


## LETTER

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# A Fungal Endophyte Alters Poplar Leaf Chemistry, Deters Insect Feeding and Shapes Insect Community Assembly

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## ABSTRACT

Fungal endophytes of grasses and other herbaceous plants have been known to provide plants with anti-herbivore defence compounds, but there is little information about whether the endophytes of trees also engage in such mutualisms. We investigated the influence of the endophytic fungus *Cladosporium* sp. on the chemical defences of black poplar (*Populus nigra*) trees and the consequences for feeding preference and fitness of herbivorous insects and insect community assembly. Endophyte colonisation increased both constitutive- and induced poplar defences. Generalist *Lymantria dispar* larvae preferred and performed better on uninfected over endophyte-infected poplar leaves, most likely due to higher concentrations of salicinoids in endophyte-inoculated leaves and the endophyte-produced alkaloid stachydrine. Under field conditions, the endophytic fungus shapes insect community assembly i. a. attracting aphids, which can excrete stachydrine. Our results show that endophytic fungi play a crucial role in the defence against insects from different feeding guilds and thereby structuring insect communities.

## 1 | Introduction

Trees are large, long-lived organisms that host a huge number of arthropod and microbial species (Basset et al. 2012; Lämke and Unsicker 2018). To protect themselves from natural enemies, trees have developed many chemical defences with toxic, repellent or anti-nutritive properties (Jander and Howe 2008), which can be constitutive or induced in response to biotic stressors (Wu and Baldwin 2010). Recent studies have focused on tree defences in response to attack by a single insect or pathogen species, but in nature trees are usually attacked simultaneously by insect herbivores and pathogens. Only a few studies have investigated tripartite interactions

of trees, pathogen and insect herbivores (Eberl et al. 2020; Eberl et al. 2019). Specialised tree metabolites were mainly studied related to interactions between trees and antagonists such as pathogenic microbes and insect herbivores although trees are also colonised by endophytic microbes, fungi or bacteria that live at least part of their lives in plant tissue without causing disease (Petrini 1991). As endophytes are widespread in the plant kingdom, their influence on plant-insect interactions has already been studied (Hyde and Soyong 2008), and endophyte-specific metabolites that may affect plant-insect interactions were detected in the plant matrix. Prominent examples are the alkaloid-producing *Epichloë* (Fries) Tulasne and Tulasne, species in fescue grasses (Clay 1988; Popay and

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Bonos 2005; Saikkonen et al. 2013). Alkaloids are known for their detrimental effects on herbivore performance (Dewick 2002; Kaur 2020). When the grass *Lolium multiflorum* was inoculated with the loline alkaloid-synthesising endophyte *E. occultans* (Moon, Scott & Chr.) Schardl, aphid (*Rhopalosiphum padi* Linnaeus) performance was reduced (Bastías, Ueno et al. 2017). Grass fungal endophytes are an outstanding example of mutualistic plant–microbe interactions (Hartley and Gange 2009; Saikkonen et al. 2010). In contrast, horizontally transmitted tree endophytes are thought to be less mutualistic and less dependent on their host. They can be categorised as transient or non-systemic endophytes (Mostert et al. 2000, Rodriguez et al. 2009, Wani et al. 2015). However, in comparison to grass endophytes, the ecological importance of tree endophytes, is less well studied (Eberl et al. 2019; Meister et al. 2006; Rodriguez et al. 2009), with some exceptions (e.g., Carroll 1986; Faeth and Hammon 1997a, 1997b; Miller et al. 2002; Miller et al. 2008; Sumarah et al. 2008, 2010; Sumarah and Miller 2009). Even less is known about the role of endophytes in the arthropod assembly in trees.

Here, we aim to test how an endophytic fungus impacts tree–insect interactions. We used black poplar (*Populus nigra* Linnaeus), a tree species native to riparian ecosystems in Europe. *P. nigra* produces phenolic compounds, especially two major groups, salicinoids and condensed tannins (Boeckler et al. 2011, 2013) for defence against herbivores and pathogens. These compounds can make up to 30% of the leaf dry weight, with salicinoids decreasing the performance of generalist insect herbivores (Boeckler et al. 2016; Hemming and Lindroth 1995; Lindroth 1991). Here, we inoculated black poplar with the endophytic fungus *Cladosporium* sp. Link, previously isolated from *P. nigra* (Walther et al. 2021). Fungi of the genus *Cladosporium* are cosmopolitan, occurring as pathogens, hyperparasites, epiphytes and moulds in numerous natural and anthropogenic habitats (Bensch et al. 2012; Ellis 1971, 1976; Heuchert et al. 2005; Inácio et al. 2002; Schubert 2005), but are also frequently isolated as endophytes (Bensch et al. 2012; Brown 1998; Riesen 1985). *Cladosporium* species are known to produce anti-fungal toxins (Bensch et al. 2012; Wang et al. 2013), but their influence on tree–insect interactions has rarely been studied.

We investigated the consequence of *Cladosporium* sp. colonisation on (a) leaf metabolome, (b) the preference and performance of common poplar herbivorous insects and (c) arthropod assembly.

## 2 | Materials and Methods

### 2.1 | Plants, Insects and Endophytic Fungus

*Populus nigra* trees were propagated as monoclonal stem cuttings under sterile conditions in a climate chamber and transferred to sand/soil mixture for further cultivation (Method S1). Generalists lepidopteran *Lymantria dispar* Linnaeus, *Orgyia antiqua* Linnaeus larvae, specialist *Chrysomela tremulae* Fabricius beetles and *Chaitophorus leucomelas* Koch aphids were reared on artificial diet or poplar leaves, respectively (Method S1). *Cladosporium* sp. (identification and growth described in

Method S1, Figure S1) was isolated from mature black poplar trees (Walther et al. 2021).

### 2.2 | Plant Treatments and Harvesting

To investigate the consequences of *Cladosporium* sp. on black poplar defence chemistry, 16 young trees were split into four treatments (*control*, *endophyte*, *herbivory* and *endophyte + herbivory*,  $n = 4$ ). Trees in the *endophyte* and *endophyte + herbivory* treatments, 9–10 leaves were sprayed with a spore solution (1.5 mL per leaf, Method S3). Comparable leaves in the *control* and *herbivory* treatment were sprayed with a mock solution. To support germination and to avoid unwanted spore spreading, trees were individually enclosed in polyethylene terephthalate tubes (150 cm long, 31 cm diameter; Toppits, Minden, Germany). Ten days *post infection* (dpi), all treated leaves were wiped with wet cellulose wipes to avoid epiphytic growth. At 15 dpi, the treated leaf pools of the *herbivory* and *endophyte + herbivory* treatments were infested with 15 4th to 5th instar *L. dispar* larvae. After 48 h, larvae were removed and leaves were photographed, flash-frozen, lyophilised and stored under room temperature. Leaf damage was analysed with Adobe Photoshop 2020 (Boeckler et al. 2013).

### 2.3 | Preference Assays

Trees were inoculated with either an endophyte spore or a control solution (described above,  $n = 4$ ) and individuals of the two treatments were placed alternately next to each other (Method S1). Two leaves, one from an endophytic plant (15 dpi) and one from a control plant, were enclosed in a cellophane bag (Griesinger, Neuenbürg, Germany) and one 2nd instar *L. dispar* larva was released therein. Several leaves of one tree were thus connected with leaves of the neighbouring tree from the other treatment (Figure S2). Larvae were allowed to feed for 48 h. Leaf damage was analysed as described above.

To test the effect of stachydrine on herbivore preferences, choice assays with 2nd instar *L. dispar* ( $n = 20$ ) and *O. antiqua* ( $n = 20$ ) larvae and adults of *C. tremulae* ( $n = 16$ ) were conducted. Leaf discs (16 mm diameter) were coated either with 20  $\mu$ L of a control solution (0.01% Triton X-100) or a solution containing stachydrine 4.74  $\mu$ g/mL, corresponding to the *in planta* concentration (Roth, Karlsruhe, Germany). Leaf discs were offered in alternating order on pins in a modified Petri dish arena (Boeckler et al. 2014). *L. dispar* larvae were allowed to feed for 48 h, *O. antiqua* and *C. tremulae* for 24 h. Leaf disc damage was determined as described above. To determine stachydrine concentrations in leaf discs, three samples of five discs each were sampled as described above (Table S1D).

### 2.4 | Performance Assay

To assess the influence of *Cladosporium* sp. on the performance of *L. dispar* ( $n = 7$ –9) and *C. tremulae* larvae ( $n = 10$ ), 2 days old larvae were placed in cages installed around single leaves (Method S1, Figure S2, Eberl et al. 2020). Fifteen days before experimental start, all plants were either treated with an endophyte spore solution or a control solution (described above). Due to generally high mortality in early larval stages, we started

with 10 individuals per species and cage and reduced to one individual 9 days (*C. tremulae*) and 15 days (*L. dispar*) later. Every 3 days, insects in their respective cages were weighed as a group and transferred to new leaves on a new tree as soon as around 80% of total leaf area was consumed. Pupae of *L. dispar* were kept at room temperature until hatching for sexing.

## 2.5 | Field Experiment on Arthropod Community Assembly

Endophyte-infected and uninfected young *P. nigra* trees (~140 cm high) were transferred 15 dpi to a natural population of mature trees (52°34'03.1"N, 14°38'06.8"E, Figure S3). A plastic fence (Grube, Bisingen, Germany) was installed to protect them from mammalian herbivory. In June 2020, insects on trees were monitored at 9.00 AM, 12.00 AM, 3.00 PM and 6.00 PM daily for 9 days by two experimenters. All insects were determined at least to order level. After 20 days, leaves were harvested (damaged and undamaged leaves kept separately), photographed, flash-frozen and lyophilised. Leaf damage was assessed as described above.

## 2.6 | Aphid Honeydew Assay

To test the presence of stachydrine in the honeydew of aphids feeding on endophyte-inoculated plants, 75 adult aphids from either control or endophyte-inoculated plants ( $n = 5$ ) were placed on a leaf in a Petri dish (petioles of leaves placed in water-filled tubes). The honeydew was collected on an aluminium foil placed in the Petri dish, washed off after 24 h with 2 mL of 80% methanol and stored at  $-20^{\circ}\text{C}$ .

## 2.7 | Quantification of Endophytic DNA

To quantify *Cladosporium* sp. abundance, genomic DNA was extracted using Invisorb Spin Plant Mini Kit following the manufacturer's instructions, then quantified with a NanoDrop 2000c Spectrophotometer (Peqlab Biotechnology GmbH, Erlangen, Germany) and diluted to 100 ng/ $\mu\text{L}$ . A qPCR was performed with primers specific for *Cladosporium* sp. (Method S2).

## 2.8 | Chemical Analysis

### 2.8.1 | Extraction and Target Analysis

Leaf material was extracted with 1 mL methanol containing internal standards for phytohormones and phenolics (Method S4). Phenolic compounds were measured with an HPLC-UV as described in Boeckler et al. (2013). Phytohormones and phenolic acids were measured with an HPLC-MS/MS system as described in Fernández-Milmanda et al. (2020) and Fabisch et al. (2019) (Method S5).

### 2.8.2 | Untargeted Metabolomics

Leaf extracts were analysed using a Dionex Ultimate 3000 series UHPLC (Thermo Scientific) and a Bruker timsToF mass

spectrometer (Bruker Daltonik, Bremen, Germany, Method S6). MetaboScape (Bruker, Germany) was used to accomplish bucketing of the raw data (Method S6). Identification was carried out by comparing mass spectra and retention times with those of known compounds of *P. nigra*.

### 2.8.3 | Stachydrine Identification and Quantification

Endophyte-inoculated and control leaf extracts were compared by using quadrupole ion-trap MS coupled to an HPLC (Method S7). For structure confirmation, fungal mycelium was extracted for analysis using a Dionex Ultimate 3000 series UHPLC and a Bruker timsToF mass spectrometer (Method S7). The target compound showed a signal fully consistent with the mass spectrum of authentic stachydrine (Figure S4).

For quantification of stachydrine, the leaf and honeydew sample extracts were diluted and equipped with amino acid standard. The measurements were conducted on the triple quadrupole HPLC-MS/MS system (Method S7).

## 2.9 | Statistics

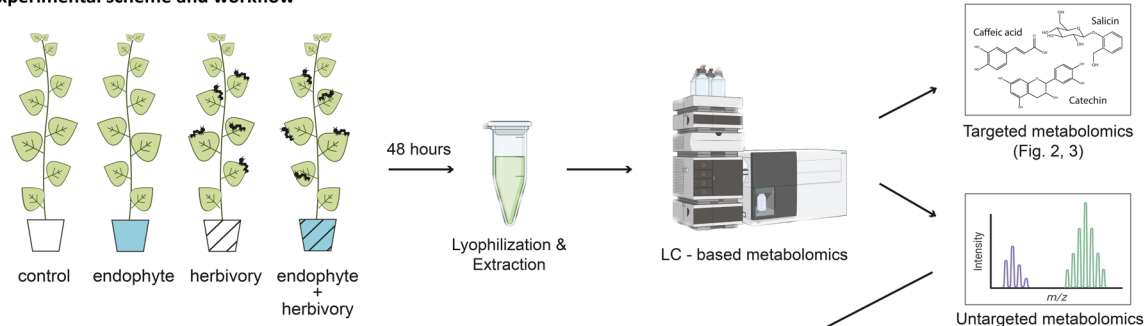
Data were checked for statistical assumptions and log- or arcsine transformed (percentage data) whenever necessary. For untargeted metabolomics, a PCA with peak intensities (peak height) was performed and heatmaps were made to highlight significant metabolites found by a two-way ANOVA analysis. Targeted chemical analysis data were tested with two-way ANOVA. Percent leaf area loss from the preference assay was analysed with a mixed effect model. Performance data were evaluated by a GLS regression. Leaf area loss in the leaf discs preference assay was analysed with a paired t-test or a Wilcoxon signed-rank test. Data on insect community were analysed by a GLM. A cumulative visitation network visualises the insect community. The difference in leaf area loss in the field was tested with Mann-Whitney U. Field stachydrine concentration was analysed with a GLMM. Analysis was done in R version 4.3.2 (R Core Team 2021), IBM SPSS statistics 25 (SPSS, Chicago, USA) and MetaboAnalyst (Pang et al. 2022). Used packages in R were pacman (Rinker and Kurkiewicz 2018), readr (Wickham et al. 2024), dplyr (Wickham et al. 2023), tidyverse (Wickham et al. 2019), bipartite (Dormann et al. 2008), Matrix (Bates et al. 2024), lme4 (Bates et al. 2014), performance (Lüdtke et al. 2021), nlme (Pinheiro et al. 2023), ggplot2 (Wickham 2016), and xlsx (Dragulescu and Arendt 2020). For statistical details see Method S8.

## 3 | Results

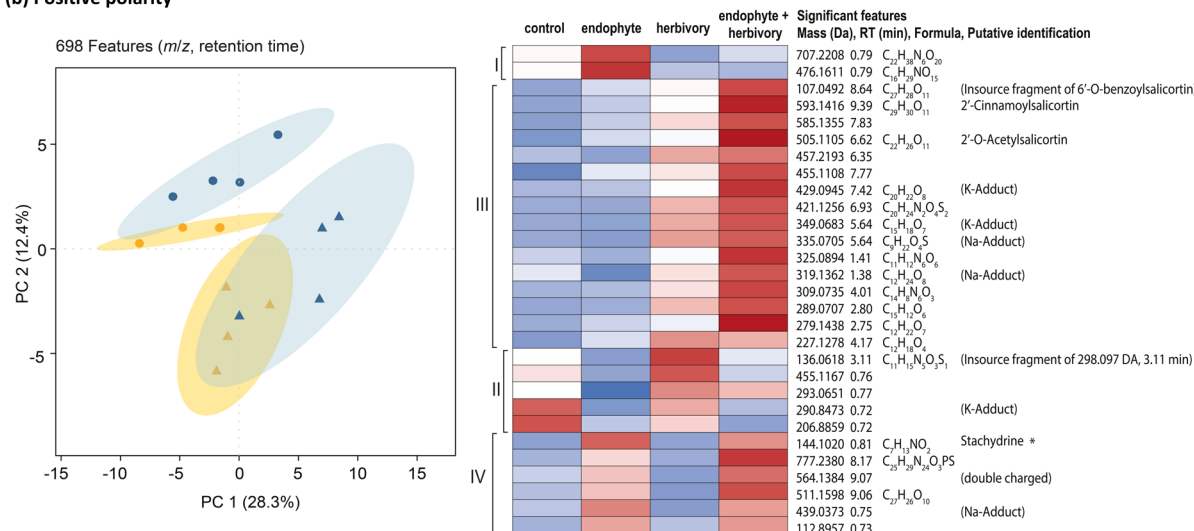
### 3.1 | Endophyte Inoculation Causes Metabolic Changes in Poplar

Metabolite analyses (Figure 1a) were performed with leaf extracts of *P. nigra* trees from four treatment groups (*control*, *endophyte*, *herbivory* and *endophyte + herbivory*). Untargeted analysis performed in positive and negative ion modes yielded two data matrices of 698 and 492 signals, respectively. The

## (a) Experimental scheme and workflow



## (b) Positive polarity



## (c) Negative polarity

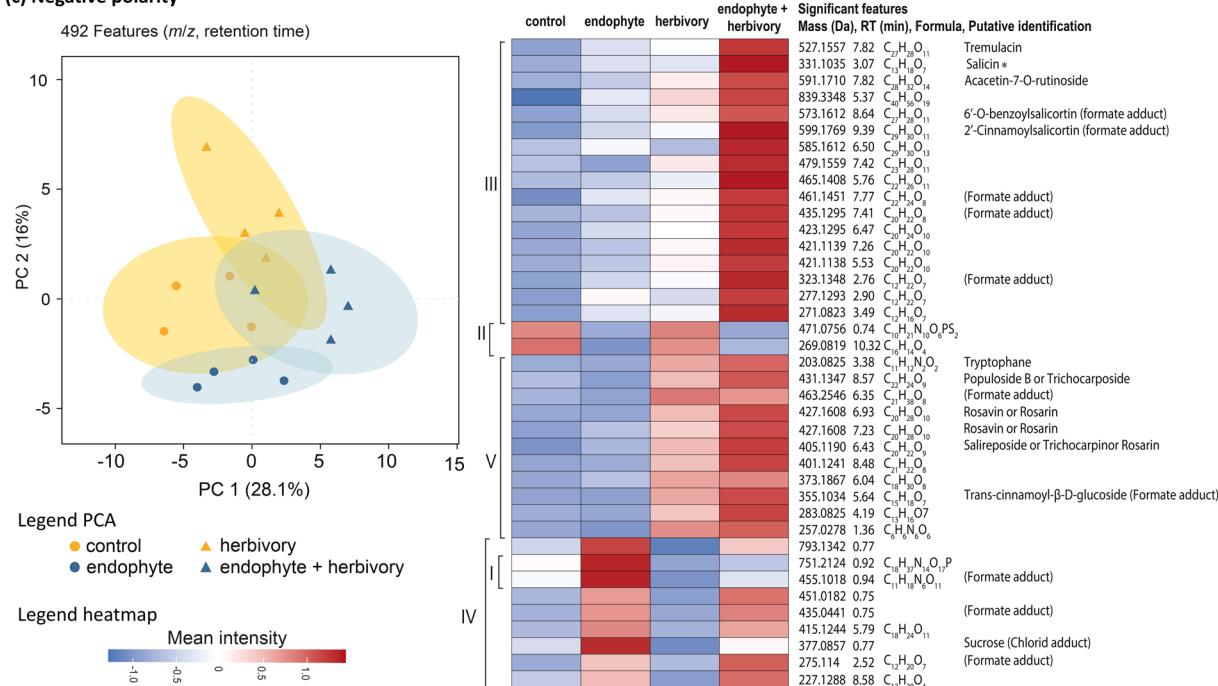


FIGURE 1 | Legend on next page.

PCA indicates differences among the metabolic profiles between the four treatments (positive mode 44%, negative mode 40.7%, Figure 1b,c, left side plot). To describe the metabolomic fingerprint, we compared the peak intensities (height)

of relevant signals. Metabolic features significantly affected by the treatments are shown in heatmaps (Figure 1b,c right-side plot). Here, up to five clusters were identified with cluster I containing features that increased (had higher peak



**FIGURE 1** | Metabolomic investigations of black poplar (*P. nigra*) leaves after fungal endophyte inoculation and herbivory by *L. dispar* larvae. (a) Trees were either inoculated with a solution of *Cladosporium* sp. spores or a control solution 15 days before the onset of larval feeding, resulting in four treatments: *Control*, *endophyte*, *herbivory* and *endophyte + herbivory*. After 48 h leaves were harvested. Different LC–MS instruments were used for targeted versus non-targeted metabolomics (see methods). The PCA analysis highlights the differences among the metabolic profiles of the various treatments acquired from LC–HRMS in positive (b) and negative (c) polarity. Heatmaps are used to visualise the significant features obtained by a two-way ANOVA. Metabolites up- and downregulated during endophyte treatment with or without herbivores are represented based on their relative peak intensities (red colour for high intensity, blue for low intensity). Clusters of significant features of the heatmap are identified on the left edge Cluster I: Features increased in *endophyte*; Cluster II: Features increased in *control* and *herbivory*; Cluster III: Features increased in *endophyte + herbivory*; Cluster IV: Features increased in *endophyte* and *endophyte + herbivory*; Cluster V: Features increased in *herbivory* and *endophyte + herbivory*. For each feature, the mass (Da), retention time (RT in minutes), molecular formula and putative identification are given. Features assigned to known black poplar defence compounds (salicin) and the fungal endophyte-produced alkaloid stachydrine are highlighted with an asterisk and verified with authentic standards.

intensities) in response to the endophyte *Cladosporium* sp. alone, and cluster II with features higher in the *control* and *herbivory* treatments. Cluster III features increased in the *endophyte + herbivory* treatment, with a slight increase in *herbivory* in positive mode, while Cluster IV features increased in the *endophyte* and *endophyte + herbivory* treatment. The well-known poplar salicinoid, salicin (Cluster III) was detected in significantly elevated amounts in *endophyte + herbivory* treatment compared to the other treatments. The signal identified as stachydrine (Cluster IV) is likely of fungal origin, as it was detected in the mycelium of the fungus- and endophyte-inoculated plants (but see below). Lastly, Cluster V features increased in the *herbivory* and *endophyte + herbivory* treatment (Figure 1b,c right-side plot).

### 3.2 | Endophyte Inoculation Increases Levels of Chemical Defence Compounds in *P. nigra* Leaves

Salicin and nigracin concentrations significantly increased in the presence of *Cladosporium* sp. both with and without herbivory (Figure 2a). The more abundant salicinoids, salicortin and homaloside D, showed a trend towards increased concentrations in the *endophyte + herbivory* compared to the other treatments, but this was not significant (Figure 2a). 6'-O-benzoylsalicortin levels were slightly, but non-significantly higher in the *endophyte + herbivory* treatment (Figure 2a).

The concentration of caffeic acid in the leaves increased significantly in the *endophyte + herbivory* treatment, leading to a significant effect on the interaction of both treatments (Figure 3). Lastly, *p*-coumaric acid and ferulic acid decreased in response to the endophyte, irrespective of herbivory, while catechin did not differ between treatments (Figure 3).

### 3.3 | The Endophyte Increases Biologically Active Jasmonates in Poplar Leaves After Herbivory

Jasmonates, the major phytohormones controlling plant responses to herbivores, significantly increased in poplar leaves after feeding by *L. dispar* (Figures S5 and S6, Tables S2 and S3). This was most pronounced for the jasmonic acid-isoleucine conjugate (bioactive form of jasmonate) in the *endophyte + herbivory* treatment compared to the *endophyte* or *herbivory* treatments alone (Tables S2 and S3). Abscisic acid (ABA) concentrations in poplar leaves significantly increased in the *herbivory* treatment, but were

unaffected by the *endophyte* treatment (Figure S6, Tables S2 and S3). Salicylic acid (SA) concentrations were not affected by the endophyte, but by herbivory (Figure S6, Tables S2 and S3).

### 3.4 | The Endophyte Reduces the Preference and Performance of an Insect Herbivore

Behavioural assays were conducted on poplar leaves with or without endophyte infection (Figure 4a, Figure S2). In the preference assay, early-instar *L. dispar* larvae preferred control over endophyte-inoculated plants (Figure 4b). In the performance assays, larvae of both the generalist *L. dispar* and the specialist *C. tremulae* gained significantly less weight when feeding on endophyte-inoculated leaves over control leaves (Figure 4b).

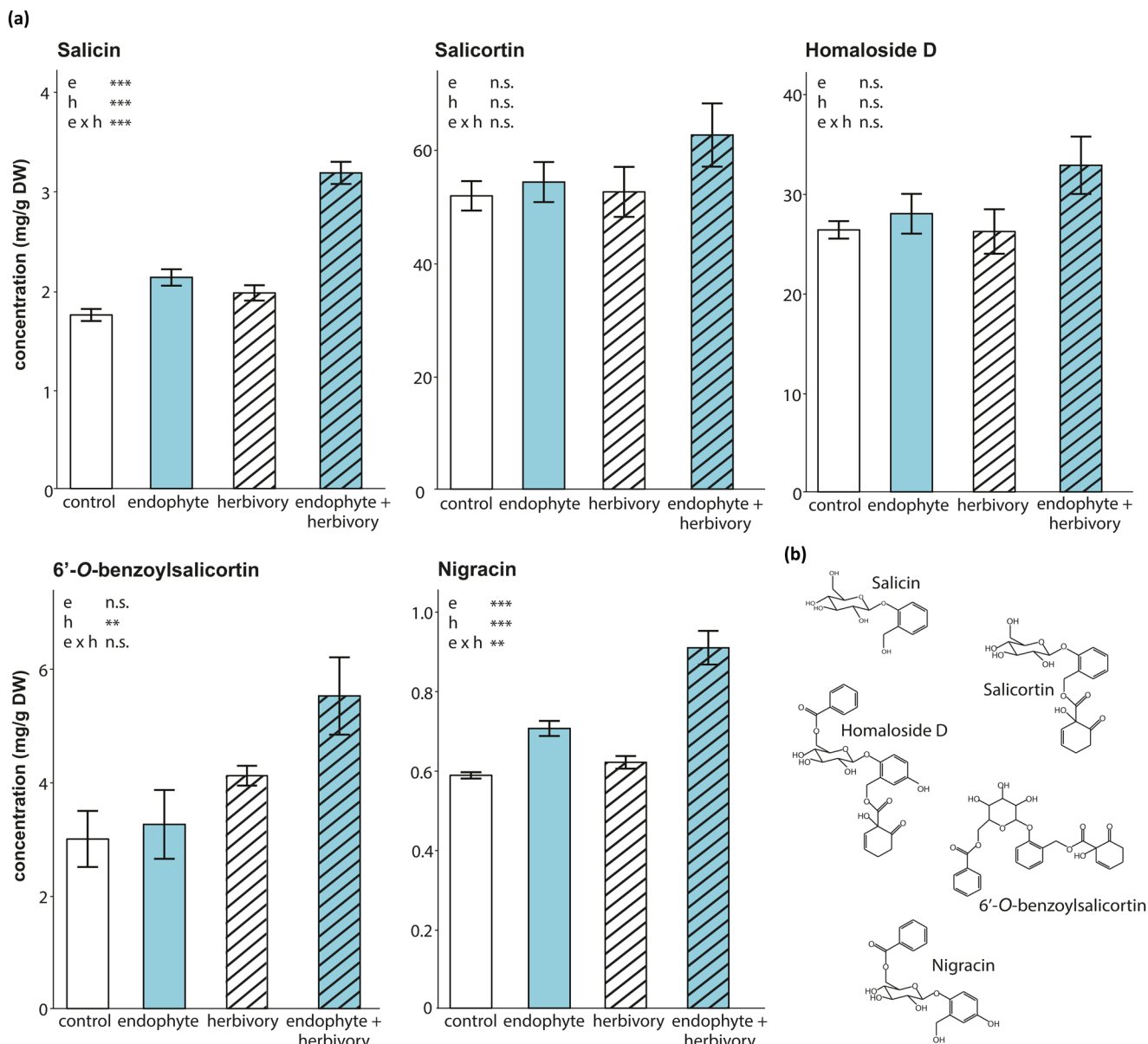
### 3.5 | The Endophyte-Produced Alkaloid Influences Insect Feeding Preference

One metabolic feature from Cluster IV present only in leaves of endophyte-inoculated poplar trees was identified as the pyrrolidine alkaloid stachydrine by comparison of its retention time and mass spectrum with those of an authentic standard (Figures 1b and 5a, Figure S4). Stachydrine, also called proline betaine, is known from other fungi, algae and higher plants (Murata et al. 2011). The endophyte mycelium was found to contain the highest amount of stachydrine with  $6050 \pm 460$  nmol/g (dw) (Figure 5b). However, readily detectable amounts of stachydrine were also found in the *endophyte* ( $34.52 \pm 8.53$  nmol/g dw) and *endophyte + herbivory* ( $45.80 \pm 8.03$  nmol/g dw) treatment. Herbivory and the interaction of herbivory and endophyte did not affect stachydrine concentration (Figure 5b).

Stachydrine was coated on leaves at natural concentration and offered together with control leaves to larvae *L. dispar* and *O. antiqua* larvae and *C. tremulae* beetles. Larvae of *O. antiqua* and *C. tremulae* beetles consumed significantly more leaf material from controls, while *L. dispar* did not show a preference (Figure 5d).

### 3.6 | Endophytic Inoculation Shapes *P. nigra* Arthropod Communities and Affects Aphid Honeydew

The amount of leaf damage in the field did not differ between endophyte-inoculated versus control trees (Figure S7), but we

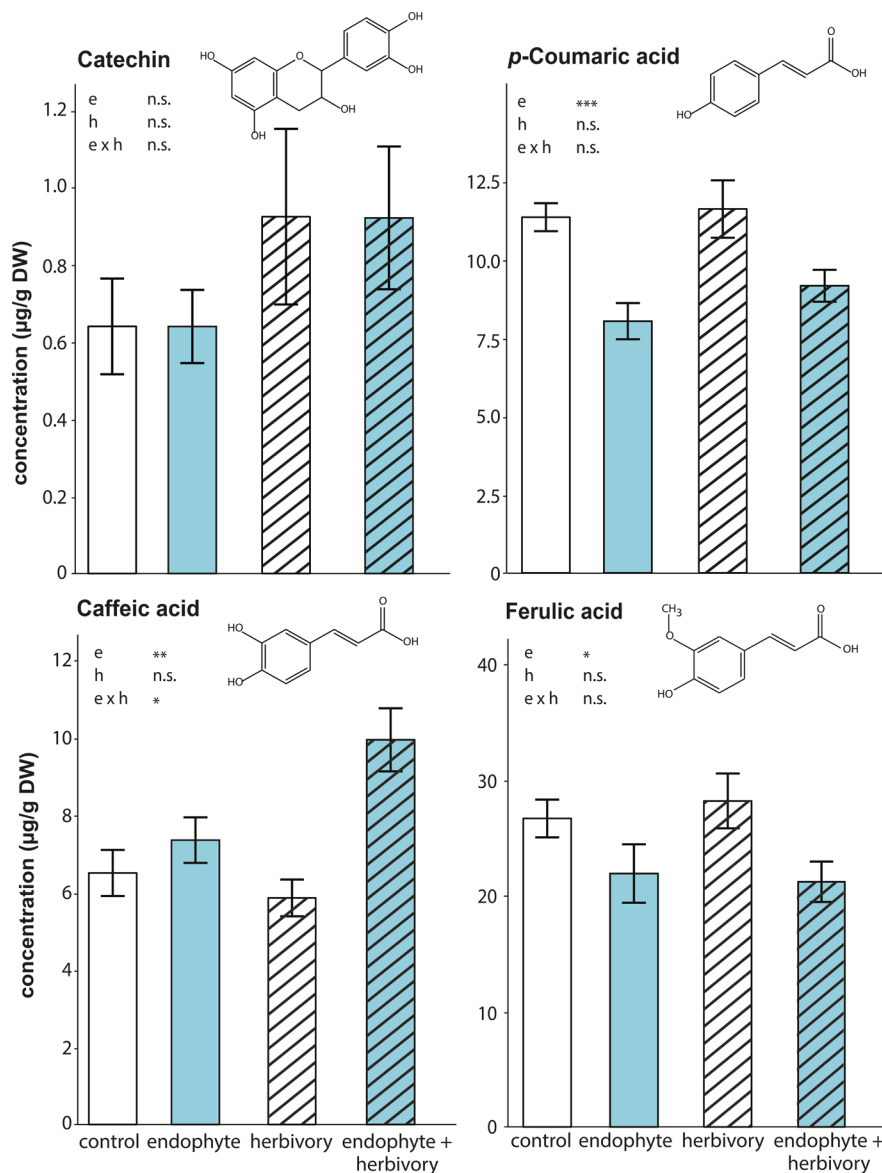


**FIGURE 2** | Effect of endophyte inoculation and herbivory on salicinoid concentrations in leaves of black poplar. (a) Trees were either inoculated with endophyte spore solution or a control solution 15 days before the onset of larval feeding, resulting in 4 treatments: *Control*, *endophyte*, *herbivory* and *endophyte + herbivory*. After 48 h of herbivory, leaves were harvested. A two-way ANOVA (top left) was used to estimate the effect of endophyte infection (e), herbivory (h) and the interaction of both (e x h), (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; n.s. = not significant). Mean  $\pm$  SE are shown ( $n = 4$ ). (b) Structures of major salicinoids of black poplar: Salicin, salicortin, homaloside D, 6'-O-benzoylsalicortin and nigracin.

observed significant treatment effects on the composition of insect communities. Coleoptera were more abundant on control plants irrespective of the time of observation (Figure 6). Endophyte-inoculated plants were visited significantly more often by hemipteran species than control plants, with aphids comprising the largest proportion (93.75%) of the visitors. Visitation was significantly influenced by the interaction of time and treatment (Figure 6, Table S4). Hymenoptera, mainly represented by Formicidae (94.16%) and a few Ichneumonidae, were found significantly more often on control plants (Figure 6, Table S4). In a laboratory experiment, significantly higher relative amounts of stachydrine were found in the honeydew of aphids feeding on endophyte-inoculated plants compared to the control trees (Figure S8).

## 4 | Discussion

In this study, we demonstrated that the endophyte *Cladosporium* sp. alters the metabolome of black poplar trees by increasing levels of specific poplar defence compounds. The endophyte also produces the alkaloid stachydrine which acts as a deterrent against a specialist leaf beetle and larvae of a lepidopteran species. Larvae of a generalist lepidopteran were deterred from feeding on endophyte-infected leaves and performed worse when forced to feed on them. A field experiment showed that the presence of *Cladosporium* sp. in young *P. nigra* saplings had no effect on herbivore damage, but shapes the arthropod community.



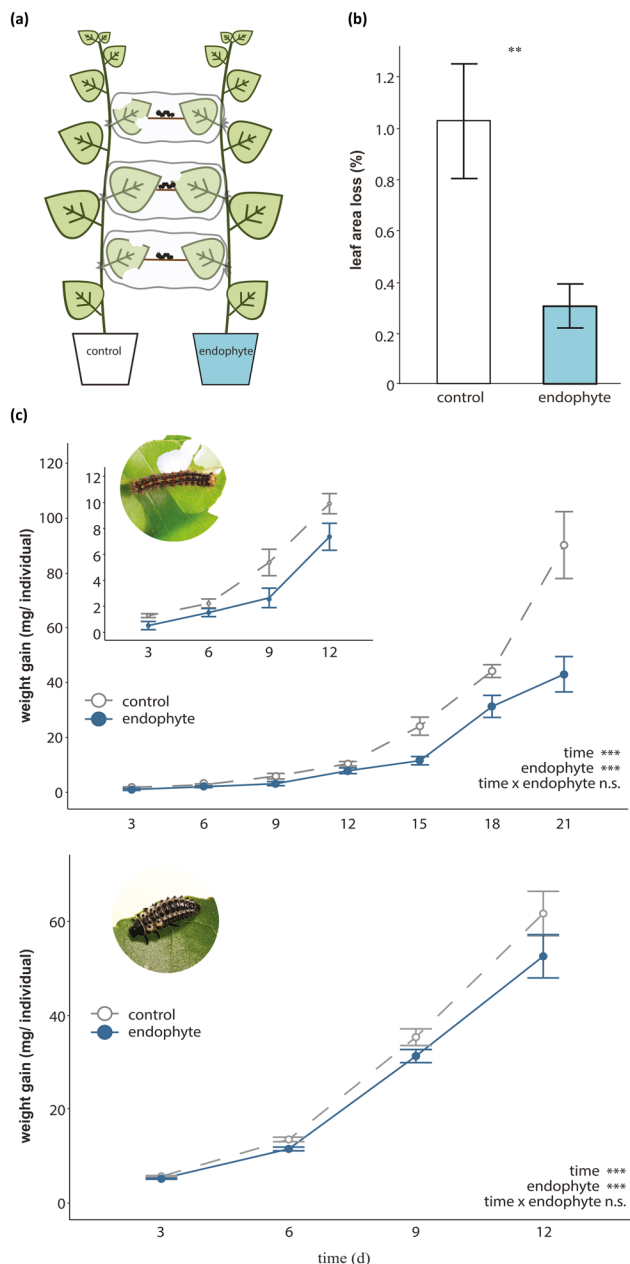
**FIGURE 3** | Effect of fungal endophyte inoculation on the levels of non-salicycinoid phenolic defence compounds in black poplar leaves. Trees were either inoculated with endophyte spore solution or a control solution 15 days before the onset of larval feeding, resulting in 4 treatments: *Control*, *endophyte*, *herbivory* and *endophyte + herbivory*. After 48 h of herbivory, leaves were harvested. A two-way ANOVA (top left) was used to estimate the effect of endophyte (e), herbivory (h) and the interaction of both (e x h), (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; n.s. = not significant). Mean  $\pm$  SE are shown ( $n = 4$ ).

#### 4.1 | An Endophytic Fungus Increases Poplar Defence Compounds

In the presence of the endophyte, the metabolome of poplar leaves changed and several metabolic features were affected solely by the endophyte. However, more changes occurred in response to insect herbivory (especially upregulation) when herbivory was combined with endophyte infection (Figure 1). Christian et al. (2020) also observed qualitative effects of an endophytic fungus *Colletotrichum tropicale* on the metabolome of *Theobroma cacao* Linnaeus, but this effect was not linked to the tree's defensive potential.

Here, we connect the effect of endophyte presence with the tree metabolome and the host's defensive abilities. We showed that *Cladosporium* sp. substantially changed levels of major poplar

defence compounds involved in protection against herbivores. Salicycinoids, which are exclusively produced by Salicaceae species, are known to be repellent or toxic especially to generalist insect herbivores (Boeckler et al. 2013; Hemming and Lindroth 1995; Lindroth 1991) with amounts variably increasing following herbivory (Boeckler et al. 2013; Fields and Orians 2006; Osier and Lindroth 2001; Ruuhola et al. 2001; Stevens and Lindroth 2005; Young et al. 2010). Salicin and nigracin concentrations increased significantly in the presence of the fungal endophyte (Figure 2a). Herbivory also led to higher concentrations of both compounds, with a significant additional increase in the presence of the fungus (Figure 2a). For other major salicycinoids, homaloside D and salicortin, a trend towards higher concentrations was observed in fungus-treated plants after herbivory. These results suggest that endophyte infection may be an advantage for poplars under herbivore attack, as it increases constitutive salicycinoid



**FIGURE 4** | Effect of fungal endophyte inoculation on preference and performance of two insect herbivores. (a) Preference assay of *L. dispar* larvae for either endophyte-inoculated or control plants. Two leaves, one from each treatment, were wrapped in a cellophane bag (blue outline) 15 dpi. Each bag contained one (2nd instar) larvae. After 48 h, larvae were removed and leaf area loss was documented. (b) Preference was evaluated as leaf area lost (%) of total leaf area. Mean  $\pm$  SE are shown ( $n=21$ ). Asterisks indicate significant difference of a linear mixed effect model with tree as a random factor ( $F=10.47$ ,  $p=0.002$ ; with outliers:  $F=3.392$ ,  $p=0.071$ ,  $n=27$ ). (c) Larvae of *L. dispar* (above) and *C. tremulae* (below) fed either on endophyte-inoculated leaves (blue line) or control leaves (grey dashed line). Data are shown from the beginning of the experiment until the first insect reached 5th instar (*L. dispar*). Results of a GLS regression (bottom right) to estimate the effect of time, endophyte and the interaction on insect performance ( $*p<0.05$ ;  $**p<0.01$ ;  $***p<0.001$ ; n.s. = not significant). Mean  $\pm$  SE are shown ( $n=7-10$ ).

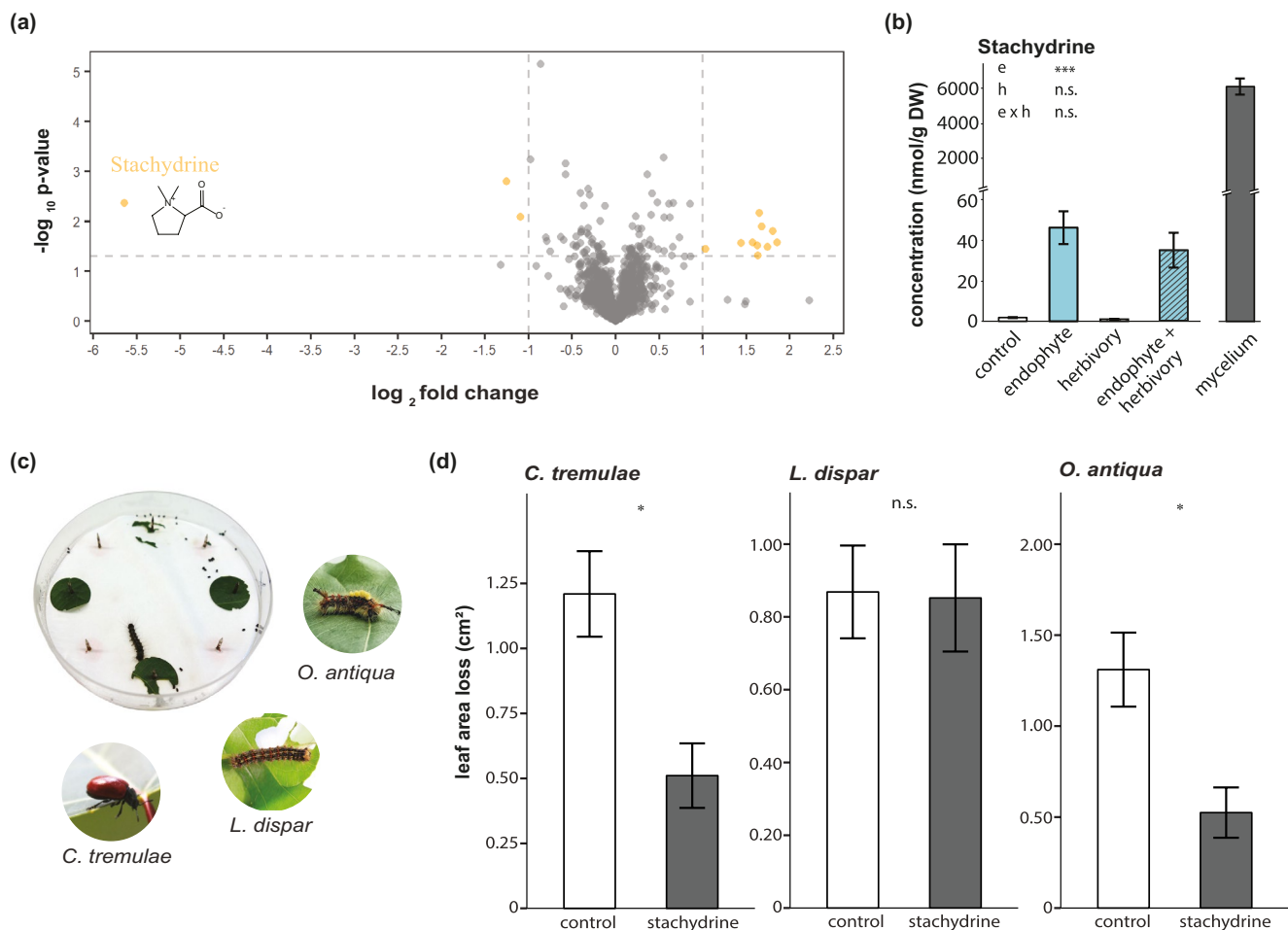
concentrations and elevates herbivore-induced concentrations. Among the simple phenolic acids, caffeic acid increased following endophyte infection, while coumaric and ferulic acids decreased significantly, irrespective of herbivory (Figure 3, Tables S2 and S3). Phenolic acids have detrimental effects on herbivorous insect performance. For instance, *p*-coumaric acid can deter the lepidopterans *Spodoptera litura* Fabricius and *Amsacta albistriga* Walker (Sambangi and Rani 2016), and caffeic acid inhibits the gut proteases of *Helicoverpa armigera* Hübner (Dixit et al. 2017). Furthermore, both compounds exhibit antimicrobial activities (Aziz et al. 1998), and serve as precursors in lignin formation, which also defends plants against pathogen infection (Xie et al. 2018).

A well-known indicator of pathogen infection in poplar is elevated levels of the flavan-3-ol catechin, an anti-fungal defence compound whose biosynthesis is linked to SA induction (Eberl et al. 2020; Ullah et al. 2019, 2017). We did not observe SA or catechin induction upon endophyte infection, indicating that the plant does not perceive the endophyte as a pathogen or the endophyte is able to actively suppress SA for successful establishment. More research on SA-related genes is needed. In general, the endophyte had only minor effects on defence hormones. In line with previous work in poplar (Boeckler et al. 2013), and other plant species (Vos et al. 2013; Wasternack and Hause 2013), only herbivory increased jasmonate and ABA concentrations. We observed a trend towards increased jasmonate concentration when endophyte-treated plants were subject to herbivory (compared to endophyte-free controls). Additionally, the endophyte + herbivory treatment significantly increased the levels of the bioactive form of jasmonate, the jasmonic acid-isoleucine conjugate. These observations may partially explain the increased chemical defence profile of endophyte-inoculated plants. Jasmonic acid was significantly elevated when grasses were infected with *Epichloë*, thus boosting resistance against chewing insects (Bastías, Martinez-Ghersa et al. 2017, but see Simons et al. 2008). Additionally, the endophyte *Sphaeropsis* sp. B301 induces ABA in cells of *Ginkgo biloba* Linnaeus, leading to increased flavonoid levels. While JA likely plays a role in the rise of defence in poplar after *Cladosporium* sp. infection, other JA-independent signalling pathways may also be involved. More research is needed to reveal how endophyte infection affects the quantities of plant-produced anti-herbivore defence compounds to determine if the ability of horizontally transmitted endophytes to enhance plant defences is a general trend.

#### 4.2 | Endophyte Inoculation Deters a Generalist Insect Herbivore and Decreases Its Performance

The negative effects of endophytes on herbivores have often been shown in grasses. However, less is known about the effect of horizontally transmitted endophytes that occur in trees. Van Bael et al. (2009) found that high endophyte concentrations negatively affect herbivore fecundity, while Quiring et al. (2019) showed that endophytes reduced the survival of eastern spruce budworm (*Choristoneura fumiferana* Clemens) feeding on white spruce (*Picea glauca* Moench). A survey linking plant chemistry



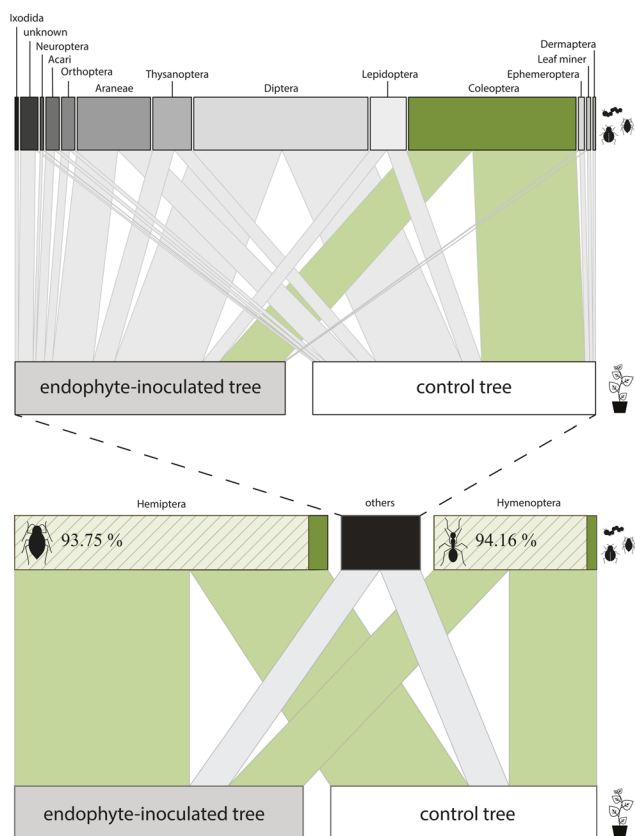


**FIGURE 5** | Detection and quantification of the alkaloid stachydrine and its effect on feeding preference of insect herbivores. (a) Volcano plot illustrates metabolite features from leaf tissue inoculated with the endophyte and those of control plants. Features were obtained from an untargeted LC-MS analysis with a QTOF mass spectrometer in positive ionisation mode. Significantly increased or decreased features are highlighted in orange, including the alkaloid stachydrine present in endophyte-inoculated plants. (b) Levels of stachydrine in leaves of black poplar trees under various treatments and in cultured endophyte (mycelium). Trees were either inoculated with endophyte spore solution or a control solution 15 days before the onset of larval feeding, resulting in four treatments: *Control*, *endophyte*, *herbivory* and *endophyte + herbivory*. After 48 h of herbivory, leaves were harvested. A two-way ANOVA (top left) was used to estimate the effect of endophyte (e), herbivory (h) and the interaction of both (e × h), (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; n.s. = not significant). Mean ± SE are shown ( $n = 4$ ). The concentration of stachydrine in the mycelium was not included in the statistical analysis. (c) Preference arena containing leaf discs coated either with a stachydrine or control solution. Beetles of *C. tremulae* and larvae of *O. antiqua* were allowed to feed for 24 h, *L. dispar* larvae fed for 48 h. (d) Results of the preference assay for *C. tremulae*, *L. dispar* and *O. antiqua* (left to right). Asterisks indicate significance differences based on related samples Wilcoxon sign rank test (*L. dispar*, *O. antiqua*,) or a paired *t*-test (*C. tremulae*) (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; n.s. = not significant). Preference was evaluated as leaf area loss (cm<sup>2</sup>). Mean ± SE are shown ( $n = 20$ ).

changes upon endophyte infection and herbivore preference and performance has been lacking until now. Young generalist *L. dispar* larvae avoided feeding on endophyte-infected plants (Figure 4b), consistent with the upregulation of several poplar defence compounds upon endophyte infection (Figures 1–3). Furthermore, the larvae feeding on control leaves gained more weight compared to individuals reared on endophyte-inoculated trees (Figure 4c), emphasising the protective effect of the endophyte against insect herbivores. These findings also support the role of salicin as feeding deterrent and toxin to generalist insect herbivores (Boeckler et al. 2016; Hemming and Lindroth 1995; Lindroth 1991). In contrast to the generalist *L. dispar*, larvae of the poplar leaf beetle *C. tremulae*, a specialist feeder, use salicin for their own protection (Strauss et al. 2014). Strikingly, *C. tremulae* larvae experienced less weight gain when feeding on

endophyte-inoculated plants. The positive effects of enhanced salicin concentration produced against predators might offset the negative impact of the endophyte on weight gain.

To link metabolic changes caused by the endophyte with the behaviour and performance of insect herbivores, we conducted preference and performance assays with leaves still attached to the tree (Figure 4a). This experimental design allows the exchange and transport of defence signals, precursors and products throughout the plant during feeding (Gange et al. 2019). Our results help explain the preference and performance of *L. dispar* larvae through changes in known poplar defence compounds. However, we could not find a correlation between the reduced growth of the poplar leaf beetle and an increase in the plant's own defence compounds. Therefore, we searched for



**FIGURE 6** | Bipartite networks of plant-arthropod interactions comparing black poplar trees infected with a fungal endophyte to control uninfected trees placed in the field. Trees were inoculated 15 days before plants were brought to the field site. To monitor visiting arthropods, trees were observed four times per day (9 AM, 12 AM, 3 PM and 6 PM) for 9 days. Bipartite networks are shown for the whole arthropod community depicting the number of arthropods per order visiting endophyte-infected versus control trees. The network above shows the results for insect orders belonging to the ‘others’ shown below. Thicknesses of lines are scaled to the abundance of individuals within an arthropod group for each treatment. Bars with shaded lines within Hemiptera and Hymenoptera represent the percentage of aphids and ants in the respective order. Green colours highlight insect orders that significantly differ among the treatments (negative binominal regression; **Coleoptera**: treatment  $p < 0.001$ , time  $p < 0.001$ , treatment  $\times$  time  $p = 0.159$ ; **Hemiptera**: treatment  $p < 0.05$ , time  $p = \text{n.s.}$ , treatment  $\times$  time  $p < 0.05$ ; **Hymenoptera**: treatment  $p < 0.05$ , time  $p < 0.001$ , treatment  $\times$  time  $p = \text{n.s.}$ ) ( $n = 10$ ).

metabolites produced by the endophyte that could serve as anti-herbivore defences.

### 4.3 | The Endophyte Produces an Alkaloid With Anti-Herbivore Properties

We identified the pyrrolidine alkaloid stachydrine in the endophytic mycelium and in endophyte-inoculated plants (Figure 5a). Stachydrine has been previously isolated from species of *Citrus* Linnaeus, *Medicago* Linnaeus, *Chrysanthemum* Linnaeus and *Stachys* Linnaeus, as well as from various algal and fungal taxa (Murata et al. 2011 and references therein). Here, we report this alkaloid from a poplar species infected with

an endophyte for the first time. Stachydrine shows a variety of pharmacological activities (Cheng et al. 2020), and in an ecological context acts in a mixture as feeding stimulant for larvae of the citrus swallowtail butterfly (*Papilio Xuthus* Linnaeus) and oviposition stimulant for their adults (Honda 1990). The role of endophytic alkaloids in the anti-herbivore defence of their host plants is well documented for many grass endophytes (Bastias, Ueno et al. 2017; Faeth and Saari 2012; Faeth and Bultman 2002), but not in other endophyte-plant associations (Faeth and Hammon 1997a, 1997b). Stachydrine concentration was higher in mycelium compared to endophyte-inoculated plants, likely due to the use of pure mycelium for analysis and the fact that the fungus was grown on nutrient-rich growth medium. It remains an open question whether the biosynthesis of stachydrine in vivo is influenced by abiotic or biotic factors, as has been described for alkaloids in *Epichloë*-grass endophytes (e.g., Fuchs et al. 2017a, 2017b).

To test the impact of stachydrine on poplar herbivores, we tested the preference of insects for leaf discs coated with stachydrine versus those coated with a control solution. Both adults of the specialist *C. tremulae* and larvae of the generalist *O. antiqua* favoured the control discs (Figure 4c). Alkaloids are usually effective against generalist insect herbivores, but specialists can often detoxify them (Saunders et al. 1992). Here, we show that an alkaloid deterred a specialist leaf beetle species and potentially reduced its performance. In contrast to the other two tested species, *L. dispar* did not show a significant preference; thus, the upregulation of poplar phenolic defence compounds most likely explains the feeding pattern and reduced weight gain of this species (Figure 4b,c). Further studies should focus on the effect of stachydrine on a broader spectrum of poplar herbivore species.

The discovery of stachydrine in endophyte-infected poplar leaves hints at the unexplored diversity of endophyte-produced defence compounds in plants. As most plant endophytes or endophyte-inoculated tissue have never been chemically screened, many other endophyte chemical compounds can be expected to contribute to host defence.

### 4.4 | The Endophyte Shapes Arthropod Communities in the Field

Most studies of interactions between plants, insects and micro-organisms are conducted either in the laboratory or in the field. Here, we combined both approaches to test whether the effects of the endophyte on plant-insect interactions observed in the laboratory are also realised under more natural conditions in the field. Coleopterans were counted more often on control trees than endophyte-infected ones in the field (Figure 6). The majority of coleopterans from the family Chrysomelidae, the family of the specialist poplar leaf beetle, were more abundant on control plants, confirming the defensive role of the endophyte observed in the lab (Table S4). However, unlike in the lab, lepidopterans did not distinguish among the two treatments (Figure 6). The lack of congruence with laboratory results may stem simply from differences in insect species or from the absence of biotic and abiotic factors in the laboratory that contribute to tripartite plant-herbivore-endophyte interactions (Hardoim et al. 2015). A lack of congruence with expectations was also

seen in a field experiment with sleepy grass (*Achnatherum robustum* (Vasey) Barkworth) containing the alkaloid-producing *Epichloë* endophytes, which revealed that endophyte infection caused increased herbivore abundance and species richness (Jani et al. 2009).

Interestingly, in our field experiment, species of the family Hemiptera were observed more on poplar plants harbouring the endophyte than on those without, with the vast majority of Hemiptera present being aphids (Aphidoidea) (Figure 6, Table S4). Numerous studies on alkaloid-producing grass endophytes showed the detrimental effects of endophytes on aphid performance (Bastías, Ueno et al. 2017; Shymanovich et al. 2015; Siegel et al. 1990). However, not every alkaloid produced by fungi negatively affects aphids, as was shown for the alkaloid ergovaline (Siegel et al. 1990). Furthermore, leaves of *Acer pseudoplatanus* Linnaeus harbouring the endophyte *Rhizoma acerinum* Schwein contained more aphids than control trees in the field (Gange 1996). Plants with ineffective alkaloids are more susceptible to sap-sucking insects, considering that endophytes may suppress SA to establish successfully (Bastías, Martínez-Ghersa et al. 2017). In our experiments, the endophyte potentially inhibits SA induction, which could explain the higher abundance of aphids (Tables S2, S3, S5).

Ants and aphids often form a mutualistic relationship in which the aphid is protected from predators by the ants and in return the ants receive sugary honeydew from the aphids (Banks 1962; Nielsen et al. 2010). We expected that the increase in aphids on endophyte-inoculated plants would also result in more ants. Hymenoptera, with 94.16% of them belonging to the Formicidae, however, were more abundant on control plants (Figure 6). Successful ant-aphid mutualism is strongly dependent on honeydew quality (Züst and Agrawal 2017). For example, aphids feeding on plants high in cardenolides are visited less often by ants, as these aphids excrete cardenolides via their honeydew. The specialist poplar aphids in our lab experiment excreted the alkaloid stachydrine via their honeydew, possibly explaining the observed reduction in ant visitation in the field (Figure S8). Further research is needed to test, whether the presence of stachydrine in the aphid honeydew repels ants and thus modifies the mutualistic relationship between aphids and ants in the field.

No differences in leaf damage were observed in the field, which may be attributed to differing herbivore species composition, the degree of herbivore specialisation or the order of arrival of insect species on the plant and the effects on subsequent herbivores (Figure S7). Furthermore, the high number of guarding ants observed on control plants might contribute indirectly to higher plant protection. Consequently, control plants might exhibit higher levels of indirect defence (protective ants), while inoculated plants were protected by direct defence from the endophyte itself (stachydrine) or endophyte-induced changes in the plant (salicoids), suggesting a trade-off between direct and indirect defence.

## 5 | Conclusion

While it has already been proposed that endophytes influence the defence of trees against herbivores, our mechanistic understanding of such endophyte-mediated effects is poor. Here we show that an

endophytic fungus increases the accumulation of plant-produced chemical defences and produces at least one effective defence compound of its own, thus increasing protection against generalist and specialist insects. In contrast, the presence of the endophyte led to higher numbers of sap-sucking insects, which are able to excrete the endophytic alkaloid. These findings suggest that endophytes may play a different role in defence against sap-sucking insects than for chewing herbivores. Given the abundance and diversity of endophytes in trees, much additional research is needed to understand the modulating role of endophytic fungi.

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## Author Contributions

Christin Walther and Sybille B. Unsicker conceived the project and designed the experiments. Christin Walther performed most of the experiments. Prajakta Giri and Christin Walther conducted the insect performance assays. Elina J. Negwer conducted the aphid honeydew collection assay. Christin Walther, Beate Rothe and Michael Reichelt performed targeted and non-targeted metabolite analyses and Marine Vallet performed the bioinformatics with the metabolome data. Christin Walther and Pamela Medina van Berkum analysed the data. Christin Walther wrote the manuscript. Jonathan Gershenzon edited the manuscript and all authors reviewed the manuscript.

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## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.3xsj3txqt>. Codes are available from Zenodo: <https://doi.org/10.5281/zenodo.13320094>. *Cladosporium* sp. sequences of the Internal Transcribed Spacer (ITS) and Translation Elongation Factor 1- $\alpha$  (Tef-1 $\alpha$ ) regions are available on the NCBI sequence database with the accession numbers PP263592.1 for ITS and PP266349.1 for Tef-1 $\alpha$  region. Raw LC-MS data are available from the same Dryad Digital Repository.

## Peer Review

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ele.70007>.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.