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Disproportionate photosynthetic decline and inverse relationship between constitutive and induced volatile emissions upon feeding of *Quercus robur* leaves by large larvae of gypsy moth (*Lymantria dispar*)

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Abstract

Gypsy moth (*Lymantria dispar* L., Lymantriinae) is a major pest of pedunculate oak (*Quercus robur*) forests in Europe, but how its infections scale with foliage physiological characteristics, in particular with photosynthesis rates and emissions of volatile organic compounds has not been studied. Differently from the majority of insect herbivores, large larvae of *L. dispar* rapidly consume leaf area, and can also bite through tough tissues, including secondary and primary leaf veins. Given the rapid and devastating feeding responses, we hypothesized that infection of *Q. robur* leaves by *L. dispar* leads to disproportionate scaling of leaf photosynthesis and constitutive isoprene emissions with damaged leaf area, and to less prominent enhancements of induced volatile release. Leaves with 0% (control) to 50% of leaf area removed by larvae were studied. Across this range of infection severity, all physiological characteristics were quantitatively correlated with the degree of damage, but all these traits changed disproportionately with the degree of damage. The net assimilation rate was reduced by almost 10-fold and constitutive isoprene emissions by more than 7-fold, whereas the emissions of green leaf volatiles, monoterpenes, methyl salicylate and the homoterpene (3*E*)-4,8-dimethy-1,3,7-nonatriene scaled negatively and almost linearly with net assimilation rate through damage treatments. This study demonstrates that feeding by large insect herbivores disproportionately alters photosynthetic rate and constitutive isoprene emissions. Furthermore, the leaves have a surprisingly large capacity for

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enhancement of induced emissions even when foliage photosynthetic function is severely impaired.

Keywords

green leaf volatiles; induced emissions; isoprene emission; large insect herbivores; monoterpene emission; photosynthesis; quantitative responses; volatile organic compounds

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Introduction

In field environments, plants are frequently exposed to a multitude of abiotic and biotic stressors. To cope with environmental and biological stressors, more than 100,000 secondary chemical products are synthesized by plants and at least 1,700 of these are known to be volatile (Bauer et al., 1998; Copolovici and Niinemets, 2016; Kesselmeier and Staudt, 1999; Pichersky and Gershenzon, 2002). These biogenic volatile organic compounds (BVOC) serve many functions such as pollinator attraction (Lucas-Barbosa, 2016) and protection of plants against herbivore attacks (Heil, 2014; Pichersky and Gershenzon, 2002; Poelman, 2015), against excess temperatures (Becker et al., 2015; Possell and Loreto, 2013), and oxidative stress, e.g. that generated by ozone exposure (Loreto and Schnitzler, 2010; Possell and Loreto, 2013; Vickers et al., 2009).

While constitutive volatile emissions occur in only a limited number of species (Fineschi et al., 2013), stress-driven volatile emissions can be induced by abiotic and biotic stresses in all plant species (Copolovici and Niinemets, 2016; Harrison et al., 2013; Niinemets, 2010). Key biotic stresses eliciting major volatile emission responses are infestations by fungi, bacteria, herbivores and insects (Copolovici and Niinemets, 2016; Niinemets et al. 2013). Different biotic stressors elicit the same major classes of volatile compounds, green leaf volatiles (such as C5 and C6 alcohols and aldehydes), ubiquitous (e.g., α-pinene, β-pinene, Δ-3-carene) and specific monoterpenes (e.g., β-ocimene, linalool), sesquiterpenes (e.g. β-caryophyllene) and homoterpenes ((3*E*)-4,8-dimethy-1,3,7-nonatriene (DMNT) and (3*E*, 7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT)) (Blande et al., 2014; Copolovici and Niinemets, 2016; Holopainen, 2011; Kleist et al., 2012). However, as comparative experiments among different stresses, e.g., among infested, mechanically damaged and jasmonic acid-treated plants (Giorgi et al., 2015), and among leaf wounding and darkening (Brilli et al., 2011), demonstrate, different biotic stresses result in different volatile emission fingerprints.

Stress-induced volatiles can directly protect plants against biotic stress by serving as repellents of herbivores (Lucas-Barbosa et al., 2011) or inhibitors of fungal and bacterial growth (Schmidt et al., 2016). They can also confer indirect defense by attracting predators to their herbivore prey or oviposition host (Arimura et al., 2000a; Dicke and Baldwin, 2010; Johnson and Gilbert, 2015; Koski et al., 2015). For both functions, the composition of the emission blend as well as the total emission rates play important roles, the first determining the specificity of the signal for repelling or attraction, and the second, determining the dispersal of the signal as well as its chemical and physiological activity. Herbivore-induced changes in emissions of volatiles have been shown in numerous papers (Dicke, 2016; Heil,

2014 for reviews), but only a few studies have focused on quantitative relationships between the degree of damage and amount of compounds emitted (Bruce, 2015; Copolovici et al., 2014a; Holopainen and Gershenzon, 2010; Niinemets et al., 2013). Presence of correlations among the degree of herbivory damage and emission of volatiles seems trivial, however, it crucially depends on local and systemic emission responses. While local response in immediately impacted leaf areas is induced rapidly, e.g., emissions of green leaves volatles (GLV) can occur within seconds due to constitutive activity of lipoxygenases (Portillo-Estrada et al., 2015), induction of emissions of mono- and sesquiterpenes that need *de novo* expression of responsible synthases is more time-consuming, from hours to days (Copolovici et al., 2014a; Copolovici et al., 2011; Pazouki et al., 2016). Also, the induction response is quenched relatively rapidly, within a few days upon relief of herbivory stress (Copolovici et al., 2014a; Copolovici et al., 2011). Thus, the whole leaf response is a complex amalgamate of local and systemic induction and quenching responses. This is biologically highly relevant considering the huge diversity of herbivore impacts plants encounter in the field. While the rate of leaf area consumption is low for small solitary herbivores, leaving plenty of time for systemic responses in non-impacted leaf areas, simultaneous presence of many small herbivores and large solitary herbivores can rapidly consume a major proportion of leaf area, implying that the systemic emission response might not even occur in the major part of the leaf. In addition, while small herbivores mainly consume the intercostal leaf parts, large herbivores can also consume major veins and lead to catastrophic dysfunction of leaf water-, nutrient- and carbohydrate-conducting networks (Sack et al., 2003; Sack et al., 2004). Infections by small herbivores can lead to compensatory increases of leaf photosynthetic activity in remaining leaf parts, but the damage of major veins could mean that feeding by large herbivores can lead to disproportionate reductions in foliage physiological activity, including photosynthetic activity and constitutive isoprenoid emissions as well as hindered elicitation of induced emissions (Copolovici et al., 2014a; Copolovici et al., 2011).

Quantitative understanding of stress-dependent elicitation of volatile organic compound emission is further relevant for large-scale processes in biosphere-atmosphere system. This is because BVOCs affect atmospheric OH radical and O_3 concentrations and participate in the formation of secondary organic aerosols (SOA) (Shen et al., 2013; Zhang et al., 2015; Ziemann and Atkinson, 2012). For example, in the Southeastern United States BVOC emissions can have a significant influence on the total aerosol burden due to condensation on acidic sulfate seed aerosols and concomitant growth of particles (Lee et al., 2012; Link et al., 2015). The current estimates of BVOC emissions and their role in atmospheric processes only consider constitutive emissions (Arneth et al., 2011; Guenther, 2013; Guenther et al., 2012), but stress-dependent elicitation of volatiles can significantly modify the overall volatile blend and amount of volatiles released into the atmosphere (Hare, 2011; Holopainen and Blande, 2013; Holopainen and Gershenzon, 2010; Niinemets et al., 2010a). In particular, under major outbreaks of feeding herbivores that regularly occur in nature (Abrams and Orwig, 1996; Dwyer et al., 2004; Mattson and Haack, 1987) induced volatiles can dominate the release of BVOC from vegetation over large areas, underscoring the importance of gaining a better knowledge of quantitative scaling of induced emissions with the degree of herbivory damage.

Pedunculate oak (*Quercus robur* L.) is a widely distributed constitutively isoprene-emitting tree species that is one of the most economically important broad-leaved forest trees in Europe. Oak forests currently exhibit declining productivity and crown dieback in several sites throughout Europe. Various abiotic and biotic factors, including herbivory by the larvae of phyllophagous insects have been shown to importantly contribute to the oak decline (Batos et al., 2014; Thomas et al., 2002; Tonioli et al., 2001). The most important defoliating insects feeding on *Q. robur* are winter moth (*Operophtera brumata* L.), tortrix moth (*Tortrix viridana* L.) and European gypsy moth (*Lymantria dispar dispar* L.) (Thomas et al., 2002). Among these, *L. dispar* is a species with large larvae that can grow to the size of 50-90 mm (Milanovic et al., 2014) and that are capable of consuming 10 cm^2 leaf area per day per larva, including the second order veins and the terminal part of the mid-rib (Milanovic et al., 2014). In the current study, we used *L. dispar* larvae as a model to characterize the influence of large insect herbivore on constitutive and induced volatile release in *Q. robur*. We tested the hypothesis that the physiological activity of *Q. robur* leaves infected with the large herbivore *L. dispar* is quantitatively associated with the degree of damage, in particular, that the infection leads to a major decline in leaf photosynthetic activity and isoprene emissions and a modest increase in induced emissions.

Materials and methods

Plant material

The field measurements were performed in Lipova forest at Arad county, Romania (46° 5′ 30″ N, 21° 41′ 30″ E) in May 2014. The site supports a broad-leaved deciduous forest that is mainly dominated by *Quercus robur* (canopy height 4-5 m), whereas *Q. petraea, Alnus glutinosa,* and *Q. rubra* are minor canopy components. The forest expands more than 6,300 ha and more than half of the trees (51%) were infected at the time of the study. The infection was spatially highly heterogeneous, and forest patches with infested (more than 50 different patches with most trees infected observed) and non-infested patches with almost all trees lacking herbivores were interspersed. The weather conditions at the time of measurements were: average air temperature of 26 ± 2 °C, relative humidity of 62% and atmospheric pressure of 102.4 kPa. The plants of *Q. robur* included for measurements were 10-12 years old and 4-5 m tall. At the time of the measurements, the length of *L. dispar* larvae was about 30-40 mm. We took all measurements with control leaves from the clean plots with healthy trees. These control leaves were further checked for presence of eggs and small larvae, and discarded if there was evidence of biotic interactions. As all experiments were performed in natural conditions, the past and current larval damage as well as the actual number of herbivores that had been feeding in the leaf could not be controlled. Therefore, we only report the relationships of leaf physiological traits with the degree of leaf damage.

Photosynthetic measurements

Foliage photosynthetic characteristics during larval feeding were determined in the field with a portable gas exchange system GFS-3000 (Waltz, Effeltrich, Germany) as in Niinemets et al. (2010b), except for minor modifications in environmental conditions as stated below. This system has an environmental-controlled cuvette with 8 cm^2 window area and a full-window leaf chamber fluorimeter for sample illumination and fluorescence

measurements. Each time, a leaf fully filling the cuvette area was enclosed and standard measurement conditions (light intensity of 1000 nmol m^{-2} s^{-1}, leaf temperature of 25°C, chamber air humidity of 70%, and CO_2 concentration of 385 μmol mol^{-1}) were established. The leaf was stabilized under the standard conditions until stomata opened and steady-state CO_2 and water vapor exchange rates were reached. Steady-state values of net assimilation (*A*) and transpiration (*E*) rates, and stomatal conductance to water vapor (g_s) were calculated according to von Caemmerer and Farquhar (1981).

Volatile sampling and GC-MS analyses

Volatile organic compounds (VOC) were sampled via the outlet of the gas-exchange cuvette at a flow rate of 200 ml min^{-1} for 20 min with a constant flow air sample pump 210-1003MTX (SKC Inc., Houston, TX, USA). The leaf chamber air was drawn through a multibed stainless steel cartridge (10.5 cm length, 4 mm inner diameter, Supelco, Bellefonte, USA) filled with Carbotrap C 20/40 mesh (0.2 g), Carbopack B 40/60 mesh (0.1 g) and Carbotrap X 20/40 mesh (0.1 g) adsorbents (Supelco, Bellefonte, USA) to quantitatively sample all volatiles in C_5-C_{15} range (Kännaste et al., 2014). Volatiles were also collected using *L. dispar* larvae without plants in the laboratory conditions using a 3 L glass chambers with a flow rate of 2 L min^{-1} similarly as in Copolovici et al. (2011b). To estimate the background VOC concentrations (blank samples), air samples were taken from empty chambers before and after enclosure of leaves or larvae.

The adsorbent cartridges were analyzed for volatile lipoxygenase (LOX) pathway products (also called green leaf volatiles, GLV), terpenes and methyl salicylate using a Shimadzu TD20 automated cartridge desorber integrated with a Shimadzu 2010 Plus GC-MS instrument (Shimadzu Corporation, Kyoto, Japan) according to the method of Toome et al. (2010) and Kännaste et al. (2014). Briefly, for tube desorption, He purge flow was set at 40 ml min^{-1}, primary desorption temperature at 250 °C, and primary desorption time was 6 min. The mass spectrometer was operated in electron-impact (EI) mode at 70 eV, with the transfer line temperature set at 240 °C and ion-source temperature at 150 °C. The identification of terpenes, green leaf volatiles and isoprene was done using NIST spectral library ver. 14 and authentic standards (Sigma-Aldrich, Taufkirchen, Germany). The absolute concentrations of compounds were calculated based on an external authentic standard consisting of known amount of VOCs as described in full detail in Kännaste et al. (2014). Briefly, 1 μl of calibration sample has been injected into the multi-bed tube followed by passing a nitrogen gas at 300 ml min^{-1} through the tube for 5 min in order to evaporate the solvent and trap the volatiles on the adsorbent. Finally, the tube has been analyzed in GC-MS using the same program as for the samples. The background (blank) VOC concentrations were subtracted from the concentrations with leaf samples and volatile emission rates were calculated according to Niinemets et al (2011).

Leaf pigment analysis

Pigment extraction was performed according to the method of Opris et al. (2013) with minor modifications. Briefly, leaf samples of 4 cm^2 were taken after leaf gas-exchange measurements and volatile sampling and immediately frozen in liquid nitrogen. The pigments were extracted in ice-cold 100% acetone with calcium carbonate (Sigma-Aldrich,

Steinheim, Germany), and centrifuged with a Hettich centrifuge (Hettich 320 R Universal, Hettich GmbH, Tuttlingen, Germany) at 0 ºC and 9500 g for 3 min. Then the supernatant was decanted, and the extraction was repeated till the supernatant remained colorless, but at least three times. The extracts were pooled and brought to a final volume of 10 mL and then filtered through a 0.45 μm PTFE membrane filter (VWR International, Radnor, PA, USA). The obtained extracts were analyzed for carotenoid and chlorophyll pigments by a high pressure liquid chromatograph (HPLC-MS Shimadzu 2010, Kyoto, Japan) equipped with a diode array detector (DAD) according to Niinemets et al. (1998) using a Lichrosorb® RP-18 column (length 125 mm, inner diameter 4 mm, film thickness 5 μm; Hichrom, UK). The column temperature was maintained at 10 ºC and the flow rate at 1.5 ml min^{-1}. The solvents used for the chromatographic elution consisted of ultra-pure water (A) and HPLC grade acetone (B) (Sigma-Aldrich, Steinheim, Germany). The chromatographic elution was started by isocratically running a mixture of 25% A and 75% B for the first 7.5 min, followed by a 9.5 min linear gradient to 100% B, which was run isocratically for 3 min. Further, the eluent was changed to the initial composition of 25% A and 75% B by a 2 min linear gradient. The HPLC was calibrated using commercially available chlorophyll a, chlorophyll b, and β-carotene standards (Sigma-Aldrich, Steinheim, Germany), and the calibration curves were developed at corresponding spectral maxima (430 nm for chlorophyll a, and 455 nm for chlorophyll b and β-carotene).

Estimation of the degree of leaf damage

The leaves were scanned at 200 dpi, and the total leaf area and the leaf area damaged by *L. dispar* larvae were estimated with the “Leaf Area Measurement” software (www.plant-image-analysis.org). Leaf dry mass was estimated after oven-drying at 70 ºC, and leaf dry mass to leaf area was calculated. Foliage photosynthetic rates, volatile emissions and pigment contents per unit leaf area were estimated after correction for the consumed leaf area.

Statistical analysis and data handling

All measurements have been done in triplicates and data points correspond to averages of three replicate leaves in each individual plant (±SE). The data were analyzed by linear and non-linear regression analyses, and the herbivory treatment effect at a certain level of damage severity relative to non-infected leaves was also tested by ANOVA. All statistical analyses were conducted with ORIGIN 10.0 (OriginLab Corporation, MA, USA) and the statistical tests were considered significant at $P < 0.05$.

Results

Effects of herbivory on foliage photosynthetic characteristics

Herbivore feeding by *L. dispar* reduced leaf net CO_2 assimilation rate (A), and even a moderate feeding, ca. 10% of leaf area removed, resulted in ca. 12% reduction of net assimilation rate, i.e. resulting in values of about 10 μmol m^{-2} s^{-1} (Fig. 1a). Net assimilation rate decreased with further increases in insect feeding, reaching values of 1-2 μmol m^{-2} s^{-1} in leaves with ca. 50% of leaf area eaten (Fig. 1a).

Differently from *A*, the stomatal conductance to water vapor (g_s) was only moderately affected by larval feeding (Fig. 1b). In fact, the average (± SE) g_s of 109 ± 20 mmol m^{-2} s^{-1} for strongly damaged leaves with 30-35% leaf area eaten was not different from the average g_s in non-damaged leaves (141 ± 6 mmol m^{-2} s^{-1}; $P = 0.18$ for the comparison among the means; Fig. 1b). Nevertheless, the regression analysis indicated that through the entire damage range of 0-50%, g_s was reduced by ca. 30%. Given the smaller g_s than *A* response to herbivory, the intercellular CO_2 concentration (C_i) increased with increasing the degree of herbivory damage (Fig. 1c).

Elicitation of volatile emissions in herbivory-infected leaves

Constitutive isoprene emission in herbivore-fed leaves was reduced from 30.3 ± 0.7 nmol m^{-2} s^{-1} (average ± SE) in healthy leaves to 4-5 nmol m^{-2} s^{-1} in heavily infected leaves (40-50% of leaf area eaten by insects), and a strong negative correlation between isoprene emission rate and percentage of leaf area eaten was observed (Fig. 2a). Larval feeding induced emissions of green leaf volatiles [GLV; primarily, (*Z*)-3-hexenol, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate and 1-hexanol), Fig. 2b], that increased with the degree of damage, reaching very high values of 3.0-4.4 nmol m^{-2} s^{-1} (sum of all GLV) in heavily eaten leaves (Fig. 2b). Herbivory also induced emissions of ubiquitous monoterpenes (α-pinene, camphene, Δ-3-carene, limonene and β-phellandrene) and typical stress-marker monoterpenes such as (*E*)-β-ocimene and linalool (Table 1). The total emission rate of monoterpenes increased with the degree of leaf damage from close to zero level in control leaves to values as high as ca. 5.3 nmol m^{-2} s^{-1} in strongly infected leaves (Fig. 2c). Herbivory feeding also led to low-level emissions of the benzenoid methyl salicylate (MeSA) and the homoterpene (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), whereas the emission rates increased curvilinearly with the percentage of leaf damage to maximum values of 0.17 nmol m^{-2} s^{-1} for MeSA and 0.08 nmol m^{-2} s^{-1} for DMNT (Figure 3).

Responses of foliage chlorophyll and β-carotene contents to herbivory

Both chlorophyll *a* and *b* contents decreased strongly with the area eaten by the larvae, e.g., the average chlorophyll *a* content decreased from 231 ± 2 mg m^{-2} in non-infected leaves to 95 ± 3 mg m^{-2} in leaves with 30-50% damage (Fig. 4). The chlorophyll *a*/*b* ratio was on average 2.48 ± 0.26 and did not depend on the degree of leaf damage ($r = 0.5$, $P > 0.05$). β-Carotene content did not significantly correlate with the degree of larval damage (Fig. 4).

Negative scaling among constitutive and induced isoprenoids

Through different damage severities, foliage net assimilation rate scaled negatively with the emissions of induced volatiles (Fig. 5a for total monoterpenes, 5b for isoprene and 5c for total chlorophylls, $r = 0.83$, $P < 0.01$ for GLV; $r = 0.89$, $P < 0.01$ for MeSA; $r = 0.87$, $P < 0.01$ for DMNT, for linear regression used). Constitutive isoprene emissions were negatively correlated with induced emissions ($r = 0.97$, $P < 0.001$ for total monoterpenes; $r = 0.88$, $P < 0.01$ for GLV; $r = 0.93$, $P < 0.01$ for MeSA; $r = 0.91$, $P < 0.01$ for DMNT).

Discussion

Changes in photosynthetic function in leaves infected by large larvae

Plant photosynthetic responses to biotic stresses such as herbivory can range from stimulating effects, no effect or even significant impairment (Attaran et al., 2014; Gog et al., 2005; Nabity et al., 2009, 2013; Roslin et al., 2006; Zhou et al., 2015). Especially, colonization by solitary small herbivores can lead to compensatory enhancement of photosynthesis in remaining leaf parts (Copolovici et al., 2014a; Copolovici et al., 2011; Delaney et al., 2008). In *Quercus robur* in our study, we observed a drastic, disproportionate reduction in net assimilation rate (Fig. 1a), consistent with the hypothesis that herbivory by large larvae capable of biting through and consumption of also major veins can seriously impair photosynthesis. Yet, stomatal conductance was surprisingly little affected, seemingly inconsistent with this hypothesis (Fig. 1b). However, the low change in calculated stomatal conductance might be an artifact due to evaporation of water from free water surfaces generated upon herbivory, as well as due to transient increases in stomatal conductance upon relaxation of epidermal tension due to damage and concomitant opening of stomata (so-called Ivanov's effect, Moldau et al., 1993).

On the other hand, longer-term reductions in photosynthesis in herbivore-fed leaves have also been associated with impaired electron transport rate (Nabity et al., 2013). In our study, the chlorophyll content decreased with leaf damage severity (Fig. 4). Such a reduction has been observed in some studies looking at diffuse massive leaf infection, e.g. mustard (*Brassica juncea*) leaf infection by phloem-sucking mustard aphid (*Lipaphis erysimi*) (Rehman et al. (2014) and tomato (*Solanum lycopersicum*) infection by cotton mealybug (*Phenacoccus solenopsis*) (Huang et al. (2013)), but not necessarily upon infection by tissue-removing herbivores. However, damage of major veins and concomitant reduction in water availability of isolated mesophyll areas can well lead to the start of senescence processes (Munné-Bosch, 2007, 2008). Given the reduction of leaf pigment content (Fig. 4), changes characteristic to programmed cell death such as inhibition of photosynthetic electron transport and reductions in the amount or activity of photosynthetic rate-limiting enzymes were also likely responsible for reduction in net-assimilation rate in larval-eaten leaves.

In contrast, β-carotene content was weakly affected by larval feeding (Fig. 4). Differently from chlorophylls, β-carotene primarily functions as antioxidant and its sustained level might serve protective function. Although carotenoids can be destroyed under severe abiotic stress conditions (Ashraf and Harris, 2013), carotenoids are maintained longer than leaf chlorophylls in leaf tissues though senescence (Garcia-Plazaola et al., 2003; Niinemets et al., 2012).

Modification of constitutive isoprene emissions by herbivory

Concomitant reductions of net assimilation rates and constitutive isoprene emissions as observed in herbivore-fed leaves in our study (Fig. 1b) have been reported in several other herbivory studies (Laothawornkitkul et al., 2008; Loivamaeki et al., 2008; Loreto et al., 2014) as well as in fungal-infected leaves (Copolovici et al., 2014b; Jiang et al., 2016). Such a simultaneous reduction might indicate that limited plastidic carbon availability or delayed

activation of alternative carbon sources can have resulted in the reduction of the pool size of the immediate isoprene precursor, dimethylallyl diphosphate (DMADP) in chloroplasts (Rasulov et al., 2011; Rasulov et al., 2009), thereby reducing the emission rate. In addition, simultaneous reduction of isoprene synthase activity and isoprene emission can indicate overall decreases in foliage primary metabolism and constitutive isoprene synthesis in non-consumed leaf areas. Such a reduction is supported by significant declines in foliage chlorophyll contents in infected leaves (Fig. 4).

On the other hand, chloroplastic isoprene and monoterpene syntheses rely on the same plastidic DMADP pool, but the in vivo effective Michaelis-Menten constant for DMADP (K_m) is much smaller for monoterpenes than for isoprene (Rasulov et al., 2014). Lower K_m for monoterpene synthesis implies that the competition for DMADP is one-sided, and thus, elicitation of monoterpene synthesis upon herbivory feeding, can also partly explain the decline in isoprene emission rates (Fig. 5b).

Induction of green leaf volatile emissions in larval-eaten leaves

As a result of an attack by insects, specific elicitor molecules are generated by chemical or physical damage to plant membranes (Heil, 2014). Our study demonstrated elicitation of all key stress-induced volatile compound classes, green leaf volatiles (GLV), mono- and sesquiterpenes, homoterpenes and methyl salicylate in herbivory-infected leaves (Table 1). GLV are synthesized in a process where free octadecanoid fatty acids (linoleic acid = 18:2 and linolenic acid = 18:3) are released from plant membranes by phospholipases. Upon release of these free fatty acids, lipoxygenases (LOX) then produce 9- or 13-hydroperoxylinoleic or -linolenic acid or a mixture of both (Matsui, 2006). A hydroperoxide lyase further catalyzes the breakdown of 13-hydroperoxylinole(n)ic acid to a C6-compound, (*Z*)-3-hexenal, and a C12-product (12-oxo-(*Z*)-9-dodecenoic acid). (*Z*)-3-Hexenal can further give rise to (*Z*)-3-hexenol, (*E*)-2-hexenol, (*E*)-3-hexenol or (*E*)-2-hexenal in consequent reactions (Feussner and Wasternack, 2002; Matsui, 2006). The emission of green leaf volatiles is a reliable marker of oxidative stress and membrane-level damage (Porta and Rocha-Sosa, 2002). GLV are rapidly released upon herbivory feeding due to constitutive activity of LOX, and their almost immediate release has been typically associated with mechanical damage upon wounding (Matsui et al., 2012; Portillo-Estrada et al., 2015; Scala et al., 2013). In our study, we observed a strong correlation between the emissions of green leaf volatiles and the percentage of leaf area damaged (Figure 2b). Similarly to our study, increases in the emission of GLV with the degree of herbivore feeding were also observed in experiments with *Caberia pusaria* feeding on grey alder (*Alnus incana*) (Copolovici et al., 2011) and in experiments with *Epirrita autumnata* feeding on hybrid aspen (*Populus tremula* x *P. tremuloides*) (Schaub et al., 2010). Given that in these studies and in our study, the degree of damage quantified as the percentage of leaf area removed includes both fresh and somewhat older damage, the question is how such a correlation can occur if GLV release is exclusively associated with immediate damage. However, conversion of (*Z*)-3-hexenal to more reduced and less toxic volatiles can also occur in non-impacted leaf areas (Matsui et al., 2012), and GLV release can continue for a certain period of time after the immediate biotic impact (Copolovici et al., 2011; Jiang et al., 2016). Thus, scaling of GLV emissions

with the degree of damage (Fig. 2b) might be explained by the sustained GLV release from non-impacted leaf areas.

There is evidence that GLV release is higher upon damage of veins than upon damage of intercostal tissues (Portillo-Estrada et al., 2015). Interestingly, the increase of GLV release with the degree of damage in leaves with minor to moderate damage of 5-20% was much less than in leaves with moderate to extensive damage of 20-50% (Fig. 2b). Such a difference might reflect the circumstance that in leaves with 5-20% damage, there was limited big vein severance by herbivores, while herbivores also bit through major veins in leaves with extensive damage. This result is in a marked contrast with the linear relationship of GLV release vs. leaf area damage in the study with small herbivores *Caberia pusaria* (Copolovici et al., 2011) and *Monsoma pulveratum* (Copolovici et al., 2014a) that are incapable of biting through major veins.

Elicitation of emissions of terpenoids and MeSA

As a widespread consensus, isoprene and monoterpenes are thought to be synthesized via 2-C-methyl-*D*-erythritol 4-phosphate (MEP) pathway in plastids (Copolovici et al., 2014a; Fineschi et al., 2013; Vranova et al., 2012) and sesquiterpenes via mevalonate (MVA) pathway in cytosol (Lombard and Moreira, 2011), although there is recent evidence of possible monoterpene synthesis in cytosol depending on substrate availability (Pazouki and Niinemets, 2016). DMNT is synthesized from the sesquiterpene (*E*)-nerolidol likely in the cytosol (Baldwin et al., 2006; Tholl et al., 2011). Although present in different cellular compartments, both isoprenoid synthesis pathways are upregulated upon herbivory stress and several terpene synthase genes involved have already been identified (Arimura et al., 2000a; Arimura et al., 2000b; Baldwin et al., 2006; Loreto et al., 2014). Thus, elicitation of emissions of mono-, sesqui-, and homoterpenes has been found in different deciduous trees under herbivore stress (Stam et al., 2014; Zhu et al., 2014 for reviews). The interplay between different terpene synthase pathways is still somewhat unclear, especially given that different compounds serve different ecological functions. In *Q. robur* infected by the moth *Tortrix viridana*, Ghirardo et. al (2012) demonstrated that the larvae were attracted to the plants releasing higher amounts of homoterpene DMNT and monoterpene (*E*)-β-ocimene, while sesquiterpenes α-farnesene and germacrene D acted as a repellent.

We observed that the monoterpene emission rate increased with increasing leaf damage (Fig. 2c), indicating that the activity of monoterpene synthases increased upon herbivore feeding. In addition, as discussed above, monoterpene synthesis could have been further favored by greater competitive capacity for chloroplastic DMADP compared with isoprene synthesis (Fig. 5b) and inhibition of pigment synthesis as evident in the reduction in leaf pigment content (Fig. 5c). Scaling of mono- and sesquiterpene emissions with the degree of damage has been observed in several experiments looking at lepidopteran larval feeding effects on volatile release (for example Copolovici et al., 2014a; Copolovici et al., 2011). As we hypothesized, there was evidence of leveling off of monoterpene and DMNT vs. damage severity relationships at higher severity of damage (Fig. 2c, Fig. 3). In fact, the ratios of monoterpene to GLV emissions and DMNT to GLV emissions decreased with increasing the degree of damage, indicating that the induction response was relatively less prominent in

more severely damaged leaves compared with the immediate stress response (or with the rapidly induced stress response). This is different from infection by small larvae (Copolovici et al., 2014a; Copolovici et al., 2011) or from infection by slowly developing biotic stresses such as fungal infections (Copolovici et al., 2014b; Jiang et al., 2016), where GLV and monoterpene and GLV emissions are almost proportional.

The emission of shikimate pathway derived compound, methyl salicylate (MeSA), is typically observed for sap-sucking herbivores such as aphids or whiteflies (Li et al., 2006; Zarate et al., 2007) and is not usually considered as part of chewing herbivore response. However, as in our study, several previous studies have demonstrated the release of methyl salicylate upon chewing herbivore attacks (Cardoza et al., 2002; Dicke et al., 1999), indicating a complex interplay between jasmonate- and salicylate-dependent signalling pathways upon herbivore infestations.

Conclusions

The results of the current study highlight a major negative scaling of foliage photosynthetic rates and constitutive isoprene emissions, and concomitant increase in induced volatile emissions upon feeding by large herbivore larvae (Fig. 1, 2, 5). While the damage-dependent increase of green leaf volatiles was disproportionately greater in leaves with extensive degree of damage than in moderately damaged leaves (Fig. 2b), emission rates of terpenoids leveled off at higher degrees of leaf damage (Fig. 2c). This suggests that faster and more severe damage, especially major vein severance by large herbivores can much more strongly influence foliage physiological activity than feeding by smaller herbivores. These contrasting herbivore responses need consideration in modeling herbivore elicited emissions in large-scale biosphere-atmosphere models.

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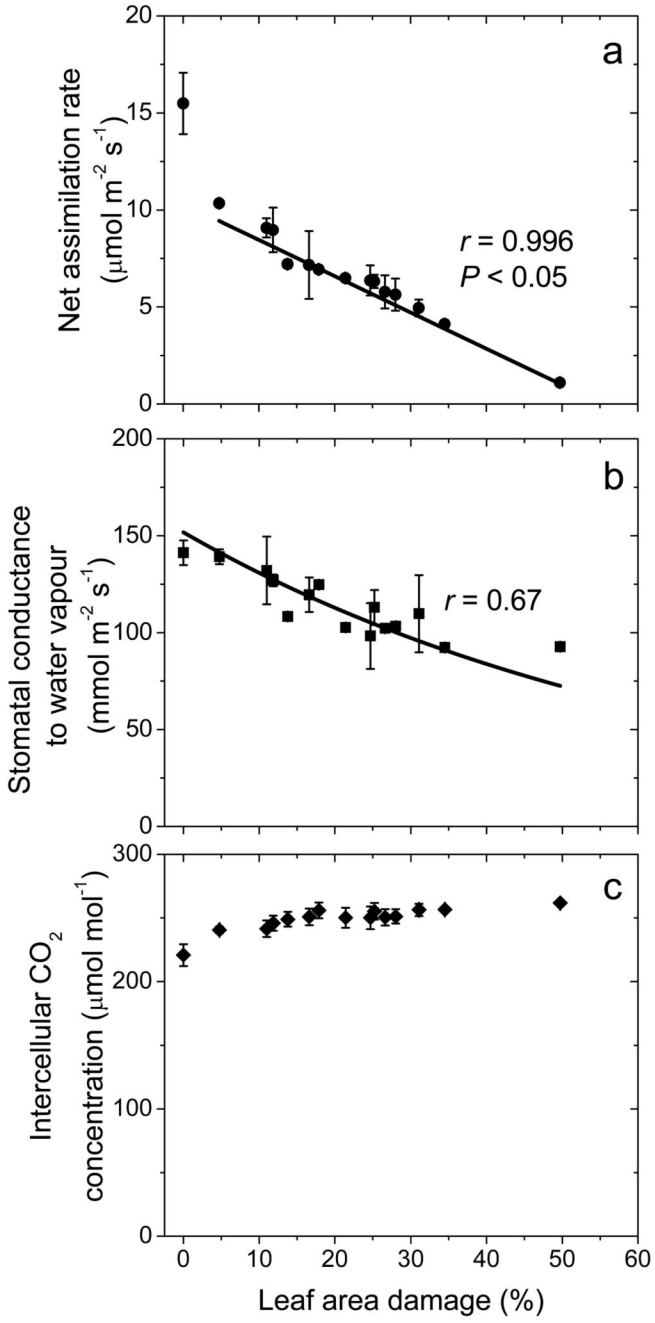

Fig. 1.

Foliage net assimilation rate (a), stomatal conductance to water vapor (b) and intracellular CO_2 concentration per unit projected leaf area in *Quercus robur* plants in relation to the degree of damage by the larvae of the lymantriid moth *Lymantria dispar* (percentage of leaf area consumed). Data points correspond to averages of three replicate leaves in each individual plant (± SE). Data were fitted by linear (a) and non-linear regression in the form $y = ab^x$ (b).

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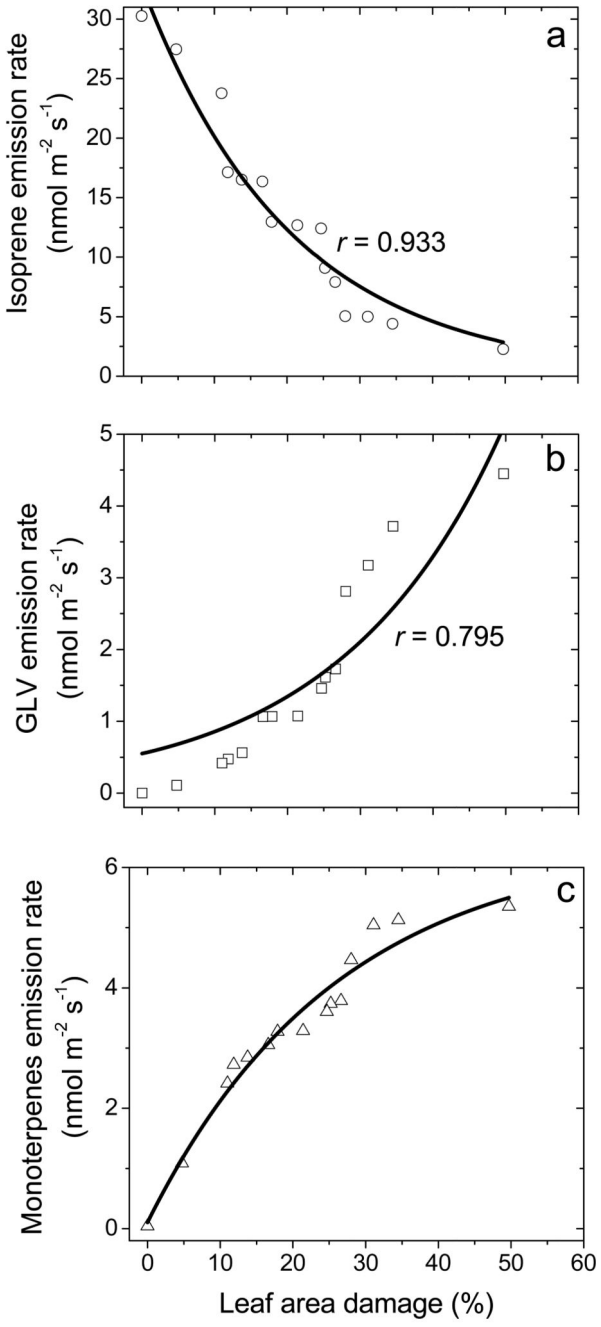

Fig. 2.

Emissions rates of isoprene (a), green leaf volatiles (b; GLV, volatiles of lipoxygenase pathway, LOX volatiles) and monoterpenes (c) from *Q. robur* leaves with different degrees of feeding by *L. dispar* larvae (replicates and data presentation as in Fig. 1). Total LOX product emission was calculated as the sum of emissions of 1-hexanol, (*Z*)-3-hexenol, (*Z*)-2-hexenal, and (*Z*)-3-hexenyl acetate and the total monoterpene emission as the sum of emissions of α-pinene, β-pinene, camphene, limonene, Δ-3-carene, *p*-cymene, and β-phellandrene. Data were fitted by non-linear regressions in the form of $y = ab^x$.

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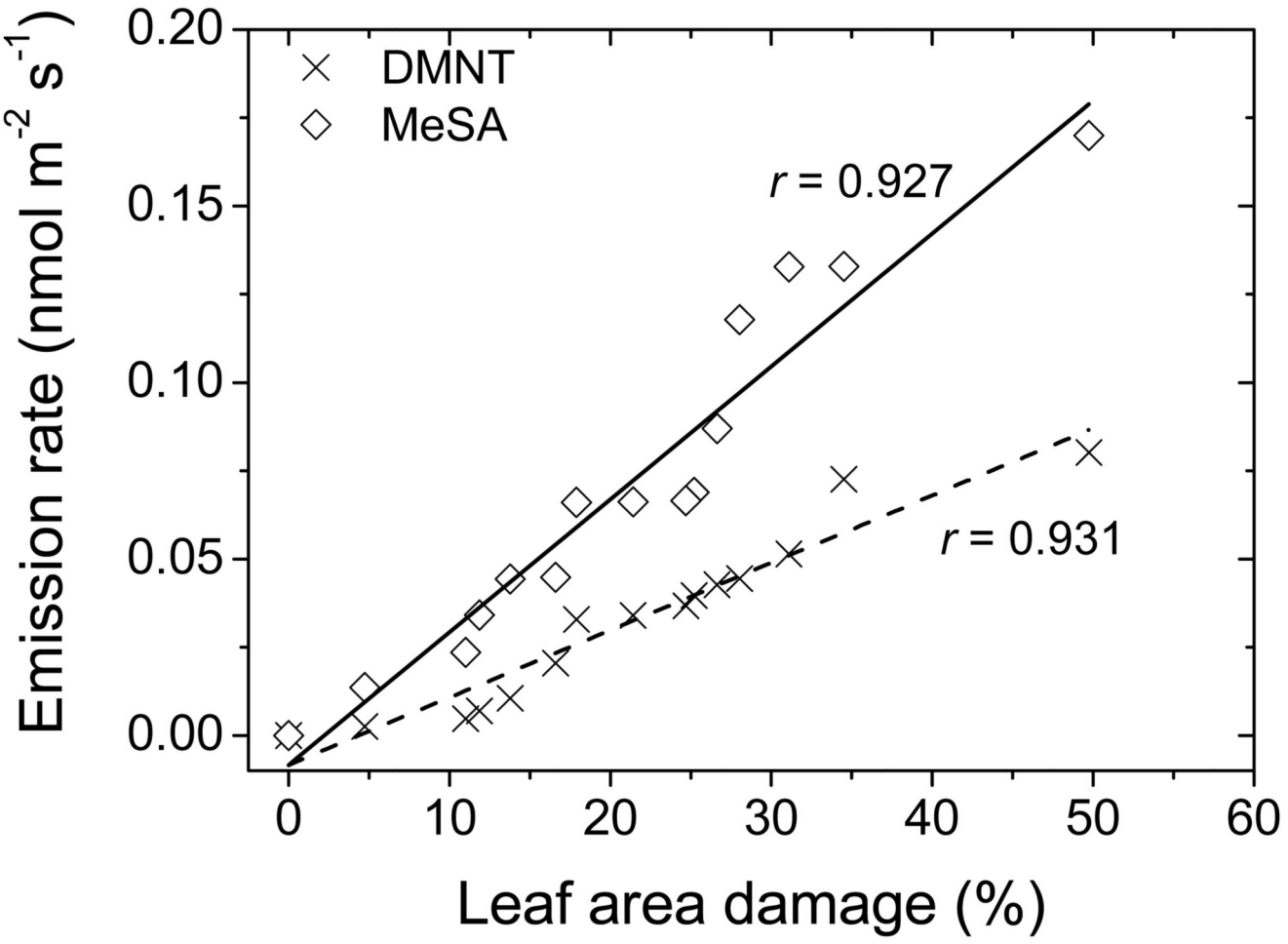

Fig. 3.

Emissions of the benzenoid methyl salicylate (MeSA) and the homoterpene (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) from leaves of *Q. robur* in relation to the degree of herbivory by *L. dispar* larvae. Data presentation and replication as in Fig. 1. The relationships were fitted by linear regressions.

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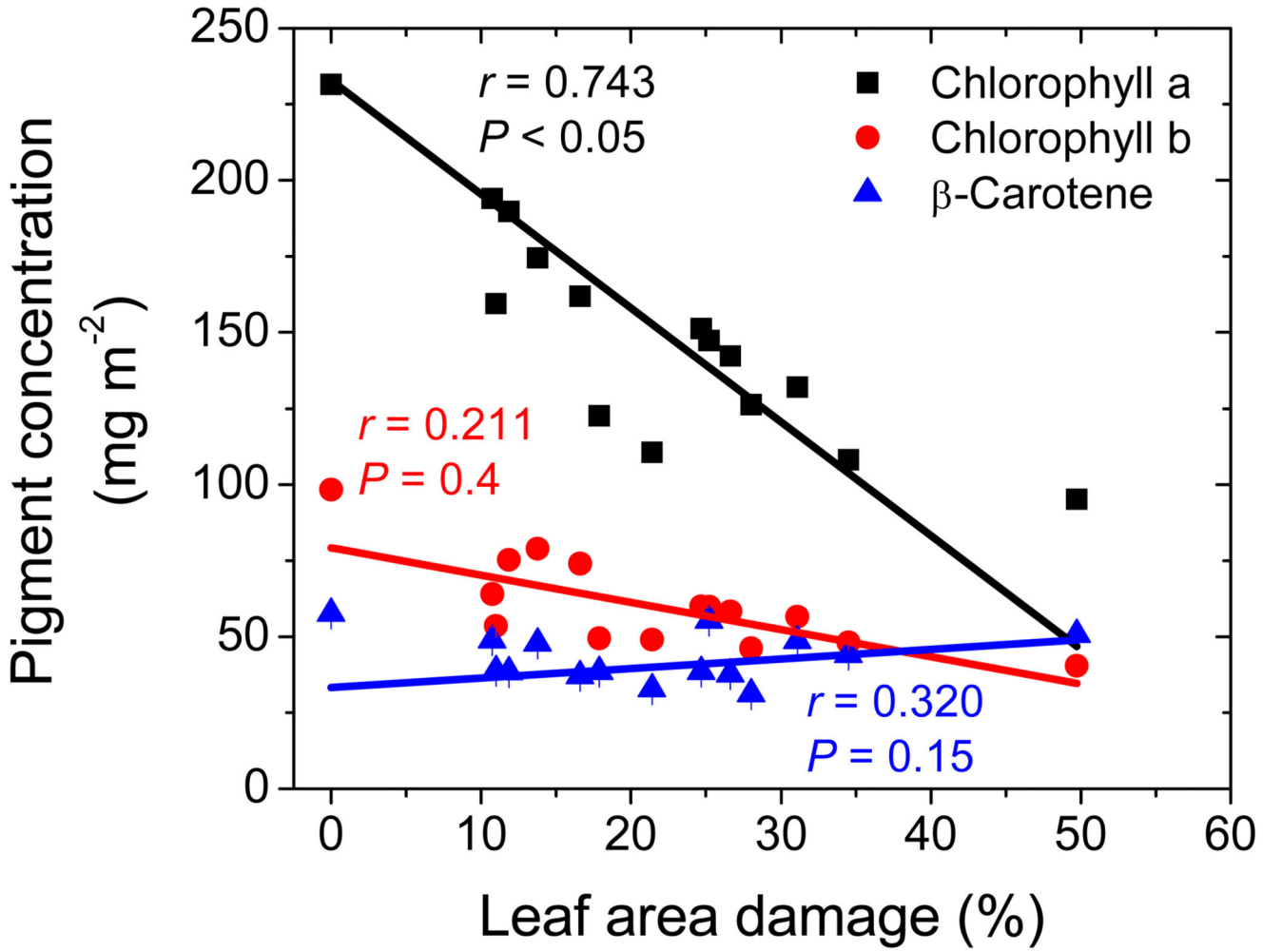

Fig. 4.

Effects of feeding by the larvae of *L. dispar* on the contents of chlorophyll *a* and *b*, and carotene in *Q. robur* leaves. Statistical replicates and data presentation as in Fig. 1. Data were fitted by linear regressions.

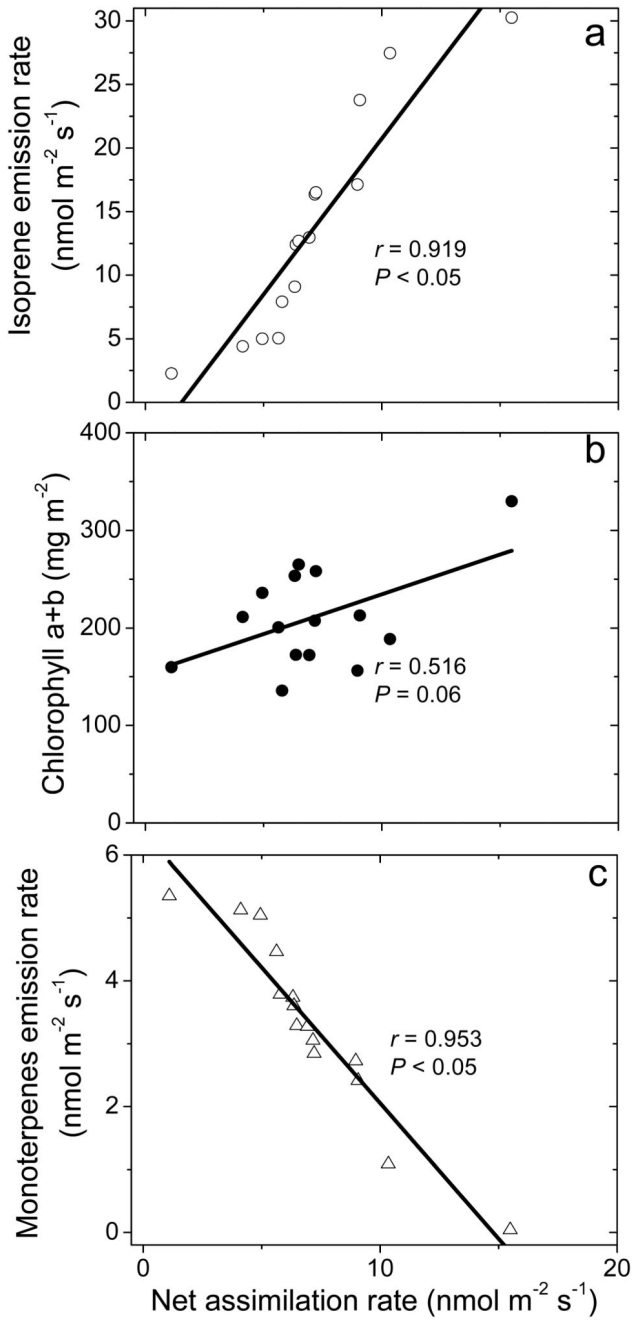

Fig. 5.

Correlations of net assimilation rate (a), isoprene emission rate (b) and chlorophyll (a+b) content (c) with monoterpene emission rate in *Q. robur* leaves with different degrees of damage by *L. dispar* larvae (Fig. 1a, 2a,c and 4 for the correlations of given traits with the degree of damage). Data were fitted by non-linear regressions.

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Table 1

Average ± SD emission rates (nmol m^{-2} s^{-1}) of different volatiles released from leaves of *Q. robur* in response to insect damage

Compound	Control	Average (±SD) degree of damage (%)		
		12.2±0.6	**18.6±1.0**	**38.4±9.9**
(*Z*)-3-hexenol	nd	0.107±0.005	0.213±0.007	0.90±0.13
(*E*)-2-hexenal	nd	0.100±0.003	0.320±0.010	0.82±0.17
(*Z*)-3-hexenyl acetate	nd	0.054±0.007	0.080±0.010	0.223±0.023
1-hexanol	nd	0.223±0.022	0.454±0.017	1.84±0.33
isoprene	30.3±3.2	19.1±3.4	14.0±1.7	3.9±1.4
α–pinene	0.0092±0.0012	0.622±0.011	0.848±0.027	1.58±0.11
camphene	nd	0.033±0.002	0.043±0.001	0.111±0.010
Δ-3-carene	0.011±0.006	0.504±0.004	0.663±0.009	1.07±0.07
limonene	0.0094±0.003	0.305±0.032	0.412±0.008	1.02±0.05
β-phellandrene	0.0106±0.007	1.12±0.14	1.09±0.13	0.98±0.15
(*E*)-β-ocimene	nd	0.052±0.001	0.098±0.001	0.297±0.044
linalool	nd	0.029±0.001	0.053±0.002	0.128±0.024
DMNT	nd	0.007±0.002	0.021±0.006	0.068±0.015
methyl salicylate	nd	0.034±0.006	0.045±0.011	0.145±0.021

nd = not detected