Rust Infection of Black Poplar Trees Reduces Photosynthesis but Does Not Affect Isoprene Biosynthesis or Emission

Franziska Eberl1, Erica Perreca1, Heiko Vogel2, Louwrance P. Wright1,3, Almuth Hammerbacher1,4, Daniel Veit5, Jonathan Gershenzon1 and Sybille B. Unsicker1*

1 Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany, 2 Department of Entomology, Max Planck Institute for Chemical Ecology, Jena, Germany, 3 Zeiselhof Research Farm, Pretoria, South Africa, 4 Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa, 5 Technical Service, Max Planck Institute for Chemical Ecology, Jena, Germany

Poplar (Populus spp.) trees are widely distributed and play an important role in ecological communities and in forestry. Moreover, by releasing high amounts of isoprene, these trees impact global atmospheric chemistry. One of the most devastating diseases for poplar is leaf rust, caused by fungi of the genus Melampsora. Despite the wide distribution of these biotrophic pathogens, very little is known about their effects on isoprene biosynthesis and emission. We therefore infected black poplar (P. nigra) trees with the rust fungus M. larici-populina and monitored isoprene emission and other physiological parameters over the course of infection to determine the underlying mechanisms. We found an immediate and persistent decrease in photosynthesis during infection, presumably caused by increased ABA levels. At the same time, isoprene emission remained stable during the time course of infection, consistent with the stability of its biosynthesis. There was no detectable change in the levels of intermediates or gene transcripts of the methylerythritol 4-phosphate (MEP) pathway in infected compared to control leaves. Rust infection thus does not affect isoprene emission, but may still influence the atmosphere via decreased fixation of CO2.

Keywords: biotrophic pathogens, disease, isoprenoids, MEP pathway, non-mevalonate pathway, plant hormones, Salicaceae, stomatal conductance

INTRODUCTION

Poplar (Populus spp.) trees are deciduous, woody plants that are widely distributed in the northern hemisphere (Stanton et al., 2010; Isebrands and Richardson, 2014). In their natural habitat, which consists of floodplain forests and riverbanks, they are of significant ecological importance as host plants for an enormous diversity of microbes, insects, and other organisms. Apart from that, poplar trees have gained increased economic attention in recent years as fast growing sources of wood, plywood, paper, and biofuels when grown in short-rotation coppices (Karp and Shield, 2008). The fast growth, which also favors agronomical use, combined with the availability of the sequenced genome (Tuskan et al., 2006) and the possibility of clonal reproduction, makes this tree...
an excellent model organism for woody plant research. However, natural populations as well as plantations of poplar regularly suffer from severe infections by biotrophic rust fungi (Polle et al., 2013). Melampsora rusts are among the most devastating diseases for poplars worldwide (Pei and Shang, 2005; Wan et al., 2013; Štochlová et al., 2016). An epidemic outbreak of rust disease can cause drastic losses over 50% in biomass production1 (Gérard et al., 2006), premature defoliation and even mortality in young stands (Aylott et al., 2008; Benetka et al., 2011; Polle et al., 2013). Upon rust infection, poplar trees activate the salicylic acid (SA) defense signaling pathway (Azaiez et al., 2009; Eberl et al., 2018), as in many other plants infected by biotrophic pathogens (Glaezebrook, 2005) leading to an enhanced production of phenolic secondary metabolites and the expression of pathogenesis-related proteins (Miranda et al., 2007; Rinaldi et al., 2007; Azaiez et al., 2009; Chen et al., 2014; Ullah et al., 2017). However, studies connecting rust infection with primary physiological processes are rare, even though it can be assumed that the biotrophic lifestyle of the rust fungus will unbalance the host’s primary metabolism even though it can be assumed that the biotrophic lifestyle of rust infection with primary physiological processes are rare, even though it can be assumed that the biotrophic lifestyle of the rust fungus will unbalance the host’s primary metabolism dramatically (Berger et al., 2007; Voegele and Mendgen, 2011). Rust infection also modifies volatile emissions of poplar, leading to increased release of monoterpenes (C10) and sesquiterpenes (C15) (Eberl et al., 2018). In addition, poplar trees emit large amounts of isoprene (C5). This small volatile hydrocarbon is emitted by a number of plant species, most of them woody. Poplar trees are amongst the strongest isoprene emitters known (Logan et al., 2000; Laathowerinkitkul et al., 2009). In the atmosphere, isoprene is involved in the formation of ozone and hydroxyl radicals, and hence plays an important role in atmospheric chemistry (Sharkey et al., 2008). Even though isoprene is emitted in considerable amounts that exceed the emission of all other biogenic volatiles (Guenther et al., 1995), its biological function is still not well understood. Isoprene is hypothesized to increase the thermotolerance of plants, protect against ozone and oxygen radicals, and act as a “safety valve” for dissipating energy under high light conditions (Loreto and Velikova, 2001; Sharkey et al., 2008; Laathowerinkitkul et al., 2009).

The biosynthesis of isoprene occurs in the chloroplasts via the methylerythritol 4-phosphate (MEP) pathway (Logan et al., 2000), an alternate route for isoprenoid production to the mevalonate (MVA) pathway, which is located in the cytoplasm. The MEP pathway is present in higher plants, algae and some bacteria, but not in fungi and animals (Hemmerlin et al., 2012). Both pathways produce dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IDP), the universal building blocks for isoprenoid formation (Rodríguez-Concepción and Boronat, 2015). The regulation of both pathways occurs at different levels, ranging from transcriptional control of genes encoding biosynthetic enzymes to post-translational modifications of pathway enzymes (Hemmerlin, 2013). The MEP pathway is tightly connected to photosynthesis, not only spatially by sharing the same compartment, but also metabolically. The MEP pathway consumes metabolic intermediates, energy and reducing equivalents drawn directly from the light and dark reactions; in return it produces the photosynthetic pigments, chlorophylls and carotenoids, and phytohormones (Hemmerlin et al., 2012). One of these hormones is abscisic acid (ABA) which controls stomatal opening and mediates responses to drought stress (Acharya and Assmann, 2009). However, ABA is also known to be involved in defense reactions against biotic stressors, but usually acts as an antagonist to SA-mediated signaling cascades (Toni et al., 2009; Cao et al., 2011).

In the past, most studies on isoprene emission have focused on its biosynthesis, its effects on atmospheric chemistry and its involvement in plant interactions with the abiotic environment. For poplar, 40% of all the articles on isoprene emission since 1990 studied its relation to abiotic environmental factors2 (January 2018), while only 3% studied its relation to biotic environmental factors (Brilli et al., 2009; Müller et al., 2015; Jiang et al., 2016).

We therefore investigated the effect of the pathogenic rust fungus Melampsora larici-populina on isoprene emission from black poplar trees (Populus nigra). We monitored temporal changes of photosynthesis and isoprene emission during infection and investigated underlying physiological mechanisms by analyzing aspects of leaf chemistry, physiology, and transcriptional changes.

MATERIALS AND METHODS

Experimental Material

Black poplar (P. nigra L.) trees were grown from hardwood cuttings (photosynthesis experiment and isoprene experiment) or softwood cuttings (transcriptome experiment) obtained from different tree genotypes growing in a common garden in Isserstedt, Germany (50°57’ 28.5”N 11°31’ 17.4”E). One genotype was used for the transcriptome experiment as well as for the photosynthesis and isoprene measurements, whereas two different genotypes were additionally used for photosynthesis measurements. The cuttings were potted in 2 l-pots, grown in the greenhouse (18/20°C, night/day, relative humidity 60%, natural light with 9–14 h photoperiod, supplement light for 12 h, SON-T Agro; Philips, Andover, MA, United States) and transferred to an environmental chamber [18/20°C, night/day; relative humidity 60%; photoperiod 16 h, MT 400 (Eye, Uxbridge, United Kingdom)] 2 days before the onset of the experiment. All three experiments (photosynthesis experiment, isoprene experiment and transcriptome experiment) were conducted separately at different time points but under the same temperature and humidity conditions. All trees were used ca. 4 months after potting and had reached a height of about 0.5 m. Trees used for photosynthesis and isoprene measurement did not show any noticeable shoot growth at time of experiment.

Uredospores of the biotrophic poplar leaf rust fungus (M. larici-populina Kleb.) were obtained from naturally infected black poplar trees growing in the above-mentioned common

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1https://ohioline.osu.edu/factsheet/plpath-tree-8

2www.webotknowledge.com
University of Freiburg, Institute of Botany, D-79104 Freiburg, Germany.

Germplasm Bank of Poplars, Department of Forest Genetics, Forstpark, 72076 Tübingen, Germany.

Current affiliation: Institute of Biophysics, Johannes Gutenberg-University Mainz, 55128 Mainz, Germany.

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Photosynthesis Measurements

Photosynthetic parameters were measured on the second mature leaf (counting from the apex) of six trees from both groups (“control” and “rust-infected”; n = 6) at six different time points: 1 day before rust infection (−1 dpi), 4 h post-infection (hpi), 1, 7, and 10 dpi. The leaf was put into a custom-made single leaf chamber (chamber: polyoxymethylene, lid: poly(methyl methacrylate), openings sealed with sponge rubber; for picture see Supplementary Figure S2), which was connected to a LI-6400XT Portable Photosynthesis System (LI-COR, Lincoln, NE, United States). For each treatment a leaf image of the rust-infected trees was visible on the abaxial side of the leaves at 7 dpi (days post-infection) (Supplementary Figure S1), which matches the time course of infection in the literature (Jacquard et al., 2011). Under natural conditions individual poplar leaves are usually exposed to many cycles of rust infection. We decided to investigate only a single cycle of infection to better determine which stage of infection influences plant processes.

Isoprene Measurements

Isoprene emission from rust-infected and control trees was measured from the second mature leaf from the apex in six trees of each treatment (n = 6). The same conditions, setup and time points (from 1 to 10 dpi) as for the photosynthesis experiment were used, but the material of the single leaf chamber was changed to aluminum (Supplementary Figure S2) to avoid volatile contamination, while the lid was still made from poly(methyl methacrylate). The ambient temperature of 20°C used in our experiments is lower than the temperature applied in comparable studies on isoprene emission (e.g., Brilli et al., 2007) but better simulates the conditions of this poplar species and the pathogen experience in the field. Furthermore, the relative humidity was reduced to 6% in order to avoid excessive transpiration inside the chamber (due to a larger leaf size compared to the photosynthesis experiment). Isoprene emission was analyzed with a proton transfer reaction mass spectrometer (PTR-MS; Ionicon Analytik, Innsbruck, Austria). A detailed description of the PTR-MS can be found in Lindinger and Jordan (1998). Before starting the analysis the PTR-MS was calibrated by using the Gas Calibration Unit, a system generating clean air mixed with precise flows of an isoprene gas standard (Ionicon Analytik). The capillary line of the PTR-MS was connected with the outflow of the leaf chamber. The proton transfer reactions occurred in the reaction chamber (drift tube) between the primary ion H3O+ coming from the ion source, and the isoprene in the sampled air. In the drift tube the pressure was in the range of 2.3 mbar.
and the E/N ratio (electric field/particle density) was 137 Td (1 Td = 10\(^{-17}\) V cm\(^2\)). Isoprene was monitored at the mass signal 69 (m/z). The raw count-rate signal intensity (\(I_0\)) of the isoprene was normalized (ncps) to the cps sum of the primary ion and water cluster, and to the drift tube pressure. Each leaf was given a 10 min equilibration period in the leaf chamber under LED light. Then the leaf chamber was connected to the PTR-MS to monitor isoprene emission for 15 min. This time was enough to reach steady-state conditions of isoprene emission. The average of cps during the steady-state period was used to calculate the emission rate, from which the background (empty leaf chamber, Supplementary Figure S2) was subtracted. Isoprene emission was normalized to the leaf area, which was determined by a picture taken at the beginning of the experiment. As the trees were not actively growing during the experiment, the leaf size was assumed to be the same throughout the whole experiment. Leaf area was calculated as described in “Photosynthesis measurements.” After the last measurement (10 dpi), the second mature leaf was flash-frozen immediately in liquid nitrogen for metabolite analysis.

Analysis of MEP Pathway Metabolites

Leaves sampled from the isoprene experiment (\(n = 6\)) were ground in liquid nitrogen and then lyophilized. The MEP pathway metabolites (see Figure 5 legend) were extracted twice with a 250 \(\mu\)l solution of 50% acetonitrile containing 10 mM ammonium acetate, pH 9.0, using 5 mg dry tissue. After vortexing and micro-centrifugation, 200 \(\mu\)l of the supernatant from both extracts were combined, transferred into a new 1.5 ml tube and dried under a stream of nitrogen gas at 40°C. The residue was dissolved in 100 \(\mu\)l of 10 mM ammonium acetate, pH 9.0, and, after vortexing, 100 \(\mu\)l of chloroform was added. The upper aqueous phase, separated by centrifugation, was transferred into a new tube and diluted with 1 volume of acetonitrile. After centrifugation for 5 min to remove any precipitate, the supernatant was transferred into an HPLC vial. MEP pathway metabolites were analyzed on an Agilent 1260 Infinity HPLC system (Agilent) connected to an API 5000 triple quadrupole mass spectrometer (AB Sciex). A 5 \(\mu\)l portion of the extract was injected and the metabolites were separated on a HILIC XBridge Amide column (150 × 2.1 mm, 3.5 \(\mu\)m; Waters, Milford, MA, United States) with a HILIC guard column containing the same sorbent (3.5 \(\mu\)m, 10 × 2.1 mm) and a SSI MT high pressure pre-column filter (Sigma-Aldrich) using two solvents: 20 mM ammonium bicarbonate adjusted to pH 10.5 with ammonium hydroxide (solvent A) and 80% acetonitrile containing 20 mM ammonium bicarbonate, pH 10.5 (solvent B). The solvent gradient profile started with 100% of solvent B which decreased to 60% in the first 15 min, followed by an isocratic gradient with solvent B. Separation was performed at 25°C with a flow rate of 500 \(\mu\)l min\(^{-1}\). The mass spectrometer operated in negative ionization mode with ion spray voltage −4500 eV, turbo gas temperature 700°C and nebulizer gas 70 psi. The MEP pathway metabolites were analyzed using the MRM conditions described by Wright et al. (2014). The metabolite concentrations were calculated by using external standard curves, and were normalized to the \([^{13}\text{C}]\)-labeled internal standards of each intermediate (González-Cabanelas et al., 2016) added to the extract after the first extraction step.

Leaf Pigment Analysis

Leaves sampled from the isoprene experiment at 10 dpi were ground in liquid nitrogen and 50 mg of fresh tissue was extracted in light-protected tubes with 1 ml of acetone by shaking for 6 h at 4°C in the dark. After centrifugation for 5 min at 2350 g at 4°C, 800 \(\mu\)l of the extract was transferred into a new light-protected tube and 200 \(\mu\)l of water was added. After spinning the samples for 1 min at 5000 rpm at 4°C, they were transferred to brown glass vials for analysis on an Agilent 1100 Series HPLC with UV/VIS diode array detector. The detector was set at 445 nm for the detection of carotenoids and at 650 nm for the chlorophylls. The pigments were separated on a Supelcosil column LC-18 (7.5 cm × 4.6 mm × 3 \(\mu\)m; Sigma-Aldrich) using an acetonite (solvent A)/1 mM NaHCO\(_3\) (in water, solvent B) gradient with a flow rate of 1.5 ml min\(^{-1}\). The initial mobile phase consisted of 65/35% (v/v) solvent A/solvent B. Then, solvent A was linearly increased to 90% within 12 min and to 100% over 8 min. 100% solvent A was kept for 2 min and then decreased to 65% again within 3 min. Quantification was done using external standard curves. Authentic standards of the chlorophylls and β-carotene (Santa Cruz Biotechnology) were analyzed in a range from 0.1 to 0.00625 mg ml\(^{-1}\). Lutein, neoxanthin, and violaxanthin were assumed to have the same response factor as β-carotene.

Transcriptome Analysis

To investigate transcriptional changes in black poplar leaves upon rust infection, eight trees grown from green cuttings were selected and half of them were inoculated with \(M. laricio-populina\) uredospores. Leaves from the trees of both treatments (“control” and “rust-infected”; \(n = 4\)) were harvested 8 dpi and flash-frozen in liquid nitrogen. RNA was isolated with the Invitrap Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany), including a DNase digestion (DNase kit, Qiagen, Hilden, Germany). RNA concentration and quality was analyzed with a NanoDrop 2000c spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) and the RNA 6000 Nano Kit on a Bioanalyzer (Agilent). Sequencing was done at the Max Planck-Genome-Centre (Köln, Germany) on a HiSeq 2500 (Illumina, San Diego, CA, United States) with 9 Mio reads per sample. Quality control measures, including the filtering of high-quality reads based on fastq file scores, the removal of reads containing primer/adapter sequences, and trimming of the read length, were carried out using CLC Genomics Workbench v9.1\(^3\). The same software was used for \(de\ novo\) transcriptome assembly, combining two replicates of each RNA-Seq treatment group, and selecting the presumed optimal consensus transcriptome as previously described (Vogel et al., 2014). The final \(de\ novo\) reference transcriptome assembly (backbone) of \(P. nigra\).

\(^3\)http://www.clcbio.com
RESULTS

Photosynthesis Decreases After Rust Infection but Sugar Levels Are Unaffected

In order to investigate the influence of fungal infection on photosynthesis in black poplar, we measured photosynthetic parameters at various times during the development of rust infection in the leaves.

The photosynthetic assimilation rate in uninfected control trees was generally stable at 9–12 µmol CO$_2$ m$^{-2}$ s$^{-1}$ during the time course of measurements (Figure 1A). The photosynthetic rate in rust-infected poplar trees, however, dropped by approximately 50% within the first 4 hpi from 12.1 ± 1.32 to 6.1 ± 0.86 µmol m$^{-2}$ s$^{-1}$. This reduction persisted throughout the later time points. The factor “rust infection” strongly influenced the photosynthetic rate, as also

![Figure 1](http://www.frontiersin.org)

**Figure 1** | Photosynthetic parameters of rust-infected black poplar trees (filled symbols) and uninfected controls (open symbols) at different time points after infection (dpi = days post-infection, hpi = hours post-infection; -1 dpi = 1 day before infection). Measurements were made on the second mature leaf counting from the apex. Shown are means ± SEM (n = 6) for (A) photosynthetic rate (µmol CO$_2$ m$^{-2}$ s$^{-1}$) and (B) stomatal conductance (mol H$_2$O m$^{-2}$ s$^{-1}$). Results from repeated measures ANOVA using the factors “Time” (time points before and post-infection), “Infection” (rust infection), and the interaction “Time x Infection” are given as insets in the graphs (ns = not significant; *P < 0.05; **P < 0.01; ***P < 0.001).
shown statistically [repeated measures ANOVA: $F_{(1, 10)} = 15.524, P = 0.003$]. However, there was also a significant interaction of time and rust infection [repeated measures ANOVA: $F_{(4, 40)} = 8.891, P < 0.001$]. The patterns of stomatal conductance were similar to those observed for photosynthetic assimilation rate. Control plants without rust infection showed a slight increase in stomatal conductance throughout the experiment from $0.09 \pm 0.003$ mol m$^{-2}$ s$^{-1}$ at the first measurement to $0.13 \pm 0.012$ mol m$^{-2}$ s$^{-1}$ during the last three measurements (Figure 1B). Rust fungus infection decreased stomatal conductance in poplar trees by more than 50% as early as 4 hpi, when it declined from $0.11 \pm 0.015$ to $0.06 \pm 0.005$ mol s$^{-1}$ m$^{-2}$. As the infection progressed, the stomatal conductance of rust-infected trees remained at around 50% of that measured from uninfected controls. The effect of “rust infection” was statistically highly significant [repeated measures ANOVA: $F_{(1, 10)} = 19.815, P = 0.001$], as was the interaction of time and rust infection [repeated measures ANOVA: $F_{(4, 40)} = 10.665, P < 0.001$]. However, the intercellular CO$_2$ levels did not differ between the different time points, nor between the two treatments, i.e., rust-infected and control black poplar leaves (Supplementary Figure S5).

After observing the reduction in photosynthetic assimilation rate in rust-infected poplar leaves, we analyzed glucose, fructose and sucrose levels in leaves harvested at 10 dpi. For these sugars, no significant changes in concentration were observed after infection of leaves by the rust fungus (Table 1).

Our results show that photosynthetic activity is downregulated in poplar leaves right after the onset of infection with the rust fungus, and remains lower over the course of infection without affecting soluble sugars.

**Salicylic Acid (SA) and Abscisic Acid (ABA) Increase in Rust-Infected Leaves**

To analyze phytohormones that might be involved in regulating anti-pathogen defense and photosynthesis, leaves were collected at 10 dpi.

Salicylic acid increased fivefold in rust-infected black poplar leaves compared to uninfected controls [331 ± 45 ng g$^{-1}$ DW in controls; 1580 ± 390 ng g$^{-1}$ DW in rust-infected trees, Figure 2A], which is highly significant [Student's $t$-test, $t_{(10)} = -4.687$, $P = 0.001$]. ABA, a phytohormone regulating stomatal opening, among other processes, also increased in rust-infected black poplar leaves, reaching a level twice as high as in the control leaves (36.2 ± 6.10 ng g$^{-1}$ DW in controls; 72.1 ± 11.34 ng g$^{-1}$ DW in rust-infected trees, Figure 2B). Additionally, ABA levels in rust-infected leaves correlated negatively with stomatal conductance and photosynthetic rate measured at 10 dpi (Table 2). In control leaves, however, no such correlation could be observed.

Taken together, we could show that rust infection increased SA as well as ABA in poplar leaves, whereas the latter shows a negative relation to photosynthetic parameters.

**Rust Infection Does Not Affect Isoprene Emission**

To evaluate the effect of pathogen infection on isoprene emission, we monitored leaves of infected and uninfected young poplar trees using a PTR-MS at different time points before and during infection with the rust fungus (*M. larici-populina*).

The isoprene emission from uninfected controls showed slight variations throughout the time course of the experiment, ranging from 2.7 to 3.4 nmol m$^{-2}$ s$^{-1}$. Infection with the rust did not significantly change emission of isoprene compared to control trees or compared to the levels measured before the pathogen inoculation (Figure 3). The fluctuations over time were similar to those in uninfected controls, leading to a significant statistical effect of “time” [repeated measures ANOVA: $F_{(4, 40)} = 1.475$, $P = 0.031$]. However, there was no significant effect of “infection” or the interaction “time × infection” on isoprene emission. This shows that rust infection does not affect the emission of isoprene from black poplar trees.

**TABLE 1 | Soluble sugar levels in control and rust-infected black poplar trees 10 days post-infection expressed in mg g$^{-1}$ dry weight.**

| Sugar      | Control  | Rust-infected | $t$     | $P$  |
|------------|----------|---------------|---------|------|
| Glucose    | 1.31 ± 0.19 | 1.36 ± 0.17   | −0.204  | 0.843|
| Fructose   | 2.02 ± 0.51 | 2.03 ± 0.36   | −0.012  | 0.991|
| Sucrose    | 35.53 ± 3.76 | 41.44 ± 3.09  | −1.213  | 0.253|

Measurements were made on the second mature leaf counting from the apex. Shown is mean ± SEM (n = 6) and t- and $P$-value of Student’s $t$-test.
**TABLE 2** | Correlation between phytohormone levels and photosynthetic parameters of control and rust-infected black poplar trees 10 days post-infection (data from Figures 1, 2).

|                        | Photosynthetic rate | Stomatal conductance |
|------------------------|---------------------|----------------------|
| Salicylic acid         | $\rho = -0.384$     | $\rho = -0.589$      |
| Abscisic acid          | $\rho = -0.257$     | $\rho = -0.186$      |
|                        | $\rho = -0.537$     | $\rho = -0.661$      |
|                        | $\rho = -0.987^{***}$ | $\rho = -0.969^{**}$ |

Measurements were made on the second mature leaf counting from the apex. $\rho$, Pearson correlation coefficient. Significant correlations are highlighted in bold and marked with asterisks ($^{***}P < 0.001; ^{**}P < 0.01; n = 6$).

**FIGURE 3** | Isoprene emission from leaves of rust-infected black poplar trees (filled symbols) and uninfected controls (open symbols) at different time points after infection (dpi = days post-infection, hpi = hours post-infection; $-1$ dpi = 1 day before infection). Measurements were made by proton-transfer reaction mass spectrometry (PTR-MS) on the second mature leaf counting from the apex. Shown are means $\pm$ SEM ($n = 6$). Results from repeated measures ANOVA using the factors “Time” (time points before and post-infection), “Infection” (rust infection) and the interaction “Time x Infection” (rust infection) and the interaction “Time x Infection” are given in the graph (ns = not significant; *$P < 0.05$).

**Rust Infection Did Not Influence the Genes, Intermediates or Most Products of the MEP Pathway**

We analyzed intermediates of the MEP pathway and levels of the photosynthetic pigments, chlorophylls and carotenoids. Like isoprene, these are also produced from DMADP and IDP originating from the MEP pathway. The analyzed leaves were sampled together with any fungal spores and mycelium present in infected leaves. The MEP pathway intermediates, DXP, MEP, CDP-ME, and MEcDP, were present at similar levels in rust-infected and control leaves (Figures 4A–D). However, the amount of DMADP and IDP (DMADP + IDP), the final products of both the MEP and MVA pathways (not separable in our LC-MS analysis), were significantly higher in rust-infected leaves [Student’s $t$-test, $t_{(10)} = -3.503$, $P = 0.006$] (Figure 4E). The concentration of DMADP + IDP in rust-infected leaves was more than double that of control leaves ($4.1 \pm 0.46 \text{ nmol g}^{-1} \text{ FW in controls}; 9.3 \pm 1.60 \text{ nmol g}^{-1} \text{ FW in rust-infected trees}$). On the other hand, many of the major isoprenoids that are known to be produced from MEP pathway-derived C5 units, including the carotenoids, lutein, neoxanthin, violaxanthin, and chlorophylls $a$ and $b$, did not change after rust infection (Table 3). β-Carotene, however, increased by more than 50% in leaves from rust-infected poplars compared to uninfected controls ($0.59 \pm 0.072 \text{ mg g}^{-1} \text{ FW in controls}; 0.93 \pm 0.090 \text{ mg g}^{-1} \text{ FW in rust-infected};$ Student’s $t$-test, $t_{(10)} = -3.100$, $P = 0.011$). The orange-colored uredospores of the rust fungus *M. larici-populina* were also analyzed separately to determine if these contained any carotenoids, and β-carotene, but none of the other poplar carotenoids, was found (Supplementary Table S1). The concentration of β-carotene was more than threefold higher in fungal spores than in uninfected poplar leaves.

DMADP and IDP, the C5 building blocks of all isoprenoids, can be produced by either the plastidic MEP pathway or the cytosolic MVA pathway (Rodríguez-Concepción and Boronat, 2015). To determine whether rust infection could influence either pathway, we carried out transcriptome analysis of uninfected and rust-infected black poplar leaves. No differential expression of genes involved in the MEP pathway was observed (Figure 5). However, genes encoding biosynthetic enzymes of the MVA pathway increased in expression in rust-infected leaves compared to uninfected controls (Figure 5). Two enzymes catalyzing early steps of the MVA pathway, acetoacetyl-coenzyme A thiolase and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, had especially higher RPKM values (reads per kilobase of transcript per million mapped reads) in leaves infected with the rust fungus. The transcriptome analysis also identified MVA pathway transcripts of fungal origin, likely from *M. larici-populina*. Contigs annotated as fungal HMG-CoA synthase and all downstream MVA pathway enzymes were detected in the transcriptome of rust-infected leaves (Supplementary Table S2), but not in control leaves.

Thus rust infection did not affect the levels of intermediates of the MEP pathway. However, the amounts of the isoprenoid building blocks DMADP + IDP increased, as a result of up-regulation of the MVA pathway. In addition, there was an increase in β-carotene, which likely arose from the isoprenoid biosynthesis by the rust fungus itself.

**DISCUSSION**

*Melampsora* rusts are among the most devastating pathogens of poplar trees (Pei and Shang, 2005). We investigated the effect of rust infection (*M. larici-populina*) on photosynthesis and the emission of isoprene in black poplar (*P. nigra*).
Our results indicate that black poplar actively downregulates photosynthesis when it is subjected to rust infection. Yet despite the spatial and metabolic connections between photosynthesis and isoprene formation, the emission of isoprene was completely unaffected by the presence of the rust fungus. Consistent with this, fungal infection also did not change the expression of biosynthetic genes or levels of intermediates of the MEP pathway, the route to formation of the \( C_5 \) units used in isoprene biosynthesis. However, an increase in the quantity of DMADP and IDP was observed, which may be attributed to the increased
activity of the MVA pathway (the alternative route to producing C5 isoprenoid units) in poplar or the fungus itself.

**Rust Infection Drastically Reduces Black Poplar Photosynthesis**

Despite the central importance of photosynthesis in supplying plants with carbon, energy, and reducing equivalents, we observed an immediate and sustained decrease in photosynthetic activity in rust-infected black poplar leaves of nearly 50%, from 4 hpi to 10 dpi (Figure 1). Infection of poplar with *Melampsora* leaf rust was earlier reported to be associated with a marked reduction in photosynthesis (Zhang et al., 2010, 2016; McKown et al., 2014; Jiang et al., 2016; Gortari et al., 2018), but the detailed temporal dynamics of this reduction had not been studied. In willow, a similar pattern of decreased net photosynthetic rate was observed after rust infection, but stomatal conductance changed only at late time points (Toome et al., 2010). A negative impact of rust on transcripts of photosynthetically relevant genes in poplar was also reported at 6 dpi (summarized in Major et al., 2010). Interestingly, we did not observe any changes in the intercellular CO₂ concentration (Cᵢ) after rust infection (Supplementary Figure S5). A decreased Cᵢ would be expected when the assimilation rate decreases after stomatal closure due to the lowered availability of CO₂. The lack of decrease of Cᵢ in our study might be explained by temporal dynamics, i.e., a fast reduction and subsequent equilibration of Cᵢ before the first measurement. Alternatively, a signal – most likely phytosphormonal – might have regulated stomatal conductance and assimilation rate simultaneously, so that reduced uptake and reduced consumption of CO₂ would balance out without any net effect on the Cᵢ. A decline in stomatal conductance...
and assimilation rate with stable C\textsubscript{i} has been observed before after herbivory (Meza-Canales et al., 2017). Also, the influence of biotic stress-related phytohormones on photosynthesis has been shown in other systems (Popova et al., 1988; Tang et al., 2017). A transcriptional analysis conducted at early time points of infection would help to better understand the physiological mechanisms leading to the rapid decline in photosynthesis.

In addition to the decrease in photosynthesis we observed elevated levels of SA and ABA (Figure 2) in poplar leaves upon rust infection. SA is known to play a central role in plant defense against biotrophic organisms such as rusts or mildew, and induces a hypersensitive response, the expression of pathogenesis-related genes and other responses (Derkxen et al., 2013). ABA, on the other hand, is primarily known to mediate responses to abiotic stresses such as drought (Tuteja, 2007) and to control stomatal closure (Acharya and Assmann, 2009). Stomata are natural openings through which the rust fungus can enter the intercellular spaces of the leaf within the first 6 h after inoculation (Hacquard et al., 2011). Yet rapid closure of stomata might prevent the pathogen from entering, as was observed in tomato leaves infected by Pseudomonas syringae (Melotto et al., 2006). A similar mechanism might occur in poplar leaves and would explain the fast decrease in stomatal conductance and the increased levels of ABA observed in infected tissue. Phytohormone analysis of earlier time points and exogenous application of ABA and SA will help to disentangle the signaling networks between these two hormones and control of photosynthesis. Considering the fast response of photosynthetic parameters to rust infection and the changes in phytohormone content, we infer an active control of stomatal closure by the plant. Previous work suggested that a mechanical disturbance by fungal hyphae could also be involved in reducing stomatal conductance (Jiang et al., 2016), perhaps especially at later time points.

**Soluble Carbohydrate Levels Are Maintained in Rust-Infected Leaves**

Although rust infection triggered a drastic decrease in the photosynthetic assimilation rate of poplar (Figure 1), soluble sugar content did not decrease in rust-infected compared to uninfected control leaves (Table 1). Given the rapid production of sucrose and hexose sugars as photosynthetic assimilates, it is surprising that their levels were not affected by the decline in photosynthesis. Soluble sugars can also originate from breakdown of storage carbohydrates or transport from other tissues. Since biotrophic pathogens utilize hexose sugars from their hosts (Voegele and Mendgen, 2011), it is assumed that infected tissues become carbon sinks even when photosynthetically active in order to satisfy the increased demand for carbon (Berger et al., 2007). This would require the mobilization of carbohydrates from other parts of plant. Such mobilization might account for the decline in wood production in poplar observed on rust infection, which causes significant economic losses in infected plantations (Frey et al., 2005; Wan et al., 2013). Another explanation for the maintenance of constant sugar levels in rust-infected leaves with a concurrent decrease in assimilation rate could be the reduced export of sugars. Further work is needed to elucidate the mechanisms responsible for maintaining sugar levels under these conditions.

**Isoprene Emission Is Not Affected by Rust Infection**

Isoprene is emitted in large amounts by poplar and other tree species, and it is assumed to protect leaves from heat stress or reactive oxygen species (Sharkey et al., 2008). However, the role of isoprene under biotic stress is poorly studied. We observed no influence of rust infection on isoprene emission from black poplar (Figure 3). Similar patterns of stable isoprene emission were observed under drought stress conditions (Pegoraro et al., 2004; Brilli et al., 2007), which also trigger ABA-mediated stomatal closure. On the other hand, studies on the effects of insect herbivory or mechanical wounding have reported variable outcomes, showing either an increased (Brilli et al., 2011), stable (Müller et al., 2015), decreased (Brilli et al., 2009; Jardine et al., 2013; Jiang et al., 2018), or time-dependent (Loreto and Sharkey, 1993; Loreto et al., 2006; Portillo-Estrada et al., 2015) isoprene emission patterns after stress application.

In contrast to our results, another study investigating the influence of rust infection on poplar trees observed lower isoprene emissions from infected compared to uninfected trees (Jiang et al., 2016) possibly due to the more severe level of infection. Although in our study rust significantly decreased photosynthetic parameters and altered hormone levels, our infected leaves did not have necrotic lesions (Supplementary Figure S6). Such necrotic lesions, however, were present on the leaves used by Jiang et al. (2016), and may have induced the reduction in isoprene emission. Necrosis leads to premature death of cells and hence could reduce the area of living tissue with which the plant synthesizes isoprene. The stability of isoprene emission under various stress conditions suggests that this compound is of vital importance to the physiology of isoprene-emitting plants. This importance might be due to a direct effect of isoprene, for example, by reducing oxidative stress, or an indirect effect by maintaining flux through the MEP pathway (Logan et al., 2000), which provides essential compounds for plant metabolism. After many years of research, the physiological role of isoprene is still to a large extent unknown.

**Involvement of Plant and Fungal MVA Pathways in Isoprenoid Production in Infected Leaves**

Considering the tight metabolic connections between photosynthesis and the MEP pathway, we expected to observe lower MEP pathway activity after rust infection due to the reduction in photosynthesis. However, the transcription of genes encoding biosynthetic enzymes of the MEP pathway did not change after rust infection (Figure 5). In addition, the stable levels of MEP pathway intermediates (Figures 4A–D) suggest a constant metabolic flux through the pathway despite
fungal infection. Consistent with this, the levels of the chlorophylls and most carotenoids, the main non-volatile products of the MEP pathway, did not change after rust infection (Table 3). Constant levels of the chlorophylls in leaves after rust infection were also recently reported in another poplar species (Gortari et al., 2018). However, the levels of DMADP and IDP (quantified together in our LC-MS analysis) increased after infection (Figure 4E). DMADP and IDP can be produced by both the plastidic MEP pathway and the cytosolic MVA pathway (Hemmerlin et al., 2012). Since the cells in the leaf were disrupted for chemical analysis, the DMADP and IDP present in both cellular compartments were analyzed simultaneously. An increased transcript abundance of genes involved in the early steps of the MVA pathway (Figure 5) suggests the increased DMADP + IDP levels observed in infected tissue might be derived from this pathway. This explanation is also supported by the recently reported increased emission of sesquiterpenes upon rust infection of poplar (Eberl et al., 2018). Alternatively, the increased DMADP + IDP levels could have resulted from the fungal metabolism in rust-infected tissue. Future research using 13C-labeled CO2 (Ghirardo et al., 2014) or glucose (Hemmerlin et al., 2012) could elucidate the biosynthetic origin of the increased DMADP + IDP.

In addition to higher DMADP + IDP-levels we also found higher amounts of β-carotene in rust-infected leaves compared to controls (Table 3). When rust spores were analyzed separately for carotenoids, high concentrations of β-carotene but no other carotenoids were found (Supplementary Table S1). We then analyzed the transcriptome of infected poplar leaves for Melampsora-specific genes involved in isoprenoid biosynthesis and found transcripts for genes encoding all steps of the MVA pathway (Supplementary Table S2). In contrast to plants where carotenoids are produced via the MEP pathway, their biosynthesis in fungi occurs via the MVA pathway (Disch and Rohmer, 1998). Taken together, it is likely that M. larici-populina produces β-carotene in its hyphae and spores. The fungus M. larici-populina in the infected tissue is therefore the most probable cause of the increased β-carotene and DMADP + IDP levels. The fungus might use this pigment in spores to attract spore dispersers (Cano et al., 2013) or in hyphae to scavenge oxygen radicals (Davoli and Weber, 2002) that are produced by plants as defenses against infection (Ferreira et al., 2006).

CONCLUSION

Our study provides new insight into the impact of a widely distributed biotrophic pathogen on photosynthesis and isoprene formation in a poplar species. The hormone-mediated closure of stomata upon infection diminishes the photosynthetic activity of infected leaves and hence reduces the ability of the tree to assimilate new carbon. However, infected leaves maintain stable carbohydrate levels and continue to emit isoprene at unchanged rates despite carbon consumption by the pathogen. Most likely, infected leaves import soluble sugars from elsewhere in the tree. In the long term, rust disease may therefore result in reduced biomass production by poplar resulting in significant declines in plantation yield. However, since the level of isoprene emission was not affected, the influence on atmospheric chemistry will likely be minimal.

AUTHOR CONTRIBUTIONS

FE designed the experiments and wrote the article. FE and EP performed the experiments and analyzed the data except for the transcriptome. HV analyzed the transcriptome data. DV designed and constructed the experimental equipment. LW, AH, SU, and JG conceived the project and complemented writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01733/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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