0.1 ± 0.1	0.002 ± 0.002	0.04 ± 0.03
Benzenoids					
38	Benzaldehyde	0.0020 ± 0.0001	0.02 ± 0.02	0.010 ± 0.002	0.63 ± 0.25
39	o-Xylene	0.010 ± 0.001	0.01 ± 0.01	0.020 ± 0.001	0.030 ± 0.001
40	p-Xylene	0.010 ± 0.006	0.02 ± 0.01	0.020 ± 0.001	0.030 ± 0.001
GGDP pathway volatiles					
41	6-Methyl-5-hepten-2-one	0.0020 ± 0.0001	0.1 ± 0.1	0.005 ± 0.001	0.3 ± 0.3
42	Geranyl acetone	0.030 ± 0.002	0.3 ± 0.1	0.10 ± 0.01	0.5 ± 0.3

As the emission rates of volatile compounds at 0.5 h since the treatments were generally the highest for 5 ppm ozone treatment, and 5 ppm ozone and wounding treatments, the data for these treatments were shown here. nd = not detected.

et al., 2003; Wildt et al., 2003), for the compound class "saturated aldehydes" we considered aliphatic saturated aldehydes with more than six carbon atoms (Kask et al., 2016). The volatile compounds were identified based on pure standards (Sigma-Aldrich, St. Louis, MO, USA) and NIST 05 library. For further details of volatile sampling and GC–MS analysis we refer to Copolovici et al. (2009) and Kännaste et al. (2014). A full list of compounds detected in control and treated leaves (wounding, 5 ppm ozone, and 5 ppm ozone and wounding) is provided in Table 1.

The leaves were scanned at 300 dpi to measure leaf area using a custom made software tool. Foliage net assimilation rate (A), and stomatal conductance to water vapor were calculated according to von Caemmerer and Farquhar (1981), and volatile emission rates according to Niinemets et al. (2011).

2.5. Chlorophyll florescence measurements

After the completion of gas exchange measurements and volatile sampling, maximum dark-adapted (10 min darkening) quantum yield of photosystem II (PSII, F_v/F_m) of each treated and untreated leaf was measured with a PAM flourometer (Walz IMAG-MIN/B, Walz GmbH, Effeltrich, Germany) by providing a 1 s saturating (pulse intensity of 7000 μmol $m^{-2} s^{-1}$) flash of blue light (460 nm).

2.6. Calculation of ozone uptake by individual leaves

The stomatal conductance for ozone was calculated as the ratio of the average stomatal conductance to water vapor divided by the ratio of the binary diffusion coefficients for ozone and water vapor (2.03). The ratio of the binary diffusion coefficients for ozone and water vapor was estimated from ozone diffusion coefficient in air of $1.267 \cdot 10^{-5}$ $m^2 s^{-1}$

at 22.84 °C (Ivanov et al., 2007) and water vapor diffusion coefficient in air of $2.569{\cdot}10^{-5}$ m^2 s^{-1} at the same temperature determined according to Chapman and Enskog (Niinemets and Reichstein, 2003). As the temperature effects on the collision integral for both water vapor and O_3 (Tucker and Nelken, 1982) were negligible (the ratio varied less than 0.1% for 25 °C used for leaf measurements and 22.84 °C), we used the non-modified ratio in our calculations (Li et al., 2017).

Average stomatal ozone uptake rate ($\Phi_{O3,S}$) was estimated as the product of average chamber O_3 concentration and average stomatal conductance for ozone for the entire 3 h fumigation period (g_{O3}) assuming that ozone uptake through the cuticular layer was negligible (Kerstiens and Lendzian, 1989) and that the intercellular ozone concentration was zero (Laisk et al., 1989). We acknowledge that this latter assumption might give slightly overestimated ozone uptake as ozone concentrations in the leaf interior are somewhat higher than zero (Moldau and Bichele, 2002), but due to lack of intercellular ozone concentrations, we cannot correct for such effects and we consider this simplified estimate of ozone flux rate as a quantitative measurement of the severity ozone stress (Beauchamp et al., 2005; Li et al., 2017). Boundary layer resistance was neglected due to high air turbulence generated by a fan in the glass chamber. Stomatal ozone uptake rates were calculated for 4, 5, and 6 ppm ozone, and also for combined ozone and wounding treatments.

2.7. Data analysis

The measurements were replicated at least thrice for each treatment and six replicates were available for control samples. Correlation analyses were carried out between ozone uptake rate and isoprene, total LOX, and total mono- and sesquiterpene emissions for 0.5, 3, 10, and 25 h since ozone exposure. The individual and interactive effects of ozone and wounding, and recovery time on LOX, volatile isoprenoids, saturated aldehydes, benzenoids, and geranylgeranyl diphosphate pathway (GGDP) volatiles were statistically tested by generalized linear models (GLM) using maximum likelihood model fitting. Data were tested for normality of distribution and homogeneity of variance and then the data were log-transformed.

Correlation analysis was carried out using SigmaPlot Version 12.5 (Systat Software Inc, San Jose, CA, USA). All other statistical analyses were conducted using SPSS Version 24 (IBM SPSS, Chicago, IL, WA). All statistical effects were considered significant at $P < 0.05$.

3. Results

3.1. Effects of ozone and wounding treatments on foliar photosynthetic characteristics

Maximum dark-adapted photosystem II (PSII) quantum yield estimated by chlorophyll fluorescence (F_v/F_m) declined in response to all ozone treatments (4, 5 and 6 ppm O_3) and in response to the combination of ozone and wounding treatments (O_3 all + W) compared to control leaves ($P < 0.001$ for ozone, and combined ozone and wounding treatments). The reduction was particularly severe when the leaves were treated with 6 ppm ozone, and combined 6 ppm ozone and wounding at all recovery times (Fig. 1A). A partial recovery of F_v/F_m was observed since 25 h of recovery for all treated leaves. There was no significant change in F_v/F_m in leaves treated with wounding alone, compared with control leaves throughout all recovery times (Fig. 1A and Table S1).

Generally, all treatments resulted in a reduction of net assimilation rate (A) ($P < 0.001$ for all comparisons), especially at 6 ppm ozone and wounding (6 ppm O_3 + W) (Fig. 1B and Table S1). Overall, after the initial response, net assimilation rate remained relatively stable for 10 h of recovery for all stress applications, and then it partly recovered at 25 h of recovery with further reduction observed at 75 h in leaves treated with 6 ppm ozone, and combined 6 ppm ozone and wounding

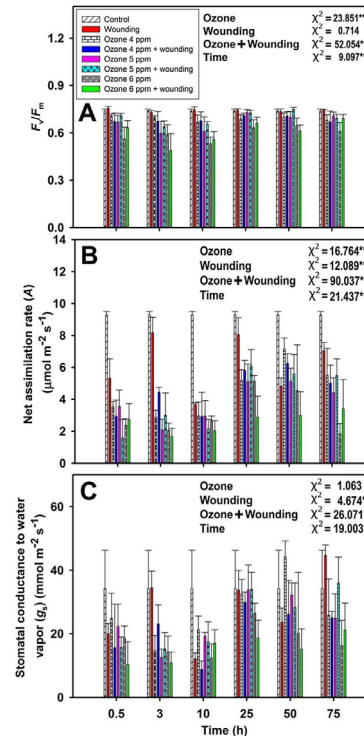

Fig. 1. Changes in dark-adapted (10 min darkening) maximum quantum efficiency of PSII photochemistry estimated by chlorophyll fluorescence (A), and net assimilation rate (B) and stomatal conductance to water vapor (C) in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (first exposed to 4, 5, and 6 ppm ozone and then wounded) leaves of *Eucalyptus globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Gas exchange measurements were conducted under stable conditions in the glass chamber with photosynthetic photon flux density at the leaf surface of 700 − 750 μmol m^{-2} s^{-1}, leaf temperature of 25–27 °C, ambient CO_2 concentration of 380–400 μmol mol^{-1}, and relative air humidity of 60–70%. All measurements were replicated at least thrice. Individual effects of ozone, and wounding, and the interactive effect of ozone and wounding were analyzed by generalized linear models (GLM) with maximum likelihood model fitting. Wald chi-squared test statistics (χ^2) and its statistical significance demonstrated as: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$.

(Fig. 1B). Generally, the reduction and recovery of net assimilation rate were associated with concomitant changes in stomatal conductance to water vapor ($P < 0.001$ for combined ozone and wounding treatments, and recovery time, and $P < 0.05$ for wounding; Fig. 1B, Fig. 1C, and Table S1). In fact, there was even almost a full recovery of g_s at 25 h for most treatments, except for leaves treated with 6 ppm ozone, and 6 ppm ozone and wounding (6 ppm O_3 + W) (Fig. 1C). However, g_s declined again for most treatments at 75 h, except for wounding, and 5 ppm ozone and wounding-treated (5 ppm O_3 + W) leaves (Fig. 1C).

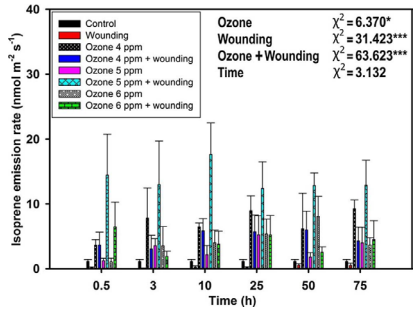

Fig. 2. Average (+SE) emission rates of isoprene in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (4, 5, and 6 ppm ozone and wounding) leaves of *E. globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Data presentation and statistical significance as in Fig. 1.

3.2. Isoprene emission in response to ozone and wounding treatments and in dependence on time of recovery

Non-treated leaves constitutively emitted isoprene at a relatively low level of ca. 1.4 ± 0.8 nmol m^{-2} s^{-1}, but isoprene emission rate achieved the highest level of ca. 17.6 ± 4.9 nmol m^{-2} s^{-1} in response to combined 5 ppm ozone and wounding treatment (5 ppm O_3 + W) at 10 h of recovery, followed by 4 ppm ozone treatment through all recovery times (Fig. 2).

Generally, isoprene emission was strongly enhanced when the leaves were subjected to combined ozone and wounding treatments, followed by separate ozone treatments ($P < 0.05$ for impact of ozone, $P < 0.001$ for ozone and wounding, and $P < 0.001$ for wounding, Table S1). However, compared to controls, wounding alone resulted in reduced isoprene emission rate through all recovery times. In fact, there was no recovery of isoprene emission after stress, implying that isoprene emissions remained either high as in ozone, and combined ozone and wounding treatments or low in the wounding treatment (Fig. 2).

3.3. Emission of total LOX, mono- and sesquiterpenes, saturated aldehydes, benzenoids, and GGDP pathway volatiles

In non-treated leaves, the baseline emission rate of total lipoxygenase pathway volatiles (LOX) varied between ca. 0.001-0.002 nmol m^{-2} s^{-1}. The maximum emission rate of average total LOX was 1.4 ± 0.5 nmol m^{-2} s^{-1}, achieved in response to 6 ppm ozone and wounding treatment (6 ppm O_3 + W) (Fig. 3A). Total emission rate of LOX was the highest immediately after stress applications and sharply decreased through the recovery phase (Fig. 3A; $P < 0.05$ for ozone, and wounding treatments, $P < 0.001$ for ozone and wounding treatments (O_3 all + W), and recovery time, Table S1).

Eucalyptus globulus is a significant constitutive emitter of mono- and sesquiterpenes under non-stressed conditions. Average total monoterpene emission by control leaves was ca. 0.20 ± 0.04 nmol m^{-2} s^{-1}. However, the rate of total monoterpene emission increased upon all stress treatments at 0.5 h since the stress application, and then it gradually decayed during the recovery phase (Fig. 3B). In particular, average total emission rate of monoterpenes increased non-linearly in response to stresses with the combined stress applications resulting in the highest elicitation rate (e.g., total monoterpene emission was increased by ca. 750-fold for combined 5 ppm ozone and wounding treatment, and by 650-fold for combined 6 ppm ozone and wounding treatment at 0.5 h of recovery), followed by ozone treatments alone. Wounding treatment alone resulted in the lowest enhancement, ca. 25-times greater monoterpene emission rate (Fig. 3B).

The average total sesquiterpene emission rate of control leaves was ca. 0.010 ± 0.002 nmol m^{-2} s^{-1} and the emissions non-linearly increased in response to all stresses applied (Fig. 3C). In contrast to isoprene and monoterpene emission responses, total sesquiterpene emission achieved the highest level of ca. 14.6 ± 9.5 nmol m^{-2} s^{-1} upon combined 4 ppm ozone and wounding treatment, followed by 6 ppm ozone and wounding (6 ppm O_3 + W) at 0.5 h since stress applications. The emission rate of total sesquiterpenes initially decayed through recovery phase with a further increase observed at 75 h of recovery from the leaves treated with 5 ppm ozone, 6 ppm ozone, and combination of 5 ppm ozone and wounding stress (Fig. 3C).

Average total emission rate of saturated aldehydes by control leaves was ca. 0.020 ± 0.004 nmol m^{-2} s^{-1}. Emission of total saturated aldehydes remained almost similar through recovery period (Fig. 3D). Benzaldehyde, *o*-xylene, and *p*-xylene (data not shown) were the benzenoids emitted by *E. globulus* leaves in all stress applications (Fig. 3E and Table 1). Total benzaldehyde emission remained elicited throughout the entire recovery period with the highest emission observed in response to combined 5 ppm ozone and wounding stress, especially at 0.5 h since the treatment. Similarly, total GGDP volatiles, made up of geranyl acetone and 6-methyl-5-hepten-2-one, showed enhanced emission rates through all recovery period in all treatments; in particular, emission rates in combined ozone and wounding treatments (O_3 all + W) were higher (Fig. 3F and Table 1).

3.4. Emission of foliar LOX volatiles in response to ozone and wounding treatments at different times of recovery

LOX emissions primarily consisted of hexanal, pentanal, (*E*)-2-hexenal, and (Z)-3-hexenol (Fig. 4 and Table 1). The emission of LOX was substantially enhanced in all ozone treatments (O_3 all, O_3 all + W), and upon wounding (W), but ozone alone led to greater emissions than wounding alone, and ozone combined with wounding (O_3 all + W) led to the greatest emissions. Separate ozone and wounding treatments had a minor effect on the emissions of primary LOX volatiles such as (*E*)-2-hexenal and (*Z*)-3-hexenol, but combined ozone and wounding treatments enhanced the emission responses by more than 25 times compared to separate wounding and ozone treatments (Fig. 4). In all treatments, the initial LOX emission rate at 0.5 h was dominated by (*Z*)-3-hexenol, followed by (*E*)-2-hexenal, hexanal, and pentanal (Fig. 4). In particular, (*Z*)-3-hexenol and (*E*)-2-hexenal declined to close to background levels at 75 h, while substantial emission responses of hexanal and pentanal were observed throughout the recovery time (Fig. 4). Thus, a considerable fraction of total LOX emission remained at all recovery times due to the major contribution of hexanal and pentanal to the emission blend (Fig. 3A). We also observed (*Z*)-3-hexenyl acetate at trace levels in response to ozone (O_3 all), and combined ozone and wounding treatments (O_3 all + W).

3.5. Emission of foliar monoterpenes upon ozone and wounding treatments and in dependence on time of recovery

Fifteen monoterpenes were observed in eucalypt emissions in this study (Fig. 5 and Table 1). Monoterpene emissions were dominated by 1,8-cineole (ca. 50% of total monoterpenes), followed by α-pinene (ca. 20% of total monoterpenes) and limonene (ca. 10% of total monoterpenes) in response to all treatments and in controls (Fig. 5). Apart from constitutively released monoterpenes, emissions of the characteristic stress-induced monoterpene, (*Z*)-β-ocimene, were detected in all treated leaves (Fig. 5H). All combined treatments of ozone and wounding had the highest (*Z*)-β-ocimene emissions followed by individual ozone, and wounding treatments. As with total monoterpenes, leaves treated with 5 ppm ozone and wounding demonstrated the highest level of (*Z*)-β-ocimene emission, 1.2 ± 1.0 nmol m^{-2} s^{-1} at

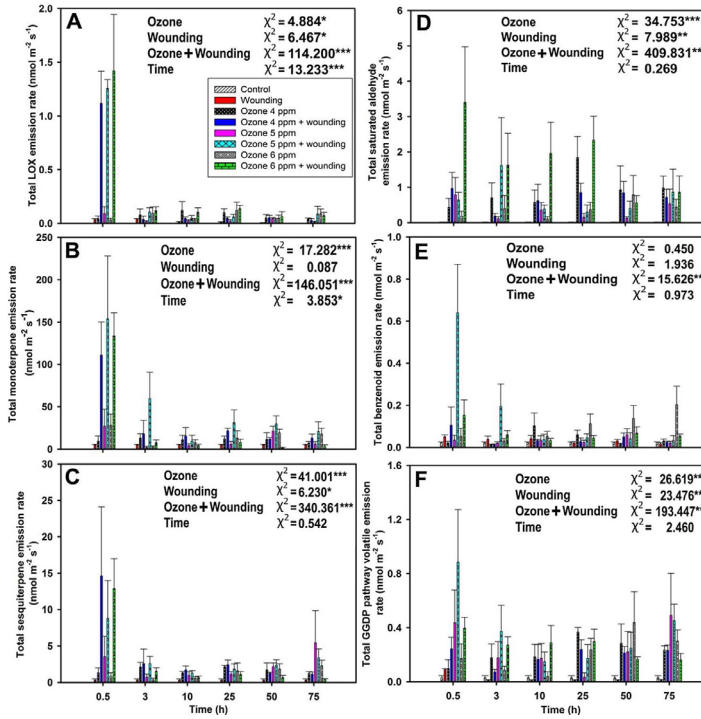

Fig. 3. Average (+SE) emission rates of total LOX volatiles (A), monoterpenes (B), sesquiterpenes (C), saturated aldehydes (D), benzenoids (E), and GGDP pathway volatiles (F) in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (4, 5, and 6 ppm ozone and wounding) leaves of *E. globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Data presentation and statistical significance as in Fig. 1.

0.5 h since stress applications, but even at 75 h of recovery, a substantial quantity of (*Z*)-β-ocimene was detected in leaves subjected to combined 5 ppm ozone and wounding, and combined 4 ppm ozone and wounding treatments (Fig. 5H).

Regarding the recovery kinetics of individual monoterpenes, four different emission patterns were observed. For most of the monoterpenes, the emissions were the highest at 0.5 h after treatment. However, for Δ^3-carene, once induced, its emission remained high throughout the recovery period in the case of most treatments, except for 6 ppm ozone treatment at 25 h and 5 ppm ozone treatment at 75 h (Fig. 5B). In addition, a secondary rise of (*Z*)-β-ocimene emission was detected in 10 h after exposure to 4 ppm ozone (Fig. 5H). For α-terpinene, the emissions remained high for most treatments at 3 h since the treatment, then the emissions declined (Fig. 5L). (*Z*)-β-ocimene was the most strongly elicited monoterpene followed by limonene, α-pinene, and 1,8-cineole. In addition, combined ozone and wounding treatments had statistically significant impact at $P < 0.001$ (Table S1) on the emission rate of all individual monoterpenes, except for α-fenchene, where $P < 0.01$ (Fig. 5 and Table S1).

3.6. Leaf sesquiterpene emissions in response to ozone and wounding treatments and in dependence on time of recovery

Eleven sesquiterpenes were detected in this study, whereas the emissions were dominated by aromadendrene (ca. 40% of total sesquiterpenes), followed by α-gurjunene (ca. 20%) and alloaromadendrene (ca. 10%) (Fig. 6 and Table 1). These three abundant sesquiterpenes comprised ca. 70% of total sesquiterpene blend at 0.5 h of recovery (Fig. 6).

As with monoterpenes, the emission rate of all individual sesquiterpenes tended to peak for combined ozone and wounding treatments (O_3 all + W), followed by separate ozone (O_3 all) and wounding (W) treatments, reflecting the stronger sesquiterpene emission responses for more severe stress (Fig. 6). In addition, ozone treatment had a statistically significant impact on the emission rate of all other individual sesquiterpenes, except for epiglobulol and γ-muurolene (Fig. 6 and Table S1). Similarly to total sesquiterpene emissions, a secondary rise of sesquiterpene emissions at 75 h was observed for several individual sesquiterpenes with particularly pronounced increase observed for α-copaene (Fig. 6D), epiglobulol (Fig. 6E), β-gurjunene (Fig. 6G), and γ-gurjunene (Fig. 6H) emissions. Overall, the elicitation of (*E*)-β-

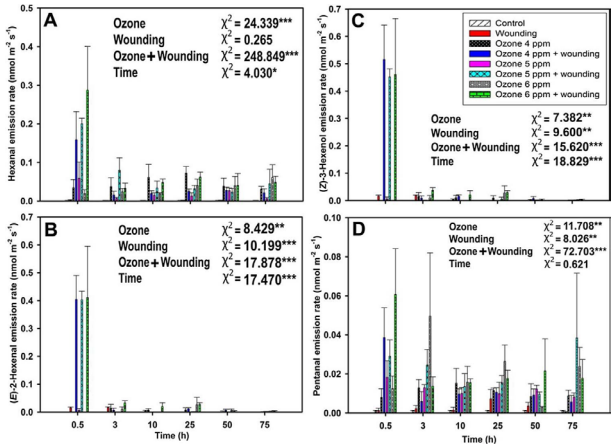

Fig. 4. Average (+SE) emission rates of individual lipoxygenase (LOX) pathway volatiles (LOX volatiles or green leaf volatiles) hexanal (A), (*E*)-2-hexenal (B), (*Z*)-3-hexenol (C), and pentanal (D) in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (4, 5, and 6 ppm ozone and wounding) leaves of *E. globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Data presentation and statistical significance as in Fig. 1.(

caryophyllene was the highest, followed by epiglobulol, γ-gurjunene, and β-gurjunene, and there was little variation in elicitation of all sesquiterpenes at all recovery times.

3.7. Emission of saturated aldehydes as affected by ozone and wounding treatments and time of recovery

Four individual compounds contributed to the pool of saturated aldehydes: heptanal, octanal, nonanal and decanal (Fig. 7 and Table 1). The blend of saturated aldehydes was dominated by decanal, followed by nonanal, and the emission pattern of these two compounds broadly reflected the variation in total saturated aldehyde emission (Figs. 3D and 7). Initially, the emission rate of all saturated aldehydes was enhanced in all stress treatments, but the enhancement was the highest for leaves treated with combined 6 ppm ozone and wounding treatment (3.40 ± 1.60 nmol m^{-2} s^{-1} for combined 6 ppm ozone and wounding treatment at 0.5 h since stress application) compared to other treatments (Fig. 3D).

3.8. Emission of isoprene, and total mono- and sesquiterpene volatiles in relation to stomatal ozone uptake

Generally, the correlations between stomatal ozone uptake rate and isoprene, total LOX, and total mono- and sesquiterpene emission rates were poor for all ozone (O_3 all) exposures (Fig. 8). For total LOX emission, the correlation with stomatal ozone uptake was negative at 0.5 h since the treatments, and non-significant for other recovery times (Fig. 8). In all cases, the correlation of isoprene with ozone uptake rate was non-significant, but marginally significant negative correlations were observed between total mono- and sesquiterpene emissions and stomatal ozone uptake rate at 0.5 h since ozone exposure (Fig. 8).

Similarly, there were poor correlations between stomatal ozone uptake rate and isoprene, total LOX, and mono- and sesquiterpene emissions for all combined ozone and wounding (O_3 all + W) treatments till 25 h since stress application (Fig. 9). However, marginally significant positive correlations were observed between stomatal ozone uptake rate and total LOX emission at 3 h and 10 h since the treatments, and a marginally significant negative correlation was observed between isoprene emission and ozone uptake rates at 0.5 h (Fig. 9). In contrast, significant or marginally significant positive correlations between stomatal ozone uptake rate and isoprene, and total mono- and sesquiterpene emission rates were observed only at 3 h since stress applications (Fig. 9). However, at 25 h since the combined ozone and wounding treatments (O_3 all + W), the correlations were negative and statistically significant between ozone uptake and total mono- and sesquiterpene emissions (Fig. 9).

4. Discussion

4.1. Changes in leaf photosynthetic characteristics due to ozone and wounding treatments

The reduction of maximum dark-adapted photosystem II (PSII) quantum yield estimated by chlorophyll fluorescence (F_v/F_m) upon ozone, and combined ozone and wounding treatments indicates that ozone induced perturbations in the reaction centers of PSII. This is consistent with previous studies where both energy dissipation activity and efficiency of PSII declined in ozone-exposed leaves (Guidi et al., 1999; Guidi and Degl'Innocenti, 2008), and the level of reduction of PSII activity scaled with the quantity of ozone entering leaf intercellular air spaces (Gerosa et al., 2009). Furthermore, the reduction of PSII activity was also associated with sustained photoinhibition (Guidi and Degl'Innocenti, 2008). In fact, the ozone-induced photoinhibition is a synergistic effect of light-dependent photodamage associated with net reduction of *de novo* synthesis of D1 protein, ultimately leading to a subsequent decline in the repair rate of PSII (Fiscus et al., 2005; Godde and Buchhold, 1992; Guidi and Degl'Innocenti, 2008). In this study, wounding treatment had no apparent effect on F_v/F_m, suggesting that it did not reduce the number of open PSII centers (Fig. 1A).

Separate and combined applications of ozone and wounding substantially reduced net assimilation rate in a time-dependent manner (Fig. 1B). Elevated ozone can directly affect photosynthesis by reducing foliage biochemical potentials (non-stomatal inhibition) or by reducing stomatal conductance (stomatal inhibition) or both (Fusaro et al., 2016). The decline of net assimilation rate upon individual and combined applications of ozone and wounding treatments could be due to the impaired electron transport capacity and irreversible damage in chlorophyll reaction centers caused by oxidative stress (Guidi and

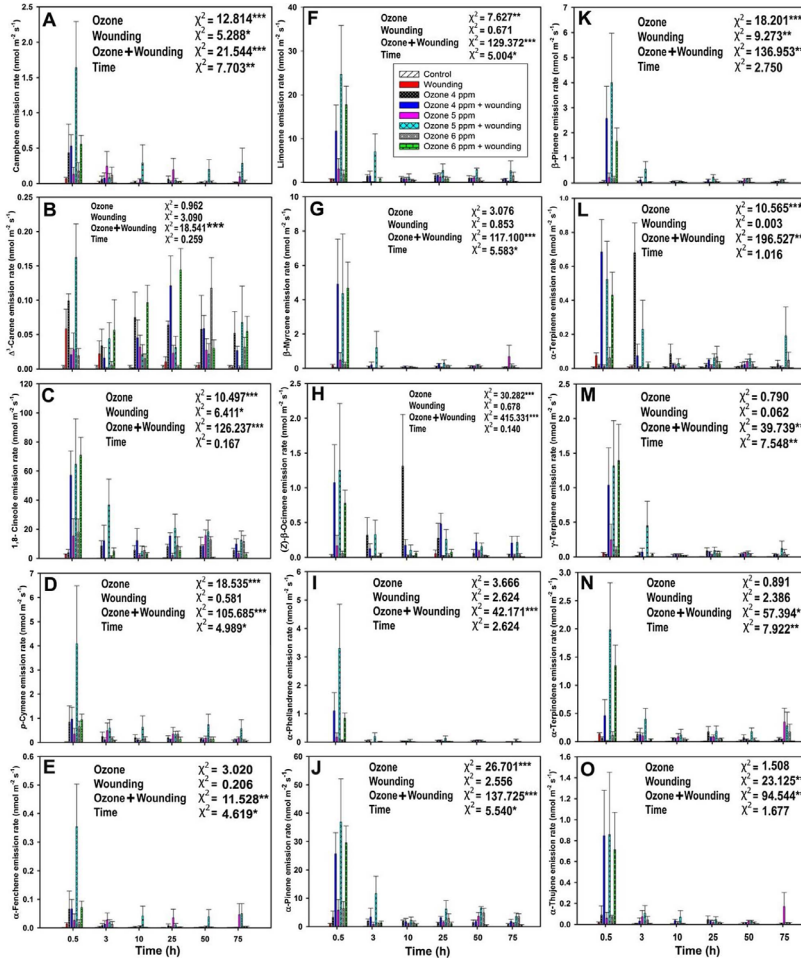

Fig. 5. Average (+SE) emission rates of monoterpenes (A–O) in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (4, 5, and 6 ppm ozone and wounding) leaves of *E. globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Data presentation and statistical significance as in Fig. 1.

Degl'Innocenti, 2008) consistent with the reduction in PSII activity in our study. In addition to suppression of PSII, elevated ozone often inhibits photosynthesis through the suppression of Calvin cycle; particularly, it reduces the activity of ribulose-1, 5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) (Guidi and Degl'Innocenti, 2008; Zhang et al., 2011). In fact, Rubisco is the primary component affected by elevated ozone in plants (Noormets et al., 2001) and both the quantity and activation state of Rubisco are reduced in ozone-exposed plants, leading to a decline in maximum rates of carboxylation (V_{cmax}) (Fusaro et al., 2016; Niu et al., 2014). For example, upon chronic ozone exposures in *Cinnamomum camphora* seedings, both V_{cmax} and the capacity for photosynthetic electron transport (J_{max}) were substantially reduced, but there was no apparent change in J_{max}/V_{cmax} ratio. The constant value of J_{max}/V_{cmax} demonstrates that there was a strong correlation between reductions in RuBP carboxylation and light-driven electron transport (Niu et al., 2014). In addition, certain evergreen woody plants

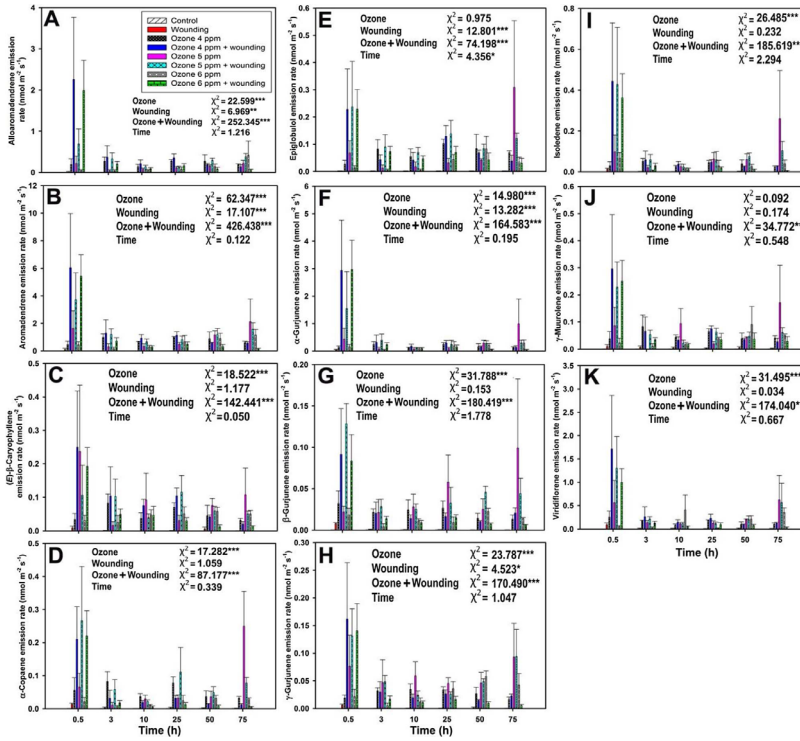

Fig. 6. Average (+SE) emission rates of sesquiterpenes (A-H) in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (4, 5, and 6 ppm ozone and wounding) leaves of *E. globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Data presentation and statistical significance as in Fig. 1.

such as *Quercus ilex* L. and *Arbutus unedo* L. have the ability to maintain photosynthetic characteristics to a normal level even during salt stress episodes by removing harmful ions from effective shoot organs (Fusaro et al., 2014).

In our study, the time-dependent reduction of net assimilation rate was also associated with the reduction of stomatal conductance to water vapor (Fig. 1B and C) as observed in several other studies (Zhang et al., 2011). This is in agreement with the evidence that acute ozone exposure exerts an inhibitory effect on guard cell K^+ channels, resulting in reduced stomatal conductance (Niu et al., 2014). However, in our study, there was even a certain overshoot in stomatal conductance at the end of the recovery period (Fig. 1C), implying that there might be compensatory responses (Copolovici et al., 2014, 2011; Niinemets, 2016).

4.2. Effects of ozone and wounding treatments on the emission of volatile isoprenoids, LOX, saturated aldehydes, benzenoids, and GGDP pathway volatiles

4.2.1. Effects of ozone and wounding treatments on isoprene emission kinetics

As biosynthesis and emission of isoprene occur simultaneously (Rasulov et al., 2009), the rate of isoprene emission is controlled by substrate availability and/or isoprene synthase enzyme activity. *Eucalyptus globulus* is a strong constitutive isoprene emitter, but stress can affect both substrate availability as well as the rate of isoprene synthase expression, thereby potentially resulting in major changes in isoprene emissions (Niinemets, 2010a). In our study, wounding reduced isoprene emission rate by ca. 85% at 0.5 h after stress application and by ca. 54% at 75 h of recovery, indicating that only a slight restoration of isoprene emission was observed (Fig. 2). However, wounding also initially reduced net assimilation rate by ca. 73% and by ca. 30% at 75 h of recovery; this is consistent with the previous report of Loreto and Sharkey (1993) showing that isoprene emission is highly sensitive to wounding stress and it responds more rapidly than photosynthesis and stomatal

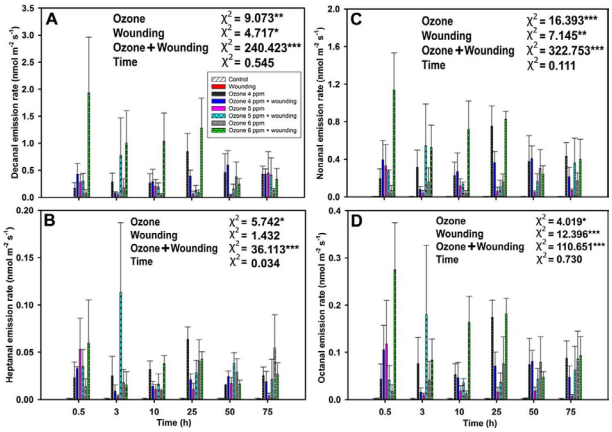

Fig. 7. Average emission rates (+SE) of saturated aldehydes (A-D) in control (0 ppm), wounded, ozone-exposed (4, 5, and 6 ppm), and ozone-exposed and wounded (4, 5, and 6 ppm ozone and wounding) leaves of *E. globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. Data presentation and statistical significance as in Fig. 1.

conductance. Although stomatal conductance declined in our study, stomata cannot control isoprene emissions (Fall and Monson, 1992), because the buildup of isoprene in the leaf gas phase upon stomatal closure compensates for reduced stomatal conductance (Niinemets et al., 2014; Niinemets and Reichstein, 2003). Indeed, isoprene emission is often even enhanced by moderate stomatal closure due to the positive effect of reduced intercellular CO_2 concentration on isoprene emission rate (Rasulov et al., 2016; Wilkinson et al., 2009). In our study, the reduction of isoprene emission upon wounding was likely associated with the downregulation of rate-limiting enzyme activities (Kanagendran et al., unpublished).

However, ozone stress, and ozone and wounding stress enhanced isoprene emission through the entire recovery period (Fig. 2), suggesting that when wounding was combined with ozone exposure, ozone might have primed for isoprene synthase gene expression and isoprene synthase enzyme activity, ultimately enhancing sustained *de novo* isoprene emission through recovery (Fig. 2). This suggestion is in line with the patterns of photosynthesis and isoprene changes in different treatments. Differently from the wounding treatment, where isoprene emission and photosynthesis rates declined together, in individual ozone, and in combined ozone and wounding stress treatments, isoprene emission rate was enhanced while net assimilation rate decreased through all recovery periods, demonstrating that enhanced isoprene emission was not associated with the changes in photosynthesis, particularly for individual ozone, and combined ozone and wounding treatments. Indeed, the enhanced isoprene emission was likely due to upregulation of isoprene synthase gene expression and enhanced rate-limiting enzyme activities through all recovery periods (Kanagendran et al., unpublished) (Figs. 1 B and 2). Elevated isoprene emissions upon ozone exposure have also been observed in other studies (Fares et al., 2006; Velikova et al., 2005). This has been considered adaptive as isoprene can act as a volatile antioxidant scavenging reactive oxygen species (ROS) and thus, enhance membrane stability of leaves upon oxidative stress (Asada, 2006; Velikova et al., 2005).

However, chronic ozone exposure inhibited isoprene emission in field-grown trembling aspen (*Populus tremuloides*) (Calfapietra et al., 2008, 2007). This was associated with downregulation of isoprene synthase gene expression and isoprene synthase enzyme level in the ozone-sensitive aspen clone (Calfapietra et al., 2008, 2007). This difference from our study further supports the suggestion that different biochemical mechanisms are activated by acute and chronic ozone exposures.

4.2.2. *Effects of ozone and wounding treatments on LOX pathway volatiles and saturated aldehydes*

LOX compounds are synthesized from free polyunsaturated fatty acids in cell membranes (linoleic acid, 18:2 and linolenic acid, 18:3) as a part of stress-dependent hypersensitive reaction (Feussner and Wasternack, 2002). In plant cells, lipoxygenases are constitutively active and therefore, release of free polyunsaturated fatty acids due to membrane damage and consequent formation of volatile LOX products is a characteristic early stress response; thus, LOX volatiles are rapidly emitted upon acute stress treatments (Jiang et al., 2016; Li et al., 2017; Portillo-Estrada et al., 2015). In our study, the total LOX emission rate was quantitatively related to the severity of stress (Fig. 3A), consistent with previous studies of Beauchamp et al. (2005), Copolovici et al. (2012), Jiang et al. (2016), and Jiang et al. (2017).

The initial LOX emission burst usually lasts for some minutes to a few hours (Jiang et al., 2016; Li et al., 2017; Portillo-Estrada et al., 2015) and consists of classic LOX volatiles such as (*Z*)-3-hexenol and (*E*)-2-hexenal. In our study, the observation of sustained emissions of these classic LOX volatiles through the recovery period suggests that acute ozone and combined ozone and wounding exposures caused severe cellular damage, consistent with only a partial recovery of photosynthesis (Fig. 1B). These acute stresses could also have elicited overexpression of additional lipoxygenases and enzymes responsible for derivatization of primary LOX compounds, ultimately supporting the longer-term emission response of LOX compounds (Liavonchanka and Feussner, 2006; Porta and Rocha-Sosa, 2002). Thus, the regulation of LOX biosynthesis might have shifted from substrate level control at early stages of recovery to gene expression level control at later stages of recovery (Figs. 3 A and 4 A–D). A change of LOX biosynthesis regulation from substrate level to gene expression level through recovery phases was further suggested by Jiang et al. (2016) in *Melampsora larici-populina*-infected poplar leaves that had high emissions of derivatized LOX volatiles (Z)-3-hexenyl acetate and (Z)-3-hexenol. Possibly, the expression of genes responsible for derivatization of C6 aldehydes regulated the emission of (Z)-3-hexenyl acetate and (Z)-3-hexenol (Jiang et al., 2016). However, we cannot rule out the formation of (Z)-3-hexenol from (*E*)-2-hexenal (or from its unstable conjugate (*Z*)-3-

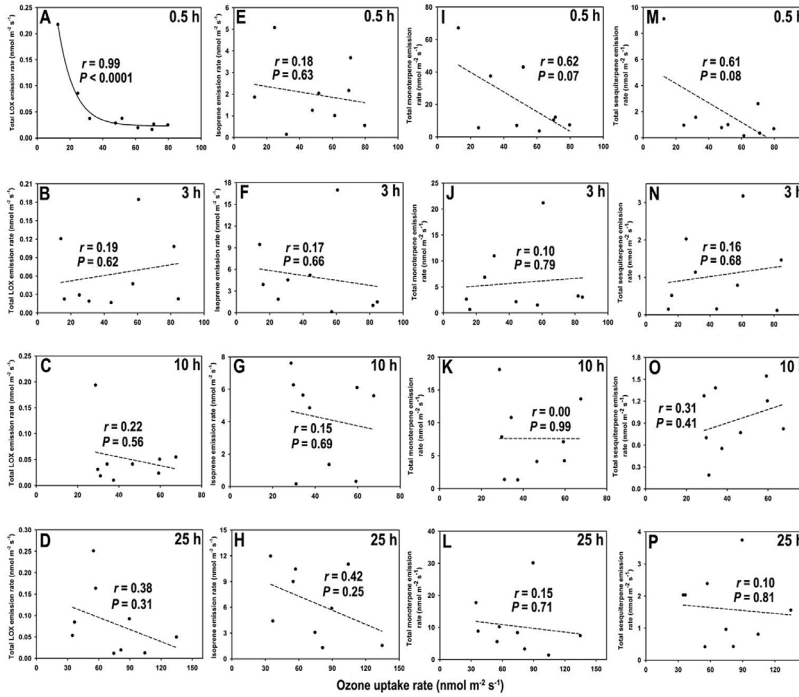

Fig. 8. Correlation of total LOX, (A-D), isoprene (E-H), total monoterpene (I-L), and total sesquiterpene (M-P) emission rates with stomatal ozone uptake rate at different recovery times (0.5, 3, 10, 25 h) after the leaves of *E. globulus* were treated with ozone (4, 5, and 6 ppm) for 3 h. Each data point indicates an individual replicate. Data were fitted by non-linear regression for Fig. 8A and linear regressions for Figs. 8B-8P.

hexenal) by a corresponding constitutively expressed alcohol dehydrogenase as reported by Davoine et al. (2006) and Farmer and Davoine (2007).

The pathway for saturated aldehydes is still uncertain (Hu et al., 2009), although the lower molecular mass compounds, C5 and C6 might partly share the same pathway with LOX (Heiden et al., 2003; Loreto and Fares, 2007; Wildt et al., 2003). In addition, ozone itself can oxidize membrane lipids and also cause oxidative degradation of lipids on the leaf surface as well (Senser et al., 1987), suggesting that some of the saturated aldehyde release might be non-biochemical. However, provided the oxidative burst is mainly confined to the initial phase of the recovery, the non-biochemical processes cannot explain the sustained release of the saturated aldehydes through the entire recovery period in our study.

In our study, emission of saturated aldehydes was ca. 2–3 higher than LOX (Figs. 3 A, D and 7). Wildt et al. (2003) also suggested that LOX volatiles could have primed for the elicitation of saturated aldehydes.

4.2.3. Influence of ozone and wounding treatments on mono- and sesquiterpene emissions

A distinguishing feature of eucalypt leaves is the presence of numerous sub-dermal secretory cavities, oil glands, where mono- and sesquiterpenes are stored (Goodger et al., 2010, 2016). In addition, leaf cuticle of *E. globulus* also stores multiple terpene products to a minor extent (Guzmán et al., 2014). As is common in terpene-storing species, there is a slow diffusion of these volatiles out of the storage compartments (so-called storage emissions, Copolovici and Niinemets, 2016; Grote et al., 2013; Grote and Niinemets, 2008). However, apart from the storage emissions, many storage-emitters also emit *de novo* synthesized mono- and sesquiterpenes in a light- and temperature-dependent manner (Grote et al., 2013; Holzke et al., 2006; Komenda and Koppmann, 2002). Ultimately, the sum of storage and non-storage emissions gives the constitutive terpene emission potential for the given species under specific ambient conditions. Furthermore, in all constitutive terpene emitters, further terpenoid emissions can be induced by abiotic and biotic stresses (Copolovici and Niinemets, 2016).

Treatment of *E. globulus* leaves with ozone, and combined ozone and wounding stress resulted in a substantial enhancement of emissions of all mono- and sesquiterpenes, albeit a strict stress dose-dependency of emission responses was not observed (Figs. 3 B, C, 5, and 6). The wounding treatment alone enhanced mono- and sesquiterpene emissions to a minor degree compared to other stress treatments through all recovery periods (Figs. 3 B, C, 5, and 6). This is surprising given that

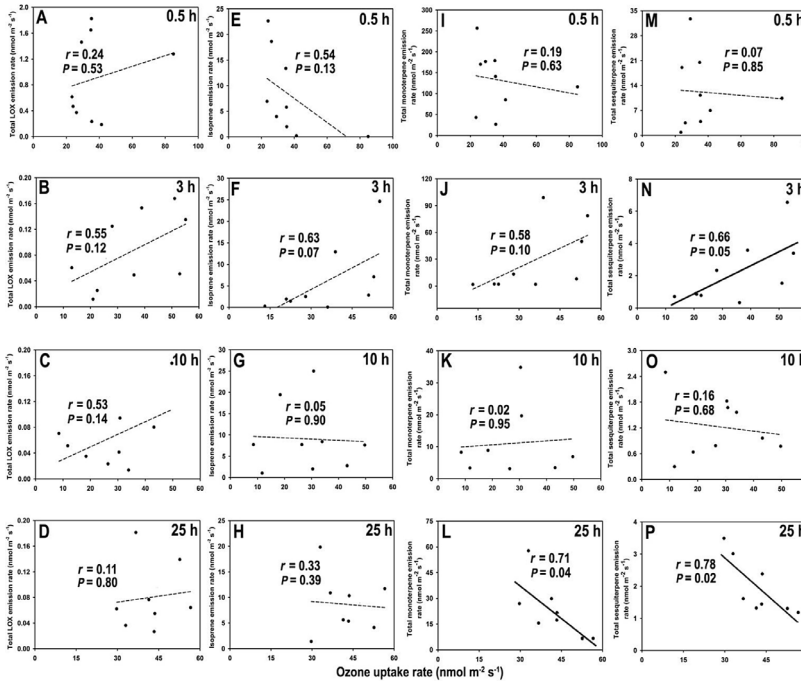

Fig. 9. Correlation of total LOX, (A-D), isoprene (E-H), total monoterpene (I-L), and total sesquiterpene (M-P) emission rates with stomatal ozone uptake rate at different recovery times (0.5, 3, 10, 25 h) after the leaves of *E. globulus* were treated with combined ozone (4, 5, and 6 ppm for 3 h) and wounding treatments. Each data point indicates an individual replicate. Data were fitted by linear regressions.

wounding leads to a direct exposure of terpene storage pools to the ambient air, while ozone stress is expected to affect less the thick-walled storage structures. Nevertheless, the highest emission burst of mono- and sesquiterpenes observed at 0.5 h of recovery could be due to the damage of epidermal cells and epithelial cells of storage glands as a result of hypersensitive response to ozone exposure (Bussotti et al., 2007), ultimately leading to increased terpene permeability of cell layers surrounding the storage structures. In our study, cellular damage was further confirmed by the burst of primary LOX emissions (Fig. 3A and Fig. 4). In addition, abrasion of leaf cuticle by acute ozone exposure could also have released some terpenoids. Association of the emission burst of mono- and sesquiterpenes with the damage of epidermal and epithelial cells of storage glands, and abrasion of cuticles at 0.5 h can explain why the emission responses were opposite to changes in net assimilation rate at the same recovery time (Figs. 1 B, 3 B, C, 5 and 6).

Once directly damaged, emissions of mono- and sesquiterpenes from free-air exposed storage structures continue for long time periods until the wounds are sealed due to terpenoid oxidization and polymerization (Loreto et al., 2000; Schuh et al., 1996). However, in our study, enhanced emissions of most mono- and sesquiterpenes were relatively short-lived (Figs. 3 B, C, 5, and 6). This suggests that either the storage structures are sealed faster or these short-living initial emissions resulted from a rapid increase in substrate availability.

Biosynthesis of monoterpenes occurs via the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway) in plastids and sesquiterpene synthesis occurs via the mevalonate pathway (MVA) in cytosol. Activity of MEP/DOXP pathway in mesophyll is primarily driven by the continuous supply of NADPH and ATP generated by photosynthetic electron transport (Niinemets et al., 2002; Rasulov et al., 2009, 2016). Thus, monoterpene emission rates and photosynthesis are often correlated (Niinemets et al., 2002; Staudt and Lhoutellier, 2011) due to direct effects of the substrate availability on monoterpene synthesis, but no such association is expected for sesquiterpene emission. In our study, there was no clear relationship between the changes in photosynthesis and monoterpene emission rates. Given that emission rates of total monoterpenes were uncoupled from photosynthesis and were broadly correlated with sesquiterpene emission rate at all recovery points, it is unlikely that substrate level control played a significant role in regulating their emission responses. Furthermore, to cope with direct damage, photosynthetic intermediates and MEP/DOXP pathway products could primarily be allocated for the biosynthesis of larger isoprenoids such as chlorophyll and carotenoids (Pazouki et al., 2016) upon ozone and wounding treatments rather than for the biosynthesis of monoterpenes, thereby temporarily reducing the rate of monoterpene synthesis until enhancement of corresponding enzyme activities as the result of the onset of gene expression responses.

In addition, as volatility of sesquiterpenes is less than that for monoterpenes, sesquiterpene pools take longer to exhaust than monoterpene pools (Copolovici and Niinemets, 2015). This may be the reason why a considerable quantity of sesquiterpenes was present throughout all recovery phases. Interestingly, we did not observe a complete cessation of mono- and sesquiterpene emissions throughout the recovery phase. Possibly, *de novo* emission of mono- and sesquiterpenes could have contributed to the volatile blend and thus, stressed leaves continuously supported higher emissions than those in control leaves at all recovery periods. However, we cannot rule out that a part of mono- and sesquiterpenes was also removed within the leaf cells in the reactions with ROS generated by oxidative stress through recovery. Therefore, we suggest that mono- and sesquiterpene emission rates can reflect three simultaneous processes: depletion of storage pools, quenching by ROS, and *de novo* synthesis.

A secondary rise of mono- and sesquiterpene emissions, especially after 10 h since stress applications (Figs. 3 B, C, 5 and 6), suggests that activation of gene expression and changes in rate-limiting enzyme activity upon ozone and wounding treatments might be responsible for the emission of individual mono- and sesquiterpenes at the later stages of recovery. In fact, several studies have demonstrated that enhanced emission of volatile isoprenoids hours to days after stress application is due to upregulation of the activity of terminal enzymes, terpene synthases (Frank et al., 2012; Pateraki and Kanellis, 2010). Given that temporal variation of volatile isoprenoid emission was associated with the emission of LOX volatiles in our study (Fig. 3A–C and Figs. 4–6), the stress-induced activation of LOX could also have induced changes in the transcript levels of lipoxygenase catalase, eventually triggering jasmonate biosynthesis. Jasmonate is a key regulator of terpene biosynthesis and can ultimately regulate the synthesis and emission of volatile isoprenoids (Schaller et al., 2004).

Among the induced responses, we observed enhanced emissions of monoterpene (*Z*)-β-ocimene (Fig. 5H) and sesquiterpene (*E*)-β-caryophyllene (Fig. 6C). Often, sesquiterpenes are stress-inducible, particularly (*E*)-β-caryophyllene (Lung et al., 2016; Toome et al., 2010). In addition, stress-induced emission of β-ocimene has been demonstrated in several studies. For example, enhanced emission of β-ocimene upon herbivory feeding of *Medicago truncatula* leaves was associated with greater transcript abundance of *M. truncatula* β-ocimene synthase (MtEBOS) (Navia-Giné et al., 2009) Analogously, enhanced β-ocimene emissions have been observed upon heat (Copolovici et al., 2012; Pazouki et al., 2016) and cold (Copolovici et al., 2012) shock in *Solanum lycopersicum*, biotrophic fungus *Melampsora* spp. infection of *Salix* spp. leaves (Toome et al., 2010), and feeding of *Alnus glutinosa* leaves by the larvae of *Cabera pusaria* (Copolovici et al., 2011). Taken together, these findings suggest that single and combined application of ozone and wounding treatments could have activated defense responses and also altered the expression rate of several terpene synthase genes and other genes in MEP/DOXP and MVA pathways, ultimately contributing to significant *de novo* emissions of (*Z*)-β-ocimene and (*E*)-β-caryophyllene.

4.2.4. Effects of ozone and wounding treatments on benzenoids and geranylgeranyl diphosphate pathway volatiles

Plant benzenoids, are biosynthesized through shikimate pathway and they are further esterified to other secondary metabolites or bound to cell walls (Zhao et al., 2010). In this study, we detected only three benzenoid compounds – benzaldehyde, *o*-xylene and *p*-xylene (Table 1) – that did not show dose-dependent emission responses (Fig. 3E). However, we did not detect the common stress-induced volatile methyl salicylate (MeSA) that triggers multiple biochemical pathways upon its release (Arimura et al., 2005; Zhao et al., 2010). To the best of our knowledge, there are no studies on the benzenoid emission from eucalypt foliage upon ozone and wounding exposure, although the impact of ozone on salicylic acid metabolism has been observed in numerous species, including tobacco (*Nicotiana tabacum*) (Beauchamp et al., 2005; Janzik et al., 2005), common bean (*Phaseolus vulgaris*) (Li et al., 2017), and beech (*Fagus sylvatica*) saplings (Betz et al., 2009; Olbrich et al., 2005), and trees (Betz et al., 2009; Jehnes et al., 2007).

In this study, GGDP pathway volatile emission was affected by both ozone and wounding stress and their combination, and the initial increase in emissions resembled that for all other compound classes (Fig. 3F) through the recovery phase. GGDP pathway products are biosynthesized in plastids (Rajabi Memari et al., 2013) and thus, the emission pattern of these volatiles probably represents the carotenoid turnover during routine plant metabolism (Beisel et al., 2010). Among the GGDP pathway volatiles, we detected the emissions of geranyl acetone and 6-methyl-5-hepten-2-one that can be emitted as a result of oxidative cleavage of carotenoids (Buttery et al., 1988; Goff and Klee, 2006; Tieman et al., 2006). Thus, the alteration in the emission pattern of these volatiles can be associated with the corresponding changes in carotenoid synthesis upon ozone and wounding exposures.

4.3. Synergistic effects of combined ozone and wounding treatments on gas exchange and stress volatile emission responses in E. globulus leaves

The simultaneous actions of several stress factors on plants typically considerably exceed the simple additive effects of the individual stresses, i.e. the simultaneous stresses act synergistically (Alexieva et al., 2003; Niinemets et al., 2017). For instance, in nature, in midsummer, high solar radiation often leads to a decline in net assimilation rate due to photoinhibition of reaction centers and photooxidation of plastid pigments, and such conditions are frequently exacerbated by concomitant heat stress, and drought (Niinemets, 2010a), implying a synergistic interaction of different stresses (Alexieva et al., 2003). However, the situation might be different for sequential stresses. Application of a short and mild stress treatment with a single stressor can alleviate the adverse effect of subsequent stress because the first stress can induce defense response (priming) (Frost et al., 2008a,b), resulting in a certain resistance to the subsequent unfavorable factors (cross-adaptation). On the other hand, a similar treatment with a single stressor could result in an increased susceptibility or hypersensitivity of the overall physiological state of the plant to the same or different stressor, and this can result in irreversible damages (cross-synergism). As the tolerance to any given stress varies strongly among species, the physiological responses upon subsequent stress applications can be species-specific. For instance, a subsequent application of low temperature stress with UV-B resulted in cross-adaptation in pea (*Pisum sativum*) plants and cross-synergism in maize (*Zea mays*) plants (Alexieva et al., 2003). In addition, recovery from multiple stresses is a complex process that cannot be predicted from the recovery of single stress (Bansal et al., 2013).

In this study, enhanced reduction of the gas exchange characteristics and increased emission responses of stress volatiles through recovery phase in combined ozone and wounding treatments (Figs. 1–7), clearly demonstrate synergistic effects. This is in accordance with previous studies that have demonstrated interactions of stressors and their impacts on stress volatile emissions, sometimes leading to highly complicated and convoluted responses. For instance, in silver birch (*Betula pendula*), the combination of elevated temperature and ozone increased the emission blend of terpenes, LOX, and MeSA, but elevated ozone alone did not change the emission of mono-, homo- or sesquiterpenes and it decreased the emission of LOX and MeSA; furthermore, elevated temperature resulted in an enhancement of total mono-, homo- and sesquiterpene, and LOX and MeSA emissions (Hartikainen et al., 2012). Similarly, combined application of drought and herbivore feeding in *Alnus glutinosa* strongly amplified the emission responses of total LOX and monoterpenes compared with both stresses applied alone (Copolovici et al., 2014). In young hybrid poplars (*Populus deltoides* cv. 55/56x *P. deltoides* cv. Imperial), combined application of elevated ozone and drought decreased isoprene emission, whereas drought

increased the emission, and ozone decreased it (Yuan et al., 2016).

Synergistic effects of combined ozone and wounding treatments can differ among species with different physiology. Since plant-produced volatile isoprenoids can quench ozone in ambient air and in leaf intercellular air spaces due to their direct reaction with ozone (Fares et al., 2010), eucalypts are expected to be highly resistant to ozone stress. However, among eucalypt species, photosynthetic characteristics differ even under unstressed conditions (Battaglia et al., 1996). In addition, there is a significant variation of constitutive and induced isoprenoid emissions among eucalypt species (He et al., 2000). Further studies across a range of eucalypt species with different constitutive and induced volatile emission capacities are needed to gain an insight into the generality of the observed synergistic wounding and ozone stress responses. Moreover, we further suggest that synergistic effect of ozone and wounding will be more severe for plant species that are low-level isoprenoid emitters or non-emitter, since the reaction of ozone with volatile isoprenoids in intercellular airspaces, inside the leaf boundary layer, and outside the leaf boundary layer is marginal in low-level isoprenoid emitters (Loreto and Fares, 2007). Thus, the actual quantity of ozone exerting oxidative stress at given ozone exposure can be considerably higher. In strong isoprenoid emitters, the severity of oxidative stress caused by given level of ozone is lower due to the higher proportion of ozone reacting with volatile isoprenoids (Loreto and Fares, 2007; Loreto et al., 2001).

4.4. Relationship between stomatal ozone uptake and stress volatile emissions

Stomatal uptake plays the main role in elicitation and emission of stress volatiles irrespective of ozone concentration around a leaf surface (Beauchamp et al., 2005). However, the ultimate damaging effect of ozone inside the leaf is determined by the balance between the quantity of ozone taken up by stomata and that scavenged by the water-soluble antioxidants within the aqueous phase in cell membrane, and by volatile isoprenoids present in leaf gas and lipid phases (Fares et al., 2010). In our study, there were no strong correlations between stomatal ozone uptake and emission rates of isoprene, total LOX, and total mono- and sesquiterpenes in treatments with ozone alone and in combination with wounding at any recovery phases (Figs. 8 and 9). Lack of clear dose-dependencies of emission responses in our study can be explained by three factors: first, the concentrations of volatile isoprenoids in intercellular spaces are typically hundreds of times higher than those above leaf boundary layer (Loreto and Fares, 2007). Therefore, once ozone enters the intercellular airspaces, it quickly reacts with volatile isoprenoids, especially with mono- and sesquiterpenes and thus, the quantity of ozone molecules entering the mesophyll cells is much lower than that taken up through stomata; second, as soon as ozone enters the surface of leaf mesophyll cells, it is also scavenged by antioxidants such as putrescine and apoplastic ascorbate in cell wall water; and third, reactive oxygen species (ROS) generated by ozone could be removed through the reaction with volatile isoprenoids (Loreto and Fares, 2007). Therefore, the quantity of ozone molecules ultimately causing a physiological stress is expected to be much lower than that entering leaf intercellular space. This is supported by our findings that at 0.5 h after stress application, the highest negative correlation was achieved for total mono- and sesquiterpenes (Figs. 8 and 9). Another implication of the possible reaction of volatiles with ROS inside the leaves is that the emission rates of isoprene, LOX, and mono- and sesquiterpenes through recovery phase were lower than the rates of their biosynthesis, further contributing to the poor correlations between volatile emission and stomatal O_3 uptake.

To further complicate the emission responses, different ozone concentrations differently affected steady-state stomatal conductance, and possibly also the rate of change in stomatal conductance. In particular, stomatal ozone uptake at 6 ppm ozone was comparatively lower than at 4 ppm and 5 ppm ozone applied both separately and in combination with wounding. This was because of lower stomatal conductance to ozone at the highest dose of ozone, contributing to the lower induction of volatile emissions at this ozone concentration (Fig. 3B and C, and Figs. 2, 5 and 6). Kinetic studies through ozone exposure period are needed to understand whether differences in the rate of stomatal closure at different ozone concentrations could play a role in poor relationships between ozone uptake and volatile emissions in the steady state.

5. Conclusion

To our knowledge, the present study is the first report of combined application of acute ozone and wounding stress on foliage gas exchange characteristics, and differential regulation of key volatile emissions from stress application through recovery. The physiological and biochemical responses of plants to a combined effect of ozone and wounding through recovery were different from the responses in plants treated with ozone and wounding separately. In particular, the reduction of photosynthetic traits such as A, g_s and F_v/F_m, and enhanced emission responses were greater for combined ozone and wounding treatments than those for separate ozone and wounding treatments. The secondary rise of mono- and sesquiterpenes through recovery phase upon all stress applications was likely associated with the upregulation of terpene synthase gene expression and/or rate limiting enzyme activity.

Collectively these data indicated that combined acute ozone and wounding treatments resulted in synergistic effects on leaf gas exchange characteristics and volatile emissions. Generally, the cross-synergism was more severe in response to 5 ppm ozone and wounding treatments than for 4 and 6 ppm ozone and wounding treatments. LOX emissions were quantitatively associated with the severity of stress, but for other volatiles, there were no quantitative relationships between the severity of stress and emission responses. The absence of stress dose vs. emission relationship was likely due to scavenging of ozone within leaf intercellular air spaces, and ROS in mesophyll cells by volatile isoprenoids.

Author's contribution

AK – Conception and design, execution of experiment, analysis and interpretation of the data, drafting of the article, and critical revision of the article for important intellectual content.

LP – Interpretation of the data and drafting of the article.

ÜN – Drafting of the article and interpretation of data.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2017.10.012.

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RESEARCH PAPER

Ozone-triggered surface uptake and stress volatile emissions in *Nicotiana tabacum* 'Wisconsin'

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Abstract

Ozone is a strong oxidant and a key stress elicitor. The immediate and longer term impacts of ozone are poorly understood in species with emission of both *de novo* synthesized and stored volatiles, such a tobacco (*Nicotiana tabacum*), which has terpene-containing glandular trichomes on the leaf surface. In this study, we exposed *N. tabacum* 'Wisconsin' leaves to acute ozone doses of 0 (control), 400, 600, 800, and 1000 ppb for 30 min and studied the effects of ozone exposure on ozone uptake, gas-exchange characteristics, and emissions of lipoxygenase pathway volatiles, monoterpenes, and sesquiterpenes. Foliage emissions of lipoxygenase pathway volatiles were quantitatively related to the severity of ozone exposure, but the stress dose *vs*. emission relationship was weaker for terpenoids. Analysis of leaf terpene content and composition indicated that several monoterpenes and sesquiterpenes were not stored in leaves and were synthesized *de novo* upon ozone exposure. The highest degree of elicitation for each compound was observed immediately after ozone treatment and it declined considerably during recovery. Leaf ozone uptake was dominated by non-stomatal deposition, and the emissions of total lipoxygenase pathway volatiles and mono- and sesquiterpenes were positively correlated with non-stomatal ozone deposition. Overall, this study demonstrates remarkably high ozone resistance of the studied tobacco cultivar and indicates that ozone's effects on volatile emissions primarily reflect modifications in the release of stored volatiles and reaction of ozone with the leaf surface structure.

Keywords: Acute ozone stress, *de novo* emission, elicitation rate, glandular trichome, LOX volatiles, monoterpenes, non-stomatal ozone deposition, sesquiterpenes, solvent extract, stomatal ozone uptake, trichome permeability.

Introduction

Ozone (O_3) is a phytotoxic gas that leads to major losses of vegetation productivity worldwide (Fares *et al.*, 2010*b*). In the troposphere, ozone formation is controlled by concentrations of NO_2 and reactive volatile organic compounds in the presence of sunlight (Fowler *et al.*, 2009; Karnosky *et al.*, 2005). Currently, tropospheric ozone concentration over most of the terrestrial surface is between 20 and 45 ppb (Sicard *et al.*, 2017), but in local pollution hotspots, much higher concentrations can be observed, e.g. in major industrial cities in China, the ozone concentration is *ca* 70 ppb (Yuan *et al.*, 2015) and in industrial cities in India, it is *ca* 50–60 ppb (Sharma *et al.*, 2013). However, even the current average

Abbreviations: BVOC, biogenic volatile organic compound; DOXP, 1-deoxy-D-xylulose 5-phosphate; GLM, generalized linear model; GLV, green leaf volatiles; LOX, lipoxygenase pathway; MEP, 2-C-methyl-D-erythritol 4-phosphate; MVA, mevalonate.

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levels of tropospheric ozone are capable of having a detrimental effect on certain sensitive crop plants, such as wheat, maize, and soybean (Rai and Agrawal, 2014).

Plants act as a sink for ozone in two ways: stomatal uptake and non-stomatal deposition (Altimir *et al.*, 2006; Fares *et al.*, 2010*b*). During stomatal uptake, ozone diffuses through the stomata into the leaf intercellular air spaces along the ozone gradient from the ambient air (Laisk *et al.*, 1989). Stomatal uptake is the primary route for ozone into the leaf mesophyll cells (Fares *et al.*, 2008). Once taken up, ozone, as a strong oxidant, can result in major oxidative stress (Schwanz *et al.*, 1996; Calfapietra *et al.*, 2013).

There is still a large uncertainty over how plant stress severity scales with ozone uptake. In particular, counterintuitively, less physiological damage has been observed in species with greater ozone uptake, especially in species with greater volatile emissions (Fares *et al.*, 2010*b*; Kanagendran *et al.*, 2018). Yet, many model species also have significant volatile-containing surface structures, in particular glandular trichomes, and it is currently unclear whether the greater ozone tolerance of species with greater ozone uptake is associated with ozone detoxification inside the leaves or on the leaf surface (Jardine *et al.*, 2012; Jud *et al.*, 2016).

Nicotiana tabacum L. is an annual herb that has terpene-filled glandular trichomes on the leaf surface and has therefore been used as a model species for ozone–plant surface reactions (Jud *et al.*, 2016). There have been several studies looking at the physiological impact of ozone on *N. tabacum* (Beauchamp *et al.*, 2005; Janzik *et al.*, 2005; Samuel *et al.*, 2005; Eltayeb *et al.*, 2006; Heidenreich *et al.*, 2006; Mancini *et al.*, 2006; Cheng and Sun, 2013; Pasqualini *et al.*, 2009; Silva *et al.*, 2012), but the majority of studies have mainly focused on short-term immediate stress responses rather than considered the full response from exposure through the recovery period. There is also a lack of knowledge of how the emissions of the lipoxygenase pathway (LOX) volatiles (also called green leaf volatiles), mono- and sesquiterpenes are regulated upon acute ozone doses from exposure through the recovery period and their relationship with foliage surface ozone uptake and elicitation rate.

There is a significant variability in ozone sensitivity among *N. tabacum* cultivars but *N. tabacum* 'Bel W3' is more sensitive to ozone than many other crop plants and therefore often used as an ozone bioindicator (Schraudner *et al.*, 1998; Krupa *et al.*, 2001). In particular, the study of Heiden *et al.* (1999) compared ozone-induced volatile emissions between the ozone-resistant cultivar 'Bel B' and ozone-sensitive cultivar 'Bel W3' and found that the elicitation of LOX volatiles and volatile isoprenoid release depended strongly on both the duration of ozone exposure and the cultivar. However, it is unclear how more resistant cultivars with potentially a greater share of surface deposition flux respond to acute ozone exposure and how immediate stress responses and gene-expression-level elicitation responses correlate with the ozone dose.

In particular, elicitation of *de novo* volatile emissions by activation of a gene-expression-level response upon ozone exposure is expected to increase through the recovery phase, while the release of volatiles from the leaf surface is expected to decrease during recovery. Thus, time kinetics of volatile responses upon ozone exposure can provide important insight into the development of oxidative stress vis-à-vis defence responses and explain non-intuitive ozone dose *vs*. physiological plant responses in different tobacco cultivars.

In this study, we used short-term acute (400–1000 ppb) ozone exposures and studied the changes in photosynthetic characteristics, emissions of LOX volatiles and mono- and sesquiterpenes, stomatal ozone uptake rates, non-stomatal ozone deposition rates, and stress-dependent elicitation rates of each volatile through the recovery phase in the ozone-resistant *N. tabacum* 'Wisconsin'. In addition, terpenoid content and composition in untreated leaves were also studied to confirm the capacity for terpenoid storage by leaves and allow separation of stored and *de novo* synthesized terpene sources. The objectives of this study were to (a) assess the relationships between ozone concentration and changes in foliage photosynthetic characteristics and emissions of LOX volatiles and mono- and sesquiterpenes from ozone exposure on through the recovery phase, and (b) distribute leaf ozone uptake between stomatal ozone uptake and surface deposition and relate the different components of ozone flux to the degree of elicitation of different volatiles. Different stressed-induced mechanisms varying from gene expression to allocation of primary intermediates can be responsible for stress-dependent changes in volatile emission.

We hypothesized that acute ozone exposure to foliage of this *N. tabacum* ozone-resistant cultivar will lead to (a) moderate reductions in gas exchange characteristics, (b) greater non-stomatal deposition rate of ozone than stomatal uptake rate, (c) alteration in LOX volatile and mono- and sesquiterpene emission rates primarily due to changes in the constitutive emissions, most likely associated with changes in trichome surface permeability, and (d) changes in stress-dependent elicitation rates of LOX volatile, mono- and sesquiterpene emissions.

Materials and methods

Plant material and growth conditions

Tobacco (*N. tabacum* 'Wisconsin') seeds were sown in 1-litre plastic pots filled with commercial potting soil (Biolan Oy, Kekkilä group, Finland). Four weeks after germination, seedlings were transplanted into 3-litre plastic pots filled with the same potting soil. The plants were maintained for the whole experimental period under a light intensity of 400–500 µmol m^{-2} s^{-1} provided by metal halide lamps (HPI-T Plus 400 W, Philips) with a 12 h photoperiod, relative humidity of 60% and day/night temperature of 24/18 °C. Plants were watered daily to soil field capacity. In addition to the nutrients provided by the soil, a liquid fertilizer (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%) was applied for optimum growth conditions. Once a week, 70 ml of diluted liquid fertilizer (*ca* 0.5 % solution) was applied to each plant until the end of the study.

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Experimental design and data collection

The experiment was conducted with the leaves of 10- to 12-week-old plants. Fully mature similar-sized leaves from similar canopy positions and a similar leaf order were used for the experiment. For each of the five ozone exposure concentrations, five arbitrarily selected leaves from a single plant were used to measure gas exchange and volatile emission rates at five recovery time points, and stomatal ozone uptake and non-stomatal ozone deposition during ozone exposures. Each leaf from each plant was separately treated with the same ozone concentration and sampled at a single recovery time point. All exposures were replicated with three individual plants including control measurements. Therefore, in total, 75 leaves (5 recovery time points × 3 plants for each exposure × 5 exposures including 0, 400, 600, 800, and 1000 ppm ozone) from 15 plants were used. In addition, there were six untreated leaves from six plants used for the estimation of contents and composition of foliage terpenoids by solvent extract analysis. This experimental design does not allow for direct assessment of leaf-to-leaf variability in emission responses, but treated leaves were measured only once to avoid possible leaf damage and release of surface volatiles when the leaves had to be removed, inserted and sealed in the leaf chamber repeatedly (see Niinemets *et al.* (2011) for discussion of the caveats of plant volatile measurements).

Experimental set-up and ozone exposure

A custom-made cylindrical double-walled glass chamber (1.2 litres) with stainless steel bottom was used for ozone treatments, gas exchange measurements and volatile sampling. As the leaf was the experimental unit in this study, individual leaves were placed in the 1.2-litre glass chamber for ozone treatment. The chamber temperature was maintained constant at 25 °C by circulating thermostatted water between the double walls of the glass chamber. Light was provided by four 50 W halogen lamps. A thermistor (NTC thermistor, model ACC-001, RTI Electronics, Inc., Anaheim, CA, USA) was used to monitor the air temperature inside the chamber, and leaf temperature was measured by a thermocouple attached to the lower side of the leaf surface. Ambient air was filtered through activated charcoal and supplied to the chamber in the form of pure and ozone-free air. A fan inserted inside the chamber provided constant high turbulence air mixing. The chamber ports could be switched between reference and measurement modes in order to take gas-exchange measurements (see Copolovici and Niinemets (2010) for further details of the glass chamber set-up). CO_2 and H_2O concentrations of the chamber inlet and outlet were measured with an infra-red dual-channel gas analyser operated in differential mode (CIRAS II, PP Systems, Amesbury, MA, USA).

Ozone was generated with a Certizon C100 ozoniser (Erwin Sander Elektroapparatenbau GmbH, Germany). Arbitrarily selected mature leaves were exposed to 400, 600, 800, and 1000 ppb ozone for 30 min, and untreated leaves were used as control. The ozone concentration in the inlet and outlet of the chamber was monitored using a UV photometric ozone detector (Model 49i, Thermo Fisher Scientific, Franklin, MA, USA).

Gas exchange measurements, volatile sampling, and gas chromatography–mass spectrometry analyses

After leaf enclosure, standard environmental conditions were established: light intensity at leaf surface of 700 µmol m^{-2} s^{-1}, leaf temperature of 25 °C, ambient CO_2 concentration of 380–400 µmol mol^{-1}, and relative air humidity of 50–60%. Once the foliage gas-exchange rates had reached a steady state, usually after *ca* 15 min of leaf enclosure, foliage gas exchange rates were recorded and volatiles were collected (control). Then the leaf was exposed to ozone for 30 min, and the measurements were repeated at 0.5, 3, 10, 24, and 48 h after the exposure. Net assimilation rate (A), and stomatal conductance to water vapour (g_s) were calculated according to von Caemmerer and Farquhar (1981).

Volatile samples were collected onto multi-bed stainless steel adsorbent cartridges connected to a portable suction pump, 210–1003 MTX (SKC Inc., Houston, TX, USA) providing a constant suction rate of 200 ml min^{-1}. A detailed description of the method and adsorbent cartridges can be found in Kännaste *et al.* (2014) and Niinemets *et al.* (2010). An air sample was taken before the leaf enclosure to estimate the chamber volatile background without the leaf. Adsorbent cartridges were analysed with a combined Shimadzu TD20 automated cartridge desorber and Shimadzu 2010 Plus gas chromatography–mass spectrometry (GC-MS) system (Shimadzu Corporation, Kyoto, Japan) for LOX volatiles, monoterpenes, and sesquiterpenes (Kännaste *et al.* (2014) and Toome *et al.* (2010) for further details about GC-MS analysis and compound identification), and the emission rates were calculated according to Niinemets *et al.* (2011).

Quantitative estimation of foliage terpene contents

Terpene content was quantitatively estimated from untreated leaves. For the chemical analyses of foliage terpene content, six fresh leaf samples (120 ± 30 mg) were randomly chosen from individual plants and weighed into a 2 ml tube with 2.8 mm stainless steel beads (Bertin Technologies, Aix-en-Provence, France). Tubes with fresh samples were instantly frozen in liquid N_2 and 1.5 ml GC-grade hexane (Sigma-Aldrich, St Louis, MO, USA) was added into each tube. The mixture was then immediately homogenized with a homogenizer (Precellys 24, Bertin Technologies) at 2400 *g* for 30 s at 25 °C. The homogenized plant material was incubated in a Thermo-Shaker (TS-100C, Biosan, Riga, Latvia) for 3 h at 1000 rpm at 25 °C. After incubation, samples were centrifuged (Universal 320R, Hettich, Tuttlingen, Germany) for 30 min at 13 200 *g* at 25 °C. An aliquot of 1 ml supernatant was pipetted into a 2 ml GC-MS glass vial (Sigma-Aldrich) for GC-MS analysis. A detailed description of GC-MS analysis and quantitative estimation of foliage terpene contents can be found in Kännaste *et al.* (2013).

Chlorophyll florescence measurements

Dark-adapted chlorophyll fluorescence yield (10 min darkening, F_v/F_m) of each ozone-treated leaf was estimated with a PAM fluorimeter (Walz IMAG-MIN/B, Walz GmbH, Effeltrich, Germany) after gas exchange measurements and volatile sampling were completed. A saturating pulse intensity of 7000 µmol m^{-2} s^{-1} was used.

Estimation of ozone uptake by individual leaves

Average stomatal ozone uptake rate and non-stomatal ozone deposition rate for the exposure period by each individual leaf at each dose of ozone was calculated from measurements of chamber incoming and outgoing ozone concentrations. First, total ozone uptake flux ($\Phi_{O3,Tot}$) was calculated as the product of chamber flow rate and difference in ozone concentrations between chamber inlets and outlets.

Average stomatal ozone uptake rate ($\Phi_{O3,S}$) was calculated as the product of average chamber O_3 concentration and average stomatal conductance for ozone (g_{O3}). The latter was estimated as the 30-min average stomatal conductance to water vapour divided by the ratio of the binary diffusion coefficients for ozone and water vapour (2.03) assuming that the intercellular ozone concentration was zero (Laisk *et al.*, 1989). This assumption might overestimate ozone uptake as ozone concentrations in the leaf interior can reach small non-zero values (Moldau and Bichele, 2002). The ratio of water vapour and ozone diffusivities was calculated using an ozone diffusion coefficient in air of 1.267×10^{-5} m^2 s^{-1} at 22.84 °C (Ivanov *et al.*, 2007) and a water vapour diffusion coefficient in air of 2.569×10^{-5} m^2 s^{-1} at the same temperature according to the theory of Chapman and Enskog (Li *et al.*, 2017; Niinemets and Reichstein, 2003). In fact, as the ratio of the collision integrals for both water vapour and O_3 changes little with temperature (calculated according to Tucker and Nelken, 1982), temperature effects on this ratio were negligible (the

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change in the ratio was less than 0.1% between 25 °C used for leaf measurements and 22.84 °C).

Cuticular ozone uptake into the leaf interior was excluded because compared with stomata, the cuticle is highly impermeable to ozone (Kerstiens and Lendzian, 1989). Ozone deposition flux was estimated as the difference between $\Phi_{O3,Tot}$ and $\Phi_{O3,S}$. A detailed description of quantifying stomatal ozone flux and non-stomatal ozone deposition can be found in Winner (1994). Stomatal ozone uptake rate and non-stomatal ozone deposition rate were calculated for each ozone exposure.

Data analyses

Measurements were replicated three times with each ozone concentration. A generalized linear model (GLM) with maximum likelihood model fitting was used to test for individual and interactive effects of ozone and the time of recovery on the emissions of total LOX volatiles, total monoterpenes, total sesquiterpenes, individual mono- and sesquiterpenes, and elicitation rate of each compound. The elicitation rates of log-transformed LOX volatiles, and mono- and sesquiterpene emission rates were calculated using the data fitted to a model selected based on the emission pattern of each compound (Beauchamp *et al.*, 2005). Here we used the sigmoidal model in the form:

$$y = y_0 + \frac{\alpha}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}.$$

In this case, the elicitation rate of each compound emission was calculated at recovery times 0.5, 10, and 48 h, compared with the emission of corresponding compounds from control leaves. Here, y_0 is the initial level of emission (emission of control) at time x_0 (0 h for control emission), a is the saturation level of emission (emission of treatment sample at each recovery point), x is the recovery time (such as 0.5, 10, and 48 h), and ($1/b$) is the elicitation rate. The elicitation rate of log-transformed emission data at each recovery point was calculated for each replicate and then the mean (±SE) elicitation rate was estimated.

Emission data were log-transformed after checking for normality of distribution of data and homogeneity of variances, in order to minimize the inequality of variances for Wald's chi-square test in the GLM. Correlation analyses of net assimilation rate, intercellular CO_2 concentration, and stomatal conductance to water vapour *vs.* ozone doses at different times through recovery were carried out. In addition, correlations of total LOX volatile, total monoterpene, and total sesquiterpene emission rates with stomatal ozone uptake rate and non-stomatal ozone deposition rate at different recovery periods were also examined by non-parametric Spearman correlations.

Elicitation rate calculation was carried out using SigmaPlot v. 12.5 (Systat Software Inc., San Jose, CA, USA). All other statistical analyses were conducted using SPSS Statistics v. 24 (IBM Corp., Armonk, NY, USA). All statistical effects were considered significant at $P<0.05$.

Results

Effects of ozone stress on foliage photosynthetic characteristics

The maximum dark-adapted photosystem II (PSII) quantum yield (F_v/F_m) significantly decreased in leaves exposed to 600–1000 ppb ozone; however, 400 ppb exposure of ozone caused only a marginal decline in F_v/F_m (Fig. 1). F_v/F_m at 0.5 h after the exposure was negatively correlated with ozone concentration (r^2=0.73, P<0.001, data not shown). For leaves exposed to 400 ppb, F_v/F_m had recovered almost fully at 48 h after exposure, but the recovery was only partial for leaves exposed to 600–1000 ppb ozone (Fig. 1).

Exposure of ozone led to a statistically significant reduction in net assimilation rate (A; Fig. 2A), stomatal conductance to water vapour (g_s; Fig. 2B), and intercellular CO_2 concentration (C_i; Fig. 2C) for all exposure concentrations. However, net assimilation rate, stomatal conductance to water vapour, and intercellular CO_2 concentration completely recovered after 48 h of recovery for all treatments (see Supplementary Fig. S1 at *JXB* online).

Emission of foliar LOX volatiles in relation to ozone dose and time of recovery

In non-treated leaves, LOX volatiles were emitted at a very low level (mean±SE=0.03 ± 0.01 nmol m^{-2} s^{-1}). Ozone treatment strongly enhanced the emission of LOX volatiles after ozone exposure (Figs 3A and 4A, B). The total emission of LOX volatiles was highest immediately after ozone exposure and decayed over the recovery phase to the pre-stress level (Fig. 3A, P<0.001 for ozone treatments and P<0.01 for the recovery time). The statistically significant ozone concentration effect reflected the highest rate of total LOX volatile emission at 1000 ppb ozone, a *ca* 160-fold higher rate compared with the control, followed by the intermediate rates at 600 and 800 ppb, and minor enhancement at 400 ppb (Fig. 3A).

The ozone-elicited LOX volatile emissions were dominated by the saturated aldehydes hexanal and pentanal throughout the time of recovery, and as for total LOX volatile emissions, the emissions were greatest at 0.5 h after the ozone exposure. In this case, ozone and recovery time had a statistically significant impact on LOX volatile emission rates (Figs 3A and 4A, B). In this study, emission of the classic LOX volatiles

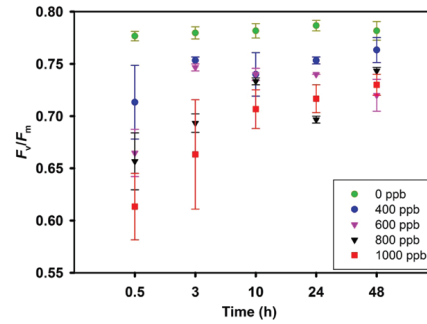

Fig. 1. Dark-adapted (10-min darkening) average (±SE) maximum fluorescence yield of photosystem II (F_v/F_m) of mature leaves of 10- to 12-week-old *Nicotiana tabacum* 'Wisconsin' at 0.5, 3, 10, 24, and 48 h of recovery following the exposure to ozone concentrations of 0, 400, 600, 800, and 1000 ppb for 30 min in a custom-made cylindrical double-walled glass chamber at 25 °C. All measurements were replicated at least three times. (This figure is available in color at *JXB* online.)

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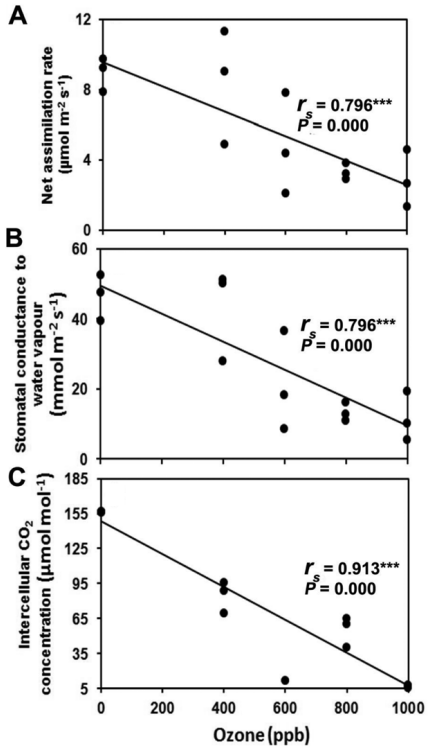

Fig. 2. Changes in leaf net assimilation rate (A), stomatal conductance to water vapour (B), and intercellular CO_2 concentration (C) of mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin' in relation to ozone concentration during exposure. The measurements were taken at 0.5 h after ozone exposure at 0, 400, 600, 800, and 1000 ppb for 30 min in a custom-made cylindrical double-walled glass chamber at 25 °C. All measurements were replicated at least three times. Gas exchange measurements were conducted at leaf temperature of 25 °C, quantum flux density of 700 μmol m^{-2} s^{-1} at leaf surface, ambient CO_2 concentration of 380–400 μmol mol^{-1}, and relative air humidity of 50–60%. Each data point corresponds to an individual replicate measurement. Data were fitted by Spearman correlations. Statistical significance of regressions is indicated as ***P<0.001.

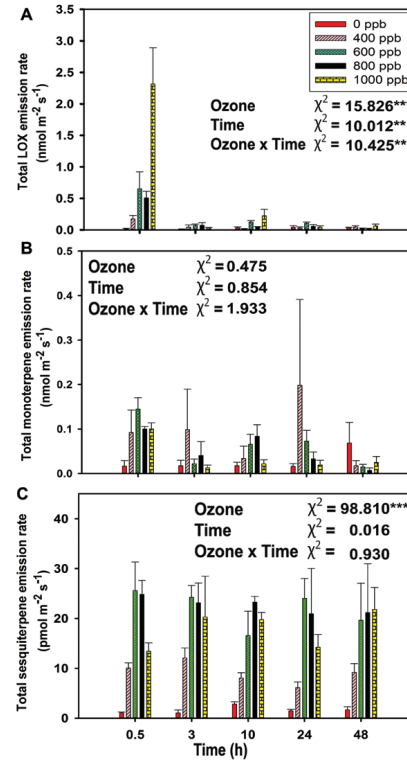

Fig. 3. Average (+SE) emission rates of total lipoxygenase pathway volatiles (LOX volatiles or green leaf volatiles, A), total monoterpenes (B), and total sesquiterpenes (C) in mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin' at 0.5, 3, 10, 24, and 48 h of recovery after ozone exposure at 0, 400, 600, 800, and 1000 ppb for 30 min in a custom-made cylindrical double-walled glass chamber at 25 °C. Data are averages of three independent replicates. Individual effects of ozone, recovery time (Time), and their interactions on emission rates were tested by GLM with maximum likelihood model fitting. Wald's chi-square (χ^2) test statistics and statistical significance are indicated as **P<0.01, ***P<0.001. (This figure is available in color at *JXB* online.)

(*Z*)-3-hexenol and (*E*)-2-hexenal was observed at trace levels only at 1000 ppb ozone dose (data not shown).

Emission responses of foliar mono- and sesquiterpenes in relation to ozone exposure and time of recovery

Non-treated leaves of *N. tabacum* were low-level emitters of monoterpenes (mean±SE rate of emission of 0.02 ± 0.01 nmol m^{-2} s^{-1}) and sesquiterpenes (1.66 ± 0.25 pmol m^{-2} s^{-1}) (Fig. 3B, C). In control leaves, the blend of emitted monoterpenes was dominated by limonene, followed by α-pinene, β-pinene, Δ^3-carene, and camphene (Fig. 5), whereas α-caryophyllene was observed as the main sesquiterpene (Fig. 6A). In addition, the monoterpene (*E*)-β-ocimene (Fig. 5D) and the sesquiterpene α-muurolene were emitted upon ozone exposure (Fig. 6B).

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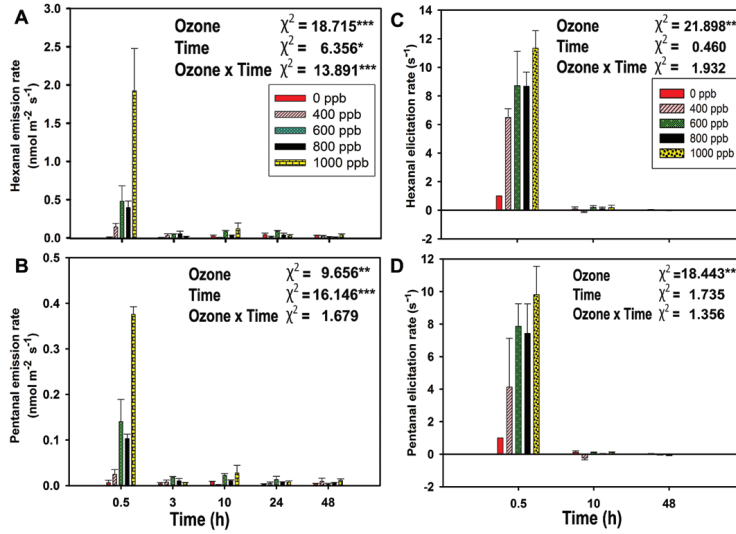

Fig. 4. Average (+SE) emission rates of the individual lipoxygenase pathway volatiles (LOX volatiles) hexanal (A) and pentanal (B) and average (+SE) elicitation rate of hexanal (C) and pentanal (D) in mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin' at different recovery times (0.5, 3, 10, 24, and 48 h for emission rate and 0.5, 10, and 48 h for elicitation rate) after ozone exposure at 0, 400, 600, 800, and 1000 ppb for 30 min in a custom-made cylindrical double-walled glass chamber at 25 °C. The elicitation rate of emissions is defined as the rate of change of emission (positive values indicate an increase of the rate and negative values a decrease of the rate). Data are averages of three independent replicates. Individual effects of ozone, recovery time (Time), and their interactions on emission rates were tested by GLM with maximum likelihood model fitting. Wald's chi-square (χ^2) test statistics and statistical significance are indicated as *P<0.05, **P<0.01, ***P<0.001. (This figure is available in color at *JXB* online.)

The effect of ozone concentration on monoterpene emissions was non-linear with the highest emissions initially observed for 600 ppb exposure at 0.5 h since the exposure (Fig. 3B). The concentration effect was more clearcut for sesquiterpene emissions to 600 ppb of exposure, and then the emission decreased with increasing ozone concentration (Fig. 3C). Monoterpene emissions of ozone-treated leaves remained similar through the recovery, except at the end of the recovery at 48 h when the emissions of treated leaves had decreased below the values observed for control leaves (Fig. 3B). In contrast, no such decline was observed for sesquiterpenes (Fig. 3C).

The time courses of individual monoterpene emissions broadly reflected the variation in total monoterpene emissions, except for Δ^3-carene, for which ozone concentration and time had a minor impact on the emissions (Fig. 5B). In addition, there were some differences in reaching peak emission and modified emission patterns through recovery for different monoterpenes. In particular, β-pinene and limonene emissions tended to peak in the leaves exposed to 400 ppb ozone at 24 h after exposure (Fig. 5C, F), but α-pinene emissions peaked at 600 ppb ozone exposure and at 0.5 h since the exposure (Fig. 5E). The emission of the characteristic stress-induced monoterpene, (*E*)-β-ocimene, was first observed after 3 h since ozone treatment, and the emission peaked for 600 ppb ozone at 10 h since the exposure (Fig. 5D).

In the case of sesquiterpenes, ozone concentration and time of recovery affected α-caryophyllene emissions similarly to total sesquiterpene emissions (Figs 3C and 6A). In contrast, there were no emission responses of α-muurolene observed from control leaves and the highest α-muurolene emissions were observed for 600 ppb ozone treatment at 0.5 h since the exposure, and the emissions decreased slightly through the recovery phase (Fig. 6B). Furthermore, we have observed γ-cadinene and γ-muurolene at trace levels in response to 600–1000 ppb ozone exposures (data not shown).

Quantitative estimation of terpene content in Nicotiana tabacum *leaves*

Four monoterpenes, Δ^3-carene, limonene, α-pinene, and β-pinene, were stored in the tissues of untreated leaves (Table 1). Δ^3-Carene was the most abundant monoterpene observed in the leaf tissues followed by α-pinene, limonene, and β-pinene. The sesquiterpenes α-caryophyllene and α-muurolene were not observed at a detectable level in leaf tissues (Table 1).

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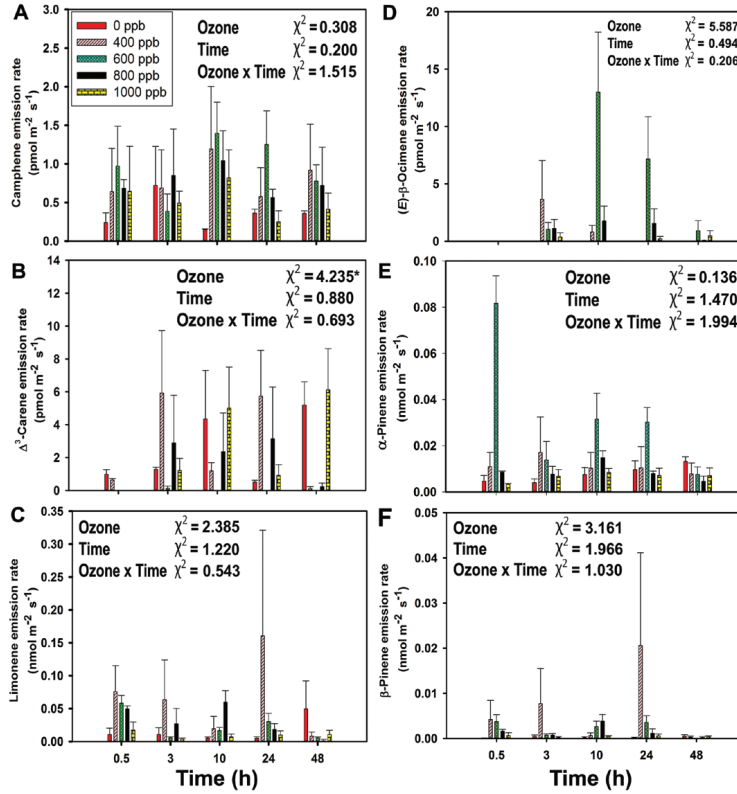

Fig. 5. Average (+SE) emission rates of key monoterpenes (A–F) from mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin' at 0.5, 3, 10, 24, and 48 h of recovery after ozone exposure at 0, 400, 600, 800, and 1000 ppb for 30 min in a custom-made cylindrical double-walled glass chamber at 25 °C. Individual effects of ozone, recovery time (Time), and their interactions on emission rates were tested by GLM with maximum likelihood model fitting. Wald's chi-square (χ^2) test statistics and statistical significance are indicated as *P<0.05. (This figure is available in color at *JXB* online.)

Elicitation rate of emissions of LOX volatiles, mono- and sesquiterpenes

At the initial enhancement, hexanal emission was *ca* five times higher than pentanal emission upon ozone stress, but the hexanal elicitation rate was almost equal to the pentanal elicitation rate. Furthermore, at 48 h of recovery, the hexanal emission rate was three times higher than the pentanal emission rate, but its elicitation rate was two times lower than the pentanal elicitation rate (Fig. 4A–D). Ozone had a statistically significant impact (P<0.001) on the elicitation rates of hexanal and pentanal (Fig. 4C, D).

β-Pinene was the monoterpene elicited to the greatest degree, followed by limonene, α-pinene, and (*E*)-β-ocimene, although limonene was the highest emitted monoterpene (Fig. 7). The lowest elicitation rate was observed for camphene. The Δ^3-carene emission rate was *ca* four times higher than the camphene emission rate but the elicitation rate of both compounds was almost equal (Figs 5 and 7). Ozone had a statistically significant impact on elicitation rates of limonene, (*E*)-β-ocimene, α-pinene, and β-pinene ((Fig. 7C–F)). The elicitation rate for (*E*)-β-ocimene was positive through the entire recovery period (Fig. 7D).

α-Muurolene was the most strongly elicited sesquiterpene, followed by α-caryophyllene. For both sesquiterpenes, ozone and recovery time significantly affected the elicitation rates (Fig. 6C, D). At 0.5 h of recovery, the α-muurolene elicitation rate was *ca* four times higher

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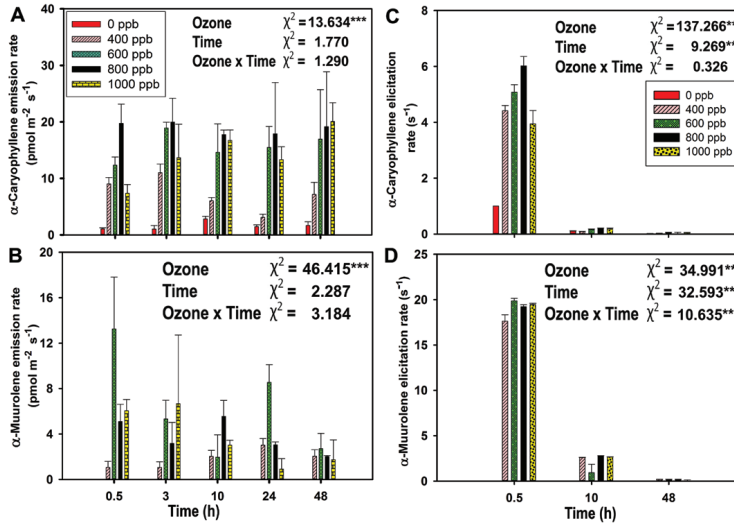

Fig. 6. Average (+SE) emission rates of α-caryophyllene (A) and α-muurolene (B) and average (+SE) elicitation rate of α-caryophyllene (C) and α-muurolene (D) in mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin' at different recovery times (0.5, 3, 10, 24, and 48 h for emission rate and 0.5, 10, and 48 h for elicitation rate) after ozone exposure at 0, 400, 600, 800, and 1000 ppb for 30 min in a custom-made cylindrical double-walled glass chamber at 25 °C. The elicitation rate of emissions is defined as the rate of change of emission (positive values indicate an increase of the rate and negative values a decrease of the rate). Data are averages of three independent replicates. Individual effects of ozone, recovery time (Time), and their interactions on emission rates were tested by GLM with maximum likelihood model fitting. Wald's chi-square (χ^2) test statistics and statistical significance are indicated as $^{**}P<0.01$, $^{***}P<0.001$. (This figure is available in color at *JXB* online.)

than the α-caryophyllene elicitation rate, although the emission rate of α-caryophyllene was *ca* two times higher than that of α-muurolene. At 48 h of recovery, the elicitation rate of both compounds reached the lowest level (Fig. 6A–D).

Emissions of LOX volatiles, mono- and sesquiterpenes in relation to stomatal ozone uptake and non-stomatal ozone deposition rates

Overall, the total LOX volatile emission rate was poorly correlated with stomatal ozone uptake rate (Fig. 8A–D), but the correlations with non-stomatal ozone deposition rate (Fig. 8E–H) were positive and statistically significant at 0.5, 10, and 24 h since the exposure (Fig. 8E, G, H). In the case of total monoterpenes, a statistically significant negative correlation between stomatal ozone uptake rate (Fig. 9D), and a marginally significant positive correlation between non-stomatal ozone deposition rate (Fig. 9H) and monoterpene emission were observed at 24 h of recovery. For sesquiterpenes, the total emission rate was negatively correlated with stomatal ozone uptake rate at all recovery times (Fig. 10A–D), and positively and significantly correlated with ozone deposition rate at 3, 10, and 24 h of recovery (Fig. 10F, G, H).

Table 1. *Terpene contents in untreated leaves of* Nicotiana tabacum *'Wisconsin'*

Data are averages (±SE) of six independent replicates. nd, not detected.

Compound	Foliage terpene content (nmol g^{-1} DM)
Camphene	nd
Δ^3-Carene	43 ± 7
Limonene	16 ± 5
(*E*)-β-Ocimene	nd
α-Pinene	20 ± 3
β-Pinene	10 ± 3
α-Caryophyllene	nd
α-Muurolene	nd

Discussion

Changes in leaf photosynthetic characteristics upon acute ozone stress

Acute ozone concentrations caused a sharp decline in the maximum dark-adapted photosystem II quantum yield (F_v/F_m), especially at the exposures of 800 and 1000 ppb (Fig. 1). A reduction in F_v/F_m is typically associated with sustained photoinhibition due to damage at PSII (Osmond

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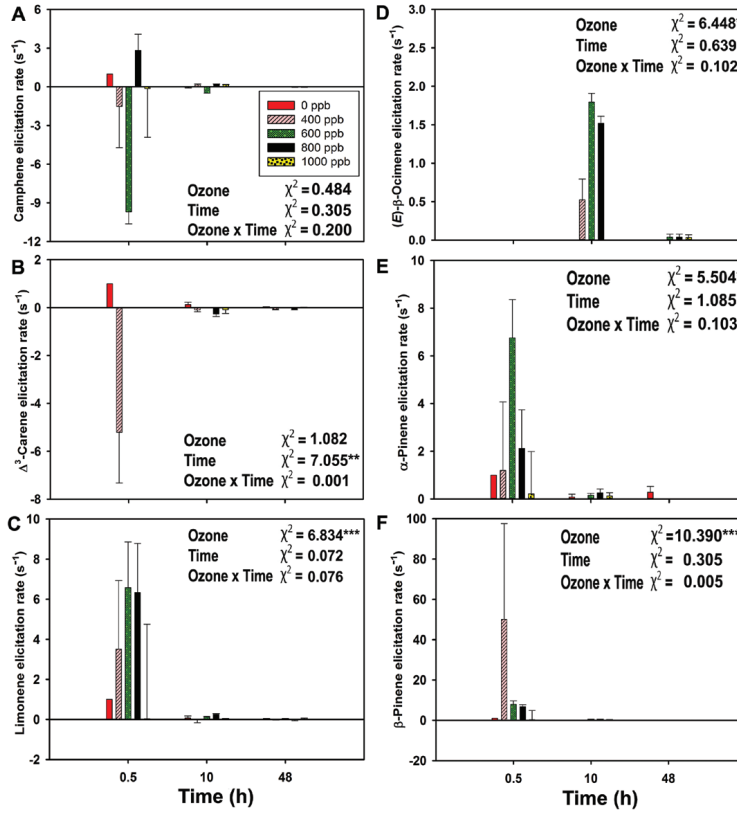

Fig. 7. Average (+SE) elicitation rate of monoterpenes in mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin' at 0.5, 10, and 48 h of recovery after ozone exposure at 0, 400, 600, 800, and 1000 ppb for 30 min in a custom-made cylindrical double-walled glass chamber at 25 °C. The elicitation rate of emissions is defined as the rate of change of emission (positive values indicate an increase of the rate and negative values a decrease of the rate). Data are averages of three independent replicates. Individual effects of ozone, recovery time (Time), and their interactions on emission rates were tested by GLM with maximum likelihood model fitting. Wald's chi-square (χ^2) test statistics and statistical significance are indicated as *P<0.05, **P<0.01, ***P<0.001. (This figure is available in color at *JXB* online.)

et al., 1999; Demmig-Adams and Adams, 2006). In fact, studies have demonstrated that exposure of leaves to elevated ozone results in increasingly deactivated PSII centres, thereby reducing the photochemical capacity of PSII (Guidi and Degl'Innocenti, 2008). Photoinhibitory effects of ozone are primarily caused by the overall net reduction of the D1 core protein of PSII (Adir *et al.*, 2003; Singh *et al.*, 2009).

There is also evidence that elevated ozone inhibits photosynthesis in a time-dependent manner due to suppression of the Calvin cycle (Guidi and Degl'Innocenti, 2008). In potato (*Solanum tuberosum*) leaves, elevated ozone inhibited the activity and reduced the quantity of ribulose-1,5-bisphosphate carboxylase/oxygenase leading to a reduced rate of photosynthesis (Dann and Pell, 1989) as was also observed in our study (Fig. 2A). However, the reductions in the photosynthetic rate and intercellular CO_2 concentration were associated with a decline of stomatal conductance to water vapour (Fig. 2A–C). Although net assimilation rate and stomatal conductance typically decrease in parallel through abiotic stresses (Copolovici *et al.*, 2012; Kask *et al.*, 2016, Kanagendran *et al.*, 2018), this

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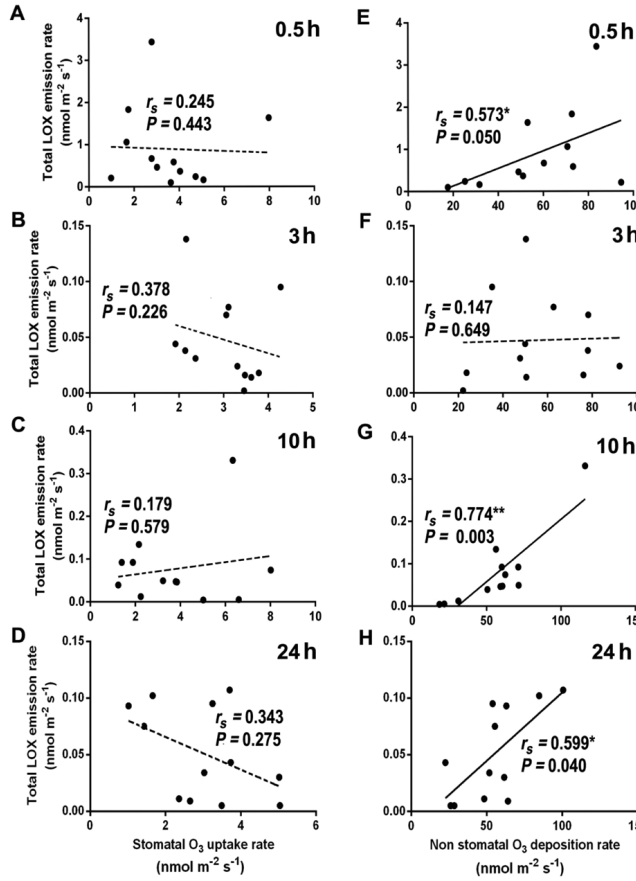

Fig. 8. Correlations of total LOX volatile emission rates with stomatal ozone uptake rate (A–D) and non-stomatal ozone deposition rate (E–H) at different times (0.5, 3, 10, and 24 h) during recovery in mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin'. Each data point indicates an individual replicate. Data were fitted by Spearman correlation. Non-significant regressions (P>0.05) are shown by dashed lines. Statistical significance is indicated as *P<0.05, **P<0.01.

result indicates that the inhibitory effect of elevated ozone on net assimilation was at least partly associated with stomatal closure.

The reductions in net assimilation rate, stomatal conductance to water vapour, and intercellular CO_2 concentration were strongly correlated with ozone concentration during the exposure (Figs 2A–C), indicating a dose-dependent response as observed in several studies (Guidi *et al.*, 2001; Degl'Innocenti *et al.*, 2002; Akhtar *et al.*, 2010). Despite treatment with high ozone concentrations, the recovery of photosynthetic characteristics at 48 h after ozone exposure indicates the high degree of ozone resistance of *N. tabacum* 'Wisconsin' used in our study (see Supplementary Fig. S1). However, from an ecological perspective, we note that the recovery times of 24–48 h are too long for a stress factor such as ozone that has a diurnal cycle. Another exposure cycle on the following day would not allow for such recovery to take place.

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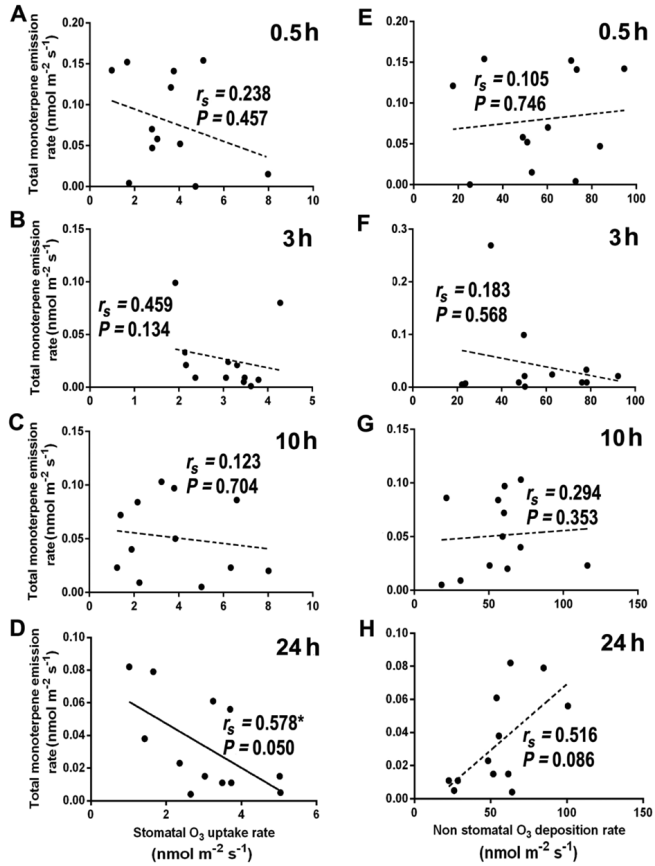

Fig. 9. Correlations of total monoterpene emission rates with stomatal ozone uptake rate (A–D) and non-stomatal ozone deposition rate (E–H) at different times (0.5, 3, 10, and 24 h) through recovery in mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin'. Data were fitted by Spearman correlation. Non-significant regressions (P>0.05) are shown by dashed lines. Statistical significance is indicated as: *P<0.05.

Effect of acute ozone exposure on the emission of lipoxygenase pathway volatiles

Multiple lipoxygenases are constitutively active in plant cells, and once their substrates, polyunsaturated fatty acids, become available, LOX volatiles are rapidly released from membranes (Feussner and Wasternack, 2002). Typically, the release of polyunsaturated fatty acids from membranes and concomitant release of LOX volatiles are considered to be an acute stress response associated with major membrane-level damage (Jiang *et al.*, 2016, 2017). However, the situation with ozone might be more complicated as ozone itself can cause oxidative degradation of membrane lipids as well as oxidize cuticular lipids on the leaf surface (Senser *et al.*, 1987; Slimestad *et al.*, 1995).

The typical LOX volatiles emitted upon acute abiotic stress are (*E*)-2-hexenal, (*Z*)-3-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate (Heiden *et al.*, 2003; Copolovici and Niinemets, 2010; Copolovici *et al.*, 2012). Indeed, Heiden *et al.* (1999) reported emission of these classical LOX volatiles upon acute ozone fumigation of 175 ppb for 5 h and

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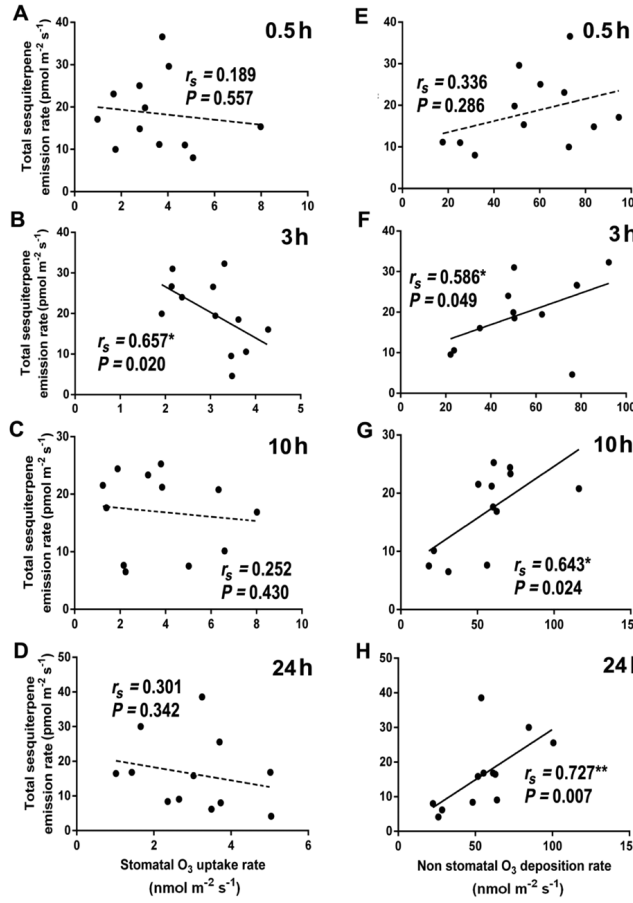

Fig. 10. Correlations of total sesquiterpene emission rates with stomatal ozone uptake rate (A–D) and non-stomatal ozone deposition rate (E–H) at different times (0.5, 3, 10, and 24 h) during recovery in mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin'. Data were fitted by Spearman correlation. Non-significant regressions (P>0.05) are shown by dashed lines. Statistical significance is indicated as: *P<0.05, **P<0.01.

150 ppb for 11 h from ozone-sensitive *N. tabacum* 'Bel W3' and ozone-resistant *N. tabacum* 'Bel B'. In our study, however, the emission of (*E*)-2-hexenal and (*Z*)-3-hexenol was observed at trace level at the highest ozone doses, and there was no emission of 1-hexanol and (*Z*)-3-hexenyl acetate upon elevated ozone. This suggests that the impact of ozone exposure on LOX volatiles depends on ozone concentration and exposure duration used and also varies among cultivars with different ozone sensitivity. In our study, we observed that the oxygenated volatile emissions were dominated by the saturated aldehyde hexanal followed by the saturated aldehyde pentanal and that these emissions were related to ozone concentration in a dose-dependent manner right after ozone exposure at 0.5 h (Figs 3A and 4A, B). The formation of hexanal is partly attributed to the breakdown of membrane lipids by lipoxygenase and hydroperoxide lyase enzymes (Heiden *et al.*, 2003), and thus the emission of hexanal following ozone fumigation may be associated with the

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direct effect of the lipoxygenase cleavage of fatty acids in the leaf membranes exposed to ozone (Paoletti, 2006). Similar to our study, Carriero *et al.* (2016) observed that the dominant LOX volatile was hexanal emitted following short-term acute ozone exposures in silver birch (*Betula pendula*). All these reports suggest that hexanal is a ubiquitous LOX volatile product emitted in relation to acute ozone stress. Nevertheless, we cannot rule out alternative biochemical reactions leading to saturated aldehyde formation (Wildt *et al.*, 2003) or direct reactions of ozone with leaf surface lipids or elicitation of LOX volatile production within leaf surface structures such as secretory cells feeding leaf glandular trichomes. At least the rapid cessation of LOX volatile emissions (Figs 3A and 4A, B) suggests that even the highest ozone exposures did not result in major cellular damage, consistent with almost full recovery of leaf photosynthetic characteristics (Figs 1 and 2 and Supplementary Fig. S1).

Effect of acute ozone exposure on the emission of mono- and sesquiterpenes

Mainly due to the presence of glandular trichomes, *N. tabacum* is a constitutive low-level terpene emitter under non-stressed conditions. Relatively low levels of terpene emissions observed for non-stressed leaves are likely associated with a limited storage capacity for monoterpenes in glandular trichomes in tobacco (Table 1; Ohara *et al.*, 2003), compared with less volatile diterpene storage in trichomes (Cui *et al.*, 2011).

Upon ozone exposure, mono- and sesquiterpene emissions were generally enhanced, but the responses varied in time for different ozone doses (Figs 3B, C, 5, and 6A, B). Although total monoterpene emission was enhanced by ozone, it peaked at ozone concentrations of 400–600 ppb at different times during recovery and ultimately decreased to levels below that observed in control leaves at 48 h after exposure (Fig. 3B). In contrast, sesquiterpene emissions were more exposure dose dependent and weakly decreased over the recovery period (Fig. 3C). This difference between the two terpenoid classes is puzzling and might be associated with different metabolic pathways responsible for synthesis of these volatiles. In the case of monoterpenes, the synthesis occurs through the plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP)–1-deoxy-D-xylulose 5-phosphate (DOXP) pathway, whereas sesquiterpene synthesis occurs through the mevalonate-dependent pathway in the cytosol (Rajabi Memari *et al.*, 2013; Rosenkranz and Schnitzler, 2013; Pazouki and Niinemets, 2016). In the mesophyll cells, the MEP–DOXP pathway activity is strongly dependent on immediate availability of NADPH and ATP generated by photosynthetic electron transport (Niinemets *et al.*, 2002; Rasulov *et al.*, 2009), and thus modifications in the activity of photosynthesis are expected to impact the substrate availability for monoterpene synthesis. In contrast, no such photosynthetic control is expected on the rate of sesquiterpene synthesis. Indeed, elevated ozone, 800–1000 ppb, significantly inhibited photosynthesis, and in turn it decreased emission rates of monoterpenes compared with the control treatment, suggesting that such a substrate-level control might be possible. However, monoterpene emission was actually initially enhanced for lower ozone concentrations and the substrate-level control also does not explain the decline of monoterpene emission, especially at 48 h when photosynthesis had almost fully recovered (cf. Figs 1, 2 and 3B, and Supplementary Fig. S1). Such a discrepancy might be associated with the circumstance that under ozone stress, a higher proportion of MEP–DOXP pathway products might have been allocated to the replacement of non-volatile larger isoprenoids, such as chlorophyll and carotenoids, oxidized upon ozone exposure (Pazouki *et al.*, 2016). In addition, as the stress might alter the allocation of photosynthates between carbon storage and biosynthesis of new chemicals (Fichtner *et al.*, 1994), full recovery of photosynthesis right after stress does not always guarantee that photosynthetic products will be high enough to support the rate of biosynthesis of secondary compounds.

An alternative explanation to the observed discrepancy between changes in photosynthesis and monoterpene emissions is that monoterpenes were primarily emitted from terpene pools stored in glandular trichomes (Table 1). Ozone exposure can enhance trichome emissions by damage to the trichome surface, thereby enhancing terpene permeance (Jud *et al.*, 2016). On the other hand, ozone itself can oxidize the terpenes released at a constant rate from trichomes (Pinto *et al.*, 2007), and this could explain the decreased emissions at the highest ozone concentrations (Fig. 3B). Furthermore, once damaged, discharge of terpene pools can be responsible for the monoterpene emission rates such that they reached values lower than those observed in control leaves. In the case of sesquiterpenes, changes in the emissions can also be explained by ozone effects on trichome surface permeability, albeit that sesquiterpenes are much less volatile than monoterpenes (Copolovici and Niinemets, 2005; Copolovici and Niinemets, 2015), and thus emptying the pools is expected to take much longer than for monoterpenes. However, this still does not explain why there was an apparent dose dependence for sesquiterpenes and not for monoterpenes.

Contribution of terpenoid synthesized de novo *and from storage pools to the terpenoid emission*

Quantitative estimation of terpene contents in tobacco leaves demonstrated that Δ^3-carene was the most abundant monoterpene stored in fresh leaf tissues followed by α-pinene, limonene, and β-pinene. However, the blend of monoterpenes emitted by both ozone-treated and untreated leaves was dominated by limonene, followed by α-pinene and β-pinene (Fig. 5). Therefore, a major portion of the emitted limonene is likely synthesized *de novo*, but camphene and (*E*)-β-ocimene were fully *de novo* synthesized in this study. Similarly, as there were no traces of α-caryophyllene and α-muurolene found in the leaf tissues, those two sesquiterpenes were ultimately *de novo* synthesized (Table 1 and Fig. 6A, B).

Ozone-dependent modification of the blend of emitted terpenoids and time kinetics of elicitation rates of LOX volatiles and terpenoids

Several studies have demonstrated that upregulation of terminal enzymes following a stress was responsible for

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the enhanced emission of volatile isoprenoids (Singh and Johnson-Flanagan, 1998; Frank *et al.*, 2009; Pateraki and Kanellis, 2010). In a study with the ozone-sensitive tobacco cultivar 'Bel W3', Beauchamp *et al.* (2005) further demonstrated significant emissions of methyl salicylate (MeSA), suggesting a major physiological stress response upon ozone exposure in this sensitive cultivar. In our study, emissions of six monoterpenes and two sesquiterpenes were observed but no traces of MeSA. Lack of MeSA emission together with the lack of characteristic LOX volatiles (see above) further suggests that the observed modifications in terpenoid emissions, particularly Δ^3-carene, α-pinene, and β-pinene, in this ozone-resistant cultivar primarily reflected changes in the constitutive emissions, most likely associated with changes in trichome surface permeability, further supported by correlation analysis and solvent extract analysis (Figs 8–10 and Table 1).

Nevertheless, lack of storage capacity and the characteristic emission response observed suggested elicitation of *de novo* synthesis of (*E*)-β-ocimene upon ozone exposure. In particular, relatively low-level emissions of (*E*)-β-ocimene were detected at later stages during recovery, with the emissions first observed at 3 h and peaking at 10 h since the exposure (Fig. 5D), suggesting that ozone exposure did activate defence responses. (*E*)-β-Ocimene has been considered an iconic stress monoterpene, and its emissions have been shown to be elicited upon biotic and abiotic stresses including exposure to high doses of ozone in *Phaseolus vulgaris* (Li *et al.*, 2017), cold and heat shock in *Solanum lycopersicum* (Copolovici *et al.*, 2012; Pazouki *et al.*, 2016), feeding by larvae of beet armyworm (*Spodoptera exigua*) in *Medicago truncatula* (Navia-Giné *et al.*, 2009), and feeding by larvae of common white wave (*Cabera pusaria*) in leaves of *Alnus glutinosa* (Copolovici *et al.*, 2011).

Sesquiterpenes, including α-caryophyllene, are also commonly considered stress-inducible (Lung *et al.*, 2016), but in our study it was α-muurolene, emission of which was lacking in control leaves and was induced during the recovery phase (Fig. 6B). Thus, this evidence suggests that ozone treatment could have changed the expression of several terpene synthase genes in our study, leading to a certain modification in the blend of emitted volatiles, albeit the influence of *de novo* gene expression on total emission was likely minor in our study.

A great variability in the emission rates of each compound, despite similar elicitation rates, can be explained by several factors governing the underlying mechanism, such as the following. (i) Ozone exposure can trigger an array of reactions and mechanisms varying from elicitation of gene expression to protein turnover and signal transduction, and all these process are time consuming. At the onset of ozone treatment, LOX volatile elicitation rate is primarily determined by the rate of free fatty acid break-down, resulting in an initial emission burst; higher emission rates of mono- and sesquiterpenes and concomitantly higher elicitation rate can further partly rely on the rapid increase of trichome permeability and, thus, the release of stored terpenes. On the other hand, the depletion of terpene contents in the storage structures leads to declining elicitation rates, ultimately even to negative values. (ii) Expression of an array of genes in the LOX, MEP, and MVA pathways is elicited after some hours of stress application and has a substantial impact on emission responses along with substrate level control (Pazouki *et al.*, 2016), especially at 10 h of elicitation in this study. However, at 48 h of recovery, the elicitation rate became negative for most of the compounds, reflecting lower emission rates than in controls. And (iii) there are physiological and physico-chemical limitations within the leaf mesophyll that control the volatile diffusion at the leaf–atmosphere interface, such as the availability of ATP, NADPH, and glyceraldehyde 3-phosphate; maximum activity of rate-controlling enzymes; compound gas-phase partial pressure; and aqueous- and lipid-phase concentrations (Niinemets and Reichstein, 2003 for a review). While physiological limitations can affect the maximum synthesis rate of volatiles, physico-chemical limitations on *de novo* synthesized volatiles, except for lipophilic compounds such as non-oxygenated terpenes, can have a substantial influence on the time lags between synthesis and emission.

Emission responses of stress volatiles in response to stomatal ozone uptake and non-stomatal ozone deposition

Ozone uptake by the leaves from the ambient atmosphere occurs by two means, stomatal uptake and by surface deposition. These means of uptake have fundamentally different physiological impacts. In addition, once taken up, the ultimate damaging effect of ozone depends on the extent to which ozone is scavenged by water-soluble antioxidants within the aqueous phase in cell walls and by volatile antioxidants within the leaf gas phase as well as by lipid-soluble volatile and non-volatile antioxidants. Among these volatile antioxidants (Kurpius and Goldstein, 2003; Goldstein *et al.*, 2004; Bouvier-Brown *et al.*, 2009), mono- and sesquiterpenes are particularly reactive with ozone (Fares *et al.*, 2010*a*).

Analysis of the distribution of ozone flux between stomatal uptake and surface deposition indicated that stomatal absorption of ozone was much lower than the non-stomatal deposition for *N. tabacum* 'Wisconsin' (Figs 8–10). We argue that the high surface deposition flux reflects the presence of terpene-filled glandular trichomes on the leaf surface, and accordingly direct reaction of ozone with unsaturated terpenoids, e.g. unsaturated semi-volatile compounds such as *cis*-abienol ($C_{20}H_{34}O$), present on the *N. tabacum* leaf surface that can act as an efficient sink for ozone and a powerful chemical barrier against stomatal ozone uptake at the leaf surface (Jud *et al.*, 2016)—but, of course, other terpenoids including mono- and sesquiterpenes can contribute to ozone detoxification. Furthermore, non-stomatal deposition, itself due to volatiles produced by the plant, not only occurs at the leaf surface, but also can occur within the leaf boundary layer and even outside the boundary layer (Altimir *et al.*, 2006; Fares *et al.*, 2008).

Comparisons of the correlations of total emission rates of LOX volatiles, mono- and sesquiterpenes with stomatal ozone uptake and with non-stomatal ozone deposition indicated that the correlations were stronger with non-stomatal

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deposition (Figs 8A–D, 9A–D, and 10A–D *vs.* Figs 8E–H, 9E–H, and 10E–H). Counterintuitively, in several cases, total emission rates of mono- and sesquiterpenes were negatively correlated with stomatal ozone uptake. There can be several explanations for these negative correlations. First, when ozone enters the leaf mesophyll cells it can rapidly react with water-soluble antioxidants such as putrescine and apoplastic ascorbate, and therefore the amount of ozone trigging stress is typically much lower than that entering through stomata (Laisk *et al.*, 1989). Second, volatile isoprenoids can react directly with ozone inside the leaves or with ozone-induced reactive oxygen species (ROS; Loreto and Fares, 2007), resulting in lower rates of emission of mono- and sesquiterpenes than their biosynthesis rates. Lack of correlation immediately after the exposure suggests that terpene reactions with ozone inside the leaves were unlikely to have played a role in these negative correlations. Yet, there is typically a secondary rise of ROS several hours after the initial stress impact (Beauchamp *et al.*, 2005; Jiang *et al.*, 2017; Li *et al.*, 2017), and it is plausible that the correlations observed 3–24 h after the ozone exposure reflected isoprenoid reactions with the endogenously generated ROS.

The positive correlations among non-stomatal ozone deposition and total LOX volatile and mono- and sesquiterpene emissions (Figs 8E–H, 9E–H, and 10E–H) are wholly consistent with the hypothesis of the surface release of volatiles from glandular trichomes or impacted secretory cells. These results collectively suggest that a significant part of volatile emission in response to ozone exposure was release from the leaf surface.

Conclusions

Ozone is believed to affect plants in a dose-dependent manner, i.e. the effects depend both on ozone concentration above the threshold value (concentration eliciting a physiological response, typically a low value of 40 ppb is assumed) and the duration of the ozone exposure (Beauchamp *et al.*, 2005; Niinemets *et al.*, 2010), but as our study demonstrates, the ozone dose responses can be complicated due to surface reactions. Taken together, these results from one stress cycle, with a long time for recovery, indicate that acute ozone exposure led to a significant reduction of foliar gas-exchange characteristics, but the reductions in all these characteristics were almost fully reversible even at the highest ozone concentrations underscoring the high ozone resistance of *N. tabacum* 'Wisconsin'. The emission rate of LOX volatiles was dose dependent, whereas the dose dependences for terpenes, especially for monoterpenes, were weaker, but *de novo* elicitation of terpenoid synthesis was moderate, again corroborating the high ozone resistance of this genotype.

In this study, most of the ozone flux to the leaf was due to non-stomatal ozone deposition, and non-stomatal ozone deposition scaled with the release of terpenes, suggesting that terpenoid emission responses to ozone mostly reflected modification of terpene release from trichomes on the leaf surface. The results further suggest that the surprisingly high ozone resistance of *N. tabacum* 'Wisconsin' is likely due to surface reactions of volatiles released upon ozone exposure. This study demonstrated that, counterintuitively, in this ozone-resistant tobacco cultivar, the quantity of ozone uptake through stomata is not proportional to the volatile emission rates. Overall, there was a substantial variability of emission rates of each compound upon similar ozone uptake, suggesting that several genetic, physiological, and physiochemical factors control elicitation and emission of volatile compounds upon acute ozone stress in a complex manner. Further studies are required to understand the complex linkages between elicitation and emission due to genetic, physiological, and physiochemical factors in multiple tobacco cultivars with varying ozone surface and stomatal deposition fluxes.

Supplementary data

Supplementary material is available at JXB online.

Fig. S1. Changes in leaf net assimilation rate (A), stomatal conductance to water vapour (g_s), and intercellular CO_2 concentration (C_i) of mature leaves of 10- to 12-week-old *N. tabacum* 'Wisconsin' in relation to ozone concentration during exposure.

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LIST OF PUBLICATIONS

1. Pazouki, L., **Kanagendran, A**., Li, S., Kännaste, A., Rajabi Memari, H., Bichele, R., Niinemets, Ü. (2016). Mono- and sesquiterpene release from tomato (*Solanum lycopersicum*) leaves upon mild and severe heat stress and through recovery: from gene expression to emission responses. *Environmental and Experimental Botany* 132, 1–15.
2. **Kanagendran**, **A**., Pazouki, L., Niinemets, Ü. (2018). Differential regulation of volatile emission from *Eucalyptus globulus* leaves upon single and combined ozone and wounding treatments through recovery and relationships with ozone uptake. *Environmental and Experimental Botany* 145, 21-38.
3. **Kanagendran**, **A**., Pazouki, L., Li, S., Liu, B., Kännaste, A, Niinemets, Ü. (2018). Ozone-triggered surface uptake and stress volatile emissions in *Nicotiana tabacum* 'Wisconsin'. *Journal of Experimental Botany*, 69, 681-697.
4. Li S, Tosens T, Harley PC, Jiang Y, **Kanagendran A**, Grosberg M, Jaamets K, Niinemets Ü (2017). Glandular trichomes as a barrier against atmospheric oxidative stress: relationships with ozone uptake, leaf damage and emission of volatiles across a diverse set of species. *Plant, Cell & Environment*, In Press.

CONFERENCE ABSTRACTS

1. **Kanagendran A**, Pazouki L, Li S, Niinemets Ü (2017). Impact of elevated ozone and wounding on foliage stress volatile emissions and gas exchange of Tasmanian blue gum (*Eucalyptus globulus*). Global Conference on Plant Science and Molecular Biology, Valencia, Spain.
2. **Kanagendran A**, Pazouki L, Li S, Niinemets Ü (2017) Effects of acute ozone exposure on the release of stress volatiles and the expression of a monoterpene synthase gene in *Nicotiana tabacum* leaves through recovery. *3rd Global Summit on Plant Science*, Rome, Italy.
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TAIMELEHTEDE LENDUVÜHENDITE EMISSIOONI INDUKTSIOON OSOONI JA METÜÜL JASMONAADI MÕJUL
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