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Volatile organic compound emissions from *Alnus glutinosa* under interacting drought and herbivory stresses

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Abstract

Plant volatile organic compounds (VOCs) elicited in response to herbivory can serve as cues for parasitic and predatory insects, but the modification of VOC elicitation responses under interacting abiotic stresses is poorly known. We studied foliage VOC emissions in the deciduous tree *Alnus glutinosa* induced by feeding by the larvae of green alder sawfly (*Monsoma pulveratum*) under well-watered and drought-stressed conditions. Drought strongly curbed photosynthesis rate and stomatal conductance, but there were no effects of insect feeding on photosynthetic characteristics. Feeding induced emissions of volatile products of lipoxygenase pathway and monoterpenes, and emissions of stress marker compounds (*E*)- β -ocimene and homoterpene DMNT. The emissions were more strongly elicited and reached a maximum value earlier in drought-stressed plants. In addition, methyl salicylate emissions were elicited in herbivory-fed drought-stressed plants. Herbivores were more strongly attracted to well-watered plants and consumed more than a four-fold greater fraction of leaf area than they consumed from drought-treated plants. Overall, this study demonstrates an important priming effect of drought, suggesting that plants under combined drought/herbivory stress are more resistant to herbivores.

Keywords

biotic stress; green leaf volatiles; herbivory damage; interacting stresses; *Monsoma pulveratum*; methyl salicylate; photosynthesis rate; stress priming; volatile organic compounds

1 Introduction

Under natural conditions, plants are often exposed to a combination of two or more simultaneous or sequential stress factors (Holopainen and Gershenzon 2010; Mittler 2006; Niinemets 2010a; Niinemets 2010b). In the case of simultaneously occurring stresses, one type of stress could weaken or enhance the effects of another simultaneous stress factor by direct physiological cumulative or interactive effects (Ibrahim et al. 2008; Niinemets 2010a). Sequential or superimposed stress effects can be further complicated by stress priming (Frost

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et al. 2008; Heil and Kost 2006; Kessler et al. 2006; Niinemets 2010b) that can induce partial acclimation responses to similar type of stress or result in metabolic changes that protect directly or indirectly against a different type of stress. For instance, abiotic stress-driven metabolic changes can affect biotic stress response, or priming in responses to one type of biotic stress can affect responses to a different type of stress (Cardoza et al. 2002; Dicke 2009; Holopainen and Gershenzon 2010; Peng et al. 2011; Thaler et al. 2002).

Plant volatile emission patterns change quantitatively and qualitatively in response to damage by herbivores (Arimura et al. 2005; Brilli et al. 2009; Dicke 2009; Kant et al. 2009; Niinemets et al., 2013; Trowbridge and Stoy, 2013). Most plants have evolved effective defense strategies in order to reduce insect attacks (reviews in Dicke 2009; Dicke and Baldwin 2010; Dicke and Loreto 2010; Holopainen and Gershenzon 2010). Presence of multiple stresses can significantly affect the volatile emissions, but so far, the interactive effects are still poorly understood. For example, simultaneous fungal and lepidopteran herbivory treatment resulted in similar emissions of herbivory-induced volatiles in *Arachis hypogea* (Cardoza et al. 2002) and ca. 50% reduced emissions in maize (*Zea mays*) (Rostás et al. 2006), but the herbivore performance was not affected (Rostás et al. 2006) or was even enhanced (Cardoza et al. 2002) in fungal-infected plants, demonstrating complex response to multiple biotic stresses. On the other hand, in *Zea mays*, herbivore-induced VOCs were reduced by 75% in the case of nutrient deficiency (Gouinguene and Turlings 2002), indicating reduced capacity for indirect defense.

The typical fast response of the plants to herbivore attacks is the emission of volatile products of lipoxygenase pathway - LOX products (also called green leaf volatiles) consisting of various C6 aldehydes and alcohols (Copolovici et al. 2011; Gosset et al. 2009; Pinto et al. 2007; Toome et al. 2010). LOX product emission is a ubiquitous stress response that has been observed in response to other biotic stresses such as fungal attacks (Steindel et al. 2005; Toome et al. 2010) and many abiotic stresses including heat and frost (Copolovici et al. 2012), flooding (Copolovici and Niinemets 2010), ozone (Beauchamp et al. 2005; Heiden et al. 2003), high light (Loreto et al. 2006) and mechanical wounding (Loreto et al. 2006). This initial rapid response is followed by emissions of induced volatile isoprenoids, in particular, mono- and sesquiterpenes (Beauchamp et al. 2005; Copolovici et al. 2012; Steindel et al. 2005; Toome et al. 2010) and in some cases by emissions of benzenoid methyl salicylate (Beauchamp et al. 2005; Cardoza et al. 2002; Zhao et al. 2010). These compounds can play a role in host detection of herbivores as well as in eliciting priming responses in plants (Choudhary et al. 2008; Peñuelas et al. 2007; Ton et al. 2007), thereby playing a major role in determining the integrated response of previously imposed biotic or abiotic stress and subsequent herbivory stress.

Potential changes in host quality and both constitutive and induced resistance induced by abiotic stresses could influence herbivores' development and health, and thereby determine the degree of herbivory damage (Lerdau et al. 1994). The share of the constitutive vs. induced response is expected to depend on plant characteristics (growth rate, defense strategy) and the specific type of stress. A key abiotic stress that strongly curtails plant carbon gain and growth is drought, but the influence of water deficiency on the strategies of plants to cope with different biotic attacks has received limited attention, and the studies

have mainly focused on non-volatile primary or secondary metabolites (e.g., Gutbrodt et al. 2012; Khan et al. 2011) or plants' growth characteristics (Eranen et al. 2009; Halpern et al. 2010). For deciduous trees in general, there are very few studies investigating the impact of multiple stresses on plant performance and, especially limited are studies combining abiotic and biotic stresses (Dicke and Loreto 2010; Holopainen and Gershenzon 2010; Niinemets 2010a; Niinemets and Monson 2013). This is an important omission because in trees with longer-living foliage and slower leaf turnover (Wright et al. 2004), the constitutive stress response is expected to be of greater significance than in herbaceous species with faster leaf turnover and more dynamic stress responses.

Here we investigated the kinetics of VOC emission in black alder (*Alnus glutinosa*) leaves in response to combined drought and herbivory by green alder sawfly (*Monsoma pulveratum*) larvae, and studied the degree of herbivory damage and plant attractiveness to herbivores in drought-stressed and well-watered plants. *Alnus glutinosa* is a relatively short-living (ca. 40 yr.) wide-spread fast-growing tree species colonizing habitats along stream banks and wet forests. It has a very low drought tolerance (Niinemets and Valladares 2006). Herbivores and pathogens have a strong impact on *Alnus* spp. altering their survival, growth and even reproduction, thereby influencing the composition of early successional alder dominated ecosystems. So far, only a few studies have investigated stress induced by herbivore attack (e.g., Blande et al. 2010; Dolch and Tschardt 2000; Giertych et al. 2006; Tschardt et al. 2001) or by drought (Arbellay et al. 2010; Francis et al. 2005; Hacke and Sauter 1996; Sundstrom and Hussdanell 1995) in *Alnus* spp, but the combined stress has not been studied in alder to our knowledge.

We tested the hypotheses that priming by drought stress results in altered kinetics of VOC emissions, with drought-stressed plants responding earlier to herbivory stress than well-watered plants and we also expected the drought-stressed plants to be less attractive and less palatable to the herbivores due to priming of defense responses by water stress.

2 Materials and methods

2.1 Plant material

Alnus glutinosa seedlings of local (Estonian) origin were grown in 5 L clay pots filled with a 1:1 mixture of commercial potting soil (Biolan Oy, Finland) and quartz sand under light intensity of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (HPI-T Plus 400 W metal halide lamps, Philips) with day/night temperatures of 24/18 °C for a 12 hr light period. The plants were watered daily and fertilized once per month with a slow release NPK (3-1-2 ratio) fertilizer containing microelements. In all experiments, we used similar-sized 2-yr-old seedlings with 20-25 leaves. The experiment was conducted with plants having fully mature leaves, and no leaf area expansion was observed throughout the experiment in both the control and insect-treated plants.

2.2 Insects

Larvae of green alder sawfly *Monsoma pulveratum* (Hymenoptera: Tenthredinidae) at final instar were used as herbivores. *Monsoma pulveratum* is a common Palearctic species

(recently also found in North America) whose folivorous larvae feed on *Alnus* species, occasionally causing total defoliation of the trees. The larvae were collected from the vicinity of Tartu, Estonia (58°22' N 26°43' E) a few days prior to the experiment and kept in plastic containers with access to fresh *Alnus* leaves. Larvae of similar size, approximately 1.2 cm in length, were selected for the experiment.

2.3 Experimental setup

Whole plants were placed in a dynamic headspace sampling cuvette system consisting of eight 3 L glass chambers similar to the system described in detail in Toome et al. (2010) and Copolovici et al. (2011). In this system, roots with the potting medium stayed outside the chambers, and above-ground plant parts were hermetically sealed in individual cuvettes. The air flow through each chamber was 0.25 L min⁻¹ and a fan (Sunon Group, Beijing, China) was installed in the chamber resulting in high turbulence. During the experiment, light was provided by Philips HPI-T Plus 400 W metal halide lamps for 12 hours photoperiod at a level of light intensity of 200 µmol m⁻² s⁻¹. After hermetic installation of the plants (day -1), all plants were watered to field capacity. Three plants were randomly chosen for drought treatment (no water provided for six days), while the remaining five were watered daily. After 48 hours (day 1), the larvae of *M. pulveratum* were placed on three drought-stressed and on three well-watered plants to initiate the herbivory stress, while two well-watered plants were left untreated as controls. Five larvae were placed on each herbivory-treated plant. After the introduction of the larvae, the plants were stabilized in the system with dynamic air flow for two more hours before VOC sampling. The larvae were left to feed on the leaves for 7 days. After five days of feeding, the plants under drought stress were watered to field capacity and their recovery was followed for another two days with the larvae still feeding. A similar experiment was performed using the same set-up with 4 plants as controls and 4 plants drought-stressed without the herbivory treatment. Both experiments were replicated twice.

2.4 Leaf structural measurements

After the last collection of VOCs, all leaves were harvested and scanned at 300 dpi. Projected leaf area was determined from the scanned images by a custom made software. Both the total leaf area after the experiment and before the feeding treatment (non-damaged area, i.e., the holes and fed margins added) were estimated. *Monsoma pulveratum* feeding mainly consisted of holes and perforations with a minor contribution of feeding at the margins. Moreover, the leaf area lost due to the herbivore damage was estimated daily by counting the new holes. Leaf fresh mass was determined immediately after the area estimation, and leaf dry mass after drying the leaves for 48 hr at 70 °C.

2.5 Photosynthesis measurements

The photosynthetic characteristics of the plants during larval feeding were monitored throughout the experiment as in Copolovici et al. (2011). Shortly, plants were placed in a thermostatable glass chamber with controlled conditions: light intensity of 200 µmol m⁻² s⁻¹, temperature of 28 °C, and ambient CO₂ concentration of 380-400 µmol mol⁻¹. CO₂ and H₂O concentrations at the chamber in- and outlets were measured with an infra-red dual-channel gas analyzer operated in differential mode (CIRAS II, PP-systems, Amesbury, MA, USA).

The rates of net assimilation (A), transpiration (E), and stomatal conductance to water vapor (g_s) were determined from the chamber in- and outgoing gas concentrations according to von Caemmerer and Farquhar (1981).

2.6 VOC sampling and GC-MS analysis

VOC sampling was performed on adsorbent cartridges via the outlets of each cuvette every day with a flow rate of 200 ml min⁻¹ for 20 min by using a constant flow air sample pump with four ports (1003-SKC, SKC Inc., Houston, TX, USA). In addition, a sample was taken from the air inlet prior to the cuvettes to estimate the background VOC concentrations. Multibed adsorbent cartridges were filled with different grades of Carbopack and were optimized for trapping of all plant volatiles between C5-C15 (Copolovici et al. 2009; Toome et al. 2010). Adsorbent cartridges were analyzed for lipoxygenase pathway products (LOX), mono-, homo- and sesquiterpene concentrations using a Shimadzu TD20 automated cartridge desorber and Shimadzu 2010Plus GC-MS instrument (Shimadzu Corporation, Kyoto, Japan) according to the GC-MS method detailed in our previous studies (Copolovici et al. 2009; Toome et al. 2010). The identifications and quantifications of different compounds were done using authentic standards (Sigma-Aldrich, Taufkirchen, Germany). The background (blank) VOC concentrations were subtracted from the emission samples with the seedlings. Separate samples were collected from the larvae without the plants. The emissions of volatile compounds from the larvae were below the detection limit of our system.

2.7 Olfactometer Bioassays

Responses of herbivores to plant-derived volatiles were assessed using a home-made Teflon Y-tube olfactometer (internal diameter of 0.65 cm; length of arms 6 cm). The olfactometer assay was carried out in the same environmental conditions as the herbivory treatments. The Y-tube was placed in the centre of a box (10 cm x 10 cm x 5 cm). Two jars enclosing plants similar to those used for the experiments, one with a well-watered plant and the other with a drought-stressed plant, were connected to each of the ends of the Y-tube olfactometer. The air was circulated through the system using a small pump as indicated in Figure 1. Before the first larva was released, the whole setup was left to stabilize for two hours. The larvae of *M. pulveratum* were kept without food for one day before the experiment started. In each time, 30 larvae were released in the box and the number of larvae choosing either one or the other plant were counted after 10 hours. The test was replicated five times with independent plants.

2.8 Statistical analysis and data handling

The experiments were replicated twice with independent plants and larvae (in total 6 plants per treatment and 4 controls). As the effect of the replicate experiment (block effect) was not statistically significant, the replicate experiments were pooled. The treatment means were statistically compared by Tukey's test and Student ANOVA post-hoc test using ORIGIN 8 (OriginLab corporation, MA, USA) and GraphPad PRISM 5 (GraphPad Software Inc., La Jolla, CA, USA).

The plants' emissions were evaluated by principal component analysis (PCA). After mean-centering, logarithmical data transformation was used. From PCA analysis, resulting loading and score plots were derived. The differences between the bouquets were tested by Monte-Carlo permutation test using redundancy data analysis (RDA). The multivariate analyses were conducted by Canoco 4.5 software (ter Braak and Smilauer, Biometris Plant Research International, The Netherlands). All statistical tests were considered significant at $P < 0.05$. Means in olfactometer assay were compared by a Kruskal-Wallis non-parametric ANOVA.

3 Results

3.1 Herbivore preference and degree of feeding damage in well-watered and drought-stressed plants

When the larvae of *M. pulveratum* could choose between well-watered and drought-stressed *Alnus glutinosa* plants in the Y-tube olfactometer test, ca. 60% of them choose the well-watered plants, and 40% the drought-stressed plants (Figure 2a). The leaf area eaten by the insects was significantly higher ($P < 0.01$) for well-watered plants than for drought-stressed plants (Figure 2b).

3.2 Herbivory effects on foliage structure and photosynthetic characteristics

Average values ($\pm$ SE) of leaf dry to fresh mass ratio of $0.265 \pm 0.023 \text{ g g}^{-1}$ and leaf dry mass per unit area of $65 \pm 6 \text{ g m}^{-2}$ were observed in *A. glutinosa* seedlings. There were no statistical differences among the treatments in both traits ($P > 0.1$).

In control (well-watered, non-herbivory treated) plants, the net assimilation rate, A , was stable at $10 - 11 \mu\text{mol m}^{-2} \text{ s}^{-1}$ throughout the experiment (Fig. 3a). In *M. pulveratum* infested well-watered plants, A was between $9 - 10 \mu\text{mol m}^{-2} \text{ s}^{-1}$ during the experiment, and did not differ from the control treatment ($P > 0.05$; Fig. 3a). In the case of infested drought-stressed plants, A decreased to a level of $1.55 \pm 0.37 \mu\text{mol m}^{-2} \text{ s}^{-1}$ after seven days of drought and recovered to a level of $10.13 \pm 0.48 \mu\text{mol m}^{-2} \text{ s}^{-1}$ after re-watering (Fig. 3a). The trend was the same for the drought-stressed plants without insects.

Stomatal conductance was stable at $85 \text{ mmol m}^{-2} \text{ s}^{-1}$ throughout the study in both control and well-watered herbivore-infested plants (Figure 3b). In the drought-stressed plants, the stomatal conductance to water vapor decreased drastically after seven days of water restriction to a very low level of $17.8 \pm 2.1 \text{ mmol m}^{-2} \text{ s}^{-1}$ in plants with the herbivore feeding and to a level $13 \pm 3 \text{ mmol m}^{-2} \text{ s}^{-1}$ in plants without herbivore feeding. After re-watering, stomatal conductance recovered to the initial level.

3.3 LOX product emissions

Volatile lipoxygenase pathway products (LOX compounds) were the first volatiles induced by stresses. Even before the larvae were added to the plants (day 0), the emission of LOX had already significantly increased in drought-stressed plants compared with well-watered plants ($P < 0.01$). After the larvae started to eat the leaves (day 1), the emission of LOX increased significantly in well-watered plants relative to controls ($P < 0.05$), but even more in the drought-stressed plants ($P < 0.001$; Fig. 4). In the following days, the emissions of

LOX compounds in well-watered plants stayed almost constant at the level of $57 \pm 8 \text{ pmol m}^{-2} \text{ s}^{-1}$ until day 8. In drought-stressed plants, LOX emission rate was on average $182 \pm 32 \text{ pmol m}^{-2} \text{ s}^{-1}$ between days 1-6 for plants with insects, and $135 \pm 32 \text{ pmol m}^{-2} \text{ s}^{-1}$ for plants without insects. LOX emissions decreased significantly ($P < 0.01$) on day 7 when the plants were re-watered (Fig. 4). At the end of the experiment on day 8, LOX emission rate of $62 \pm 8 \text{ pmol m}^{-2} \text{ s}^{-1}$ in infested drought-stressed plants was not different from LOX emissions in infested well-watered plants, but the emissions were significantly different ($P < 0.01$) in non-infested drought-stressed plants (Fig. 4).

3.4 Monoterpene emissions

The monoterpenes detected were α -pinene, β -pinene, camphene, limonene, 3-carene, *p*-cymene, β -phellandrene and (*E*)- β -ocimene. Low emissions of terpenes ($0.03 \text{ nmol m}^{-2} \text{ s}^{-1}$), close to the baseline level, were observed in control plants (Fig. 5). On the first day after the larvae were added to the plants (day 1), no significant increase in monoterpene emissions was observed ($P = 0.13$, Fig. 5). On day 2, the emission of monoterpenes from the drought-stressed larvae-infested plants increased drastically, reaching a level of $1547 \pm 51 \text{ pmol m}^{-2} \text{ s}^{-1}$ while the emission from well-watered infested plants were still low, and not significantly different from controls ($P = 0.32$, Fig. 5). On the same day, the emissions from non-infested drought-stressed plants increased to a level of $752 \pm 69 \text{ pmol m}^{-2} \text{ s}^{-1}$, being significantly different ($P = 0.32$) from control and drought-stressed larvae-infested plants. On day 3, the emissions of monoterpenes from drought-stressed plants had increased even more, and monoterpene emissions from well-watered infested plants were also significantly elevated ($P < 0.01$, Fig. 5). The differences among the treatments - the emissions in drought-stressed infested plants higher than in the two other treatments, and in well-watered infested plants higher than in control plants - were maintained on the following days (Fig. 5). On day 7, when the plants under drought were re-watered, the monoterpene emissions decreased to a value of $1070 \pm 170 \text{ pmol m}^{-2} \text{ s}^{-1}$ and the monoterpene emission rates were no longer significantly different among drought- and well-watered herbivory-stressed treatments (Fig. 5). The emission from drought-stressed non-infested plants became also significantly lower ($257 \pm 58 \text{ pmol m}^{-2} \text{ s}^{-1}$) than that from infested well-watered plants.

3.5 Emissions of herbivory-stress marker compounds (*E*)- β -ocimene, DMNT and MeSA

Temporal variation in (*E*)- β -ocimene emissions was similar to total monoterpene emissions with significant emissions elicited immediately after the start of feeding under drought stress, and with a delay in well-watered plants. However, differently from total monoterpenes, (*E*)- β -ocimene emission increased continuously until reaching a maximum on day 4 in drought-stressed plants and on day 5 in well-watered plants (Fig. 6a). The maximum level of (*E*)- β -ocimene emission was much lower, $30.6 \pm 0.7 \text{ pmol m}^{-2} \text{ s}^{-1}$ in well-watered infested than in drought-stressed infested plants ($44.6 \pm 1.0 \text{ pmol m}^{-2} \text{ s}^{-1}$). Furthermore, differently from total monoterpenes that started to decrease in drought-stressed plants after re-watering and reached a similar level to that in well-watered plants on day 7, (*E*)- β -ocimene emissions reached a similar level in both infested treatments on day 6 (cf. Fig. 5 and Fig. 6a).

The emission of nerolidol-derived homoterpene 4,8-dimethyl-nona-1,3,7-triene (DMNT) started on the first day when the larvae were added (Fig. 6b). For the first two days (day 1 and 2) of insect treatment, the emissions were not statistically different between the drought-stressed and well-watered plants ($P > 0.5$). The emission maximum was reached on day 2 in drought-stressed plants and on day 4 in well-watered plants. From this day on, the DMNT emissions from the well-watered herbivore-infested plants exceeded the emissions from drought-stressed herbivore-infested plants (Fig. 6b). The emission of DMNT from plants under drought stress alone was not statistically different from control plants ($P > 0.5$).

Methyl salicylate (MeSA) emission from plants under drought stress was strongly increased on day 1 of the insect treatment and remained at the high level (on average $63 \pm 17 \text{ pmol m}^{-2} \text{ s}^{-1}$) until the plants were re-watered on day 6, after which the MeSA emissions first decreased to $14 \pm 5 \text{ pmol m}^{-2} \text{ s}^{-1}$ on day 7 and then to the background level on day 8 (Fig. 6c). The same trend in MeSA emission was evident in plants under drought stress alone with a maximum emission of $40 \pm 17 \text{ pmol m}^{-2} \text{ s}^{-1}$ on day 1. MeSA emissions of well-watered insect-infested plants did not significantly differ from the control plants (Fig. 6c).

3.6 Changes in the emission blends

Larval feeding had a great impact on the emitted VOC content and composition in both well-watered and drought-stressed *A. glutinosa* plants (Fig. 7a and 7b). During the experiment, the composition of the VOC bouquet did not change in control plants (Monte Carlo test, $P > 0.05$). In the case of drought stress, the plant emission blend changed already on the first day of drought (D0 in Fig. 7b). Moreover, on the next day (D1 in Fig. 7b), feeding of larvae for only two hours was sufficient to cause considerable changes in the emissions of *A. glutinosa* plants (Monte Carlo test, $P < 0.01$). The bouquet of drought-stressed plants changed again after the plants were re-watered (Monte Carlo test, $P < 0.01$). During the first days of infection in well-watered plants (from W-1 to W1, Fig. 7b), the emission bouquet of well-watered plants was similar to that in control plants (Monte Carlo test, $P > 0.05$), and considerable modifications were discovered from the second day on (day W2, Fig. 7b, Monte Carlo test, $P < 0.05$).

4 Discussions

4.1 Attractiveness and palatability of water-stressed vs. well-watered plants to herbivores

Plant photosynthesis and growth rates are strongly reduced by drought due to stomatal closure, and our results are consistent with these general patterns (Chaves et al. 2009; Chaves et al. 2011; Flexas and Medrano 2002; Centritto et al. 2011). Stopping watering of drought-sensitive species *Alnus glutinosa* reduced photosynthesis and stomatal conductance by more than 80% during the seven days of drought treatment (Fig. 3). Olfactometer experiments indicated that the *Monsoma pulveratum* larvae preferred the well-watered plants compared with the drought-stressed plants (Fig. 2a). The drought-stressed plants were apparently also less palatable as suggested by more than four-fold greater amount of leaf area consumed by herbivores in well-watered plants (Fig. 2b). This result is in agreement with other studies demonstrating that the insects perform better on healthy plants (Huberty and Denno 2004; Huberty and Denno 2006) and might suggest induction of enhanced

investment in defense metabolites such as tannins and polyphenols in drought-stressed plants. However, past studies in deciduous trees have actually suggested that drought leads to reduced phenolic compound concentrations (Shure et al. 1998; Thomas and Schafellner 1999), but the data are limited. We suggest that more work is needed to analyze modifications in investment in secondary metabolites in drought-stressed plants.

4.2 Herbivory effects on photosynthetic characteristics

Previous studies have shown that net assimilation rate and stomatal conductance can be stimulated, suppressed or not affected by the herbivore feeding (Copolovici et al. 2011; Kerchev et al. 2012; Woolery and Jacobs 2011). In our study, well-watered plants with only herbivore stress maintained the photosynthetic rates and stomatal conductance at the same level as control plants through the entire experiment (Fig. 3a, b). Leaf damage due to herbivory typically results in suppressed photosynthesis in the immediate vicinity of the damaged leaf parts, but also in reduced photosynthesis in surrounding areas due to free water evaporation and desiccation stress (Aldea et al. 2006; DeLucia et al. 2008). Thus, constancy of net assimilation rates and stomatal conductance suggests a compensatory response. Such a compensatory response may result from alteration of the strength of the source-sink relationships with increasing the absolute demand of sinks for carbohydrates (Copolovici et al. 2011). Recovery of photosynthetic rates of drought-stressed insect-fed plants to the level before the drought stress suggests that if there was compensatory photosynthesis, it occurred similarly in drought-stressed and well-watered plants.

4.3 Elicitation of LOX products

Damage of *A. glutinosa* leaves by *M. pulveratum* larvae elicited characteristic emissions of lipoxygenase pathway compounds (LOX) with the maximum emission rates already achieved in the first day of the feeding (Fig. 4). Induction of LOX emissions by herbivory treatment is in agreement with numerous studies demonstrating enhanced emissions in herbivore-fed plants.

The emissions under water limitation were much higher despite lower degree of herbivory damage (Fig. 2b, Fig. 4). Thus, a clear priming effect of drought was found on LOX emissions. This evidence suggest that a given level of herbivory stress was “more severe” in the case of drought-stressed plants. Provided that LOX are likely released in response to early signaling events including formation of reactive oxygen species (ROS) and electrolyte leakage at the site of insect damage (Arimura et al. 2011; Bruinsma et al. 2010; Wu and Baldwin 2009) it might be that drought-driven formation of ROS (Chaves et al. 2009) sensitized the LOX response.

Induction of terpenoid emissions—Leaves of *A. glutinosa* plants do not emit terpenes (monoterpenes, sesquiterpenes, or terpenoid derivatives), and do not accumulate any of these compounds under non-stressed conditions (Copolovici et al. 2011) and this was confirmed by very low, close to the detection limit, emission of terpenoids in control plants. Insect feeding resulted in induction of moderately high monoterpene emissions from well-watered and drought-stressed plants, but monoterpene emissions were induced later than LOX emissions. Analogous induction of monoterpene emissions has been observed in several

lepidopteran larvae feeding experiments (Copolovici et al. 2011; Holopainen et al. 2013; Schaub et al. 2010; Trowbridge and Stoy 2013).

As with LOX emission, monoterpene emissions were higher in drought-stressed plants, and the emissions increased faster, again suggesting that drought primed the plants to more sensitive herbivory response (Gouinguene and Turlings 2002; Takabayashi, et al. 1994). Such enhancement of monoterpene emissions by drought has been demonstrated in constitutively monoterpene-emitting species (Blanch et al. 2009; Llusà and Peñuelas 1998; Nogués et al. 2012; Ormeño et al. 2007; Peñuelas et al. 2007), but to our knowledge such a drought enhancement has not been demonstrated for induced emissions.

4.4 Induction of stress-specific compounds

Three key compounds implicated in herbivore response, acyclic monoterpene (*E*)- β -ocimene, homoterpene DMNT, and methyl salicylate were apparently regulated differently than either LOX or other monoterpenes (cf. Figs. 4-5 and Fig. 6). Differently from LOX or non-stress-specific monoterpenes, emission of (*E*)- β -ocimene and DMNT were characterized by a maximum indicating downregulation in these emissions at later stages of herbivory. Furthermore, although these emissions were induced earlier in drought-stressed plants, the differences among the treatments were much smaller than the differences in either LOX or ubiquitous monoterpenes. Such similarity in the emissions is in agreement with our previous study, where no herbivory “dose” effect was found for DMNT and a weak effect was observed for (*E*)- β -ocimene (Copolovici et al. 2011), suggesting that emissions might serve as signals for the presence of herbivores, but are not indicative of the number of herbivores.

It has been demonstrated that increased emission of (*E*)- β -ocimene in herbivore-damaged plants of *Medicago truncatula* is associated with accumulation of transcripts of corresponding *M. truncatula* (*E*)- β -ocimene synthase (MtEBOS), indicating that herbivore feeding increases volatile release by inducing terpene synthase gene expression (Navia-Gine et al. 2009). Herbivore-elicited (*E*)- β -ocimene emissions have been shown to occur via the plastidial 2-C-methyl-D-erythritol 4-P (MEP) pathway (Arimura et al. 2000) while the emissions of DMNT likely occur through mevalonate pathway (Bartram et al. 2006). Both isoprenoid synthesis pathways are upregulated under herbivory (Arimura et al. 2000; Bartram et al. 2006). Earlier induction of these two compounds in drought-stressed plants furthermore emphasizes the priming effect of drought, possibly involving upregulation of the two isoprenoid synthesis pathways.

The most interesting result is the strongly enhanced MeSA emissions in drought-stressed plants during herbivory. It has been shown that oxidative stress can induce premature senescence of mature leaves and salicylic acid may be involved in the regulation of this process (Elizabeth Abreu and Munné-Bosch 2008; Munné-Bosch and Peñuelas 2003). On the other hand, the genes which are regulated by salicylic acid are defense-related genes, and they can participate in plant responses to biotic and abiotic stresses (Morris et al. 2000). Methyl salicylate is commonly elicited in response to fungal pathogens (Cardoza et al. 2002) and attacks by sap-sucking herbivores such as aphids or whiteflies (Li et al. 2006; Zarate et al. 2007), but it may also be elicited downstream the cascade of jasmonic acid induced

responses after chewing herbivore attacks (Cardoza et al. 2002; Dicke et al. 1999; Rodriguez-Saona et al. 2001). Thus, MeSA emissions following the start of herbivory in drought-stressed plants reflect interactions among defense pathways that clearly deserve further study.

We did not study tritrophic interactions, but alteration of volatile blends by drought stress might serve as an important signal for enemies of herbivores as well. The natural enemies of the herbivores use different chemical compounds that can be detected at long distances from attacked plants (see for reviews Dicke and Baldwin 2010; Dicke and Loreto 2010). Differences in the emission blend between well-watered and drought-stressed plants, in particular the apparent repellence to herbivores of the odor of drought-stressed plants may be informative of the quality of prey for the parasitoids.

5 Conclusions

These data provide evidence that *Alnus glutinosa* plants exposed to mild drought prior to herbivore attack become primed to subsequent different type of stress. In this context, alder trees become less sensitive to herbivory by the larvae of *M. pulveratum*. Also, changes in the emission blend by drought that clearly change the attractiveness of plant odor may play an important role in tritrophic interactions. These data collectively suggest that abiotic stresses can strongly interact with biotic stresses and such stress interactions can play a major role in determining the ultimate degree of insect damage as well as alter tritrophic interactions.

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Fig. 1.

Illustration of the custom-made Y-tube olfactometer (Teflon tubing, internal diameter of 0.65 cm; length of arms 6 cm) to assess the responses of herbivores to plant volatiles. For the olfactometric bioassay, the Y-tube was positioned in the centre of a box (10 cm x 10 cm x 5 cm), and two jars, one enclosing a well-watered plant and the other a drought-stressed plant, were connected to each of the Y-tube olfactometer ends.

Fig. 2.

Average ($\pm$ SE) behavioral responses of alder sawfly (*Monsoma pulveratum*) larvae in a Y-type olfactometer to odors of well-watered and drought-stressed *Alnus glutinosa* plants (a) and average leaf area eaten ($\pm$ SE) by five larvae of *M. pulveratum* at the end of the experiment in the well-watered and drought-stressed treatments (b). Means in (a) were compared by a Kruskal-Wallis non-parametric ANOVA ($n = 5$), and in (b) by a standard ANOVA ($n = 6$) and different letters indicate means that are statistically different at $P < 0.05$.

Fig. 3.

Changes in net assimilation rate (a) and stomatal conductance to water vapor (b) in seedlings of *Alnus glutinosa* without insects and well watered – control (■), under drought without insects (○), and with *Monsoma pulveratum* larvae, either well-watered (●) or under drought stress (△). In herbivory treatments, five larvae of *Monsoma pulveratum* were placed on each plant. The measurements were conducted at a quantum flux density of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, air temperature of 28°C and at ambient CO_2 concentration of $380 \pm 20 \mu\text{mol mol}^{-1}$. The data are expressed per unit projected leaf area. Each data point is the mean ($\pm$ SE) of 6 independent replicate plants.

Fig. 4. Emission time-courses (in $\text{pmol m}^{-2} \text{s}^{-1}$) of total LOX products for non-treated plants (control), and plants fed by *M. pulveratum* larvae, and either well-watered or drought-stressed. Means were separated by Tukey's test and different letters indicate means that are statistically different with $P < 0.05$. Experimental conditions as in Fig. 2.

Fig. 5. Emission time-courses (in $\text{pmol m}^{-2} \text{s}^{-1}$) of total monoterpene emissions per unit leaf area for control plants (non-herbivory treated, well-watered), and plants fed by *M. pulveratum* larvae, and either well-watered or drought-stressed. Experimental conditions as described in Fig. 2, and data presentation and statistical analysis as in Fig. 3.

Fig. 6. Temporal variations in monoterpene (*E*)- β -ocimene (a), homoterpene 4,8-dimethylnona-1,3,7-triene (DMNT) (b) and benzenoid methyl salicylate (c) emissions (in $\text{pmol m}^{-2} \text{s}^{-1}$) from *A. glutinosa* control plants (well-watered, non-fed) and plants fed by *M. pulveratum* larvae and either well-watered or drought-stressed. Experimental conditions and treatments as in Fig. 2 and data presentation as in Fig. 3.

Fig. 7.

Loading (a) and score (b) plots of principal component analysis (PCA) based on the time-courses of emitted volatiles (in $\text{pmol m}^{-2} \text{s}^{-1}$) for non-treated plants (C), and plants fed by *M. pulveratum* larvae, and either well-watered (W) or drought-stressed (D). In the score plot, each symbol represents a mean bouquet of 6 replicate plants (numbers after the treatment symbol denote the day of the experiment), while in the loading plot, the numbers represent different volatiles as following: **1.** 1-Hexanol; **2.** 2-(*E*)-Hexenal; **3.** 3-(*Z*)-Hexenol; **4.** 3-(*Z*)-Hexenyl acetate; **5.** Bornyl acetate; **6.** Camphene; **7.** *para*-Cymene; **8.** 3-Carene; **9.** Limonene; **10.** Linalool; **11.** (*E*)- β -Ocimene; **12.** (*Z*)- β -Ocimene; **13.** β -Phellandrene; **14.** α -Pinene; **15.** β -Pinene; **16.** DMNT; **17.** Geranyl acetone; **18.** 6-Methyl-5-heptene-2-one; **19.** Methyl salicylate (MeSA); **20.** Nonanal. In the loading plot, the amount of the emitted compound increases with the distance from the origin of the coordinate system.