

Leaf rust induced volatile organic compounds signalling in willow during the infection

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Received: 17 March 2010 / Accepted: 7 April 2010 / Published online: 24 April 2010
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Abstract Plants are known to emit volatile organic compounds (VOC) in response to various biotic or abiotic stresses. Although the VOC emission in the case of insect attacks is well described, there is only little known about the impact of pathogens on plant emission. In the present study, we used a willow-leaf rust system to describe the effects of a biotrophic fungal infection on the VOC emission pattern of willow leaves. We detected that isoprene emissions from rust-infected leaves decreased threefold compared to control. The total monoterpene emissions did not change although a stress-signalling compound (*Z*)- β -ocimene showed an increase in infected plants on several days. The infection also increased the emission of sesquiterpenes and lipoxygenase products (LOX) by factors of 175-fold and 10-fold, respectively. The volatile emission signals showed two clear peaks during the experiment. At 6, 7 and 12 days post-infection (dpi), the relative volatile emission signal increased to about sixfold compared to uninfected plants. These time points are directly connected to rust infection since at 6 dpi the first rust pustules appeared on the leaves and at 12 dpi necrosis had developed around several pustules. We present correlations between LOX and sesquiterpene emission signals, which suggest at least two different steps in eliciting the volatile emission.

Keywords Leaf rust infection · Lipoxygenase pathway products · Plant stress response · VOC signalling · Willow

Abbreviations

GC–MS	Gas chromatography–mass spectrometry
LOX	Lipoxygenase pathway products
MEP	Methylerythritol phosphate pathway
MVP	Mevalonate pathway
VOC	Volatile organic compounds

Introduction

Rust diseases are common on various herbal plants all over the world. Rust fungi are obligate parasites, which mean that they absorb nutrients from their host plants without killing them. They are known as highly specialised pathogens (Staples 2000). The members of the rust family *Melampsora* have been studied very intensively during the last two decades, mainly because the majority of them occur on willows (*Salix* spp.) and poplars (*Populus* spp.) (Cummins and Hiratsuka 2003). These tree species are nowadays used as renewable bioenergy crops in temperate regions, and leaf rust infections can significantly reduce their biomass yield (McCracken and Dawson 1998; Åhman and Wilson 2008; Toome et al. 2009, 2010) since rusts absorb the photosynthesis products of the plants (Voegele and Mendgen 2003).

It is known that plants communicate via volatile organic compound (VOC) signals that are released as a response to attacks by insects or pathogens (Baldwin et al. 2006; Heil and Silva Bueno 2007). The presence of VOC emissions

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from the genus *Salix* has been reported and entered into emission inventories (Isebrands et al. 1999; Simon et al. 2006; Smiatek and Steinbrecher 2006), but there are only few studies available describing the emission of specified volatiles (Hakola et al. 1998; Rinne et al. 1999; Hakola 2001). The outcome of those works characterises willow as a predominantly high isoprene-emitting species that emits monoterpenes as well. However, detailed information on sesquiterpenes and other volatiles emitted from willows is scarce.

While many studies have been focusing on the plant–insect interactions (Röse and Tumlinson 2005; Blande et al. 2007; Schaub et al. 2010), little is known about the VOC signals that are connected with fungal infections in general (Cardoza et al. 2002; Mendgen et al. 2006; Vuorinen et al. 2007; Jansen et al. 2009b), and infections by *Melampsora* rust in particular. Typically, volatile compounds like specific monoterpenes, sesquiterpenes and lipoxygenase pathway products (LOX) are emitted in connection to both biotic and abiotic stresses that affect plants (Agarwal and Grover 2006; Jansen et al. 2009b; Yuan et al. 2009). Since rusts, as biotrophs, need living host tissue to survive, it is important for them to avoid the recognition by the host plant and following resistance reactions (Heath 1997). We assume that the response of the plant to rust is different as compared to aggressive pests such as insects or non-obligate parasites that have been studied so far (Singh et al. 1998; Jansen et al. 2009b), which show temporal dynamic VOC emission patterns (Holopainen 2004; Arimura et al. 2005). To our knowledge, there are no data available about the VOC emission patterns during the infection process with the biotrophic fungus *Melampsora* spp. Therefore, the aim of this experiment was to study the effect of an obligate parasitic fungal infection on the emission of VOC, using willow-leaf rust system.

Materials and methods

Experiment setup

The experiment was conducted on rooted cuttings of the hybrid willow clone ‘Gudrun’ (*Salix burjatica* Nasarow × *S. dasyclados* Wimm.) from Svalöf Weibull AB (Svalöf, Sweden). The plants were grown from dormant cuttings using regular soil mix with no additional fertilisers and watered every second day. In a growth chamber, the daylight period was 12 h, and the photosynthetic active radiation flux was in average $1,300 \mu\text{mol m}^{-2} \text{s}^{-1}$ above the canopy. Four-week-old plants were approximately 25 cm high, and had 10–12 fully expanded leaves. They were placed in a multi-chamber cuvette system with constant humidified airflow of 0.45 L min^{-1} . Each glass

chamber was of 3 L volume and allowed to place the whole shoot into the enclosure while pot and soil stayed outside. The system was air tightened by plastiline gaskets, allowing a total exchange of air in the chamber by 6.7 min, and had a separate filtrated outlet to prevent possible contaminations by rust spores and to avoid plant–plant signalling. To avoid water condensation on the chamber walls with subsequent solvation of VOC and to ensure a proper air mixing within the chambers, each of them had a fan installed. The light regime followed a 12-h daylight regime, with photosynthetic active radiation fluxes ranging from 200 to $1,300 \mu\text{mol m}^{-2} \text{s}^{-1}$. After installation, the plants were left untreated for 2 days for stabilisation.

In the end of the second day after the placement in the chambers, two plants were chosen to be controls and three were infected with willow leaf rust. The lower side of all leaves were inoculated with fresh rust spores from single spore isolate of *Melampsora epitea* collected from clone ‘Gudrun’ in Estonia in August 2008. Rust spores were applied on willow leaves with a brush using spores from fresh mature uredinia. At 14 days post-inoculation (dpi), all leaves were removed from the plants, their area was measured and the number of rust uredinia on every leaf was determined. During the experiment, the total leaf area of each studied plant was measured every second day.

Photosynthesis measurements

To monitor the state of the plants during the rust infection, we conducted photosynthesis measurements on leaf level using the GFS 3000 Portable Gas Exchange System (Walz, Effeltrich, Germany). The photosynthesis measurements were carried out about 2 h after the VOC sampling. For that one leaf was placed into the Walz cuvette and applied an ambient CO_2 mixing ratio of 380 ppm and photosynthetic active radiation flux of $1,000 \mu\text{mol Quanta m}^{-2} \text{s}^{-1}$. The Walz cuvette system was set to hold the leaf temperature constant during the measurements at 25°C , a relative air humidity of 80% with a flow through the cuvette of 750 ml min^{-1} . Leaves covered the Walz cuvette area totally; therefore, the leaf area during photosynthesis measurements equals 8 cm^2 . To prevent contamination by rust spores while measuring the photosynthetic parameters, the cuvette foam rubber gaskets were covered with Teflon tape, which was changed after each measurement procedure.

VOC sampling and gas chromatography mass spectrometry (GC–MS) analysis

Unlike for photosynthesis measurement, VOC sampling was made from every chamber containing a whole willow branch with several leaves. Isoprene, LOX products, mono

and sesquiterpene emissions from leaves were analysed after concentrating 4 L of the air from the chamber outlet into a multibed stainless steel cartridge (10.5 cm length, 3 cm inner diameter, Supelco, Bellefonte, PA, USA) filled with Carbotrap C 20/40 mesh (0.2 g), Carbopack C 40/60 mesh (0.1 g) and Carbotrap X 20/40 mesh (0.1 g) adsorbents (Supelco). The adsorption was carried out every morning for each plant at a flow rate of 200 ml min⁻¹ for 20 min using a constant flow air sample pump (1003-SKC, SKC Inc., Huston, TX, USA). An additional sample was taken from the inlet air stream prior to the chambers to give a background value, which was subtracted from emission samples. Before the collection of volatiles, the traps were cleaned by the passage of a stream of ultra pure helium at a flow rate of 200 ml min⁻¹ and at temperature of 250°C for 2 h.

Adsorbent cartridges were analysed with a combined Shimadzu TD20 automated cartridge desorber and Shimadzu 2010 plus GC–MS instrument (Shimadzu Corporation, Kyoto, Japan); the method has been described previously (Copolovici et al. 2009). The GC carrier gas was He (99.9999%, Elmer Messer Gaas AS, Tallinn, Estonia) with a total flow in the column of 1.48 ml min⁻¹. The following TD20-parameters were used: He purge flow 40 ml min⁻¹, primary desorption temperature 250°C, primary desorption time 6 min, second stage trap temperature during primary desorption: -20°C, second stage trap desorption temperature 280°C, hold time 6 min. Adsorbent cartridges were back flushed with high purity He during thermal desorption. A ZB-624 capillary column (0.32 mm i.d. × 60 m, 1.8 µm film Zebron, Phenomenex, Torrance, CA, USA) was employed for the volatile separation using the following GC oven programme: 40°C for 1 min, 9°C min⁻¹ to 120°C, 2°C min⁻¹ to 190°C, 20°C min⁻¹ to 250°C, 250°C for 5 min. The mass spectrometer was operated in electron-impact mode (EI) at 70 eV, in the scan range *m/z* 30–400, the transfer line temperature was set at 240°C and ion-source temperature at 150°C. Compounds were identified by use of the NIST spectral library and based on retention time identity with the authentic standard (GC purity, Sigma-Aldrich, St. Louis, MO, USA). The absolute concentrations of isoprene, terpenes and LOX products were calculated based on an external authentic standard consisting of known amount of VOCs.

Field measurements

In order to verify the emission patterns to be applicable in the field conditions, additional measurements were made in a willow plantation where the same willow clone ‘Gudrun’ is planted as short rotation coppice. Six healthy-looking leaves (used as controls) and six rust-infected leaves were chosen, and their photosynthesis activity was measured

with the Walz cuvette system with the measurement procedure described above. In difference to the VOC sampling in the laboratory, the adsorption was done directly using an aliquot of 200 ml min⁻¹, split out of the cuvette air stream via a T-piece; thus, we used the same leaf to measure photosynthesis parameters and to obtain the VOC sample. For background correction, several empty cuvette samples were adsorbed.

Statistical analysis and data handling

All data shown in the figures and table are means derived from replicates on different plants. The treatment means were statistically compared by 1-way and Student post-hoc ANOVA using ORIGIN 8 (OriginLab Corporation, Northampton, MA, USA). Significantly different means ($P < 0.05$), derived from two control plants or three rust-infected plants are indicated with different letters for data series. To express the relative changes in the emission rates, we normalised the rust-infected data according to the control plants datasets. This procedure enabled a comparison independent of the signal amplitude that varied by individual plants. These analyses were conducted in Matlab 7.5 (MathWorks Inc., Natick, MA, USA).

Results

Rust infection

The first rust uredinia appeared on the leaves at 5 and 6 dpi. All three infected plants developed severe disease symptoms, which led to several necrotic lesions on the leaves for 11 dpi. After 14 days, there were in average 6.6 uredinia cm⁻². There were no rust pustules or necrotic lesions detected on the leaves of control plants during this experiment.

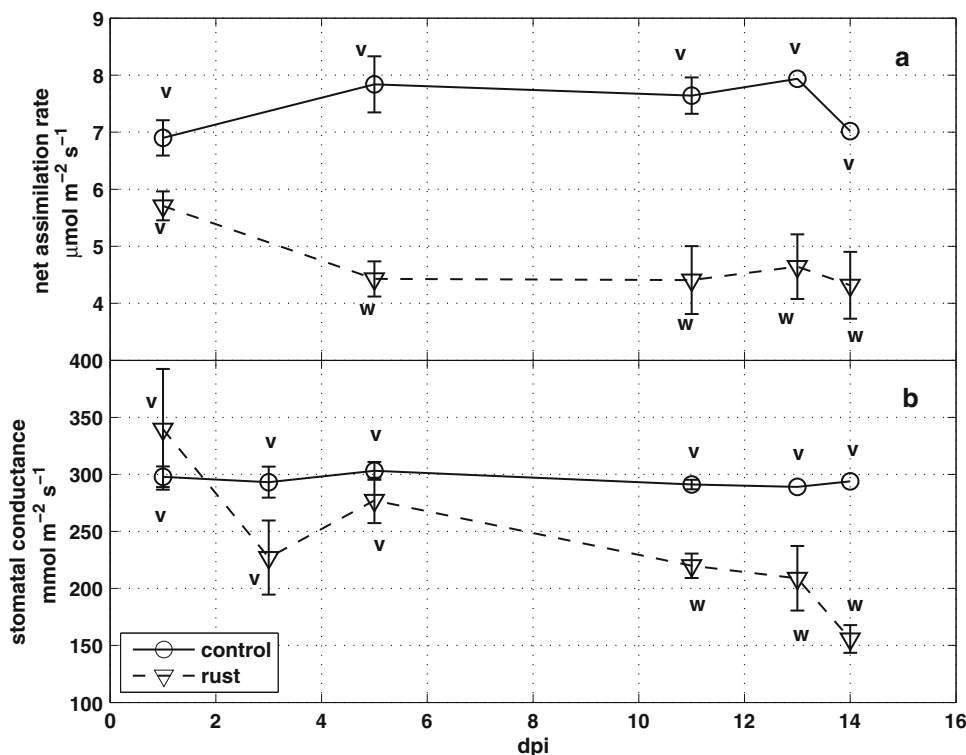
Photosynthesis

The results of the photosynthesis measurements are compiled in Fig. 1. Net assimilation rate was found to be stable at an interval between 7 and 8 µmol m⁻² s⁻¹ for the control plants, while in case of infected plants it showed a significant decrease and dropped by almost to 50% (4–5 µmol m⁻² s⁻¹) as compared to the value before fungal infection ($P < 0.05$) (Fig. 1a). During the 2-day stabilisation period before the infection, there was no difference between all plants ($P > 0.05$) (data not shown).

The stomatal conductance showed the same pattern as the net assimilation rate. The control plants stayed stable with conductivity of 300 mmol m⁻² s⁻¹ throughout the study, while the diseased plants showed a significant

Fig. 1 The net assimilation rate (a) and the stomatal conductance (b) of control and infected plants at 1–14 dpi.

Letters indicate the statistically significant differences inside the time point



decline in stomatal conductance ($P < 0.05$), resulting with a minimum of $150 \text{ mmol m}^{-2} \text{ s}^{-1}$ at the end of the experiment. At the first stage of the experiment (1–5 dpi), the stomatal conductance of the infected plants did not show significant differences, even though it fluctuated with rather high amplitude between 230 and $340 \text{ mmol m}^{-2} \text{ s}^{-1}$. However, the conductivities measured from 11 to 14 dpi were significantly lower compared to the control plants ($P < 0.05$) (Fig. 1b).

VOC emission

The GC–MS analysis showed that the total emission of different VOCs was influenced by rust infection. Isoprene emission was greatly reduced in case of infected plants compared to controls ($P < 0.05$), and dropped to almost 40% of the emission rate found in the control plants. The total monoterpene emission was about a factor of 40 smaller than the isoprene emission, and no significant difference between the infected and control plants were detected. The average emission rates of LOX compounds were tenfold higher in case of the infected plants; sesquiterpenes emission was detected only in rust-infected plants and never in control plants. The general emission rate of isoprene or monoterpenes was in $\text{nmol m}^{-2} \text{ s}^{-1}$, whereas LOX and sesquiterpenes were emitted in three orders of magnitude higher levels in $\text{pmol m}^{-2} \text{ s}^{-1}$ (Fig. 2).

When examining the emission day by day during the infection, there were some days that monoterpene (*Z*)- β -

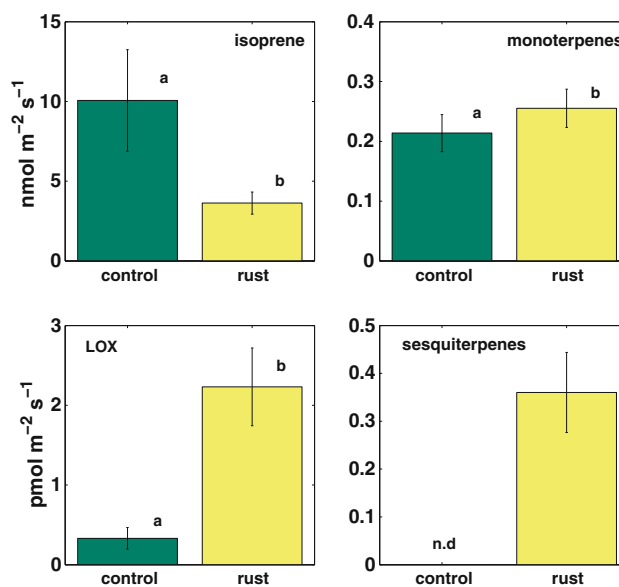


Fig. 2 Average of total volatile organic compounds emission rate for control and infected plants over the time of emission. Letters indicate statistically significant difference between the bars. Error bars denote standard errors, $n = 14$

ocimene, LOX and sesquiterpene compounds were detected at higher rates from infected plants. The first peak of the emission of these stress-related compounds was around 6 dpi, and the second peak at 12 dpi. (*Z*)- β -ocimene emission had two very clear peaks; its emission was higher compared to the control first at days 6 and 7 dpi and then

later in the infection process at 12 dpi. Higher sesquiterpenes rates were detected since 3 dpi until the end of the experiment, having highest emission at 6 dpi. Higher LOX compound rates were detected since 5 dpi and resembled the ocimene pattern, having two peaks, one at 6 dpi and the other at 12 dpi (Fig. 3).

A list of identified volatile compounds detected during the experiment is compiled together in Table 1. Especially α -pinene, Δ^3 -carene and limonene dominated the monoterpene emissions in both infected and uninfected plants. Sesquiterpenes were found to be emitted only by rust-diseased plants from where α -copaene, (*E,E*)- α -farnesene and α -murolene were identified (Table 1).

The field measurements confirmed our findings that infected leaves emit less isoprene. Uninfected leaves emitted isoprene with a rate of $5.8 \pm 0.8 \text{ nmol m}^{-2} \text{ s}^{-1}$ while the emission of infected leaves was about 50% lower, only $3.3 \pm 0.2 \text{ nmol m}^{-2} \text{ s}^{-1}$. Monoterpene emissions were found to be substantially higher in the plantation compared to the laboratory experiment. Total monoterpene emission rate was $3.5 \pm 0.9 \text{ nmol m}^{-2} \text{ s}^{-1}$ for uninfected leaves and $2.5 \pm 1.2 \text{ nmol m}^{-2} \text{ s}^{-1}$ for rust-infected leaves. Under field conditions, the LOX compounds and sesquiterpenes could not be detected. Moreover, the photosynthesis

pattern showed the same trend as the laboratory measurements, being lower in the case of infected leaves. Net assimilation rate was found to be $9.4 \pm 0.65 \mu\text{mol m}^{-2} \text{ s}^{-1}$ for the uninfected leaves and $8.8 \pm 0.46 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in case of the infected leaves, and the stomatal conductance was $182 \pm 10.6 \text{ mmol m}^{-2} \text{ s}^{-1}$ and $168 \pm 15.1 \text{ mmol m}^{-2} \text{ s}^{-1}$ for uninfected and infected leaves, respectively.

Discussion

The results of our experiment demonstrated that willow responds very clearly to leaf rust infection by emitting several stress-related volatile compounds during the different stages of the infection. The interaction between the leaf rust and willow plant seems to be very specific since the response of the plant is not following known patterns of other biotic stressors. The specificity of similar interaction has been shown before by transcriptome studies where the response of the plant to the infection depends greatly on the species or even on the *forma speciales* of the rust (Laurans and Pilate 1999; Rinaldi et al. 2007; Azaiez et al. 2009).

Fig. 3 The relative change in emission of LOX compounds, (*Z*)- β -ocimene and sesquiterpenes in the infected plants relative to the control plants. Pictures above demonstrate rust-infected leaves at 0, 6 and 12 dpi

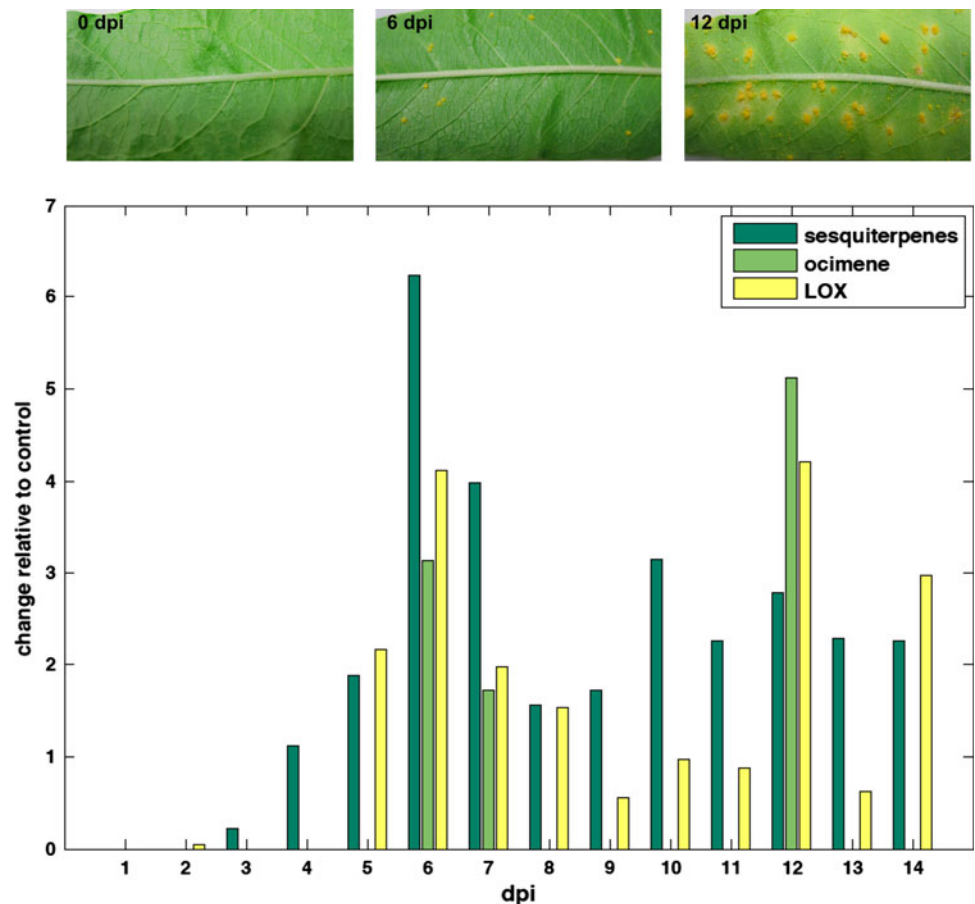


Table 1 Average emission rates \pm SE throughout the experiment of major constituents from control and rust-infected willow plants and the *P* value for their statistical difference in Student's test

Constituents	Emission rate averages – control leaves		Emission rate averages – infected leaves		<i>P</i> value
	nmol m ⁻² s ⁻¹	μg gDW ⁻¹ h ⁻¹	nmol m ⁻² s ⁻¹	μg gDW ⁻¹ h ⁻¹	
Isoprene	11.0 \pm 3.0	71 \pm 23	3.6 \pm 0.6	23 \pm 4	0.0013
(<i>Z</i>)-3-Hexenol + (<i>E</i>)-2-hexenal	2 \times 10 ⁻⁴ \pm 9 \times 10 ⁻⁵	2 \times 10 ⁻³ \pm 9 \times 10 ⁻⁴	0.0011 \pm 0.0009	0.011 \pm 0.009	0.0031
1-Hexanol	3 \times 10 ⁻⁴ \pm 5 \times 10 ⁻⁵	3 \times 10 ⁻³ \pm 5 \times 10 ⁻⁴	0.0016 \pm 0.0002	0.016 \pm 0.002	0.0023
α -Pinene	0.16 \pm 0.03	2.27 \pm 0.42	0.13 \pm 0.01	1.85 \pm 0.14	0.234
Camphene	0.0050 \pm 0.0009	0.071 \pm 0.013	0.0055 \pm 0.0007	0.078 \pm 0.010	0.758
β -Pinene	0.024 \pm 0.003	0.34 \pm 0.04	0.015 \pm 0.004	0.21 \pm 0.06	0.185
α -Phellandrene	ND	ND	1.8 \times 10 ⁻⁴ \pm 0.9 \times 10 ⁻⁴	2.5 \times 10 ⁻³ \pm 1.3 \times 10 ⁻³	
Δ^3 -Carene	0.097 \pm 0.014	1.38 \pm 0.20	0.095 \pm 0.007	1.35 \pm 0.10	0.683
5-Hepten-2-one	0.041 \pm 0.018	0.58 \pm 0.25	0.070 \pm 0.010	0.99 \pm 0.14	0.235
Limonene	0.11 \pm 0.05	1.6 \pm 0.7	0.12 \pm 0.03	1.7 \pm 0.4	0.896
(<i>Z</i>)- β -Ocimene	1 \times 10 ⁻⁴ \pm 0.3 \times 10 ⁻⁴	1.4 \times 10 ⁻³ \pm 0.4 \times 10 ⁻⁴	1 \times 10 ⁻³ \pm 3 \times 10 ⁻⁴	0.014 \pm 0.0005	0.022
α -Copaene	ND	ND	1.3 \times 10 ⁻⁵ \pm 0.3 \times 10 ⁻⁵	1.2 \times 10 ⁻³ \pm 0.5 \times 10 ⁻³	
(<i>E,E</i>)- α -Farnesene	ND	ND	1.5 \times 10 ⁻⁴ \pm 0.2 \times 10 ⁻⁵	3.6 \times 10 ⁻² \pm 6 \times 10 ⁻⁴	
α -Murolene	ND	ND	1.3 \times 10 ⁻⁴ \pm 0.1 \times 10 ⁻⁵	5.5 \times 10 ⁻² \pm 5 \times 10 ⁻⁴	

ND values below the detection limit

Decreased photosynthetic parameters

Photosynthesis measurement results revealed that all plants continued their physiological activities during the experiment. Although the infected plants showed a reduction in net assimilation and stomatal conductance, all the plants maintained green leaf tissues and turgor until the end of the experiment. Furthermore, previous studies have shown that plant pathogens, including rusts, significantly reduce the photosynthesis and increase the evaporation of their host (Staples 2000; Robert et al. 2005; Major et al. 2009). It has also been detected that infected willow leaves have lower chlorophyll content than healthy ones (Abd El-Ghany et al. 2009). As in this study an online gas exchange measurement system was used, the drop in net photosynthesis rate could be caused also by an increased net respiration rate of the leaf. The growing fungus can add non-photosynthetically active tissues that increase the rate of respiration.

Changes in VOC emission signals during infection

Monitoring the VOC emissions during the infection revealed a large reduction in emitted isoprene from infected plants. As formed by the methylerythritol phosphate (MEP) chloroplastid pathway, isoprene formation relies on the input of triose phosphates, originated from photosynthetic carbon fixation. Therefore, isoprene reduction is likely to be caused by the drain out of carbohydrates from the plant cells by the fungal feeding structures (Voegelé and Mendgen 2003; Schübler et al. 2006). Monoterpenes,

as well synthesised via the MEP pathway, however, did not show a significant change in their total emission throughout the experiment. Moreover, we found (*Z*)- β -ocimene, which is known as a stress-signalling monoterpene in plant–herbivore interactions (Jansen et al. 2009b), at much higher levels in infected plants at 6–7 and 12 dpi. Such increase in the monoterpene emission would further reduce isoprene emission as they share the same pathway. The so called “green leaf volatiles” or LOX compounds, originated from the lipoxygenase pathway, as well as sesquiterpenes, formed via the mevalonate pathway (MVP) were clearly amplified during the fungal infection. According to transcript profiling studies, terpene synthase-related genes are up-regulated during the rust infection (Azaiez et al. 2009). This can be connected to sesquiterpene cyclase, which could mediate the increase in sesquiterpene emissions.

Studies with poplars have demonstrated that rust infection induces the expression of several stress-related genes over time (Rinaldi et al. 2007; Azaiez et al. 2009). These studies also provide possible explanations to the most important result of this study, the pattern of VOC emission of infected plants during 2 weeks. As shown in Fig. 3, there were clear emission peaks detected in case of stress signals. A transcriptome study with rust-infected poplar leaves showed that at 6 dpi (which was the day the rust pustules appeared), there were several genes up-regulated that play a role in secondary metabolism and signalling (Azaiez et al. 2009). One of these highly expressed genes was connected to terpene synthase, which can explain the terpene emission pattern in our study. We also detected the

first emission peak of signalling compounds at 6 dpi, which was shortly after the appearance of the first rust pustules. Unfortunately, there are no expression data available about interaction studies longer than 9 dpi to correlate with the second emission peak at 12 dpi in our study. All those expression studies were made with detached leaves (Rinaldi et al. 2007; Azaiez et al. 2009), and therefore, the legibility of their results *in vivo* was doubtful. However, the data from the literature seem to correlate rather well with the data obtained from attached leaves in our study, and therefore, the correlation between the results obtained from detached and attached leaves is possible.

VOC emissions are different under field conditions

The field measurement results were in accordance to the laboratory experiment in terms of photosynthetic parameters and isoprene emissions. Monoterpene emissions were found to be substantially higher than under laboratory conditions. This could be due to a higher rate of other stresses under field conditions, most probably due to water deficiency and high irradiance, which cause changes in the emission pattern (Hakola 2001; Hakola et al. 2001; Peñuelas and Llusià 2001; Staudt et al. 2002). Additionally, seasonality and leaf age have an influence on the development of the leaf tissues and alters VOC emission patterns (Fischbach et al. 2002; Niinemets et al. 2009). The failure to detect the LOX products and sesquiterpenes under field conditions was most probably caused by high background values of these compounds. There are some willow clones with higher rust susceptibility growing in the vicinity of clone ‘Gurdrun’; thus, high background levels of rust-induced volatiles are likely. Under greenhouse conditions, VOCs have been used to monitor plant health status in a model study (Jansen et al. 2009a); however, this is very complicated under field conditions because the lifetime of some VOCs might be very short, and this limits their detection. Furthermore, long-chained volatiles have a lag phase between synthesis and emission that ranges from hours to days (Noe et al. 2010), whereby a clear detection of the time of eliciting is not possible.

Correlation between LOX and sesquiterpene emission signals

The data shown in Fig. 3 demonstrate that sesquiterpene and LOX emission signals are timely correlated. We found that both relative sesquiterpene and LOX emissions change around 5–8 and 10–14 dpi substantially. When correlating these relative changes, we found a positive relationship although the steepness of the slopes depended on the stage of infection. During the onset of the infection (2–8 dpi), the sesquiterpene signal was strongly coupled to the LOX

signal, i.e. a change in LOX emission signal was reflected almost with the same amplitude in a change of the sesquiterpene emission signal. In the later stage of the infection (11–14 dpi), this correlation was not found anymore (Fig. 4). While LOX fluctuated only with a small interval, sesquiterpene changes span over the whole range. A possible explanation for that could be connected to an elicitor scheme, covering fungal elicitors and wounding that has been proposed earlier (Singh et al. 1998). They described that as a response to fungal attack, LOX compounds are activated and affect positively sesquiterpene cyclase activity and formation, which subsequently lead to enhanced sesquiterpene production and emission. Whereas wounding also causes enhanced sesquiterpene production, it results in a different sesquiterpene composition as found by the fungal eliciting (Singh et al. 1998). Combining these results with our finding allows us to assume that during the infection onset the fungal-elicited LOX-induced emissions dominate. Whereas with the infection development and rising amounts of uredinia breaking through the cuticula and the appearance of necrotic lesions on the leaf, the wounding component dominates the emission pattern, causing the uncoupling of the sesquiterpene from the LOX emission signal.

Conclusion

This study is, to our knowledge, the first attempt to describe the willow volatile emission in response to rust

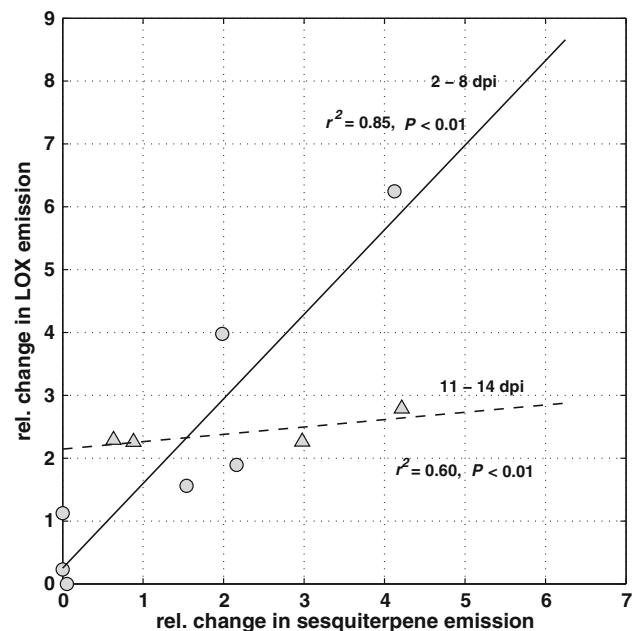


Fig. 4 Correlation between relative LOX and sesquiterpene emissions during 2–8 dpi (balls) and 11–14 dpi (triangles)

infection. The most important results were the change in volatile signalling during the infection and that very few stress-related compounds were emitted during the first days. LOX and sesquiterpene emission, which is triggered by several elicitors, also depended on the stage of rust infection. Since the interaction between obligate parasites and plants is very specific, it is important to conduct further studies to detect if the VOC emission in response to rust infection also depends on the willow genotype.

Acknowledgments The financial support by the Estonian Science Foundation grants ETF 8110, JD101, SF1090065s07 and SF0170057s09 are gratefully acknowledged. We thank Gregory Heller for improving the English of our manuscript.

References

- Abd El-Ghany TM, Eman MET, El-Sheikh HH (2009) Efficacy of fungal rust disease on willow plant in Egypt. *Aust J Basic Appl Sci* 3:1527–1539
- Agarwal S, Grover A (2006) Molecular biology, biotechnology and genomics of flooding-associated low O₂ stress response in plants. *Crit Rev Plant Sci* 25:1–21
- Åhman I, Wilson F (2008) Symptoms of pests, rust and other disorders on leaves of willow fertilised with wastewater, urine or sewage sludge. *Biomass Bioenergy* 32:1001–1008
- Arimura G-I, Kost C, Boland W (2005) Herbivore-induced, indirect plant defences. *Biochim Biophys Acta* 1734:91–111
- Azaiez A, Boyle B, Levee V, Seguin A (2009) Transcriptome profiling in hybrid poplar following interactions with *Melampsora* rust fungi. *Mol Plant Microbe Interact* 22:190–200
- Baldwin IT, Halitschke R, Paschold A, von Dahl CC, Preston CA (2006) Volatile signalling in plant-plant interactions: “talking trees” in the genomics era. *Science* 311:812–815
- Blande JD, Tiiva P, Oksanen E, Holopainen JK (2007) Emission of herbivore-induced volatile terpenoids from two hybrid aspen (*Populus tremula* × *tremuloides*) clones under ambient and elevated ozone concentrations in the field. *Global Change Biol* 13:2538–2550
- Cardoza YJ, Alborn HT, Tumlinson JH (2002) In vivo volatile emissions from peanut plants induced by simultaneous fungal infection and insect damage. *J Chem Ecol* 28:161–174
- Copolovici L, Kännaste A, Niinemets Ü (2009) Gas chromatography-mass spectrometry method for determination of monoterpene and sesquiterpene emissions from stressed plants. *Stud Univ Babes Bol* 54:329–339
- Cummins GB, Hiratsuka Y (2003) Illustrated genera of rust fungi. American Phytopathological Society, Minnesota
- Fischbach RJ, Staudt M, Zimmer I, Rambal S, Schnitzler J-P (2002) Seasonal pattern of monoterpene synthase activities in leaves of the evergreen tree *Quercus ilex*. *Physiol Plant* 114:354–360
- Hakola H (2001) Biogenic volatile organic compound (VOC) emissions from boreal deciduous trees and their atmospheric chemistry. Finnish Meteorological Institute, University of Helsinki, Helsinki
- Hakola H, Rinne J, Laurila T (1998) The hydrocarbon emission rates of tea-leafed willow (*Salix phylicifolia*), silver birch (*Betula pendula*) and European aspen (*Populus tremula*). *Atmos Environ* 32:1825–1833
- Hakola H, Laurila T, Lindfors V, Hellén H, Gaman A, Rinne J (2001) Variation of the VOC emission rates of birch species during the growing season. *Boreal Environ Res* 6:237–249
- Heath MC (1997) Signalling between pathogenic rust fungi and resistant or susceptible host plants. *Ann Bot Lond* 80:713–720
- Heil M, Silva Bueno JCS (2007) Within-plant signaling by volatiles leads to induction and priming of an indirect plant defence in nature. *Proc Natl Acad Sci USA* 104:5467–5472
- Holopainen JK (2004) Multiple functions of inducible plant volatiles. *Trends Plant Sci* 9:529–533
- Isebrands JG, Guenther AB, Harley P, Helmig D, Klinger L, Vierling L, Zimmerman P, Geron C (1999) Volatile organic compound emission rates from mixed deciduous and coniferous forests in Northern Wisconsin, USA. *Atmos Environ* 33:2527–2536
- Jansen RMC, Hofstee JW, Wildt J, Verstappen FWA, Bouwmeester HJ, Posthumus MA, Henten EJV (2009a) Health monitoring of plants by their emitted volatiles: trichome damage and cell membrane damage are detectable at greenhouse scale. *Ann Appl Biol* 154:441–452
- Jansen RMC, Miebach M, Kleist E, EJV Henten, Wildt J (2009b) Release of lipoxygenase products and monoterpenes by tomato plants as an indicator of *Botrytis cinerea*-induced stress. *Plant Biol* 11:859–868
- Laurans F, Pilate G (1999) Histological aspects of a hypersensitive response in poplar to *Melampsora larici-populina*. *Phytopathology* 89:233–238
- Major I, Nicole M-C, Duplessis S, Séguin A (2009) Photosynthetic and respiratory changes in leaves of poplar elicited by rust infection. *Photosynth Res*. doi:10.1007/s11120-009-9507-2
- McCracken AR, Dawson WM (1998) Short rotation coppice willow in Northern Ireland since 1973: development of the use of mixtures in the control of foliar rust (*Melampsora* spp.). *Eur J Forest Pathol* 28:241–250
- Mendgen K, Wirsler SGR, Jux A, Hoffmann J, Boland W (2006) Volatiles modulate the development of plant pathogenic rust fungi. *Planta* 224:1353–1361
- Niinemets U, Diaz-Espejo A, Flexas J, Galmes J, Warren C (2009) Role of mesophyll diffusion conductance in constraining potential photosynthetic productivity in the field. *J Exp Bot* 60:2249–2270
- Noe SM, Niinemets Ü, Schnitzler JP (2010) Modeling the temporal dynamics of monoterpene emission by isotopic labeling in *Quercus ilex* leaves. *Atmos Environ* 44:392–399
- Peñuelas J, Llusà J (2001) Seasonal patterns of non-terpenoid C₆–C₁₀ VOC emission from seven Mediterranean woody species. *Chemosphere* 45:237–244
- Rinaldi C, Kohler A, Frey P, Duchaussoy F, Ningre N, Couloux A, Wincker P, Le Thiec D, Fluch S, Martin F, Duplessis S (2007) Transcript profiling of poplar leaves upon infection with compatible and incompatible strains of the foliar rust *Melampsora larici-populina*. *Plant Physiol* 144:347–366
- Rinne J, Hakola H, Laurila T (1999) BVOC emissions at the canopy scale: micrometeorological flux measurements. In: Laurila T, Lindfors V (eds) Biogenic VOC emissions and photochemistry in the boreal regions of Europe—Biphorep. Commission of European Communities, Luxembourg, pp 127–150
- Robert C, Bancal M-O, Bertrand N, Lannou C (2005) Wheat leaf photosynthesis loss due to leaf rust, with respect to lesion development and leaf nitrogen status. *New Phytol* 165:227–241
- Röse U, Tumlinson J (2005) Systemic induction of volatile release in cotton: how specific is the signal to herbivory? *Planta* 222:327–335
- Schaub A, Blande JD, Graus M, Oksanen E, Holopainen JK, Hansel A (2010) Real-time monitoring of herbivore induced volatile emissions in the field. *Physiol Plant* 138:123–133
- Schüßler A, Martin H, Cohen D, Fitz M, Wipf D (2006) Characterization of a carbohydrate transporter from symbiotic glomeromycotan fungi. *Nature* 444:933–936

- Simon V, Dumergues L, Ponche J-L, Torres L (2006) The biogenic volatile organic compounds emission inventory in France: application to plant ecosystems in the Berre-Marseilles area (France). *Sci Total Environ* 372:164–182
- Singh G, Gavrieli J, Oakey JS, Curtis WR (1998) Interaction of methyl jasmonate, wounding and fungal elicitation during sesquiterpene induction in *Hyoscyamus muticus* in root cultures. *Plant Cell Rep* 17:391–395
- Smiatek G, Steinbrecher R (2006) Temporal and spatial variation of forest VOC emissions in Germany in the decade 1994–2003. *Atmos Environ* 40:166–177
- Staples RC (2000) Research on the rust fungi during the twentieth century. *Annu Rev Phytopathol* 38:49–69
- Staudt M, Rambal S, Joffre R, Kesselmeier J (2002) Impact of drought on seasonal monoterpene emissions from *Quercus ilex* in southern France. *J Geophys Res* 107:4602–4608
- Toome M, Heinsoo K, Ramstedt M, Luik A (2009) Rust severity in bioenergy willow plantations treated with additional nutrients. *Forest Pathol* 39:28–34
- Toome M, Heinsoo K, Luik A (2010) Relation between leaf rust (*Melampsora epitea*) severity and the specific leaf area in short rotation coppice willows. *Eur J Plant Pathol* 126:583–588
- Voegele RT, Mendgen K (2003) Rust haustoria: nutrient uptake and beyond. *New Phytol* 159:93–100
- Vuorinen T, Nerg A-M, Syrjälä L, Peltonen P, Holopainen J (2007) *Epirrita autumnata* induced VOC emission of silver birch differ from emission induced by leaf fungal pathogen. *Arthropod Plant Interact* 1:159–165
- Yuan JS, Himanen SJ, Holopainen JK, Chen F, Stewart CN (2009) Smelling global climate change: mitigation of function for plant volatile organic compounds. *Trends Ecol Evol* 24:323–331