1 ASPEN LEAVES AS A “CHEMICAL LANDSCAPE” FOR FUNGAL ENDOPHYTE 2 DIVERSITY – CAN NITROGEN AND HERBIVORY SHAPE THE COMMUNITY 3 COMPOSITION IN CONTROLLED CONDITIONS? 4 5

6 **Running title:** Fungal Endophytes in Aspen Leaves
7
8

9 **Authors:** Johanna Witzell¹, Vicki Huizu Guo Decker², Marta Agostinelli¹, Carmen Romeralo
10 Tapia¹, Michelle Cleary¹ and Benedicte Riber Albrechtsen²
11

12 ¹ Department of plant physiology, Umeå University, Umeå Plant Science Center, Umeå

13 ² Swedish University of Agricultural Sciences, Southern Swedish Forest Research Centre,
14 Alnarp
15

16 **Correspondence:** Benedicte Riber Albrechtsen, email:

17 benedicte.albrechtsen@plantphys.umu.se
18

19 **Keywords:** *Populus tremula*; phenolics; condensed tannins; fungal endophytes; *Chrysomela*
20 *tremula*; heterogeneity-diversity relationship hypothesis
21
22
23

ABSTRACT

The endophytic microbiome may influence the ecological performance of plants, including forest trees. Various abiotic and biotic factors may shape the endophyte communities directly but also indirectly, by modifying the quality of host plants as a substrate. We hypothesized that potentially antifungal or fungistatic condensed tannins (CTs) would determine the quality of aspen (*Populus tremula*) leaves as a substrate for endophytic fungi. By subjecting the plants to nitrogen fertilization (N) or herbivory (H; leaf beetles) we aimed to change the internal “chemical landscape”, especially the CT levels, in aspen leaves. We expected that this would lead to changes in the fungal community composition, in line with the predictions of heterogeneity-diversity relationship hypothesis. To test this we conducted a greenhouse study where aspen plants were subjected to N and H treatments, individually or in combination. The chemical status of the leaves was analysed using GC/MS (114 metabolites), LC/MS (11 phenolics) and UV-spectrometry (CTs) and the endophytic communities were characterized using culture-dependent sequencing. Nitrogen treatment, alone or in combination with herbivory had a suppressing effect on the concentration and within-treatment variation in the CT precursors, catechins, and resulted in similar trend also in CTs. Nitrogen increased the concentration of certain amino acids, and it also tended to increase most of the analysed sugars. Herbivory had a negligible effect on chemical traits. In N-treated plants, the endophyte richness and abundance were higher than in plants exposed to H, but in general, the diversity of the culturable endophytes remained stable despite the subtle changes in leaf chemistry.

Introduction

In natural ecosystems and cultivated environments, communities of fungal endophytes form a hidden stratum of biodiversity, imbedded inside plant tissues (Wilson & Lindow, 1994; Bailey et al., 2005; Albrechtsen et al., 2010). Currently, studies on endophyte communities are in the frontline of plant ecology (e.g., Hardoim et al., 2015) and interest in these communities is also boosted by the prospects of utilizing the biosynthetic capacities of the endophytes in plant protection and plant growth promotion in agriculture and forestry (Busby et al., 2016; Witzell & Martín, 2018; Terhonen et al. 2019; Blacutt et al., 2020), as well as in medical or industrial applications (Nisa et al., 2015; Gouda et al., 2016). Advancement of molecular

tools has made it possible to obtain more detailed information about the taxonomic structure of these hidden communities (Jumpponen & Jones 2009; Albrechtsen & Witzell, 2012; Unterseher et al., 2016), but investigations are still challenged by temporal and spatial dynamics, the taxonomic complexity of the fungal community, and the lack of knowledge about the ecological roles of fungal species and their inter- and intraspecies interactions inside the plants (Witzell et al. 2014). Especially in the case of large-sized and long-lived forest trees the mechanisms determining endophyte community composition have remained puzzling (Ragazzi et al., 2003; Albrechtsen & Witzell, 2012; Witzell & Martín, 2018).

The structure of endophyte communities has rarely been examined through theoretical frameworks of community ecology that aim to explain species diversity, abundance, and composition of species in communities, and the factors and processes underlying these patterns. Vellend (2010) states that all mechanisms that underpin patterns in ecological communities involve only four distinct processes, namely dispersal (movement of organisms across space); drift (stochastic changes in species abundance); speciation (creation of new species); and selection (which involves biotic and abiotic interactions including intra- and inter-specific interactions). The theoretical approaches to community ecology have been developed mainly for landscape level processes and for other organisms than fungi, but especially the selection process can be applied to the micro-environment inside plants. One of the central hypotheses in community ecology is that environmental heterogeneity promotes species richness, because it increases opportunities for niche partitioning (Ben-Hur & Kadmon, 2020). Translated to the spatial scale of a single plant, it predicts that a spatially or temporally heterogenic, internal chemical environment could support a more diverse endophyte community, and vice versa (a more uniform chemical environment would offer fewer nutritional niches).

In forest trees, endophyte communities are likely to be shaped by numerous environmental and host plant internal factors and processes. Trees receive their endophytes mainly from the surrounding environment (horizontal spreading; Clay & Holah, 1999; Petrini et al., 1993), and the influence of season and climatic conditions on the community structure has been found to be high (Gomes et al., 2018). Edaphoclimatic factors can strongly determine the dispersal and establishment of endophytes in trees (Carroll, 1995; Sieber, 2007), either directly by influencing the quality and quantity of the available inoculum, or indirectly, through effects on the host plant as a substrate for the fungi. Although landing of viable

fungal spores on potential host plants may occur stochastically, the chemical environment on and inside the plant is likely to act as a selective factor, supporting the germination and growth of some fungi and suppressing others. For instance, several studies have found a connection between endophyte infections and phenolic plant metabolites in trees (Bailey et al., 2005; Albrechtsen et al., 2010; Martín et al., 2013; Albrechtsen et al., 2018). These ubiquitous plant metabolites are potentially antifungal or fungistatic (Witzell & Martín, 2008), but some fungi may also be able to use them as a carbon source (Blumenstein et al., 2015). Soil fertility is known to influence production of phenolics in plants, and especially the availability of soil nitrogen tends to be negatively correlated with phenolic concentrations in plant tissues (Bandau et al., 2015). Phenolic metabolism is known to readily increase in response to stress, and several phenolics have been suggested to have antioxidant, antimicrobial or anti-herbivore properties (Witzell & Martín, 2008; Gourlay & Constabel, 2019). Because phoretic associations between fungi and insects are likely to be common (Lewinsohn et al., 1994; Rice et al., 2007), insect herbivores may influence the fungal community in host tissues by acting as carriers of the fungal inoculum (Albrechtsen et al., 2018). On the other hand, endophytic fungi may also influence the herbivores (Coblentz & Van Bael, 2013), further complicating the interpretation of interactions.

The goal of our study was to increase the current understanding of how foliar endophyte assemblages in woody plants vary in response to multiple, interacting factors. Our basic hypothesis was that the intrinsic status of phenolic metabolites, dominant defensive chemicals in most boreal and temperate zone trees species, would strongly influence the quality of plants as a substrate for endophytic fungi, and thus shape their community structure (Agostinelli et al., 2018; Albrechtsen et al., 2018). Moreover, we anticipated that modification of the phenolic status using abiotic or biotic factors would result in changes in the fungal community. To test this hypothesis, we isolated endophytic fungi from leaves of vegetatively propagated plants of twelve aspen (*Populus tremula* L.) genotypes that were subjected to nitrogen amendment and leaf beetle herbivory, alone and in combination. The genotypes represented a similar chemotype in terms of their content of salicinoid phenolic glycosides (SPGs, low molecular weight phenolics that are characteristic for Salicaceae plants; Keefover-Ring et al. 2014), but differed with regards to concentration of high-molecular weight phenolics, condensed tannins (CTs; Robinson et al. 2012). The plant material thus allowed us to specifically focus on the importance of the latter compounds, which have earlier been identified as potentially antifungal compounds in aspen (Ullah et al. 2017).

We expected that fertilization would generally reduce the concentrations of phenolic metabolites and particularly CTs (Witzell & Shevtsova, 2004; Bandau et al., 2015; Decker et al., 2016) in aspen leaves. Furthermore, we anticipated that it would also reduce the within-group variation in phenolic concentrations, i.e. the phenolic concentration would vary less between N-treated replicate plants as compared to the control plant group (Edenius et al., 2012; Albrechtsen et al., 2018). We expected that this quantitative response could lead to a more homogenous nutritional niche that would support a less diverse endophytic community than what is found in control plants. In contrast, we hypothesized that herbivory could lead to locally induced accumulation of constitutive or induced phenolics (Sampedro et al., 2011; Massad et al. 2014), or oxidation of phenolics due to tissue damage (Constabel et al. 2000; Salminen & Karonen, 2011). We expected that this would result in a more variable (multi-directional) and compartmentalized chemical environment in leaf endosphere, leading to higher diversity of nutritional niches and thus more diverse fungal communities, as predicted by the heterogeneity-diversity relationship hypothesis (Ben-Hur & Kadmon, 2020). The possible phoretic interactions could add to the diversity of fungal communities in H treated plants, especially in the strictly controlled greenhouse environment with limited environmental inoculum. We used the classical isolation method and molecular identification based on ITS1 and ITS2 regions that are widely used as the standard DNA barcode marker for fungi (Badotti et al. 2017) to describe the endophyte community. Despite the well-known limitations of this approach we expected it to be robust enough to capture the fast-growing fraction of the total fungal community that would most readily respond to the treatment effects in our short-term experiment. To study the metabolic status of the plants, we conducted a global metabolite analysis using GC/MS and completed it with a targeted LC/MS analysis of low-molecular weight phenolics. We also analyzed the concentrations of condensed tannins. The results are discussed within the framework of the heterogeneity-diversity relationship hypothesis (Ben-Hur & Kadmon 2020).

MATERIAL AND METHODS

Plant material

Plants belonging to twelve aspen (*Populus tremula* L.) genotypes were chosen from the SwAsp collection (genotypes number 4, 6, 7, 26, 41, 51, 69, 72, 79, 92, 98, and 100,

Robinson et al. 2012). The selected clones represented the tremuloides-like chemotype, with four dominating SPGs (salicin, tremuloidin, salicortin and tremulacin) (Robinson et al. 2012; Keefover-Ring et al., 2014). Aspen plants were produced from in vitro tissue cultures (Umeå Plant Science Centre, Umeå, Sweden) and planted into 5 L pots in May 23th 2014. The pots where placed in the greenhouse 20°C, 60% R.H., and a 16:8 h D/N cycle. Lateral branches were removed within the first four weeks after planting to promote apical growth.

Experimental set-up

Two individual plants from each of the 12 genotypes were randomly assigned to one of four treatments: control (C), nitrogen fertilization (N), herbivory (H), and their combination (NH) (n=24 plants per treatment). The whole experiment thus comprised a total of 96 plants. For N treatment, a Weibulls Rika-S ® solution (containing 84gr/liter nitrogen, NH₄NO₃) was applied weekly starting from June 17th for four weeks. This resulted in a final N input that corresponds to the level of industrial forest fertilization in Sweden, 150 kg N ha⁻¹ y⁻¹ (Decker et al., 2016; Nohrstedt, 2001). The first fully expanded leaf on each plant was marked on June 17th and this leaf was later used to assess baseline leaf chemistry (see below).

After four weeks of fertilization treatment, a mousseline fabric net was placed over the first three fully expanded leaves that had developed above the marked leaf after the first fertilization event. Five adult aspen leaf beetles (*Chrysomela tremula* Fabricius) were placed into the nets after the last fertilization event and allowed to feed for five days (treatments H and NH). The beetles had been reared in the laboratory at Umeå Plant Science Centre since the beginning of May and belonged to the second generation of a culture captured close to Ekebo, in southern Sweden. Empty nets were placed in similar position also on plants in C and N treatments to control for the effect of the mousseline bag.

Sampling

After five days of herbivory treatment, the experiment was ended. Leaves were collected from each plant for phytochemical analysis (the leaf below the netted leaves) and for fungal endophyte analysis (the netted leaves). All the leaves were morphologically at the same stage, i.e., fully expanded, and thus expected to have comparable activity of secondary metabolism.

Leaves for the chemical analyses were flash-frozen in liquid nitrogen, freeze-dried and stored at -20°C until they were ground to fine powder using a bead mill (MM 301 Vibration Mill, Retsch GmbH and Co. KG, Haan, Germany) at 25 Hz for 3 minutes. The fine powder was stored in vials at -20°C until chemical analyses. Leaves designated for endophyte analysis were collected and stored at 4°C for a maximum of three days prior to surface sterilization and endophyte isolation. In case the petiole of a netted leaf was chewed by the beetle (causing wilting or the death of the leaf), those leaves were discarded from further analyses. A total of 90 plants were included in the endophyte analysis. The growth of plants and insects was recorded (Table S1).

Phytochemical analysis

For the global GC/MS analysis, 6.00 ($\pm$ 1.00) mg of leaf powder was extracted in 1 ml methanol:chloroform:water (v:v:v) at 4 °C. Deuterated salicylic acid [$^2\text{H}_6$] (Isotec, Miamisburg, USA) was used as an internal standard in all samples. Samples were centrifuged at 4 °C and 100 μl of the resulting supernatant was evaporated in vacuum. The residues were resolved in 30 μl of methoxyamine (15 $\mu\text{g}/\mu\text{l}$ in pyridine) and 30 μl of MSTFA. Methyl stearate (30 μl of 15 $\text{ng}/\mu\text{l}$ in heptane) was added before analysis, and 1 μl of each aliquot injected by a CTC Combi Pal autosampler (CTC Analytics AG, Switzerland) into an Agilent 6890 gas chromatograph. The chromatograph was equipped with a 10 m x 0.18 mm fused silica capillary column with a chemically bonded 0.18 μm DB 5-MS UI stationary phase (J&W Scientific). The compound detection was performed in a Pegasus III time-of-flight mass spectrometer, GC/TOFMS (Leco Corp., St Joseph, MI, USA). MATLAB™ R2011b (Mathworks, Natick, MA, USA) was used to quantify the mass by means of integrated peak areas. All pre-treatment data procedures, such as base-line correction, chromatogram alignment, data compression and Hierarchical Multivariate Curve Resolution (H-MCR), were performed using custom scripts according to Jonsson et al., (2005). The extracted mass spectra were identified by comparisons of their retention index and mass spectra with libraries of retention time indices and mass spectra (Schauer et al., 2005). Identification of compounds was based on comparison with mass spectra libraries (in-house database) as well as Kovats retention index. The identified chemicals were quantified by peak area and assigned to metabolite classes (phenolics, amino acids, fatty acids) (Table S4).

For the targeted LC/MS analysis, samples were prepared as above, but the residue was suspended in 25 μ l of methanol and 25 μ l of 0.1% v/v aqueous formic acid. A 2.0 μ l aliquot was injected onto a C18 UPLC™ column (2.1 $\times$ 100 mm, 1.7 μ m) and chromatographic separation was performed on a LCT Premier TOF/MS in negative mode (Waters, Milford, MA, USA), following the method by Abreu et al., (2011). The standard compounds used in this analysis were from our in-house library (the Swedish Metabolomics Centre: SMC, Umeå, Sweden) including salicinoid standards (salicin, tremulacin, salicortin, and tremuloidin). MassHunter™ Qualitative Analysis software package (version B06.00, Agilent Technologies Inc., Santa Clara, CA, USA) was used to acquire the Mass Feature Extraction (MFE) and extracted features were aligned and matched between samples using Mass Profiler Professional™ 12.5 (Agilent Technologies Inc., Santa Clara, CA, USA). The concentrations of salicinoids were quantified according to the peak area of each compound using linear standard curves. Masses of either or both of the deprotonated ion ([M-H]⁻) and the formate adduct ([M-H + FA]⁻) were assessed, based on molecular weights according to Abreu et al., (2011) and Keefover-Ring et al., (2014) and guided by retention times where available (Table S4).

Condensed tannin concentrations were assessed based on a modified Porter's assay (acid: butanol method (Porter et al., 1985; Bandau et al., 2015): 10 $\pm$ 1 mg fine powder of freeze-dried leaf sample was added to 800 μ l of an acetone/ ascorbic acid solution (70% acetone, 30% Milli-Q water, with 10mM ascorbic acid) then incubated with an iron and acid-butanol reagent in boiling water for 1h. Absorbance was measured on a Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA) at A 550 nm. Procyanidin B2 (C₃₀H₂₆O₁₂, Sigma-Aldrich®, St. Louis, MO, USA) was used as the tannin standard (Table S4).

Fungal endophyte isolation and identification

Endophytic fungi were isolated following the method described by Albrechtsen et al. (2010). From each leaf (three leaves per plant in C and N treatments; one to three leaves per plant in H and NH treatments, a total of 228 leaves), ten segments (about 1x1 cm) were cut using a flame-sterilized scalpel under aseptic conditions (in the laminar flow bench). The segments were surface-sterilized using 95% ethanol and 2% sodium hypochlorite, and rinsed in sterile water before placing them individually on potato dextrose agar (PDA-agar; Sigma- Aldrich®, St. Louis, MO, USA) amended with streptomycin (12x12 cm dishes), as described in

Albrechtsen et al. (2010). A thin strip of Parafilm was used to keep the lid of the dishes on place. The cultures were checked for fungal growth every second day for one month. Emerging colonies were transferred to new Petri dishes (PDA) to obtain pure cultures. The plates were opened only inside the laminar, the fungi were transferred using flame-sterilized tools, and utmost care was taken to not contaminate the dishes with external fungi.

The recovered fungal cultures (n=1642) were classified into 30 distinct morphotypes (MTs) based on their visual morphological traits and growth rate estimated on a four step scale (fast, medium, slow, very slow). In order to obtain information about the taxonomy of the fungal community, a total of 59 cultures representing the 30 MTs were selected for sequencing. From each culture, plugs of PDA containing growing mycelium were transferred to malt extract broth. After an incubation period of two weeks (room temperature, darkness), mycelia were filtered, placed in 50 mL Falcon tubes (Fisher Scientific, Waltham, Massachusetts, US) and lyophilized for 48 hours. Freeze-dried samples were then pulverized in a FastPrep®-24 homogenizer (MP Biomedicals, Santa Ana, USA). Total genomic DNA was extracted using the E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek, Inc., Norcross, USA). The extracted nuclear DNA was measured using NanoDrop® ND-1000 (Wilmington, USA). PCR amplification of the internal transcribed spacer (ITS) region of the rDNA was performed with the primers ITS1 and ITS4 (White et al., 1990). Each 50 µl PCR reaction mixture contained 5 µl of 10x PCR buffer, 0.4 µM of each primer, 0.2 mM dNTP, 1.5 mM MgCl₂, 1U Taq polymerase and 10 ng fungal DNA. PCR program consisted of 94°C for 5 minutes; 33 cycles of 94°C for 30sec, 55°C for 30sec, 72°C for 30 sec and 72°C for 10 min. PCR samples were kept at 4°C before analyzing via gel electrophoresis on 1.5% Agarose (BIO-RAD) gel and visualized under UV light with 0.1% GelRed (GelRed™ Nucleic Acid Gel Stain, 10,000X in DMSO). PCR products were purified with the HT ExoSAP-IT High-throughput PCR Product Cleanup (Affymetrix, Santa Clara, USA) following manufacturer's instruction. After quantification of DNA concentrations with Qubit fluorometer 3.0 (Life Technologies, Carlsbad, USA), samples were sent to the National Genomics Infrastructure (NGI) at Science for Life Laboratory (SciLifeLab, Uppsala, Sweden) Visualization of raw sequence data was done with the software Chromas (version 2.4.4, Technelysium, South Brisbane, Australia). All sequences were aligned and manually edited using BioEdit (Ibis Biosciences, Carlsbad, CA, USA). Sequencing data were blasted against best matches in the reference database at NCBI (<http://www.ncbi.nlm.nih.gov/>). The ITS sequence homology for delimiting fungal taxa was set to >98.5% for presumed species (Table S2).

Data analysis

The Kruskal-Wallis test was used to test the treatment effects on specific chemicals and statistical analyses. The images including PCA plots (for 114 targeted and non-targeted metabolites from LC/MS and GC/MS) and 11 targeted phenolics (mainly SPGs from LC/MS) were generated with help of R version 3.6.1 (2019-07-05) packages: ggplot2 (<https://cran.r-project.org/web/packages/ggplot2/>), FactorMineR (<https://cran.r-project.org/web/packages/FactorMineR/index.html>) (Lê et al., 2008), and factoextra (<https://cran.r-project.org/web/packages/factoextra/index.html>).

Fungal diversity, (richness, S; Whittaker, 1972) was tracked as the number of distinct morphotypes (MTs) found in each plant, and the MT abundance (frequency) was recorded as the number of isolates of a given MT per sampled plant (n=24 plants for C and N, and 21 plants for H and NH treatments). Venn diagram was constructed to illustrate the number of shared and unique MTs per treatment (Shade & Handelsman; 2012). Relative abundance was determined for each MTs as the number of isolates of the given morphotype divided by the total number of isolates emerged from all samples (n=1642). To examine the possible shifts in the community composition among the different treatments (C, N, H and NH) a permutational multivariate analysis of variance (Permanova) of relative abundances was performed with the adonis function in vegan package (<https://cran.r-project.org/web/packages/vegan/index.html>) using 999 permutations, followed by a pairwise comparison with pairwise.adonis (Martinez, 2019).

MT richness values were used to create sample-size-based rarefaction (interpolation) and extrapolation (prediction) curves (Colwell et al., 2012, Chao et al., 2014, Hsieh et al., 2016) with an end point of 42 individuals and 100 bootstrap repetitions. The curves were generated with iNEXT (iNterpolation and EXTrapolation) R package (<https://cran.r-project.org/web/packages/iNEXT/index.html>) and visualized with ggiNEXT, the ggplot2 extension for iNEXT. To compare the complexity of communities, Simpson's (D) and Shannon (H') diversity indices and Pielou's index for evenness (J') were calculated using the Vegan package (Oksanen et al., 2016; Table S3). The effect of the treatments (C, N, H, NH) on the ecological diversity indexes and on the fungal richness (S) was assessed with an analysis of variance followed by a multiple comparison test (residuals were checked for normality, homoscedasticity and linearity). When the data did not meet these requirements, a

robust ANOVA based on trimmed means was performed with WRS2 package (<https://cran.r-project.org/web/packages/WRS2/index.html>). Before all the analyses were performed, data was balanced using a decision tree to generate new samples from the minority classes (H and NH treatments) with an oversampling algorithm (random walk oversampling) from the imbalance package in R (<https://cran.r-project.org/web/packages/imbalance/index.html>).

SUPPLEMENTAL MATERIAL

Supplementary file 1 (Tables S1, S2, S3; PDF), Supplementary file 2 (Table S4, Excel file).

RESULTS

Treatment effects on aspen chemistry

The score plot (PCA) of global metabolite analysis (data included 114 metabolites) showed a slight separation between fertilized and non-fertilized treatments (Fig 1a). The plot of targeted metabolites (11 phenolics, including catechin but excluding condensed tannins) did not show any clear distinction between fertilized and non-fertilized plants (Fig. 1b).

Fig 1.

The concentration of condensed tannins tended to decrease due to N and H, and for the CT precursor, catechin, the effect of N (but not H) was significant (Table 1; Fig. 2 a, b). Nitrogen treatment resulted in the lowest mean concentration and the lowest within-treatment variation in CTs was found in N treated plants (Table 1; Fig. 2 e), despite of the varied genetic background of the trees (12 clones). Overall, the effect of N on chemical characters appeared stronger compared to the effect of H (Table 1; Figs 2a-f).

The concentrations of amino acids increased in plants receiving N treatment (see the examples in Table 1, Fig 2 a-b). Most sugars tended to be reduced by H and increased by N treatment (data for glucose is shown as an example; Table 1, Fig 2 c). A deviating pattern was found only for maltose, which was reduced by treatments, especially by N, alone or in combination with H (Table 1, Fig. 2d).

Table 1.

Fig 2.

Endophyte diversity in aspen leaves

Among the 30 MTs, 18 were identified to class (2 MTs), genus (10 MTs) or species (6 MTs) level (Table 1, Table S2). Of the identified isolates, 17 belonged to *Ascomycota* (class *Dothideomycetes*, *Eurotiomycetes*, *Hypocreales*, and *Sordariomycetes*) and one to *Basidiomycota* (MT25: *Rhodotorula* sp.). The three most common morphotypes across the treatments, *Ramularia* sp. (MT1), and the unidentified morphotypes MT2 and MT3 had 576 isolates (35% of all isolates), 336 (20% of all isolates) and 329 (20% of all isolates), respectively. A total of 18 MTs were captured as a maximum of three isolates (< 0.2% relative abundance) and were thus considered as rare MTs.

The sample size-based rarefaction and extrapolation curves (Fig. 3a) are predicted to accurately estimate the number of MTs per sampling unit (tree) within the double number of species when compared to the reference sample size (Chao et al. 2014). At least two fungal isolates were recovered from each plant, and the highest increase of diversity was obtained when increasing the number of sampled trees from 1 to 5 within all the treatments. The non-asymptotic rarefaction curves suggest that additional sampling efforts would yield more MTs, especially in H treatment. However, the coverage-based rarefaction and extrapolation curves (Fig. 3b) suggested that the sample size (24 trees for C and N each, and 21 for H and NH) allowed a high completeness with the method that was used: the sample coverage values were 0.95, 0.97, 0.91 and 0.93 for C, N, H, and NH, respectively.

Figs 3a and 3b.

Treatment effects on endophytes

The treatments caused significant differences in endophyte richness (Robust ANOVA, $F=3.70$, $p=0.02$) and relative abundance (Permanova, $F=1.67$, $p=0.04$). These differences were located between N and H treatments ($p=0.01$ for richness; and $F=2.92$, $p=0.02$, for abundance), while the rest of combinations did not result in significant differences. Together,

the isolates belonging to the three dominating MTs made up 81% of all isolates in C plants, but their proportion tended to decrease by treatments: in N and H treatments they made up 73% of all isolates, and in NH 71%. At the same time, the relative abundance of the other (less frequent, and rare) morphotypes increased correspondingly (Table 1).

The Shannon index (H') did not differ among the treatments (ANOVA, $F=1.54$, $p=0.21$), with mean ($\pm$ se) and SE values $1.35 (\pm 0.07)$ for C; $1.50 (\pm 0.06)$ for N, $1.24 (\pm 0.10)$ for H; and $1.36 (\pm 0.08)$ for NH. Likewise, the Simpson index (D) values did not vary among treatments (0.68 ± 0.02 for C; 0.72 ± 0.02 for N; 0.62 ± 0.04 for H; and 0.68 ± 0.03 for NH; robust ANOVA, $F=0.41$, $p=0.74$), nor did the values of Pielou's measure of evenness (J) (0.85 ± 0.02 for C; 0.84 ± 0.02 for N, 0.82 ± 0.03 for H and 0.86 ± 0.02 for NH; robust ANOVA, $F=1.03$, $p=0.39$).

In total, 11 MTs were shared among all four treatments (Fig 4.), of which six morphotypes were identified at least to genus level (*Ramularia* sp., *Cladosporium* sp., *Penicillium olssonii*, *Arthrinium* sp. and 2, *Penicillium* sp.) and five remained unknown (Table 1). Three rare MTs were recovered only from C plants, including a Dothideomycetes and a *Coleosporium* species, three others from N treated plants (a Dothideomycetes, *Physalospora scripi*, and an unknown species). Three rare MTs, including the identified *Aureobasidium* species (*A. microstictum* and *A. pullulans*) were found only in treatments that included H (Table 2).

Fig 4. Venn diagram

Table 2

DISCUSSION

Nitrogen treatment induced the expected changes in the quality of aspen leaves: in particular the global metabolite analysis and condensed tannin levels highlighted the difference in the chemical profile between fertilized and non-fertilized aspen plants. A characteristic change in the global metabolite pool seemed to be due to increased concentrations of amino acids and sugars, which could reflect the expected positive effect of N on growth of the plants (Table S1, growth data). In accordance with results of several previous studies (Ibrahim et al., 2011; Bandau et al., 2015; Decker et al., 2016), we found lower concentration of CTs in leaves of

fertilized plants, and N treatment also reduced the variation in CT concentration among the studied trees. Thus, nitrogen fertilization seemed to render the leaves to a more nutritious and less toxic environment (assuming antifungal effects of polyphenolics), but also a more homogenous substrate for the fungi.

In contrast to our initial expectation, however, these quantitative changes were not accompanied by a more uniform fungal community in aspen leaves when compared with the control plants. Thus, our findings did not support for the classic heterogeneity–diversity relationship hypothesis among the endophyte community in aspen leaves (i.e., the apparently more homogenous chemical environment, especially in terms of condensed tannins, was not accompanied by a lower endophyte diversity). However, recent studies suggest that heterogeneity–diversity relationships may be non-linear and more complex than expected from the niche-based perspective (Ben-Hur & Kadmon, 2020). A deeper taxonomic analysis, using culture-independent approaches, may be needed to reveal these relationships. Clearly, more detailed information about nutritional niches and functional dynamics of different endophytic fungi (Blumenstein et al. 2015; Paungfoo-Lonhienne et al., 2015) would facilitate analyses of the niche-dependent mechanisms behind the observed patterns in fungal diversity.

We expected that leaf herbivory would increase both the compositional (magnitude) and configurational (spatial) chemical heterogeneity in the leaf tissues by inducing changes in phenolic metabolism, and that this change would lead to a more complex community structure among the relatively fast growing, culturable fraction of endophyte communities. However, we did not find evidence for the expected changes in CTs or other phenolics in response to herbivory. Possibly, the impact of herbivory could have been stronger in younger leaves (a systemic induction), or after a longer feeding period. Moreover, the MT richness in H treated plants did not increase as compared to C plants. Interestingly, we found that three of the MTs were found only in connection to herbivory, supporting the view that insects may influence the fungal diversity by carrying specific fungi to the plants (Albrechtsen et al., 2018). Wounding by herbivores may also open up more entry points for the ubiquitous fungi such as *Aureobasidium pullulans*, which was identified among the three H-treatment specific MTs, and is commonly found as an epiphyte and endophyte in different environments (Martín et al. 2013; Albrechtsen et al., 2018).

The combined effect of N and H on the chemical traits or fungal diversity seemed to be dominated by the N effect. The longer impact time of N was likely to accentuate its effect as a stronger bottom-up force and allowed it to shape the quality of leaves as a substrate for fungi more than the short term herbivory. Fungi transmitted to plants by the feeding beetles would probably need a longer incubation time than what was possible in our study, before they can be captured using the culturing approach. It should also be noted that in our study the insects were reared in laboratory conditions and thus exposed to only a limited environmental inoculum. Therefore, the results of this study may underestimate the importance of insect-mediated facilitation of infections that occurs in natural environments.

In accordance with the universal pattern of species abundance distribution (McGill et al. 2007), we found that the culturable endophyte community in aspen leaves was composed of a few common species (the main community member *Ramularia* spp. and two unknown ones) but many rare species. The identified taxa represented *Ascomycota* commonly found as endophytes in trees, including aspen (Arnold et al., 2007; Rodriguez et al., 2009; Unterseher, 2011). The only *Basidiomycota* species identified, *Rhodotorula* sp., has been reported as a common endophyte in trees in previous studies (Unterseher, 2011; Jumpponen and Jones, 2010; Rajala et al., 2014) and it has also been linked to herbivory treatment (Albrechtsen et al., 2018). In our study, however, only weak link was found between *Rhodotorula* sp. and herbivory. Intriguing was also that some common aspen endophytes, such as *Alternaria* and *Phomopsis* (Albrechtsen et al., 2010; Crous and Groenewald, 2013; Martín et al., 2013), were not detected, possibly because the greenhouse acted as a filter for the environmental inocula and reduced the frequency of otherwise common endophytes. Moreover, the time point of sampling in early summer may be reflected in the community composition: the species diversity is likely to increase during the season as the infections accumulate. Among the other genera identified, *Cladosporium*, *Ramularia* (anamorph *Mycosphaerella*) and *Physalospora* have been previously described as plant pathogens (Thomma et al., 2005; Tadych et al., 2012; Videira et al., 2016), but no signs of pathogen attacks were found on the leaves, suggesting that the conditions in our experiment supported endophytic lifestyle of these and other fungi.

In conclusion, our results indicate that the culturable fraction of fungal endophyte community in aspen leaves is rather stable against the directional alterations induced in the chemical “landscape” within the leaves by nitrogen fertilization or disturbances due to herbivory. This is in line with the results of the study by Christian et al. (2016) who reported high resistance

among endophyte communities against biotic and anthropogenic perturbations. However, more research is needed to better comprehend the nutritional requirements of endophytes, and the signals influencing the dynamics of the microbial communities in the endosphere. It should also be acknowledged that different fertilization levels and sampling times could result in stronger responses in the chemical environment and fungal communities. Thus, our results should be generalized with caution. While our results did not agree with the expectations we made based on the heterogeneity-diversity relationship hypothesis, we propose that by implementing theoretical frameworks of community ecology it is possible to gain new insights into the processes and traits driving the structure and functions of endophyte communities in perennial plants, such as forest trees.

ACKNOWLEDGMENTS

We thank Sylvia S. Chen for assistance in the experiments. The research was supported by FORMAS, a Swedish Research Council for Sustainable Development (2012-01358 and 2016-00907) to JW, and The Royal Swedish Academy of Agriculture and Forestry to MA.

REFERENCES

- Abreu IN, Ahnlund M, Moritz T, Albrechtsen BR (2011). UHPLC-ESI/TOFMS determination of salicylate-like phenolic glycosides in *Populus tremula* leaves. J. Chem. Ecol. 37:857–870. doi:10.1007/s10886-011-9991-7.
- Agostinelli M, Cleary M, Martín JA, Albrechtsen BR, Witzell J (2018). Pedunculate oaks (*Quercus robur* L.) differing in vitality as reservoirs for fungal biodiversity. Front. Microbiol. 9:1758. doi:10.3389/fmicb.2018.01758.
- Albrechtsen BR, Witzell J (2012). “Disentangling functions of fungal endophytes in forest trees,” in Fungi: types, environmental impact and role in disease, eds. A. Paz Silva and M. Sol (Huntington, NY: Nova Science Publishers), pp. 235–246.
- Albrechtsen BR, Björkén L, Varad A, Hagner Å, Wedin M, Karlsson J, Jansson S (2010). Endophytic fungi in European aspen (*Populus tremula*) leaves - diversity, detection, and a suggested correlation with herbivory resistance. Fungal Div. 41:17–28. doi:10.1007/s13225-009-0011-y.

- 517 Albrechtsen BR, Siddique AB, Decker VH, Unterseher M, Robinson KM (2018). Both plant
518 genotype and herbivory shape aspen endophyte communities. *Oecologia* 187:535–545.
519 doi:10.1007/s00442-018-4097-3.
- 520 Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R (2007). Diversity and phylogenetic
521 affinities of foliar fungal endophytes in loblolly pine inferred by culturing and
522 environmental PCR. *Mycologia* 99: 185–206. doi:10.3852/mycologia.99.2.185.
- 523 Badotti F, de Oliveira FS, Garcia CF, (2017) Effectiveness of ITS and sub-regions as DNA
524 barcode markers for the identification of Basidiomycota (Fungi). *BMC Microbiol.* 17, 42
525 doi: 10.1186/s12866-017-0958-x
- 526 Bailey JK, Deckert R, Schweitzer JA, Rehill BJ, Lindroth RL, Gehring C, Whitham TG
527 (2005). Host plant genetics affect hidden ecological players: links among *Populus*,
528 condensed tannins, and fungal endophyte infection. *Can. J. Bot.* 83:356–361.
529 doi:10.1139/b05-008.
- 530 Bandau F, Decker VH, Gundale MJ, Albrechtsen BR (2015). Genotypic tannin levels in
531 *Populus tremula* impact the way nitrogen enrichment affects growth and allocation
532 responses for some traits and not for others. *PLoS ONE* 10(10): e0140971.
533 doi:10.1371/journal.pone.0140971.
- 534 Ben-Hur E, Kadmon R (2020). Heterogeneity-diversity relationships in sessile organisms: a
535 unified framework. *Ecol. Lett.* 23:193–207. doi:10.1111/ele.13418.
- 536 Blacutt A, Ginnan N, Dang T, Bodaghi S, Vidalakis G, Ruegger P, Peacock B, Viravathana P,
537 Vieira FC, Drozd C, Jablonska B, Borneman J, McCollum G, Cordoza J, Meloch J, Berry
538 V, Salazar LL, Maloney KN, Rolshausen PE, Roper MC (2020). An in vitro pipeline for
539 screening and selection of citrus-associated microbiota with potential anti-“*Candidatus*
540 *Liberibacter asiaticus*” properties. *Appl Environ Microbiol* 86:e02883-19.
541 <https://doi.org/10.1128/AEM.02883-19>.
- 542 Blumenstein K, Albrechtsen BR, Martín JA, Hultberg M, Sieber TN, Helander M, Witzell J
543 (2015). Nutritional niche overlap potentiates the use of endophytes in biocontrol of a tree
544 disease. *BioControl* 60: 655–667. doi:10.1007/s10526-015-9668-1.
- 545 Busby PE, Peay KG, Newcombe G (2016). Common foliar fungi of *Populus trichocarpa*
546 modify *Melampsora* rust disease severity. *New Phytol.* 209:1681–1692.
547 doi:10.1111/nph.13742.
- 548 Carroll G (1995). Forest endophytes: pattern and process. *Can. J. Bot.* 73:1316–1324.
549 doi:10.1139/b95-393.

550 Chao A, Gotelli NJ, Hsieh TC, Sander EL, Ma KH, Colwell RK, Ellison AM (2014).
551 Rarefaction and extrapolation with Hill numbers: a framework for sampling and estimation
552 in species diversity studies. *Ecol. Monogr.* 84: 45–67.

553 Christian N, Sullivan C, Visser ND, Clay K (2016). Plant host and geographic location drive
554 endophyte community composition in the face of perturbation. *Microb Ecol.* 72:621–32. doi:
555 10.1007/s00248-016-0804-y.

556 Clay, K (1988). Fungal endophytes of grasses: a defensive mutualism between plants and
557 fungi. *Ecology* 69:10–16.

558 Coblenz KE, Van Bael SA (2013). Field colonies of leaf-cutting ants select plant materials
559 containing low abundances of endophytic fungi. *Ecosphere* 4:1–10. doi:10.1890/ES13-
560 00012.1.

561 Colwell RK, Chao A, Gotelli NJ, Lin S-Y, Mao CX, Chazdon RL, Longino JT (2012). Models
562 and estimators linking individual-based and sample-based rarefaction, extrapolation and
563 comparison of assemblages. *J. Plant Ecology* 5: 3–21.

564 Constabel CP, Yip L, Patton JJ, Christopher ME (2000). Polyphenol oxidase from hybrid
565 poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiol.*
566 124:285–295. doi:10.1104/pp.124.1.285

567 Crous PW, Groenewald JZ (2013). A phylogenetic re-evaluation of *Arthrrium*. *IMA Fungus*
568 4: 133–54. doi:10.5598/ima fungus.2013.04.01.13.

569 Decker VH, Bandau F, Gundale MJ, Cole CT, Albrechtsen BR (2016). Aspen
570 phenylpropanoid genes' expression levels correlate with genet's tannin richness and vary
571 both in responses to soil nitrogen and associations with phenolic profiles. *Tree Physiol.* 37:
572 270–279. doi:10.1093/treephys/tpw118.

573 Edenius L, Mikusinski G, Witzell J, Bergh J (2012) Effects of repeated fertilization of young
574 Norway spruce on phenolics and arthropods: implications for insectivorous birds' food
575 resources. *For. Ecol. Manage.* 277: 38–45. doi:10.1016/j.foreco.2012.04.021.

576 Faeth SH., Sullivan TJ (2003). Mutualistic asexual endophytes in a native grass are usually
577 parasitic. *Am. Nat.* 161: 310–325. doi: 10.1086/345937.

578 Gomes T, Pereira JA, Benhadi J, Lino-Neto T, Baptista P (2018). Endophytic and epiphytic
579 phyllosphere fungal communities are shaped by different environmental factors in a
580 Mediterranean ecosystem. *Microb Ecol* 76:668–679. doi: 10.1007/s00248-018-1161-9.

581 Gouda S, Das G, Sen SK, Shin H-S, Patra JK (2016). Endophytes: a treasure house of
582 bioactive compounds of medicinal importance. *Front. Microbiol.* 7: 1–8.
583 doi:10.3389/fmicb.2016.01538.

584 Gourlay G, Constabel P (2019). Condensed tannins are inducible antioxidants and protect
585 hybrid poplar against oxidative stress. *Tree Physiol.* 39: 345–355, doi:
586 10.1093/treephys/tpy143

587 Hardoim PR, van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, Döring M,
588 Sessitsch A (2015). The hidden world within plants: ecological and evolutionary
589 considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.*
590 79: 293–320. doi:10.1128/MMBR.00050-14.

591 Hsieh TC, Ma KH, Chao A (2016). iNEXT: An R package for rarefaction and extrapolation of
592 species diversity (Hill numbers). *Meth. Ecol. Evol.* 7: 1451–1456. doi: 10.1111/2041-
593 210X.12613.

594 Ibrahim MH, Jaafar HZE, Rahmat A, Rahman ZA (2011). Effects of nitrogen fertilization on
595 synthesis of primary and secondary metabolites in three varieties of Kacip Fatimah (*Labisia*
596 *pumila* Blume). *Int. J. Mol. Sci.* 12: 5238–54. doi:10.3390/ijms12085238.

597 Jonsson P, Johansson AI, Gullberg J, Trygg J, A J, Grung B., Marklund S, Sjöström M, Antti
598 H, Moritz T (2005). High-throughput data analysis for detecting and identifying differences
599 between samples in GC/MS-based metabolomic analyses. *Anal. Chem.* 77: 5635–5642.
600 doi:10.1021/ac050601e.

601 Jumpponen, A, Jones KL (2009). Massively parallel 454 sequencing indicates hyperdiverse
602 fungal communities in temperate *Quercus macrocarpa* phyllosphere. *New Phytol.* 184:438–
603 448. doi:10.1111/j.1469-8137.2009.02990.x.

604 Jumpponen A, Jones KL (2010). Seasonally dynamic fungal communities in the *Quercus*
605 *macrocarpa* phyllosphere differ between urban and nonurban environments. *New Phytol.*
606 186:496–513. doi:10.1111/j.1469-8137.2010.03197.x.

607 Keefover-Ring K, Ahnlund M, Abreu IN, Jansson S, Moritz T, Albrechtsen BR (2014). No
608 evidence of geographical structure of salicinoid chemotypes within *Populus tremula*. *PLoS*
609 *ONE* 9(10): e107189. doi:10.1371/journal.pone.0107189.

610 Lê S, Josse J, Husson F (2008). FactoMineR: An R Package for Multivariate Analysis. *J. Stat.*
611 *Soft.* 25:1–18. doi. 10.18637/jss.v025.i01.

612 Lewinsohn D, Lewinsohn E, Bertagnolli CL, Patridge AD (1994). Blue-stain fungi and their
613 transport structures on the Douglas-fir beetle. *Can. J. For. Res.* 24: 2275–2283.
614 doi:10.1139/x94-292.

615 Martín JA, Witzell J, Blumenstein K, Rozpedowska E, Helander M, Sieber TN, Gil L (2013).
616 Resistance to Dutch elm disease reduces presence of xylem endophytic fungi in elms
617 (*Ulmus* spp.). *PLoS ONE* 8(2): e56987. doi:10.1371/journal.pone.0056987.

618 Martinez Arbizu P (2019). pairwiseAdonis: Pairwise multilevel comparison using adonis. R
619 package version 0.3.

620 Massad TJ, Trumbore SE, Ganbat G, Reichelt M, Unsicker S, Boeckler A, Gleixner G,
621 Gershenzon J, Ruehlw S (2014) An optimal defense strategy for phenolic glycoside
622 production in *Populus trichocarpa* – isotope labeling demonstrates secondary metabolite
623 production in growing leaves. New Phytol. 203:607-619. doi:10.1111/nph.12811.

624 McGill BJ, Etienne RS, Gray JS, Alonso D, Anderson MJ, Benecha HK, Dornelas M, Enquist
625 BJ, Green JL, He F, Hurlbert AH, Magurran AE, Marquet PA, Maurer BA, Ostling A,
626 Soykan CU, Ugland KI, White EP (2007). Species abundance distributions: moving beyond
627 single prediction theories to integration within an ecological framework. Ecol. Lett. 10:995-
628 1015. doi:10.1111/j.1461-0248.2007.01094.x.

629 Nisa H, Kamili AN, Nawchoo IA, Shafi S, Shameem N, Bandh SA (2015). Fungal endophytes
630 as prolific source of phytochemicals and other bioactive natural products: A review.
631 Microb. Pathog. 82:50–59. doi:10.1016/j.micpath.2015.04.001.

632 Nohrstedt H-Ö (2001). Response of coniferous forest ecosystems on mineral soils to nutrient
633 additions: a review of Swedish experiences. Scand. J. For. Res. 16:555–573.
634 doi:10.1080/02827580152699385.

635 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin P, O’Hara R, et al. (2016). vegan:
636 Community Ecology Package. R package version 2.4-0.

637 Paungfoo-Lonhienne C, Yeoh YK, Kasinadhuni NRP, Lonhienne TGA, Robinson N,
638 Hugenholtz P, Ragan MA, Schmidt S (2015). Nitrogen fertilizer dose alters fungal
639 communities in sugarcane soil and rhizosphere. Sci. Rep. 5, 8678. doi:10.1038/srep08678.

640 Petrini O, Sieber TN, Toti L, Viret O (1993). Ecology, metabolite production, and substrate
641 utilization in endophytic fungi. Nat. Toxins 1: 185–196. doi:10.1002/nt.2620010306.

642 Porter JK, Bacon CW, Cutler HG, Arrendale RF, Robbins JD (1985). In vitro auxin production
643 by *Balansia epichloë*. Phytochemistry 24:1429–1431. doi:10.1016/S0031-9422(00)81037-7.

644 R Development Core Team (2016). R: A language and environment for statistical computing.
645 Vienna, Austria: R Foundation for Statistical Computing Available at: [https://www.r-](https://www.r-project.org/)
646 [project.org/](https://www.r-project.org/).

647 Ragazzi A, Moricca S, Capretti P, Dellavalle I, Turco E (2003). Differences in composition of
648 endophytic mycobiota in twigs and leaves of healthy and declining *Quercus* species in Italy.
649 For. Pathol. 33: 31–38. doi:10.1046/j.1439-0329.2003.3062003.x.

650 Rajala T, Velmala SM, Vesala R, Smolander A, Pennanen T (2014). The community of needle
651 endophytes reflects the current physiological state of Norway spruce. *Fungal Biol.* 118:
652 309–15. doi:10.1016/j.funbio.2014.01.002.

653 Rice AV, Thormann MN, Langor DW (2007). Mountain pine beetle associated blue-stain fungi
654 cause lesions on jack pine, lodgepole pine, and lodgepole × jack pine hybrids in Alberta.
655 *Can. J. Bot.* 85: 307–315. doi:10.1139/B07-014.

656 Robinson KM, Ingvarsson PK, Jansson S, Albrechtsen BR (2012). Genetic variation in
657 functional traits influences arthropod community composition in Aspen (*Populus tremula*
658 L.). *PLOS ONE* 7(5): e37679, doi:0.1371/journal.pone.0037679.

659 Rodriguez RJ, White JF, Arnold AE, Redman RS (2009). Fungal endophytes: diversity and
660 functional roles. *New Phytol.* 182: 314–30. doi:10.1111/j.1469-8137.2009.02773.x.

661 Salminen JP, Karonen M (2011). Chemical ecology of tannins and other phenolics: we need a
662 change in approach. *Funct. Ecol.* 25: 325-338. doi:10.1111/j.1365-2435.2010.01826.x

663 Sampedro L, Moreira X, Zas R (2011). Costs of constitutive and herbivore- induced chemical
664 defences in pine trees emerge only under low nutrient availability. *J. Ecol.* 99:818-827.
665 doi:10.1111/j.1365-2745.2011.01814.x.

666 Schauer N, Steinhauser D, Strelkov S, Schomburg D, Allison G, Moritz T, Lundgren
667 K, Roessner-Tunali U, Forbes MG, Willmitzer L, Fernie AR, Kopka J (2005). GC–MS
668 libraries for the rapid identification of metabolites in complex biological samples. *FEBS*
669 *Letters*, 579. doi: 10.1016/j.febslet.2005.01.029.

670 Shade A, Handelsman J (2012). Beyond the Venn diagram: the hunt for a core microbiome.
671 *Environ. Microbiol.* 14, 4–12. doi:10.1111/j.1462-2920.2011.02585.x.

672 Sieber TN (2007). Endophytic fungi in forest trees: are they mutualists? *Fungal Biol. Rev.* 21,
673 75–89. doi:10.1016/j.fbr.2007.05.004.

674 Tadych M, Bergen MS, Johnson-Cicalese J, Polashock JJ, Vorsa N, White JF (2012).
675 Endophytic and pathogenic fungi of developing cranberry ovaries from flower to mature
676 fruit: diversity and succession. *Fungal Div.* 54: 101–116. doi:10.1007/s13225-012-0160-2.

677 Terhonen E, Blumenstein K, Kovalchuk A, Asiegbu F (2019). Forest tree microbiomes and
678 associated fungal endophytes: Functional roles and impact on forest health. *Forests* 10: 42.
679 doi:10.3390/f10010042

680 Thomma BPHJ, Van Esse HP, Crous PW, De Wit PJGM (2005). *Cladosporium fulvum* (syn.
681 *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on
682 plant pathogenic Mycosphaerellaceae. *Mol. Plant Pathol.* 6: 379–393. doi:10.1111/j.1364-
683 3703.2005.00292.x.

684 Ullah C, Unsicker SB, Fellenberg C, Constabel P, Schmidt A, Gershenzon J, Hammerbacher
685 A (2017). Flavan-3-ols are an effective chemical defense against rust infection. *Plant*
686 *Physiol.*;175:1560–1578. doi:10.1104/pp.17.00842.

687 Unterseher M (2011). Diversity of fungal endophytes in temperate forest trees. In: *Endophytes*
688 *of Forest Trees: Biology and Applications*, eds. AM Pirttilä and AC Frank (Springer,
689 Dordrecht), 31–46. doi:10.1007/978-94-007-1599-8_2.

690 Unterseher M, Siddique AB, Brachmann A, Peršoh D (2016). Diversity and composition of the
691 leaf mycobiome of beech (*Fagus sylvatica*) are affected by local habitat conditions and leaf
692 biochemistry. *PLoS ONE* 11(4): e0152878. doi/10.1371/journal.pone.0152878.

693 Vellend M (2010). Conceptual synthesis in community ecology. *Quart. Rev. Biol.* 85:183-206

694 Videira SIR, Groenewald JZ, Braun U, Shin HD, Crous PW (2016). All that glitters is not
695 *Ramularia*. *Stud. Mycol.* 83:49–163. doi:10.1016/J.SIMYCO.2016.06.001.

696 White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal
697 ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and*
698 *applicaitons*, ed. TJ Innis, MA, Gelfand DH, Sninsky JJ. Academic Press Inc., New York,
699 pp 315–322.

700 Whittaker RH (1972). Evolution and measurement of species diversity. *Taxon* 21: 213–251.

701 Wilson M, Lindow SE (1994). Coexistence among epiphytic bacterial populations mediated
702 through nutritional resource partitioning. *Appl. Environ. Microbiol.* 60: 4468–77.

703 Witzell J, Shevtsova A (2004). Nitrogen-induced changes in phenolics of *Vaccinium myrtillus*
704 - implications for interaction with a parasitic fungus. *J. Chem. Ecol.* 10:1919-1938. doi:
705 10.1023/b:joec.0000045587.75128.a4.

706 Witzell J, Martín JA (2008). Phenolic metabolites in the resistance of northern forest trees to
707 pathogens — past experiences and future prospects. *Can. J. For. Res.* 38:2711–2727.
708 doi:10.1139/X08-112.

709 Witzell J, Martín JA (2018). Endophytes and forest health. In: *Endophytes of forest trees*. Eds.
710 AM Pirttilä and CA Frank. *Forestry Sciences* 86. Springer International Publishing AG,
711 Springer Nature, pp 261–282. doi:10.1007/978-3-319-89833-9_12.

712 Witzell J, Martín JA, Blumenstein K (2014) Ecological aspects of endophyte-based biocontrol
713 of forest diseases. In: VC Verma & AC Gange (eds.), *Advances in Endophytic Research*,
714 pp. 321- 333. Springer India. doi: 10.1007/978-81-322-1575-2_17.

Table 1. Kruskal-Wallis statistics (chi-square, df = 3; p-value and significance $\alpha < 0.05$) on treatment effects on selected phenolic compounds (condensed tannins and their precursor catechin), amino acids (phenylalanine and tryptophan) and sugars (glucose and maltose).

Chemicals	chi-square	p-value	Significance
<i>Phenolics</i>			
Condensed tannins	5.73	0.1255	n.s.
Catechin	25.46	1.239e-05	***
<i>Amino acids</i>			
Phenylalanine	31.45	6.81e-07	***
Tryptophan	42.04	3.928e-09	***
<i>Sugars</i>			
Glucose	4.86	0.1817	n.s.
Maltose	21.30	9.122e-05	***

Table 2. Morphotype identity and abundance (nr of isolates belonging to each morphotype per plant) detected in the leaves of aspen plants in different treatments treatments (control, C; herbivory, H; nitrogen fertilization, N; nitrogen fertilization and herbivory, NH).

Morphotype	Identity	Treatment			
		C	N	H	NH
MT1	<i>Ramularia sp.</i>	8.83	6.88	5.14	4.33
MT2		4.71	5.13	1.48	3.29
MT3	<i>Fusarium oxysporum</i>	0.04	0.04	0.00	0.05
MT4		0.00	0.04	0.05	0.00
MT5		4.83	3.92	2.71	2.95
MT6		0.17	0.08	0.05	0.05
MT7		0.04	0.00	0.00	0.00
MT8	<i>Dothideomycetes 1</i>	0.00	0.08	0.00	0.00
MT9	<i>Dothideomycetes 2</i>	0.04	0.00	0.00	0.00
MT10		0.00	0.00	0.05	0.05
MT11		0.00	0.00	0.05	0.00
MT12	<i>Penicillium olssonii</i>	0.42	0.67	0.57	0.81
MT13	<i>Cladosporium sp. 1</i>	1.08	1.29	0.71	0.76
MT14	<i>Cladosporium sp. 2</i>	0.04	0.00	0.00	0.00
MT15	<i>Penicillium 1</i>	0.54	0.54	0.10	0.52
MT16		0.00	0.04	0.00	0.00
MT17	<i>Penicillium brevicompactum</i>	0.00	0.46	0.19	0.00
MT18		0.88	0.83	0.38	0.43
MT19		0.04	0.04	0.05	0.00
MT20	<i>Penicillium sp 2</i>	0.00	0.00	0.00	0.05
MT21	<i>Penicillium sp. 3</i>	0.04	0.08	0.00	0.00
MT22	<i>Arthrimum sp 1</i>	0.54	0.75	0.52	0.52
MT23	<i>Arthrimum sp 2</i>	0.08	0.25	0.05	0.05
MT24	<i>Arthrimum sp 3</i>	0.00	0.00	0.05	0.00
MT25	<i>Rhodotorula sp.</i>	0.00	0.21	0.14	0.00
MT26		0.29	0.29	0.43	0.71
MT27	<i>Aureobasidium microstictum</i>	0.00	0.00	0.00	0.14
MT28	<i>Aureobasidium pullulans</i>	0.00	0.00	0.05	0.05
MT29		0.00	0.00	0.00	0.05
MT30	<i>Physalospora scirpi</i>	0.00	0.04	0.00	0.00
Total nr of isolates		543	520	268	311

Fig. 1. Multivariate analyses of metabolites in the leaves of aspen (*Populus tremula*) plants exposed to environmental endophytic inoculum in controlled, greenhouse-conditions: untreated control plants (C) and plants subjected to nitrogen addition (N), herbivory (H), or nitrogen addition and herbivory in combination (NH) (n = 24 plants per treatment). Shown are the Principal Component Analysis score plots of a) 114 targeted and non-targeted foliar metabolites (global analysis, data acquired using gas chromatography/mass spectrometry) and b) 11 targeted metabolites (the main salicinoid phenolic glycosides and catechin; data acquired using liquid chromatography).

Fig. 2. Concentration (mg g^{-1} DW) or level (measured as normalized peak area, pa) of individual chemical compounds in the leaves of aspen (*Populus tremula*) plants exposed to environmental endophytic inoculum in controlled, greenhouse-conditions: untreated control plants (C) and plants subjected to nitrogen addition (N), herbivory (H), or nitrogen addition and herbivory in combination (NH) ($n = 24$ plants per treatment). Shown are selected examples for phenolics (a-b: concentrations of condensed tannins and their precursor catechin), aminoacids (c-d: phenylalanine and tryptophan), and sugars (e-f: glucose, maltose). The data was acquired using liquid chromatography, except for condensed tannins which we analysed using a spectroscopy.

Fig 3. Sample-size-based (a) and (b) coverage-based rarefaction (solid line) and extrapolation (dotted line) curves comparing fungal morphotype richness in the leaves of greenhouse-cultivated aspen (*Populus tremula*) plants (n=96): untreated control plants (C) and plants subjected to nitrogen addition (N), herbivory (H), or nitrogen addition and herbivory in combination (NH) (n = 24 plants per treatment). The shaded areas represent the 95% confidence intervals. The different symbols represent the reference samples.

Fig. 4. Venn diagram showing the number of unique and shared morphotypes of fungal endophytes in the leaves of greenhouse-cultivated aspen (*Populus tremula*) plants untreated control plants (C) and plants subjected to nitrogen addition (N), herbivory (H), or nitrogen addition and herbivory in combination (NH) (n = 24 plants per treatment).