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Research paper

Impact of ectomycorrhizal colonization and rust infection on the secondary metabolism of poplar (*Populus trichocarpa* × *deltoides*)

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Fungal colonization can significantly affect the secondary metabolism of the host plants. We tested the impact of a common below-ground symbiosis, i.e., ectomycorrhiza formation, on poplar leaf chemical components that are involved in the defence against a common disease, i.e., rust fungi, in N-deficient soil. A rust-susceptible poplar clone (*Populus trichocarpa* × *deltoides* 'Beaupré') was (a) non-associated with ectomycorrhizal fungus (EM) *Hebeloma mesophaeum* (Pers.) Quélet MÜN and non-infected with rust fungus *Melampsora larici-populina* Kleb. (isolate 98AG31), (b) associated with EM, (c) inoculated with rust fungus and (d) associated with EM and inoculated with rust fungus. Poplar leaves were analysed by photometric and mass spectrometric techniques (liquid chromatography–tandem mass spectrometry (LC-MS/MS), pyrolysis–field ionization mass spectrometry (Py-FIMS)). Both rust infection and mycorrhiza formation led to increased proportions of condensed tannins in relation to total phenolics (13% in the control, 18–19% in the fungal treatments). In contrast, salicylic acid concentration (6.8 μg g^{-1} in the control) was higher only in the rust treatments (17.9 and 25.4 μg g^{-1} with rust infection). The Py-FIMS analysis revealed that the rust-infected treatments were significantly separated from the non-rust-infected treatments on the basis of six flavonoids and one lipid. The relative abundance of these components, which have known functions in plant defence, was decreased after rust infection of non-mycorrhizal plants, but not in mycorrhizal plants. The results indicate that the ectomycorrhizal formation compensated the rust infection by a decrease in the flavonoid syntheses. The study provides new evidence for an interactive response of mycorrhizal colonization and infection with rust fungi in the metabolism of poplar.

Keywords: condensed tannins, flavonoids, *Melampsora larici-populina*, phenolics, salicylic acid.

Introduction

Plants respond to fungal colonization by chemical defence, regardless of the acting fungi, pathogenic or symbiotic (Pozo et al. 2009). Both primary and secondary plant metabolism can be affected by fungal colonization, e.g., initiated by fungal production of phytohormones, N-alcyl-l-homoserine lactones or volatile organic compounds (Ortíz-Castro et al. 2009). Plant defence can result in a stimulation of the carbon fluxes from the primary to the secondary metabolic pathway, including a shift of the available resources in favour of the synthesis of secondary metabolites (Iriti and Faoro 2009).

Secondary metabolites usually belong to one of three large chemical classes: terpenoids, phenolics and alkaloids. Among these, phenolics are primarily produced via the shikimic acid and malonic acid pathways in plants, and include a wide variety of defence-related compounds including flavonoids, anthocyanins, phytoalexins, tannins, lignin and furanocoumarins (Agrawal et al. 1999). Defence-related compounds may protect plants against microbial and herbivore attack or UV irradiation (vom

Endt et al. 2002). Plants resist the infection by pathogenic fungi, e.g., by accumulation of phenolics (Lattanzio et al. 2009). The colonization by symbiotic ectomycorrhizal fungi can also result in accumulation of phenolics (Baum et al. 2009).

Ectomycorrhiza (EM) formation is common on poplars (*Populus* spp.) (Anderson and Cairney 2007). This suggests that EM formation could affect the defence against pathogens of *Populus* spp.

Among the most important leaf pathogens of poplars are rust fungi (*Melampsora* spp.) (Frey et al. 2005). After *Melampsora* infection of *Salix myrsinifolia* (Salisb.) an enhanced synthesis of catechin, a precursor of condensed tannins, and other phenolics was found (Hakulinen 1998). Also in a *Populus* hybrid clone the pathway of condensed tannins was highly induced by *Melampsora medusae* (Thünen) (Miranda et al. 2007).

To our knowledge, the interaction of ectomycorrhizal colonization and rust infection of poplar has not been investigated so far, although the consideration of this physiological aspect might be highly relevant in future strategies for sustainable poplar management. The above-to-below-ground interactions are unclear especially for perennial plants such as trees, although a re-allocation of primary metabolites or a systemic resistance expression appears to be a common mechanism (van Dam and Heil 2011). We hypothesized that the rust-triggered increase of secondary metabolite syntheses of mycorrhizal poplars was decreased by an enhanced energy sink as a consequence of the combined symbiont–parasite occurrence.

An alternative hypothesis is that EM colonization compensates the rust-triggered effects on secondary metabolites of the host plant by an improved nutrient supply of the host.

In the present study, model interactions were investigated in a rust-susceptible hybrid poplar clone (*Populus trichocarpa* × *deltoides* 'Beaupré') with and without colonization of an ectomycorrhizal fungus (*Hebeloma mesophaeum*) and infection of a species-specific rust fungus (*Melampsora larici-populina*), both in a single and combined treatment, to explore the hypotheses. *Hebeloma* spp. are common EM partners of field-grown poplar clones (Selle et al. 2005). Poplar rust, caused by the basidiomycete fungus *Melampsora larici-populina*, is the main disease in commercial poplar cultivation in Europe, since the pathogen has overcome all the resistance genes released so far (Frey et al. 2005) including the R7 resistance gene carried by the cultivar 'Beaupré' (Xhaard et al. 2011).

Materials and methods

Experimental design

A pot experiment was designed with four treatments: (i) a non-mycorrhizal and non-infected control (poplars received sterile mycorrhizal inoculum to compensate for possible chemical or physical changes in the substrate caused by the inoculum), (ii) an EM treatment (poplars in association with the EM fungus *H. mesophaeum*), (iii) a rust-infected treatment (poplars inoculated with *M. larici-populina* isolate 98AG31) and (iv) a combined EM and rust-infected treatment (poplars inoculated with *H. mesophaeum* and *M. larici-populina*). Each treatment was established in four replicates.

Soil, plant material and EM fungal cultures

A sandy nutrient-poor soil (71% sand, 24% silt and 5% clay) was taken from a topsoil of an arable site (0–30 cm soil depth). It was sieved and dried, but not sterilized before the experiment to allow interactions with autochthonous microorganisms, too. The soil had a pH ($CaCl_2$) of 6.1 and the following elemental concentrations: C_{org} 7.1 g kg^{-1}, N_t 0.8 g kg^{-1}, P_t 0.5 g kg^{-1}, K_t 0.9 g kg^{-1}, Mg_t 1.0 g kg^{-1}. A strain of the EM fungus *H. mesophaeum* (MÜN) was initially cultivated on Petri dishes with potato dextrose agar at 24 °C in darkness. After 1 month, the cultures were used as inoculum for the substrate production in a mixture of vermiculite and sphagnum peat (9 : 1 v/v) with 200 ml modified Melin Norkrans medium (Kottke et al. 1987) per litre final substrate. The inoculated substrates for subsequent soil and root inoculation were maintained for another month at 24 °C in darkness. After that, 200 ml of this inoculum substrate was mixed into pots with 4 kg (dry weight) of the soil.

Cuttings (5 cm) of the poplar clone *Populus trichocarpa* × *deltoides* 'Beaupré' were planted into these pots and cultivated in a greenhouse (16 h photoperiod; temperature: day 21 °C, night 17 °C) for 4 months to ensure the mycorrhizal establishment on the roots. The plant height ranged from 92.4 to 101.4 cm, whereas the leaf number was 25–30 in average.

The controls had received an EM inoculum that had been sterilized in advance by autoclaving (20 min, 121 °C, 1 bar) to compensate for possible chemical or physical impacts of the inoculum substrate on the plant growth.

Rust fungus inoculation

For the in vivo infection, three fully expanded leaves from the medium high of 4-month-old plants (four plants per treatment) were used. The leaves were inoculated on the abaxial side by using an air brush pistol (Conrad, HP 320, Hirschau, Germany). The inoculum contained a water–agar (0.1 g l^{-1}) mixture with 8×10^5 urediniospores ml^{-1} of *M. larici-populina* (isolate 98AG31). This isolate, collected in a 'Beaupré' poplar stand in 1998, possesses the Vir7 virulence that overcomes the R7 rust resistance gene, i.e., the interaction is compatible (Pinon et al. 2006). After inoculation, the plants were placed in a plastic tent for 12 h in darkness to promote germination of the rust spores (Pinon et al. 2006). After 12 h, the tents were opened at the top to allow free gas exchange and prevent cross-contamination of plants. After 2 days, the tents were completely removed. Rust infection was assessed by the evidence of rust pustules on the abaxial leaf side. Leaves were harvested 10

days after inoculation in the middle of the day within their light period. In rust-infected treatments only leaves with visible spore pustules were harvested, and in non-infected treatments the leaves were taken from the same position on the stem.

Chemical analyses

The total biomass was determined of dried leaves (at 45 °C) and stems (at 60 °C). For all chemical analyses, leaves were ground in a Retsch mill (Retsch GmbH, Haan, Germany) to 0.5 mm size. The total phenolics and condensed tannins were analysed according to the method of Kraus et al. (2004), with the modification that centrifugation was replaced by filtering. Fifty milligrams of the ground material were extracted and shaken with 10 ml of acetone (50%) for 24 h. After vortexing the extracts repeatedly within 1 h, they were filtered with Whatman 2V filters and the filters were washed with additional 10 ml of acetone (50%). From these extracts the concentrations of total phenolics were measured using the Folin-Ciocalteu assay (Scalbert et al. 1989) with $\lambda = 760$ nm and the concentrations of condensed tannins by using the butanol–HCl method of Porter et al. (1986) with $\lambda = 550$ nm. Gallic acid was used as the standard for both methods to create a calibration curve.

The salicylic acid (SA) was extracted as described in Baum et al. (2009). One hundred milligrams of dried and ground leaves were mixed with 10 ml methanol (50%). Each sample was vortexed for 1 min instead of shaking. After that, the samples were filtered through 2V filters (Whatman) and the filters were washed with additional 5 ml methanol (methanol 50%). The SA concentration was measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS) on a MAT TSQ 700 (Finnigan Electron, Dreieich, Germany).

Total N (N_t) of the ground samples was determined by dry combustion using a Vario EL CNS analyser (Elementar Analysensysteme GmbH, Hanau, Germany).

For the pyrolysis–field ionization mass spectrometry (Py-FIMS) analysis, 0.4 mg of the ground sample was transferred to a quartz crucible, placed into the micro-heater and introduced into a MAT 95 mass spectrometer (Finnigan, MAT, Bremen, Germany). Samples were heated step-wise from 50 to 700 °C in steps of 10° over a time period of 18 min. The individual spectra and thermograms of total ion intensity were summed and averaged out over three analyses per sample (12 analyses per treatment). The data were normalized per mg dry sample. For identification of the most discriminating features, a rust-infected sample was measured with Py-FIMS at high resolution ($R = 5000$, 10% valley).

Estimation of mycorrhizal colonization

The fine roots (<2 mm diameter) were washed carefully from the soil. Living roots were identified on the basis of a turgid appearance and possession of white cortical cells. All living root tips in the samples were classified as either non-mycorrhizal or ectomycorrhizal. Ectomycorrhiza frequencies were calculated microscopically (numbers of EM root tips × 100%/total numbers of root tips). A minimum of 500 root tips was investigated per sample (2000 per treatment).

Statistics

The data of the Py-FIMS analysis were analysed by discriminant analysis. Based on this, 40 *m/z* signals with the highest discriminating power were selected based on the univariate Wilks' lambda score calculation and tested for significance at the $P \leq 0.05$ level by the corresponding multiple *F*-test. Significant differences in the portions of signal markers were identified between the treatments. For more information about the Py-FIMS methodology and statistical evaluation, see Schulten (1996) and Schlichting and Leinweber (2009).

The effects of the ectomycorrhizal colonization and rust infection as well as their interactions on the concentrations of the total phenolics and condensed tannins were tested with analysis of variance (ANOVA). Significance was determined at $P < 0.05$. Fisher's least significant difference test was used to determine significant differences between the treatments. The data of the concentrations of SA were statistically evaluated by the Mann–Whitney *U test*. All statistical analyses were computed using the software STATISTICA 7.0 (StatSoft Inc. 1984–2004).

Results

Growth and foliar chemistry

The total above-ground biomass per plant ranged from 12.6 to 17.6 g dry matter and was similar across treatments. Ectomycorrhizal colonization occurred only in the inoculated plants with and without rust infection. The percentage of ectomycorrhizal roots was significantly higher in the mycorrhizal treatment without rust infection (26 ± 4%) than in the mycorrhizal treatment with rust-infected leaves (17 ± 5%) ($P < 0.05$).

The concentrations of total phenolics were significantly lower in the treatment with single rust infection than in all other treatments (Table 1). The concentrations of condensed tannins in the foliage were not significantly affected by the fungal treatments. However, the proportion of condensed tannins on the total foliar phenolics was higher in all fungal treatments than in the control. The foliar concentrations of SA were significantly increased in rust-infected treatments (Table 2). The foliar N concentrations were higher in the control and in the rust-infected treatments than in the mycorrhizal treatment.

The total composition of the poplar foliage summarized in 10 compound classes of organic compounds (carbohydrates, phenols and lignin monomers, lignin dimers, lipids such as alkane, alkene, n-alkylester, n-alkyldiester lipids, alkylaromatics, N-containing compounds, sterols, peptides, suberins and fatty

Table 1. Chemical leaf concentrations of 4-month-old plants of *P. trichocarpa* × *deltoides* with different fungal treatments: non-treated (control), ectomycorrhizal inoculation (EM) and rust (R) infection and their interactions (EM + R) (means with standard deviation in parentheses, $n = 4$, different letters within a row indicate significant ($P < 0.05$) differences between the treatments).

Leaf chemical components	Treatment			
	Control	EM	R	EM + R
Total phenolics (mg g^{-1})	97.8 (18.4) b	97.3 (12.7) b	78.9 (18.9) a	99.8 (20.2) b
Condensed tannins (mg g^{-1})	12.5 (3.0) a	18.4 (3.6) a	14.8 (4.4) a	17.5 (6.1) a
Ratio of condensed tannins to total phenolics (%)	12.8 (2.9) a	18.9 (1.1) b	18.8 (0.4) b	17.5 (1.2) b
Salicylic acid (µg g^{-1})	6.8 (3.6) a	4.6 (4.5) a	17.9 (10.6) b	25.4 (11.5) b
N (mg g^{-1})	12.0 (0.1) b	11.0 (0.0) a	12.0 (0.1) b	11.0 (0.1) a

Table 2. The effects of ectomycorrhizal colonization (EM), rust infection (R) and their interaction (EM × R) on the foliar concentration of total phenolics, condensed tannins, SA and nitrogen of 4-month-old plants (*P. trichocarpa* × *deltoides*) in the two-factorial ANOVA.

Source	DF	*F* value	*P*
Total phenolics			
EM	1	0.314	0.590
R	1	0.011	0.918
EM × R	1	0.140	0.718
Error	12		
Condensed tannins			
EM	1	1.05221	0.335
R	1	0.38986	0.549
EM × R	1	1.24316	0.297
Error	12		
Salicylic acid			
EM	1	2.89058	0.114
R	1	27.09301	<0.001
EM × R	1	3.46778	0.087
Error	12		
Nitrogen			
EM	1	0.3049	0.590
R	1	0.1679	0.689
EM × R	1	0.1759	0.682
Error	12		

DF, degrees of freedom.

Table 3. Volatile matter (%), TII and intensities of important compound classes (10^6 ions per mg sample) and their proportions (% of total ion intensity) in the leaves of *P. trichocarpa* × *deltoides*) with different fungal treatments: non-treated (control), ectomycorrhizal inoculation (EM) and rust (R) infection and their combination (EM + R) (10 days after rust infection). Means with standard deviations, $n = 4$.

Trait	Treatment			
	Control	EM	R	EM + R
VM	80.7	83.0	81.5	80.7
TII	436.6	533.1	483.0	461.7
Compound class				
CARB	45.8 (6.0) a	61.5 (14.0) b	51.8 (15.4) a	46.8 (7.5) a
PHLM	38.3 (8.2) a	46.1 (5.0) a	39.9 (8.1) a	38.3 (7.1) a
LDIM	9.9 (2.3) a	10.3 (3.3) a	8.7 (2.1) a	12.1 (2.8) a
LIPID	42.4 (15.7) a	54.0 (15.4) a	45.1 (17.6) a	50.3 (16.7) a
ALKYL	30.7 (7.0) a	37.4 (5.7) a	31.4 (7.2) a	31.7 (5.4) a
NCOMP	23.9 (5.7) a	26.4 (6.1) a	24.5 (6.4) a	24.0 (4.9) a
STER	12.2 (4.1) a	18.4 (1.7) b	17.9 (6.9) ab	16.4 (4.4) ab
PEPT	21.2 (2.7) a	24.5 (2.9) a	23.1 (4.3) a	20.6 (1.4) a
SUBE	1.2 (0.3) a	1.7 (0.3) b	1.6 (0.7) ab	1.6 (0.4) ab
FATTY	9.2 (5.0) a	10.9 (1.6) a	10.8 (2.4) a	9.7 (3.7) a

Different letters within a row indicate significant differences between the treatments ($P < 0.05$).
Control, non-inoculated; VM, volatile matter in %; TII, total ion intensity; CARB, carbohydrates; PHLM, phenols and lignin monomers; LDIM, lignin dimmers; LIPID, alkane, alkene, n-alkylester, n-alkyldiester lipids; ALKYL, alkylaromatics; NCOMP, N-containing compounds; STER, sterols; PEPT, peptides; SUBE, suberins; FATTY, fatty acids n-C_{16} to n-C_{34}.

acids) is presented in Table 3. The proportion of carbohydrates was significantly increased in the EM treatment compared with the control. The proportion of sterols and suberins was generally increased in the fungal treatments.

Signal markers of rust infection

The results of the discriminant analysis showed that the non-mycorrhizal rust variant was separated from the other three treatments (control, EM, EM + R) (Figure 1). These treatments clustered together. The control and the EM treatment slightly overlapped with the rust treatment whereas the EM + R treatment was clearly separated from this single rust-infected treatment.

Based on a load plot (not shown) this separation of the rust treatment was largely affected by marker signals of flavonoids (*m/z* 308, 298, 300, 268, 270) and one lipid (*m/z* 392: cyclooctacosane), which yielded positive scores on principal component 1. The *m/z* values of the marker signals measured with high resolution are listed in Table 4 together with their tentative assignments based on compounds typically found in poplar leaves. One flavanol (catechin hydrate), four flavones (5-hydroxy-4′,7-dimethoxyflavone, trihydroxy-methoxyflavone, 5-hydroxy-7-methoxyflavone, and 5,4′,7-trihydroxyflavone) and one flavanon (pinostrobin chalcone) were identified. The *m/z* ratio 270 could be assigned either to 5,4′,7-trihydroxyflavone or pinostrobin chalcone.

The relative abundances of these marker signals are presented in Figure 2. The relative proportion of these masses was lower in the rust treatment than in the other treatments. The mycorrhizal treatment did not differ significantly from the control plants.

Discussion

In ectomycorrhizal poplar plants, modulations of the leaf physiology were indicated in the primary metabolism by an increased relative abundance of carbohydrates and in the secondary metabolism by enhanced relative abundance of sterols and suberins (see Table 3). Significant changes in the primary and secondary metabolism in poplar leaves in response to EM colonization were in line with the results of Luo et al. (2011), who investigated the impact of colonization of *P.* × *canescens* with the EM fungus *Paxillus involutus* (Batsch) Fr. on the leaf physiology. The combination of changes in the primary and secondary metabolism can be explained by the regulation of the secondary metabolism combined with the signals of the primary metabolism (Berger et al. 2007). Rust infection of non-mycorrhizal poplar plants led to a significant decrease in the phenol content of the leaves, which was compensated in ectomycorrhizal plants.

Primary metabolism

In contrast to Luo et al. (2011), we found an increased abundance of carbohydrates in combination with increased concentration of selected secondary metabolites (e.g., sterols) in the foliage of EM poplar. This might be explained by the EM species-specific differences observed for *Salix* spp. by Baum et al. (2009). Furthermore, the generally low foliar N concentrations (11–12 mg g^{-1}, see Table 1) indicate N deficiency in all treatments. Under N deficiency ectomycorrhiza formation can lead to significantly reduced N assimilation by the host caused by the retained N on the external mycelia of EM fungi in the soil (Colpaert et al. 1996). This was in line with the present results of decreased foliar N concentrations of the EM treatment (see Table 1). The higher requirement for photosynthetically fixed carbon from the leaves in the roots can be differently regulated in EM plants dependent on fungi species (Baum et al. 2009). The tested *H. mesophaeum* strain led to increased concentrations of carbohydrates in the leaves. Other EM species, like *P. involutus*, decreased the foliar carbohydrate concentrations of their host plants under N-deficiency (Luo et al. 2011).

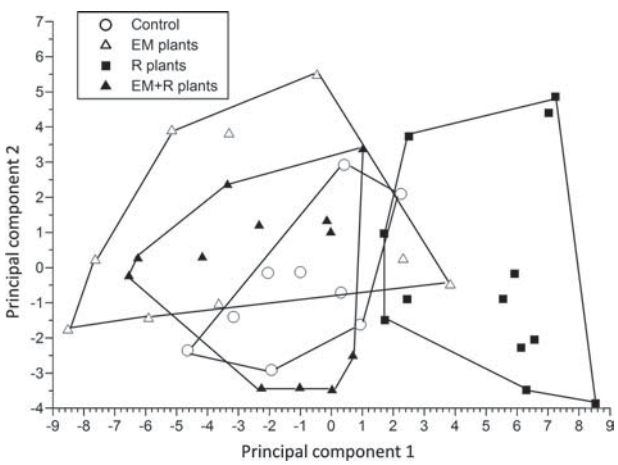

Figure 1. Score plot of the principal component 1 vs. 2 from discriminant analysis calculated from the molecular chemical composition of leaves from non-mycorrhizal and non-infected control, ectomycorrhizal (EM), rust-infected (R) and ectomycorrhizal and rust-infected (EM + R) *Populus trichocarpa* × *deltoides*.

Fatty acids play an important role in pathogen defence of plants. They derive from the fatty acid pathway in the primary metabolism and serve, e.g., as precursors for cuticular components (Kachroo and Kachroo 2009). However, in the present study no significant effect was observed in relation to the relative abundance of the fatty acids in the leaves by rust infection.

Secondary metabolism

An additional finding was the significantly enhanced concentration of sterol in the EM formation. Sterol is one of the brassinosteroids which has a function in multiple regulatory activities such as elongation and cell division or stimulation of ethylene biosynthesis, and furthermore sterol interacts with the phytohormones auxin and abscisic acid (Gross and Parthier 1994). It is also found in the epicuticular wax in willows and poplars (Cameron et al. 2002) and it seems that the synthesis of sterol is induced by the ectomycorrhizal formation. Decreased foliar N concentration in combination with increased synthesis of sterols in the EM treatment was in line with the observed negative correlation of foliar N and wax concentrations of *Picea abies* (L.) H. Karst observed in fungal treatments by Mrnka et al. (2009).

Table 4. Elemental composition of the most discriminating mass/charge (*m/z*) ratios (listed in a descending order) from high-resolution pyrolysis–field ionization mass spectra (Py-FIMS) and their tentative assignments to a molecular formula and a compound.

m/z measured	*m/z* calculated	Formula	Compound	Compound class
308.091	308.090	$C_{15}H_{16}O_7$	Catechin hydrate	Flavonoid (flavanol)
298.096	298.084	$C_{17}H_{14}O_5$	5-Hydroxy-4′,7-dimethoxyflavone	Flavonoid (flavone)
300.068	300.063	$C_{16}H_{12}O_6$	Trihydroxy-methoxyflavone	Flavonoid (flavone)
392.429	392.438	$C_{28}H_{56}$	Cyclooctacosane	Lipid (alkane)
268.067	268.074	$C_{16}H_{12}O_4$	5-Hydroxy-7-methoxyflavone	Flavonoid (flavone)
270.055	270.053	$C_{15}H_{10}O_5$	5,4′,7-Trihydroxyflavone	Flavonoid (flavone)
270.084	270.089	$C_{16}H_{14}O_4$	Pinostrobin chalcone	Flavonoid (flavanon)

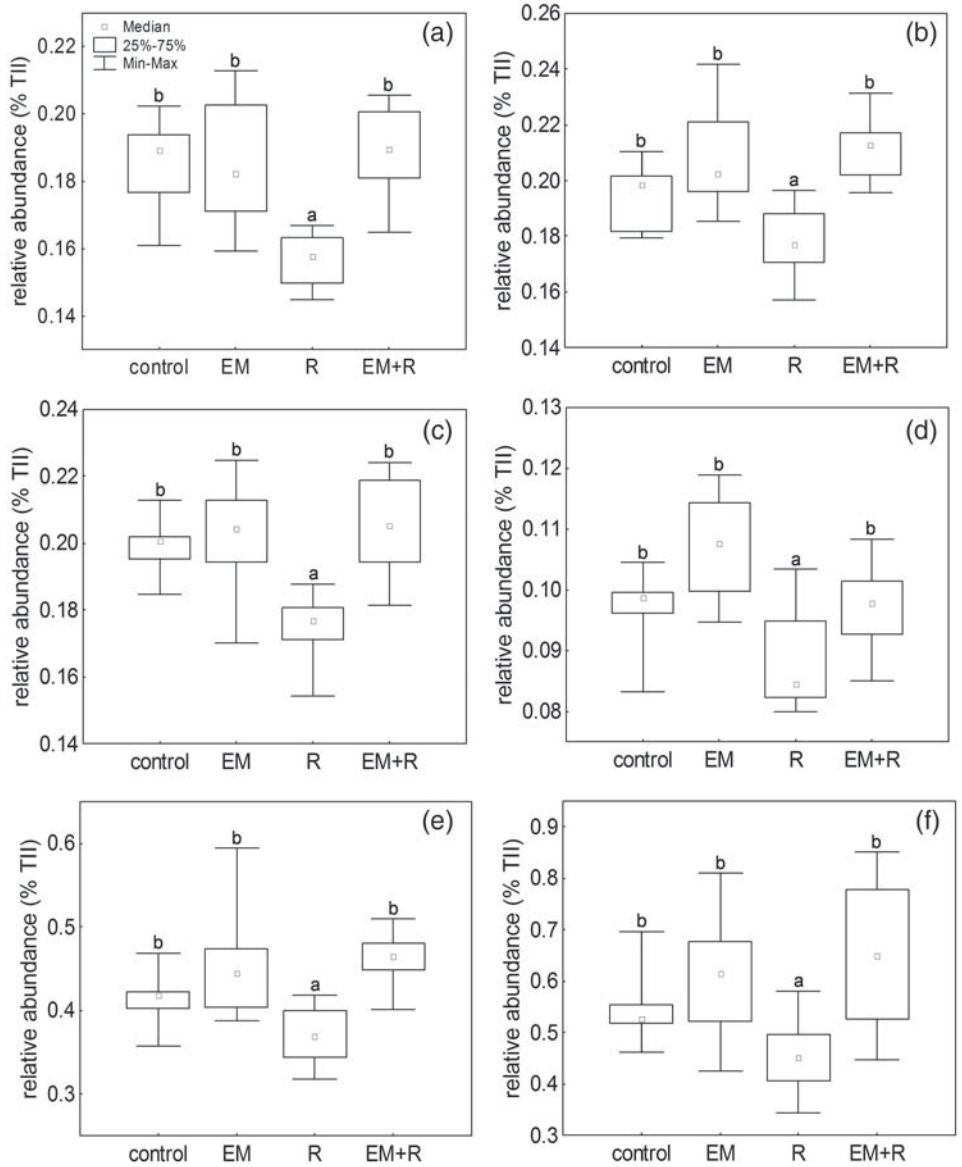

Figure 2. Relative abundance (percentages of total ion intensities, % TII) of the leaf components with the mass/charge (*m*/*z*) ratios 308 (a), 298 (b), 300 (c), 392 (d), 268 (e) and 270 (f) from high-resolution pyrolysis–field ionization mass spectrometry (PY-FIMS) in leaves from non-mycorrhizal and non-infected control, ectomycorrhizal (EM), rust-infected (R) and ectomycorrhizal and rust-infected (EM + R) *Populus trichocarpa* × *deltoides*. Different letters on the box plots indicate significant differences between the treatments ($P < 0.05$).

The infection of the poplar clones with rust caused a decrease of the total phenolic concentration. Phenolics refer to a wide range of diverse compounds including condensed tannins and other flavonoids. Interestingly, the concentration of flavonoids, but not condensed tannins, was also lower in rust-infected poplars. Here, the poplar clones were able to synthesize higher concentrations of defence substances, as indicated by the higher portions of condensed tannins after rust infection (Table 1). Evidence for such a mechanism was reported by Miranda et al. (2007), who found up-regulation of genes encoding enzymes required for condensed tannin synthesis after infection by rust fungi.

The SA concentration was significantly increased in response to the rust attack, both in the single and in the combined treatment with mycorrhizal fungus. This agrees with its key function as the signalling component for the activation of plant defence (Durner et al. 1997, Zhao and Qi 2008). It seems that the formation of SA is preferred because its synthesis takes place after the shikimate pathway, or more specifically in the phenylpropanoid pathway. Our results are in line with Azaiez et al. (2009), in which the activation of the SA-dependent pathway genes was observed as a result of rust attack with *M. larici-populina* or *M. medusae* f. sp. *deltoidae* on hybrid poplar clones. By mycorrhiza colonization the foliar SA concentration in

willows was increased in some mycorrhizal clones, which indicated a high specificity of host–fungus combinations (Baum et al. 2009).

The concentration of cyclooctacosane, an alkane, was significantly decreased in poplar leaves after rust infection. To our knowledge, cyclooctacosane has not yet been documented in relation to the interaction with rust fungus. However, alkanes were found in the cuticular wax layer of willow and poplar leaves (Cameron et al. 2002), where they reinforce the hydrophobic wax layer of the cuticle to protect against pathogens. During the rust invasion in the leaves, the structures of the cuticle seem to be degraded due to release of cell wall degrading enzymes by the fungus (Tian et al. 2009).

In the present study, the poplar clones responded to fungal treatments with enhanced concentration of suberin. This compound is a complex heteropolymer consisting of both phenolic and lipid constituents which act as a barrier of wound-healing plant tissues protecting against moisture loss and susceptibility to pathogens (Facchini et al. 2002). In general, pathogen attack includes the fast accumulation of phenolic substances and the reinforcement of cells with suberin at the site of injury (Blanchette and Biggs 1992). The suberin results are consistent with those of other studies (Baayen et al. 1996, Rioux and Baayen 1997, Jabaji-Hare et al. 1999), and suggest that the enhanced concentration in plants is a result of defence reaction to form a physical barrier (Jabaji-Hare et al. 1999).

In this study, three subgroups of this chemically diverse group were found: flavones, flavanons, flavanols. Within the flavones the hydroxy and methoxy substitutions are noticeable as well as the chalcones within the flavanons and the flavanols to have antifungal activity (Harborne and Williams 2000). However, the content of these compounds was low after rust infection. This result differs from other studies in which the accumulation of such phenolics has been reported as a sign of activated defence mechanism (Harborne and Williams 2000, Miranda et al. 2007, Azaiez et al. 2009).

The secondary metabolism is influenced by the changes in the primary processes of the carbon metabolism. The synthesis of defensive phenolic substances such as condensed tannins is likely very energy- and carbon-intensive for the plant (Major et al. 2010). Condensed tannins are produced, e.g., to suppress fungal colonization of the plant by enzyme inhibition and substrate deprivation, action on membranes and metal ion deprivation (Haslam 1989). In contrast, the production of SA is carbon-inexpensive (Bryant et al. 1983). Consequently, it seems that the formation of SA is preferred as defence substance, because this takes place after the shikimate pathway, but very early in the phenylpropanoid metabolism. A fungistatic and in higher concentrations fungicidal effect of SA was already described by Amborabé et al. (2002). The condensed tannins are metabolized after these pathways as the last step of the flavonoid biosynthesis.

In total, the impact of the rust fungus on the foliar chemistry was much stronger in comparison with the ectomycorrhizal colonization; however, ectomycorrhizal colonization was able to compensate for the decrease of flavonoids by rust infection. Promotion of other pigment (carotenoids) syntheses was also described after inoculation of *P. abies* with two other EM species [*Hebeloma bryogenes* Vesterh. and *Cladophora finlandica* (Wang & Wilcox) Harr. & McNew] by Mrnka et al. (2009). The EM impact on pigment syntheses and their significance for plant resistance to rust infection will be a challenge for future investigations.

Conclusions

We found evidence for our alternative hypothesis, i.e., the promotion of the host plant against the parasite by the symbiotic fungus. This was revealed by the compensation of the decreased flavonoid syntheses by ectomycorrhiza formation. However, we also found an indication of resource competition as measured in a decreased ectomycorrhizal formation of rust-infected plants. Furthermore, resource competition between the symbionts (host plant and EM fungus) was indicated at the nutrient-deficient soil by decreased N concentrations of EM plants. The results provide new evidence for the interaction between effects of below-ground mycorrhizal formation, above-ground rust fungi infection and host plant metabolism. However, these results were obtained with one model system, and complex interactions between, e.g., host genotype and EM species (e.g., Baum et al. 2009) may result in different effects on metabolism and defence functions depending on the specific combination of host genotype, EM fungi and rust strain.

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Conflict of interest

None declared.

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