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The effects of an endophytic fungus on the performance of insect herbivores on Poplar trees

Master's Thesis

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- List of abbreviations

ANOVA	Analysis of variance
Approx.	Approximately
c	Black poplar treated with a Control solution
c + h	Control solution treated Black poplar with herbivory treatment
conc	Concentration
C. tremulae	*Chrysomela tremulae*
C. *cladosporioides*	*Cladosporium cladosporioides*
DAD	Diode array detector
df	Degrees of freedom
DW	Dry weight
e	Black poplar treated with endophyte solution
e.g.	Example
e + h	Endophyte solution treated Black poplar with herbivory treatment
fw	Forward
g	Gram
gDNA	Genomic DNA
GLS	Generalized least squares
h	Hour
HPLC	High-performance liquid chromatography
ind	Individual
IS	Internal standard
L	Litre
L. dispar	*Lymantria dispar*
lm	Linear regression model
mg	Milligram
min	Minute
mL	Millilitre
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite

ng	Nanogram
nmol	nanomoles
n.s	Non-significant
PDA	Potato dextrose agar
PET	Polyethylene terephthalate
P. nigra	*Populus nigra*
qPCR	Quantitative real-time polymerase chain reaction
rev	Reverse
RP	Reversed-phase
Rpm	Revolutions per minute
RRF	Relative Response factor
RT	Retention time
s	Seconds
SEM	Standard error of the mean
UV	Ultraviolet
µL	Microlitre
µmol	Micromoles

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1 Introduction

Plants constantly interact with other organisms in nature. These interactions can be symbiotic (e.g. legumes and symbiotic bacteria (Hawkins & Oresnik, 2022), competitive (e.g. plant-plant interactions; Gruntman et al., 2017), or parasitic (e.g. plants and nematodes; García-Arenal & Fraile, 2013). Plants can either benefit from or be harmed by such interactions. Herbivory is one such interaction between plants and insects where the plant often suffers severe damage from insect infestation which also impacts the net primary productivity in natural ecosystems (Bastias et al., 2017).

The plants and insects have been coexisting for almost 400 million years, during which they have developed refined interactions between biochemical and population genetics (Fürstenberg-Hägg et al., 2013). Insects often feed on all plant parts, including the roots, wood, stems, leaves, floral buds, flowers, and fruits. The herbivores that feed on the plant by biting the chunks of plant tissue are said to be chewing insects, and those who feed on the phloem sap are referred to as piercing or sucking insects. In addition, some herbivores feed inside the stem or leaf as mining insects, some produce galls on plants, and some feed on the tissue between the leaf veins, skeletonising the leaf. Herbivores do not just display a variety in their feeding habits, but also in the range of their host plant. Insects that feed on a wide range of plants across different families are said to be generalists or polyphagous, and those that feed on closely related plants (species of the same family or genus) are referred to as specialists or oligophages (Ali & Agrawal, 2012).

Plants being sessile have evolved a broad range of defence mechanisms against herbivores. These defence strategies can be grouped into constitutive or induced, that can be direct or indirect, and functions (chemical, morphological, physiological). Constitutive defences are permanently present (e.g. morphological structures such as prickles, thorns, thick cell walls, and chemical compounds such as alkaloids and phenolics). Whereas, the induced defences are only formed upon attack which can involve a third trophic level like parasitoids where the plants cooperate with the predators of the herbivores that attacked it (e.g. upon attack by herbivores, the plant's anatomical traits like extra floral nectars and food bodies can be induced to attract parasitoids like ants; Heil, 2008a; Mithöfer & Boland, 2012). In addition to morphological plant traits, induced defences can also involve volatile organic compounds (VOCs), which are often released by plants after being attacked by herbivores. Activation of which requires the recognition of herbivore-associated molecular patterns (HAMPs) or pathogen-associated molecular patterns (PAMPs), such as chemical substances from oviposition fluids or from oral secretions of attacking insects (Basu et al., 2018; Heil, 2008a; Mithöfer & Boland, 2012). Plants

are capable of distinguishing between herbivory and mechanical damage, and in response to the damage, they produce a range of defence compounds, including volatiles (such as 6-carbon aldehydes, terpenoids, and alcohols), hormones (including jasmonate hormones like JA), and phenolic compounds (Fürstenberg-Hägg et al., 2013).

In nature, plants also harbour and interact with an enormous diversity of microbial species. These microbes can be antagonists in the form of pathogens (e.g. pathogenic fungi) or mutualists (e.g. mycorrhiza) to plants. Apart from the various defence strategies that plants use against herbivores, the mutual association of plants with microbes modulates the plant-herbivore relationship (Bastias et al., 2017). The plant-fungal symbiotic relationship has been extensively examined, focusing on mycorrhizal fungi and fungal endophytes in herbaceous plants (Hartley & Gange, 2009).

Every plant contains endophytic fungi that live inside the host tissues without producing any symptoms of disease (Koricheva et al., 2009). Often these endophytes originate from soil, air or insects or animals that serve as vectors. The endophytes that are transferred via seeds from one plant to the next generation are vertically transmitted. This method of transmission is often found in grass endophytes (eg: *Epichloë* sp.) belonging to the family *Clavicipitaceae*. The nonclavicipitaceae group of endophytes which are often found in trees are transmitted horizontally via spores, hyphal fragmentation, and by wind, rain, or by herbivores as vectors (Crawford et al., 2010; Eberl et al., 2019; Hartley & Gange, 2009a). The spores of endophytic fungi germinate by producing hyphae that enter the host plant via stromata and other leaf openings and undergo a quiescent state after infection (Eberl et al., 2019). It has also been speculated that fungal endophytes can become pathogenic upon the arrival of favourable environmental conditions (Sieber, 2007).

Endophytes are known to increase host resistance against herbivores by increasing the production of chemical defences by the host (Hartley & Gange, 2009a). Their presence in many grass species has been shown to reduce herbivore performance significantly (Crawford et al., 2010). Fungal endophytes produce various chemical compounds that can negatively affect insect herbivores(Eberl et al., 2019). They also produce alkaloids, polyphenols, steroids, and other secondary metabolites that were once known to be produced only by plants (Bastias et al., 2017; Bastías et al., 2021; Sumarah & David Miller, 2009). Studies on the interactions between grass-endophyte symbioses have been well documented (Crawford et al., 2010; Hartley & Gange, 2009a). However, there is inconsistent data on tree-endophyte interaction (Eberl et al., 2019). Tree fungal endophytes are often horizontally transmitted by spores and therefore, a single tree can harbour a large number of fungal species with many possible

consequences on plant-herbivore interactions (Ahlholm et al., 2002). Taking into account the beneficial aspects of fungal endophytes, they are now considered a good source of microbial biological control agents (Collinge et al., 2022). Since trees harbour a variety of insect herbivores and microbes, it makes them an important species of the forest ecosystem. Yet, very little data is available on the role of endophytic fungi in tree-herbivore interactions.

1.1 Aim of the study

The purpose of this study was to investigate how insect herbivores perform on a model woody plant, the black poplar (*Populus nigra*), when it is infected with an endophytic fungus (*Cladosporium cladosporioides*). To achieve this, the study focused on two types of herbivores: a generalist herbivore *(Lymantria dispar*) and a specialist herbivore (*Chrysomela tremulae*). The study also aimed to determine how the fungal endophyte affects the phytochemistry of poplar leaves, both in the presence and absence of insect herbivores. This was achieved through a chemical analysis of the leaves. Endophytic fungi are known to produce secondary chemical compounds, so the study also aimed to determine the amount of stachydrine, an alkaloid produced by the endophyte *Cladosporium cladosporioides,* and the abundance of the fungal endophyte, giving the analysis a molecular approach.

2 Model organisms used in the study

To understand how the fungal endophyte might affect the tree-herbivore interaction, the model organisms used in this study were young *Populus nigra* plants (Black Poplar), the fungal endophyte *Cladosporium cladosporioides*, a generalist herbivore of Black Poplar *Lymantria dispar*, and a specialist herbivore *Chrysomela tremulae*.

Populus nigra which is commonly known as the Black poplar are found throughout the Northern Hemisphere. The trees belong to the *Salicaceae* family, members of this genus are fast-growing, wind-pollinated and deciduous (Philippe & Bohlmann, 2007). The wood being soft, is used for items of furniture and in the paper industry. Black Poplar can be propagated vegetatively (by cuttings) and generatively i.e., wind and water-dispersed seeds (De Rigo et al., 2016). These trees along with other members of Salicaceae inhabit floodplain forests and riverbanks. More importantly, they can withstand flooding and can grow in poor soil conditions, which allows them to colonise disturbed sites, making these early successional species an important component of riparian ecosystems (Bradshaw et al., 2000; De Rigo et al., 2016; Philippe & Bohlmann, 2007). Due to their ecological impact, Poplars are subject to interactions with a variety of insect herbivores, microbes and other organisms (Eberl et al., 2018). The trees are one of the threatened species in Europe, due to various reasons including habitat

degradation, and gene flow from cultivated poplar plantations into wild populations. The trees are also susceptible to attacks from insect herbivores, and diseases (De Rigo et al., 2016; Eberl et al., 2018). As these trees encounter many pathogen attacks and herbivore infestation, poplars have also developed a broad spectrum of defence mechanisms. Salicinoids are the major group of anti-herbivore chemical defence compounds produced by the members of the Salicaceae family. The production of falvan-3-ols against *Melampsora* rusts fungus is well documented. Additionally, they also emit volatile organic compounds (VOCs) to attract parasitoids (Boeckler et al., 2016; Eberl et al., 2018; McCormick et al., 2014; Philippe & Bohlmann, 2007; Ullah, Tsai, et al., 2019). The Black poplar's ability to withstand various pathogen and herbivore attacks, its potential for clonal propagation, and its ecological significance make it an ideal candidate for research as a woody plant organism. Additionally, the complete genome of *Populus trichocarpa*, a closely related species of Black poplar, has been sequenced, providing molecular information to better understand the interactions of poplar with other organisms (McCormick et al., 2014).

Lymantria dispar which is commonly known as the gypsy moth or spongy moth is known to cause serious infestation across the forests of Europe, North America and in some of the northern parts of Asia. The caterpillars often feed on more than 300 species of woody plants (Ponomarev et al., 2023; Stoyenoff et al., 1993.). These caterpillars are polyphagous, lepidopteran herbivores of poplars, and many other deciduous tree species like oak and birch (Soukhovolsky et al., 2023). This herbivore has a four-stage life cycle (egg, larva, pupa and moth or the adult stage, Hajizadeh et al., 2011). The eggs hatch during April and May, after which they crawl to the tree crown or balloon if it is windy weather. The caterpillar then feeds on the tree crown, causing heavy defoliation. The caterpillar has five to six instars (depending on the sexes), followed by the pupation stage, which lasts up to two weeks (Hajizadeh et al., 2011; Ponomarev et al., 2023; Zúbrik et al., 2021).

Chrysomela tremulae commonly known as the small poplar leaf beetle is a leaf beetle and a specialist herbivore of Poplar and Salix trees. Both the larvae and adults feed on the leaves of young trees, which often leads to growth reduction of the trees (Leplé et al., 1995). The larvae of this genus possess nine pairs of defensive glands on their abdominal region through which they release defensive secretions after being touched or disturbed (Burse et al., 2009a; Rgen Kuhn et al., 2004). These beetles by feeding on Salicaceae members sequester phenol glycosides (salicinoids) like salicin and salicortin (which are otherwise toxic to generalist herbivores) and are converted into salicylaldehyde which acts as an anti-predator defence compound (Burse et al., 2009b; Kuhn et al., 2007; Zvereva et al., 2017).

The genus *Cladosporium* is characterized by a global distribution and is recognized for its capacity to safeguard plants against both biotic and abiotic stressors (Răut et al., 2021). Morphologically, the colonies of these genera are dark-greyish, to dark-greenish in colour, and possess smaller conidia, with or without septum (Islam et al., 2019). It inhabits the inner layers of leaves and can also stimulate the accumulation of secondary metabolites in plants. These fungi also produce secondary compounds like plumbagin, taxol, polyketides and stachydrine, which are of pharmaceutical interest (Walther et al, 2021, Lv et al., 2023). *C. cladosporioides* fungi are also reported to be saprophytic and pathogenic to different plants (Lv et al., 2023), while at the same time, the species is well documented to have anti-inhibitory activity against pathogenic fungi of strawberry and chrysanthemum plants (Răut et al., 2021; Wang et al., 2013). The fungus also has entomopathogenic activity against aphids, moths and many other insect herbivores (Islam et al., 2019). *Cladosporium's* potential as a biocontrol agent and its widespread distribution has attracted the attention of numerous researchers, making it a promising candidate for further study as a fungal endophyte.

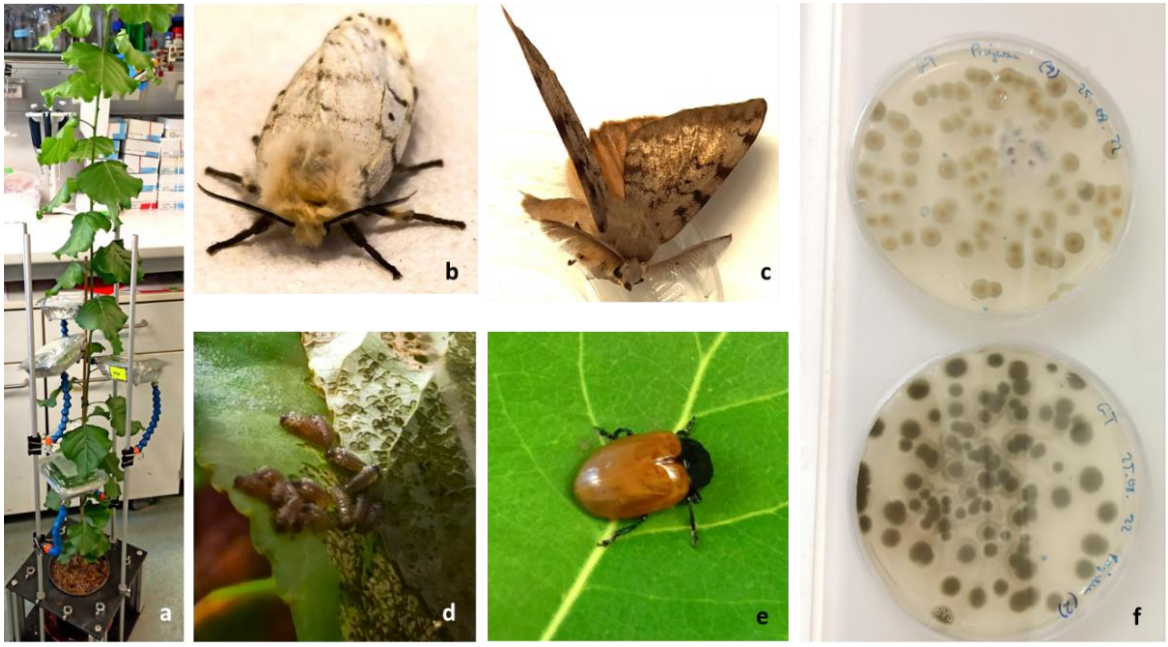

Figure 5*:* The study organisms (a-f), respectively**. (a)** Black poplar along with the clip cages placed along the supporting stand in the laboratory. **(b)** adult female of *L. dispar*. **(c)** adult male of *L. dispar*. **(d)** larvae of the beetle *C. tremulae* feeding on young Poplar trees. **(e)** adult *Chrysomela tremulae* beetle, **(f)** cultures of *C. cladosporioides* grown in petri dishes, the colour of the colonies varies from yellowish to olive greenish colour in the above figure.

3 Material and methods

3.1 The Study Organisms

Plant Material

The young Black Poplars used in the present study were grown from stem-cutting in the lab. Initially, the trees were obtained from a natural population of Black Poplar located in northeastern Germany (52°34'1" N;14°38'3" E). The plants were grown in climate chambers under sterile conditions by immersing the stem cuttings (Approx. 10 cm) into a 100 mL of water solution which had 20 mL of 2% NaOCl and 1.2 mL Tween 20 for 3 min, the cuttings were then surfaced-sterilized, followed by rinsing for five times with sterile distilled water. After removing the ends of the cuttings, the 1 cm long cuttings were transferred into a petri dish containing a growth media (McGowan's Woody Plant media WPM, Duchefa, Haarlem, Netherlands). On the onset of root development, the cuttings were further placed into Magenta™ boxes (Sigma-Aldrich, St. Louis, USA) which had the same growth media, and were placed in the growth chambers (day/night: 21/19 °C, photoperiod 16 h). Eight weeks later, the plants were placed into 1 L pots filled with a sand and soil mixture (1:1, Klasmann potting substrate; Klasman-Deilmann, Geeste, Germany) and were cultivated in a climate chamber (21/19 °C Day/night, photoperiod 16 h). After four weeks, the trees were shifted to another climate chamber (20/ 18°C Day/ night, photoperiod 16 h, humidity 60%). Two weeks after the tree's height had reached approximately 50 cm, the leaves (about 10 per plant) were treated with either a control solution or an endophyte solution, as specified below.

The Insects

The egg batches of *Lymantria dispar* were provided by the US Department of Agriculture, Buzzards Bay, MA, USA, and the larvae were reared on an artificial diet provided by Frontier Scientific Services Agriculture, Newark, USA in a climate chamber (20°C, photoperiod 14 h, 60% humidity). The *Chrysomela tremulae* beetle's larvae were reared on Black poplar saplings under laboratory conditions at the Max Planck Institute of Chemical Ecology, Jena, Germany. The plants were regularly watered and the twigs of poplar were covered in a polyethene terephthalate (PET) bag (Toppits, Minden, Germany) closed with cable binders at the upper and lower ends to avoid the escape of the larvae.

The endophyte

The endophyte *C. cladosporioides* cultures were grown on PDA (Potato Dextrose agar) which was originally obtained from the natural population of black poplar trees as mentioned in (Walther et al., 2021)**.** For the preparation of the spore solution, approximately 12 mL of a solution containing 0.01% Triton X-100 (Sigma-Aldrich), 0.9% (w/v) NaCl (Roth), 0.2% (w/v)

peptone (Roth) and 0.3% (w/v) glucose (Roth) was added to the Petri dishes containing grown cultures of *C. cladosporioides*, which was then scratched off using a pipette. This spore solution was then filtered through a sterile glass wool to separate the spores and the mycelium. Using a Hemocytometer the spores were counted from the filtrate, which was then diluted to 10^7 spores per mL. At the same time, the remaining endophyte spore solution was diluted to 100 spores/ mL and was poured on the four Petri dishes containing PDA media which was sterilized using an autoclave (Abortechnik, Gmbh, Germany) to grow back the endophyte which would then ensure the successful preparation of the endophyte spore solution (spore germination test). These Petri dishes were kept in the incubator (Heraeus Instruments, Germany) at 25° C.

3.2 Experimental design

To study the performance of herbivores on black poplar inoculated with *C. cladosporioides*, and to determine the effects of the same endophytic fungus on the defence of black poplar, a total of 28 trees were used in the study which was categorized into four groups of treatments. 1. Control treatment (c): the trees that were inoculated by a control solution, 2. Control + herbivory treatment (c + h), 3. Endophyte treatment (e): the trees that were inoculated by fungal endophyte solution and 4. Endophyte + herbivory (e + h). Out of these 28 trees, six trees did not receive herbivory (i.e., c and e), while the remaining 22 trees were used for determining the herbivore's performance (c + h) and (e + h).

For the inoculation procedure healthy, developed, and fully expanded 7- 10 leaves were selected from each tree. The selected leaves were then sprayed approximately 1.5 mL per leaf with either a fungal endophyte solution or a control solution (free of fungal spores, made up of 0.01% Triton X-100, 0.9% (w/v) NaCl, 0.3% (w/v) glucose and 0.2% (w/v) peptone). Both sides of the leaves were spray-inoculated using a spray bottle. The plants were then enclosed individually in a polyethene terephthalate (PET) bag (Toppits, Minden, Germany) closed with cable binders at the upper and lower ends to avoid contamination and to ensure spore germination. The bags were opened two days after endophyte inoculation (**Figure 3**).

3.3 Insect Performance Assay

To study the performance of insect herbivores on black poplar each selected leaf was installed with box-shaped clip cages which were perforated to allow air exchange after 15 after endophyte inoculation (**Figure 3**). Two-day-old (n = 7-9) caterpillars of *L. dispar* and larvae of beetle *C. tremulae* (n = 10) were weighed and placed in the clip cages of each leaf of the plants (c+h and e+h treatments, respectively), where n is the number of caterpillar and larvae of the beetles feeding on either control or endophyte inoculated tree. Initially, the number of insects

in each clip cage was five. To avoid complete defoliation, and due to naturally high mortality in early instar, the number of individuals was reduced from five individuals per clip cage to one in 15 days after the start of the performance experiment for caterpillars of *L. dispar* and after nine days for *C. tremulae* beetle larvae. After every three days, the insects from the clip cages were carefully transferred into the solo cups with either a size zero paint brush or with entomological tweezers to avoid injuring the insects (initially, the insects were weighed as a group). The insects were then switched to a new leaf on the same tree. When around 70% of the total leaf area was consumed, the insects were then placed on a new tree with a similar clip-cage arrangement. Both the insects after pupation were kept at room temperature until hatching for sexing.

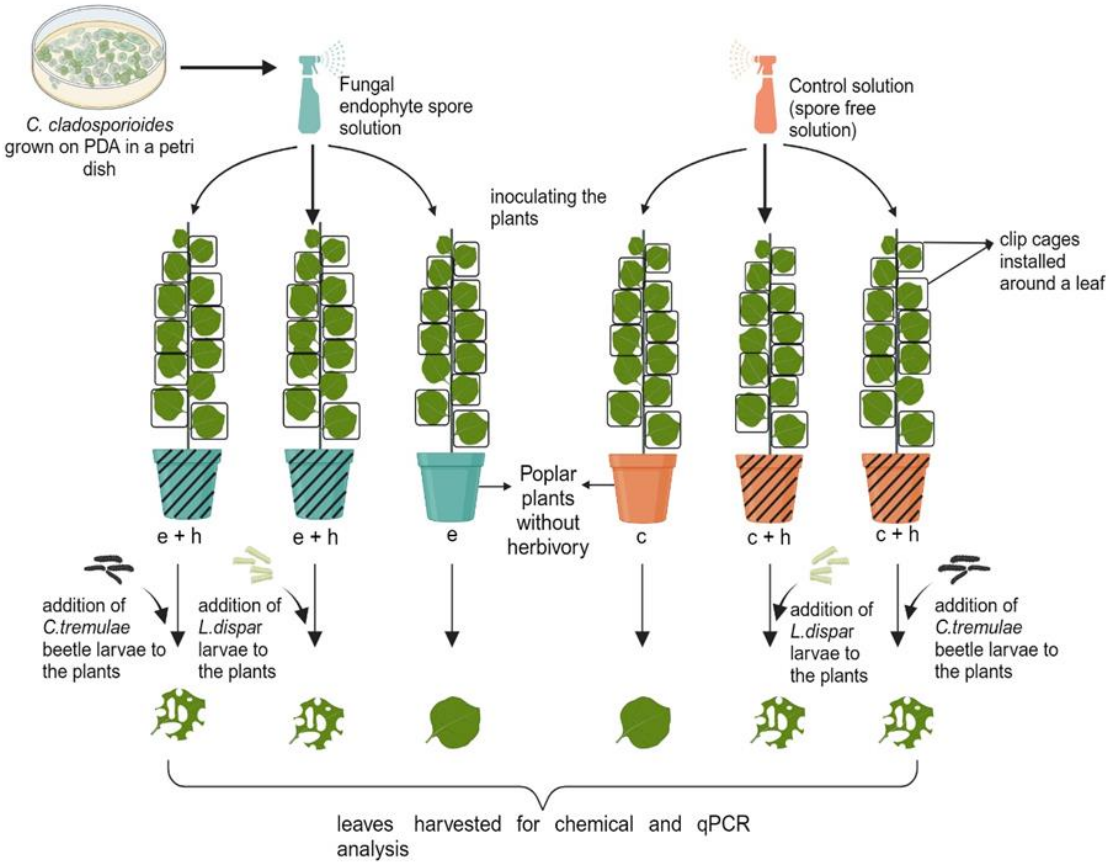

Figure 6: An illustration of the Experimental design. *C. cladosporioides* was grown on PDA media. The spore solution from the fungal endophyte and the control solution (spore-free) was prepared as mentioned above. The trees were divided into four groups "c" = control trees, "e" = fungal endophyte inoculated trees; both the groups were without herbivory, "c+h" = the control trees with herbivory, and the "e+h" = fungal endophyte inoculated trees with herbivory). The leaves of the trees without herbivory (c) and (e) were harvested for chemical analysis and to check the fungal abundance 42 days after endophyte inoculation. While the leaves of the trees with herbivory were harvested after 70% of the leaf damage.

Figure 7: Black poplar trees during inoculation and experimental setup. **(a)** Poplar trees are tied individually with PET bags along with the cable binders at the bottom before the inoculation process. The trees were then inoculated with endophyte (spore solution) or control (spore-free solution) and the individual trees were wrapped in PET bags with the top and bottom being closed by the cable binders to avoid contamination. **(b)** The Poplar trees were then kept in the climate chambers and the PET bags were opened (2 days after endophyte inoculation) at the top to ensure the exchange of air. **(c)** The Poplar trees along with the clip cages installed around each inoculated leaf and supported by a stand 15 days after endophyte inoculation. The clip cages were perforated to allow air exchange and the respective insects were then placed on each leaf to examine their performance.

3.4 Leaf sampling

The damaged leaves of trees from the treatments (c+h and e+h) i.e., the trees that were utilised to determine the performance assay, along with the trees of the treatment (c) and (e) were then harvested for further analysis (**Figure 4**). The leaves were carefully cut using a scissor. The midribs were removed, and the left and right parts of the leaf were then weighed and stored in 5 mL vials (Sarstedt, Nümbrecht, Germany). Three technical replicates were used from a single tree, which consisted of a pool of three or four leaves without petiole and midrib i.e., a total of nine or ten leaves were harvested per tree. In the trees with seven intact inoculated leaves, a total of two technical replicates were used consisting of a pool of three and four leaves, respectively. These vails were then frozen in liquid nitrogen and lyophilized for further analysis. The lyophilized leaf material was ground into a fine powder using a paint shaker (Skandex SO-10 m Shaker, Fluid Management Europe, Sassenheim, The Netherlands) and 5-6 stainless steel balls (Approx. diameter 3 mm).

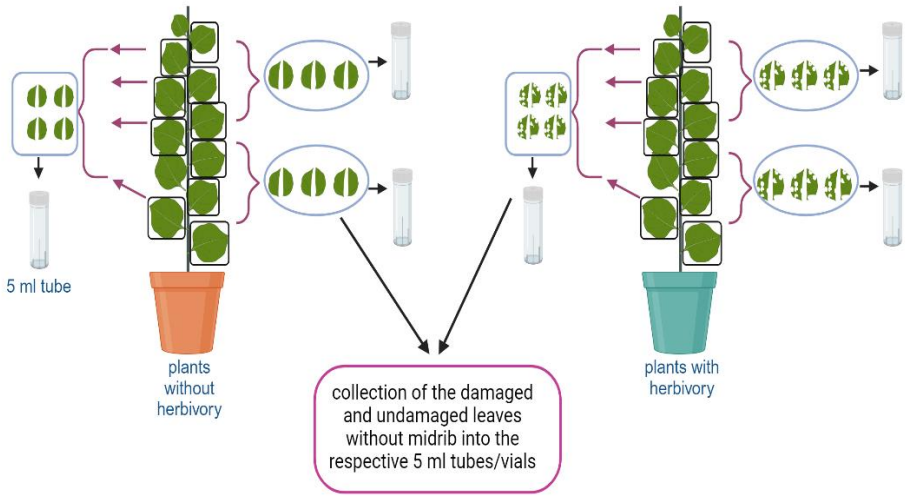

Figure 8: An illustration of Leaf sampling of black poplar trees. The trees with and without herbivory were harvested, and the inoculated leaves were cut from top to bottom. Depending on the number of intact inoculated leaves present, seven to ten leaves per plant were cut without the petiole and midribs. Three technical replicates were used, which had a pool of three to four leaves (in the case of the tree that had nine to ten intact leaves). Two technical replicates were used which had a pool of three and four leaves (in case the trees had only seven intact leaves). The pool of leaves was weighed and placed into the 5 mL vials/tubes and frozen in liquid nitrogen.

3.5 DNA extraction and Quantification of fungal genomic DNA

For the extraction of DNA, 30 mg freeze-dried leaf sample powder was weighed and then subjected to the extraction process using Invitek Diagnostics InviSorb Spin Plant Mini Kit by following the manufacturer's protocol manual. The extracted genomic DNA was then quantified using a NanoDrop 2000c Spectrophometer (Peqlab Biotechnology GmbH, Erlangen, Germany).

The abundance of endophyte fungus in the leaves was determined by Quantitative Polymerase Chain reaction (qPCR). This was done using CFX Connect Real-Time PCR Detection System (Bio-Rad, California, USA) for which the extracted genomic DNA was diluted to 100 ng/ µL. Poplar ACTIN2-specific primers were used for normalization and, the primers specific to the Internal transcribed spacer (ITS) region of *C. cladosporioides* were used to quantify the fungal endophyte DNA. Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent) along with forward and reverse primers (10 µmol each) and DNA (1 µL) were also included in the reaction mixture. The program was set to initiation and activation of polymerase 95 °C (3 min), 40 cycles of 95 °C (10 s) + 60 °C (20 s), melt curve from 65 to 95°C. In each run, a non-template control

was included to check the primer efficiencies. The analysis of the qPCR data was done with the help of the software Bio-Rad CFX Manager 3.1 (ΔΔcq).

3.6 Analysis of Stachydrine

The pyrrolidine alkaloid stachydrine is known to be produced by the fungal endophyte *C. cladosporioides*. To quantify the amount of stachydrine in the plant sample 10 mg of leaf sample powder was extracted using 1 mL of methanol. The samples were then homogenized by using a paint shaker (Skandex SO-10 m Shaker, Fluid Management Europe, Sassenheim, The Netherlands) for approximately 30 seconds to one minute, followed by 30 min shaking at 240 rpm on a horizontal shaker (IKA® Labortechnik, Steifen, Germany) and centrifuged at 3600 rpm for three minutes. The supernatant was then used for the quantification of stachydrine.

The extracted samples were diluted 1:10 using ultrapure water (also known as Milli-Q® water) which had internal standards containing isotopically labelled amino acid mixture which also had proline (^{13}C, ^{15}N labelled amino acid mixture with a concentration of 10 µg per mL) obtained from Isotec, Miamisburg OH, USA. HPLC-MS/MS system was utilized for the quantification, with a reversed-phase 100 mm x 2.1mm, 1.8 µm Zorbax Eclipse XDB-C18 column. Formic acid (0.05%) diluted with water along with the acetonitrile was used as the mobile phase A and B, respectively to elute the samples. The solvent B gradient was set to (min/ % acetonitrile): 0-1.0/3; 1.0-2.7/3-100;2.7-3.0/100; 3.1-6.1/3. Positive ionization mode was used at 5500 eV ionization energy with a curtain gas of 40psi, 70psi of electrospray and drying gas, with the temperature of drying gas set to 60 ºC. Using the relative peak area of the internal standard U-^{13}C, ^{15}N-proline, the stachydrine amount was quantified with the help of the equation given below; equation (1).

3.7 Analysis of the Phenolic Compounds

To quantify the Phenolic compounds in the plant sample, 10 mg of leaf sample powder was extracted using 1 mL of methanol along with the internal standards (0.8 mg/ mL phenyl-β-glucopryranoside (Sigma Aldrich, St. Louis, USA). The samples were then homogenized by using a paint shaker (Skandex SO-10 m Shaker, Fluid Management Europe, Sassenheim, The Netherlands) for approximately 30 seconds to one minute, followed by 30 min shaking at 240 rpm on a horizontal shaker (IKA® Labortechnik, Steifen, Germany) and centrifuged at 3600 rpm for three minutes. The supernatant was transferred into a new tube for further analysis using HPLC-UV.

The supernatant was diluted 1:2 with ultrapure water (Milli-Q® Synthesis A10). The phenolic compounds (salicinoids, flavan-3-ols) were analysed on an HPLC-ultraviolet light (UV) system

HPLC 1100 Series (Hewlett Packard, Berlin, Germany) and detected using a diode array detector (DAD). A nucleodur sphinx RP column (5 µm, 250 mm x 4.6 mm, Macherey-Nagel, Düren, Germany) was used to separate the analytes on a reverse-phase column. The mobile phase consisted of Milli-Q® water and acetonitrile as solvents A and B, respectively. The solvent B gradient was set to (min/ % acetonitrile): 0/14; 22.00/58; 22.10/100; 25.00/100; 25.10/14; 30.00/14 with a constant flow rate of 1 mL/min. The column oven temperature was set at 25 °C. Using the relative peak area of the internal standard phenyl-β-glucopyranoside, the analytes were quantified according to the equation given below; equation (1).

$$\text{conc}_{(\text{analyte})} = \frac{\left(\frac{\text{Peak area}_{(\text{analyte})} \text{ x Relative Response Factor}}{\text{Peak area}_{(\text{I.S})} \text{ x } \text{conc}_{(\text{I.S})} \text{ x } \textit{Dilution factor}}\right)}{\text{sample weight}} \quad \text{.....equation (1)}$$

3.8 Statistical analysis

The effects of fungal endophyte on stachydrine concentration in the leaves were done by linear regression model using the lmtest package (version 0.9-40). Where "stachydrine" was the response variable and "fungal gDNA abundance" was the explanatory variable (lm(stachydrine ~ fungal gDNA abundance)). The weight gain of the herbivores was calculated as ($mg(t_x)$ – $mg(t_0)$), where t_x = timepoint 2 (day6) and t_0 = timepoint 0 (day 1), followed by testing the effects using Generalized least squares regression models (GLS) using the package nlme (version 3.1-163). The data was log10 transformed. Where, "log10(delta +1)" was the response variable, "time_point" and "endophyte", and "time_point x endophyte" interaction was the explanatory variable (gls(log10(delta +1) ~ time_point + endophyte)). The model assumptions were checked using the package ggResidpanel (version 0.3.0). The effects of endophyte and gender on the weights of pupae and adults of *L. dispar* were done by performing two-way ANOVA. To test the difference between the weights of pupae for *C. tremulae* a One-way ANOVA was applied. To analyse the time taken by caterpillars and the larvae to enter pupation, and the gender ratio of the caterpillars were analysed by the Mann-Whitney U test. To analyse the individual phenolic compounds and the sum of salicinoids and flavonoids in the two treatments, One-way ANOVA was used. The data was checked for normal distribution by the Shapiro-Wilk test, when the assumptions for normality were not met, the Kruskal-Wallis test was applied. All the data was evaluated in R version 2023.12.0 +369, © Posit Software, PBC. All the figures were created using the ggplot2 package (version 3.4.4). To tidy up the graphs ggeffects package (version 1.3.1) and package tidyverse (version 2.0.0) were used whenever required.

4 Results

4.1 Abundance of fungal endophyte and alkaloid Stachydrine in the black poplar trees without herbivory

Approximately 10±12 days after endophyte inoculation, the successful inoculation was confirmed by the presence of brownish fungal growth on the leaves of fungal endophyte-inoculated plants. The genomic DNA (gDNA) of *C. cladosporioides* was quantified by qPCR and normalised to plant gDNA of black poplar. The comparison between the two treatments was done by One-way ANOVA (F =93.054, ***p = <0.001, df =1).

Alkaloid Stachydrine analysis

Quantification of the amount of the alkaloid in the leaves of the control and endophyte-inoculated black poplar was done with the help of HPLC-UV. The data was analysed using One-way ANOVA. The effects of endophyte on the stachydrine were tested by linear regression (F =34.78, ***p = <0.05, R^2 = 0.68), mean ± SEM (n =3), where n is the number of trees per treatment **(Fig 6).**

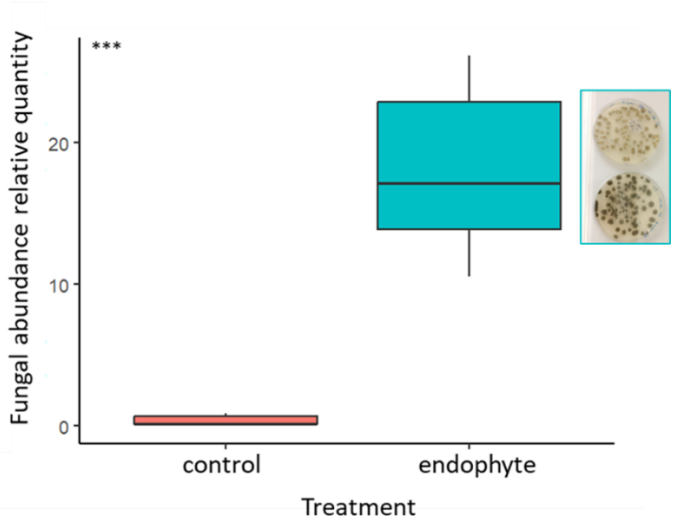

Figure 5: Fungal endophyte abundance in black poplar leaves of control (c) and fungal endophyte (e) treatments. The leaves were inoculated with either a fungal spore solution or a control solution. The leaves were sampled at 42 days after endophyte inoculation, from control (c) and endophyte (e) treated black poplar trees without midribs. The abundance of gDNA of *C. cladosporioides* is shown as relative abundance compared to plant gDNA. The data is shown as Mean ±SE (n = 3), where n = number of trees per treatment. The data was analysed by One-way ANOVA (F = 93.054, ***p = <0.001, df = 1), significant differences between the two treatments are marked asterisks (*** p <0.001) at the top left corner.

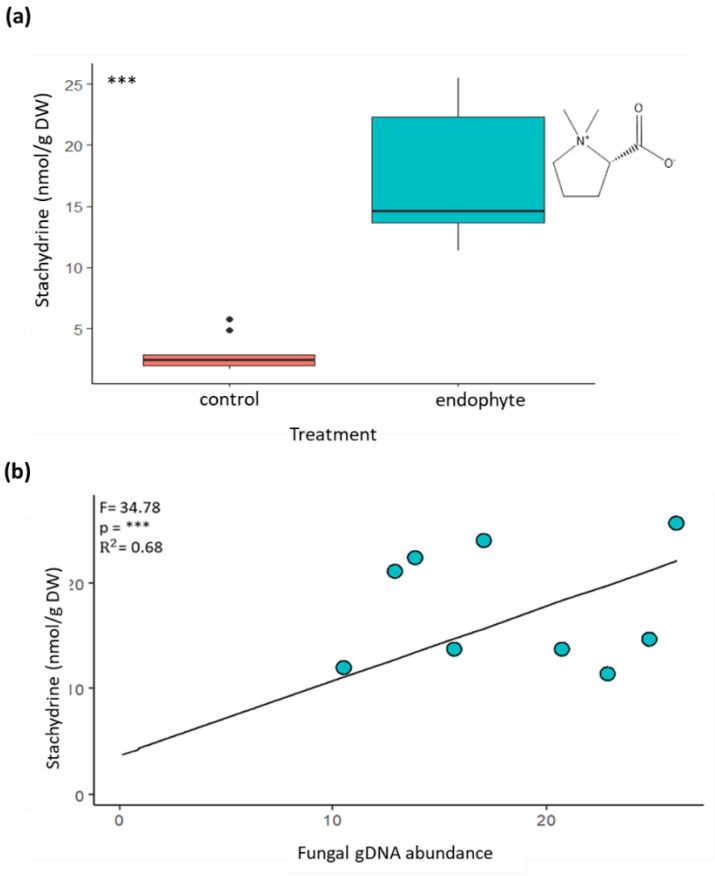

Figure 6*:* **Abundance of alkaloid Stachydrine. (a)** The amount of the alkaloid was quantified using HPLC-UV, and the leaves were harvested from the control and endophyte-inoculated Black poplar plants after 42 days after endophyte inoculation. The leaves inoculated with the endophyte show the amount of stachydrine in comparison with the data of the control plants, which contain very few traces of the alkaloid. This can be due to the possibility of cross-contamination. The difference was analysed using One-Way ANOVA (F =58.28, ***p = < 0.05, df = 1). **(b)** The effects of fungal endophyte on stachydrine were analysed using linear regression (F = 34.78, p = < 0.05, R^2 = 0.68), mean ±SEM (n =3), where n = number of trees. The significance is marked in the upper left corner.

4.2 Performance assay of the insect herbivores

4.2.1 Abundance of fungal endophyte and alkaloid stachydrine in the black poplar trees with herbivory

The herbivory treated i.e., control + herbivory (c+h) and endophyte + herbivory (e+h) black poplar leaves were harvested when the trees were damaged 70% by herbivory. Once the leaves were harvested, the herbivores were given a new poplar tree to feed. Therefore, the leaves harvest was done on different days to quantify the fungal endophyte abundance for *L. dispar* and *C. tremulae*. The genomic DNA (gDNA) of *C. cladosporioides* was quantified by qPCR and normalised to plant gDNA of Black poplar. To quantify the fungal endophyte abundance in the trees that were fed to the generalist herbivore *L. dispar*, the leaves harvest was done on

24, 30, 39, 42, 50 and 54 days after endophyte inoculation. The Kruskal-Wallis test was performed to determine the different loads of endophyte abundance between the (c+h) and (e+h) treated Black poplar trees that were fed to *L. dispar*. Data shows mean ± SEM (n = 6), where n is the number of trees per treatment. For the trees that were fed specialist herbivore *C. tremulae*, the leaves harvest was on 21, 24 and 39 days after endophyte inoculation. One-way ANOVA was performed to determine the different loads of endophyte abundance between the two treatments (c+h) and (e+h) respectively. Data shows mean ± SE (n = 3), where n is the number of trees per treatment.

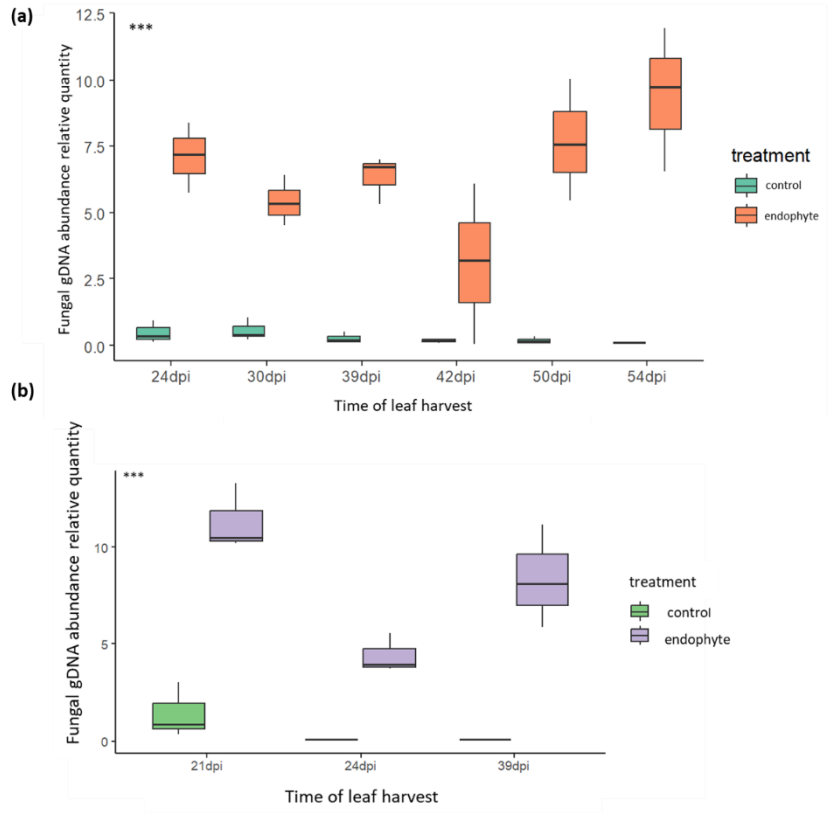

Figure 7: Fungal endophyte abundance in black poplar leaves of control + herbivory (c+h) and endophyte + herbivory (e+h) treatment harvested on different days after endophyte inoculation. The abundance of gDNA of *C. cladosporioides* is shown as relative abundance compared to plant gDNA. **(a)** Data shown here is from the trees that were fed to *L. dispar*. The leaves were harvested on 24, 30, 39, 42, 50 and 54 days after endophyte inoculation respectively. The results of the Kruskal-Wallis test with (X^2= 20.75, p = <0.001, df = 1) are presented with significant differences between the two treatments marked asterisks (*** p <0.001) at the top left corner, mean ±SE (n =6). **(b)** Data shown here is from the trees fed to *C tremulae.* The leaves were harvested at 21, 24 and 39 days after endophyte inoculation. The results of One-Way ANOVA are presented with (F =39.9, ***p = <0.001, df = 1). The significant differences between the two treatments are marked asterisks (*** p <0.001) at the top left corner, mean ±SE (n =3).

The variation in the relative fungal abundance in the leaves harvested from the endophyte + herbivory (e+h) treated black poplar trees for both the insect herbivores was determined by

One-way ANOVA, followed by Tukey's posthoc test. In the case of trees fed to *L dispar*, the amount of fungal endophyte abundance differed between the leaves harvested on 42 and 54 days after endophyte inoculation respectively (F = 3.31, * p = <0.05, df = 12), mean ± SEM (n = 6), where n is the number of trees per treatment. For the trees fed to *C tremulae*, a marginal difference was observed between the amount of fungal endophyte abundance between 24 and 39 days after endophyte inoculation and a significant difference was observed between 21 and 24 days after endophyte inoculation, respectively, (F = 9.83, ** p = <0.01, df = 2), mean ± SE (n = 3), where n is the number of trees per treatment.

Alkaloid Stachydrine analysis

For the quantification of the amount of stachydrine in the trees subjected to herbivory, the leaf harvest was done on 24, 30, 39, 42, 50 and 54 days after endophyte inoculation for *L. dipsar* and 21, 24 and 39 days after endophyte inoculation for *C. tremulae*. The data was analysed using one-way ANOVA.

Table 1: Statistical results of a One-way ANOVA for quantifying the amount of Stachydrine (nmol/g DW) in the black poplar with herbivory. For the trees in the case of *L. dispar*, the leaf harvested was done on 24, 30, 39, 42, 50 and 54 days after endophyte inoculation, mean ± SEM (n =6), where n is the number of trees per treatment, and in the case of *C. tremulae* the leaf harvest was done on 21, 24, and 39 days after endophyte inoculation, mean ± SEM (n= 3), where n is the number of trees per treatment.

Insect herbivore	control	endophyte	df	F-value	p-value
L. dispar	6.36 ± 1.04	15.25 ± 1.47	1	24.10	**< 0.05**
C. tremulae	19.71 ± 11.74	44.52 ± 8.23	1	11.53	**0.003**

(* p < 0.05; **p < 0.01; ***p <0.001; n.s = non-significant).

4.2.2 Performance of the herbivores on the control and endophyte-treated black poplar trees

To investigate the performance of both the herbivores on the control (c+h) and endophyte-inoculated (e+h) black poplar trees, various parameters like weight gain, larval period (time until pupation), pupal weight, adult weight, and sex ratio were determined. To test the effects of endophyte on the performance of caterpillars of *L. dispar* and larvae of *C. tremulae*, the data of both were analysed using a GLS regression using the factors "time", "endophyte" and "time

x endophyte" interaction. For the caterpillars, the weight gain was calculated from 15 days after endophyte inoculation till the caterpillars reached instar four, mean ± SEM (n =7-9), where seven is the number of caterpillars feeding on the endophyte-inoculated black poplar tree and nine is the number of caterpillars feeding on the control black poplar tree. For the beetle larvae, the weight gain is calculated from 15 days after endophyte inoculation till 27 days after endophyte inoculation, mean ± SEM (n = 10), where ten is the number of larvae feeding on control and endophyte-inoculated black poplar trees **(Fig 8).** The statistical results are represented in **(Table 2)**.

Table 2: Results of GLS regression analysis on the performance of caterpillars of *L. dispar* and larvae of *C. tremulae* on the black poplar plants inoculated with a control solution or an endophyte solution. The weight gain of the caterpillars is considered for analysis from 15 days after endophyte inoculation till the caterpillars reached instar four, mean ± SEM (n =7-9). The weights of the beetle larvae were considered for analysis from 15 days after endophyte inoculation till 27 days after endophyte inoculation, mean ± SEM (n = 10).

Insect herbivore	**Factor**	**df**	**F-value**	**p-value**
L.dispar	time	**1**	721.12	**<.0001**
	endophyte	**1**	22.35	**<.0001**
	time x endophyte	**1**	0.65	0.420
C.tremulae	time	**1**	2091.58	**<.0001**
	endophyte	**1**	16.37	**<.0001**
	time x endophyte	**1**	0.90	0.344

(* p < 0.05; **p < 0.01; ***p <0.001; n.s = non-significant).

The caterpillars feeding on the control tree took a period of 48 ± 60 days after endophyte inoculation to enter the pupation stage (n = 9), while those feeding on the fungal endophyte-inoculated tree took 56 ± 64 days after endophyte inoculation for the same (n = 7). The time taken by the caterpillars to enter the pupation stage (time until pupation) while feeding on control and endophyte-inoculated black poplar trees was analysed by the Mann-Whitney U-test (W = 7.5, p = 0.010), mean ± SEM (n =7-9), where seven is the number of caterpillars feeding on the endophyte inoculated black poplar trees and nine is the number of caterpillars feeding on the control black poplar trees. The weights of the pupae and adults of *L. dispar* in both treatments were analysed by a Two-way ANOVA with "weight" being the response variable,

"gender" and" endophyte" being the explanatory variables along with their interaction, mean ± SEM (n =7-9).

Table 3: Results of two-way ANOVA of the pupal and adult weight of *L. dispar* feeding on control and endophyte inoculated Black poplar trees, with "weight" being the response variable, "gender" and" endophyte" being the explanatory variable along with their interaction, mean ± SEM (n =7-9).

	Factor	df	F-value	p-value
Pupae	gender	1	16.05	**0.001**
	endophyte	1	0.24	0.630
	gender x endophyte	1	0.006	0.937
Adult	gender	1	67.41	**< 0.001**
	endophyte	1	0.13	0.723
	gender x endophyte	1	0.00	0.993

(* p < 0.05; **p < 0.01; ***p <0.001; n.s = non-significant).

Out of nine caterpillars feeding on the control tree (c+h), seven were males and two were females. Out of seven caterpillars feeding on the endophyte-inoculated tree (e+h), six were females and only one was male. Due to the unbalanced sex ratio, the difference between the ratio of the two treatments control and endophyte was evaluated using the Mann-Whitney U-test (W = 51.5, p = < 0.017), mean ± SEM (n = 7-9). The larvae of beetles *C. tremulae* took a period of 30 ± 39 days after endophyte inoculation to enter the pupation stage in both treatments (c+h) and (e+h), respectively. The time taken by the beetles to enter the pupation stage (time until pupation) while feeding on control and endophyte-inoculated black poplar trees was analysed by the Mann-Whitney U-test (W= 46.5, p = 0764). The weights of the pupae of beetles *C. tremulae* however did not show differences in their weights when reared on the control and endophyte-inoculated trees. The data was analysed by One-way ANOVA (F =1.10, p = 0.308, df = 1), mean ± SEM (n =10), where ten is the number of pupae on control and endophyte inoculated black poplar trees **(Fig 9)**

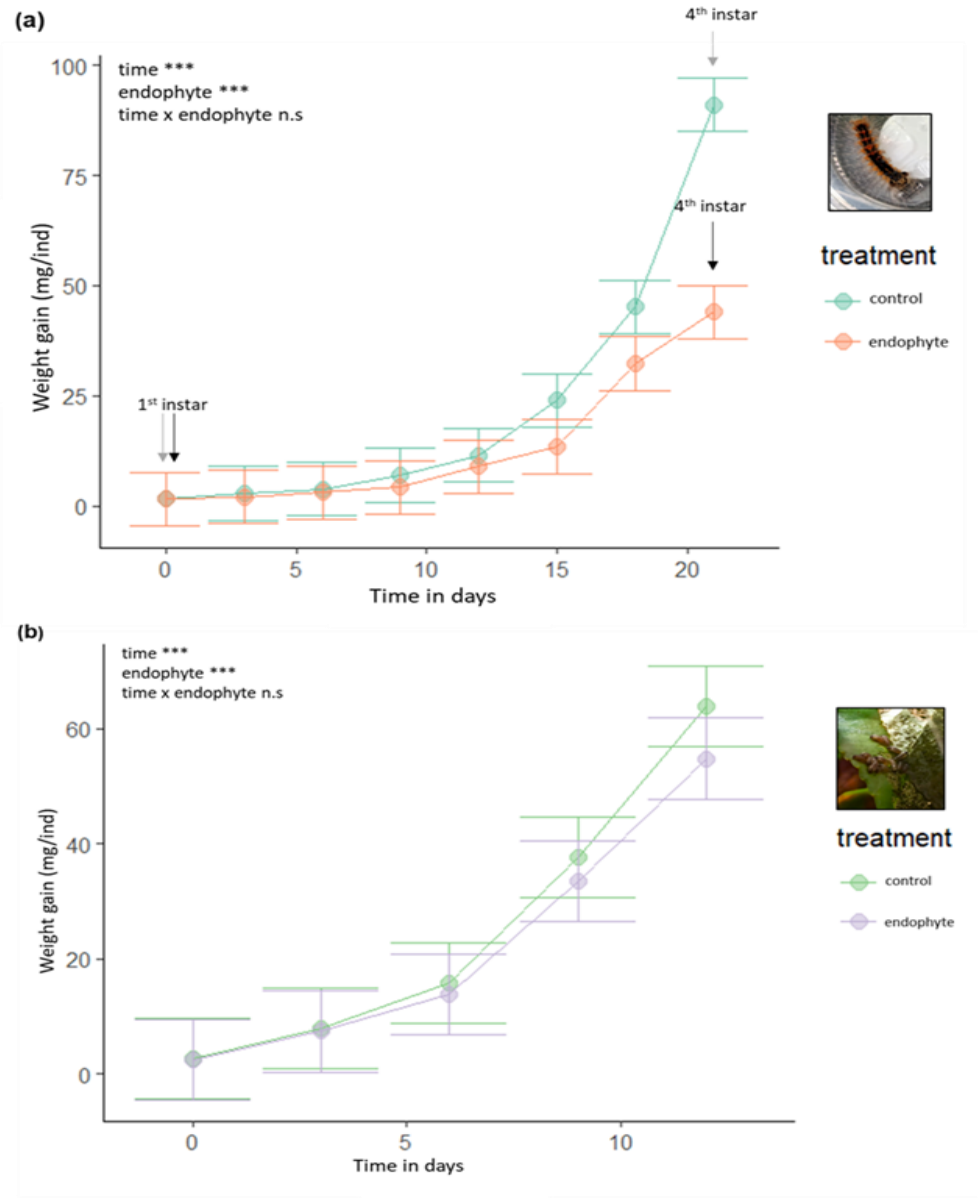

Figure 8: Performance of insect herbivores on control and endophyte-inoculated black poplar trees. (a) Performance of the caterpillars (*L. dispar*) on control (green line) and fungal endophyte (orange line) inoculated black poplar trees. The two-day-old caterpillars were placed on the control and endophyte-inoculated trees 15 days after endophyte inoculation. The weight of each caterpillar was recorded every three days. Data are shown from the onset of the performance experiment till caterpillars were in the 4th instar. The grey and black arrow indicates the caterpillar's instar state (depending on their weight gain) on both the control and endophyte inoculated trees, mean ± SEM (n = 7-9). Results of the GLS model are given in the top left corner for the factors of "time", "endophyte" and their interaction. **(b)** Performance of the larvae of *C. tremulae* on control (green line) and fungal endophyte (purple line) inoculated trees. The weights of each larva were recorded every three days. Data are shown from the onset of the performance experiment till 27 days after endophyte inoculation, mean ± SEM (n =10). Results of the GLS model are given in the top left corner for the factors of "time", "endophyte" and their interaction. For both graphs (a) and (b), the x-axis represents time in days from the start of the performance experiment, while the y-axis represents the weight gain in mg per individual. (* $p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s = non-significant).

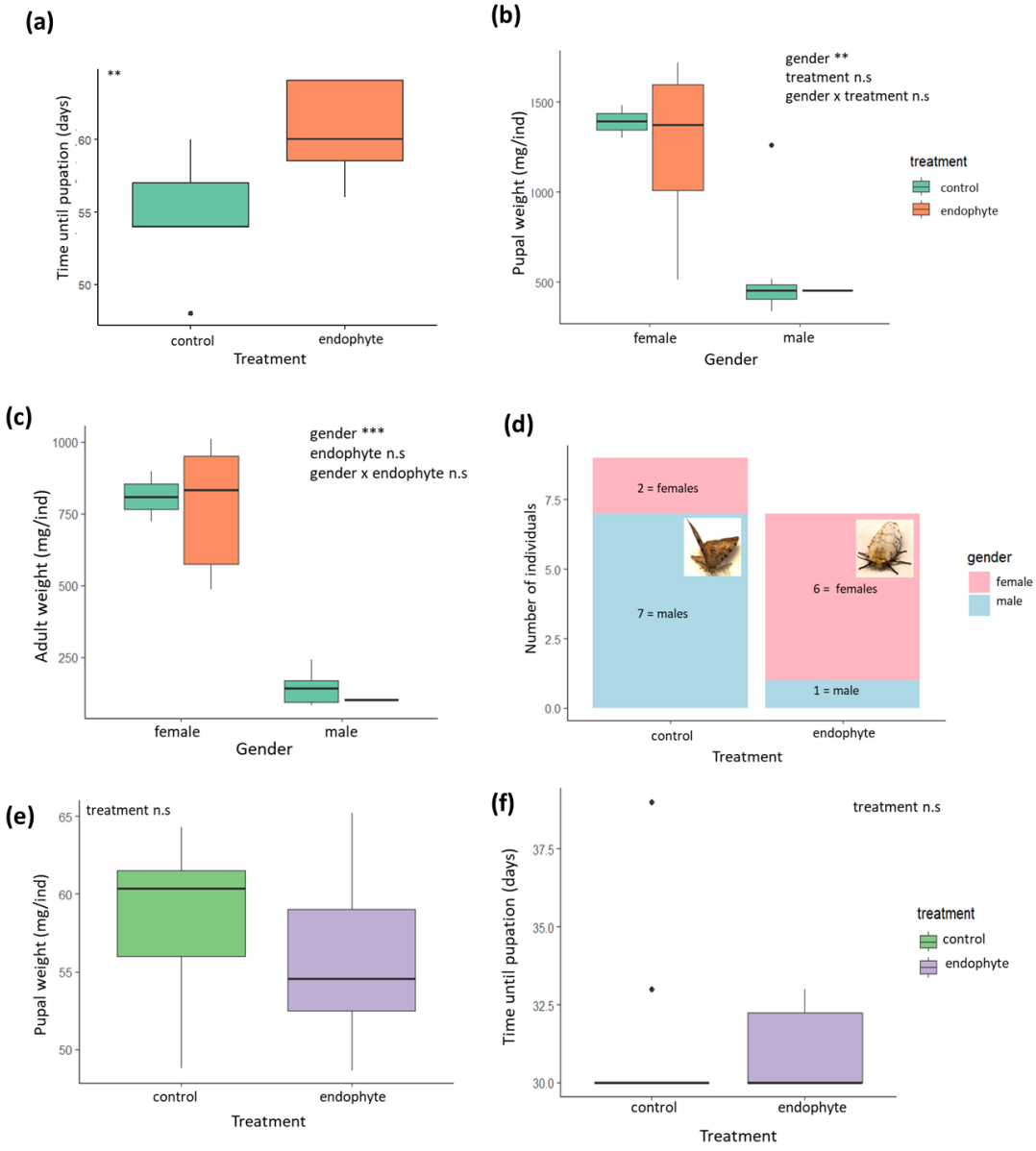

Figure 9: Development of caterpillars and larvae feeding on the control and endophyte-inoculated black poplar trees. (a) Time until pupation of caterpillars on both treatments. The results shown are from the start of the experiment till the caterpillar entered the pupation stage. The results were analysed using the Mann-Whitney U-test, significant difference is marked as asterisks at the top left corner (W =7.5, p = <0.01). **(b)** Pupal weights and **(c)** Adult weights of caterpillars in both treatments, the results were analysed using Two-way ANOVA with factors of "gender", and "endophyte" and their interaction. The weight was measured in mg/individual. The significant differences are given at the top right (* p < 0.05; **p < 0.01; ***p <0.001; n.s = non-significant). **(d)** Sex ratio of *L. dispar*. The sex ratio was analysed using the Mann-Whitney-U test (W =51.5, p = < 0.01), mean ±SEM (n =7-9), and the stacked bar graph shows the proportion of males and females in the two treatments. **(e)** Pupal weights of beetles in both treatments were measured in mg/ individual. The data was analysed by ANOVA (F= 1.10, p = n.s, df =1), mean ±SEM (n =10) and the significance is marked at the top left corner. **(f)** Time until pupation of the larvae of *C. tremulae,* the data was analyzed by the Mann-Whitney U test (W =46.5, p = n.s), the significance is marked at the top right corner.

However, after hatching into adults, the *C. tremulae* beetles were kept for sexing by keeping the emerging adults (reared on control and endophyte-inoculated trees) into separate boxes which were perforated for the airflow along with poplar leaves to serve the purpose of food. The adults were colour-coded by using paint and paintbrush. In due course of time, the beetles started losing their colour code which made it difficult to determine their gender.

4.3 Phenolic compounds of black poplar

To analyse the levels of phenolic compounds in the leaves of black poplar after the endophyte inoculation and herbivory attack, the leaves were collected from trees with control treatment (c), endophyte treatment (e), and from the trees that were subjected to herbivory treatment i.e., (c+h) and (e+h) respectively. The (c) and (e) treated trees were harvested after 42 days after endophyte inoculation, while the leaves from the trees subjected to herbivory were harvested when the trees received 70% of the leaf damage. The phenolic compounds analysed were Salicinoids (comprising salicin, salicortin, homaloside D, nigracin and 6'-o-benzoylsalicortin) and flavan-3-ols (catechin and its dimer proanthocyanidin B1).

Among the Salicinoids for the trees without herbivory (c) and (e), quantitatively abundant, Salicortin amounted to 41.40 ±1.67 mg/g DW in the control plants (c) and 40.84 ± 1.90 mg/g DW in the endophyte inoculated trees (e), and homaloside D amount to 21.65 ± 0.95 mg/g DW in the control trees (c) and 21.39 ± 1.34 mg/g DW in the endophyte inoculated trees (e). Both of these salicinoids showed an increased trend in trees with control + herbivory (c+h) and endophyte + herbivory (e+h). The quantitatively less abundant salicinoids; salicin and nigracin showed an increased trend in trees with endophyte + herbivory (e+h) in the case of *L. dispar* and *C tremulae,* respectively. However, in the case of trees that were fed to the larvae of *C tremuale,* the abundance of salicin increased with marginal significance ($F = 4.19$, $p = 0.057$, $df = 1$). The quantitatively less abundant 6'-o-benzoylsalicortin was slightly more in the control trees (c) in comparison with the (e) endophyte-inoculated trees. In the case of trees with herbivory (c+h) and (e+h), the 6'-o-benzoylsalicortin showed an increased trend, specifically, in the trees fed to *L. dispar,* the quantity was slightly higher in endophyte + herbivory treated trees (e+h) than in control +herbivory trees plants (c+h).

In the case of flavan-3-ols, proanthocyanidin B1 (PAB1) and catechin were quantitatively lesser in the plants without herbivory i.e., in control (c) and endophyte inoculated (e) plants.

Table 4: Statistical results of One-way ANOVA or Kruskal Wallis test of the phenolic compounds from the leaves harvested from control (c) and endophyte (e) black poplar trees (i.e., trees without herbivory). The leaves were harvested 42 days after endophyte inoculation. Mean ± SEM (n =3), where n is the number of trees per treatment.

Phenolic compounds (mg/g DW)	**control (c)**	**endophyte (e)**	**F/X^2- value**	**p-value**
Salicin	2.78 ± 0.14	2.75 ± 0.18	0.01	0.903
Salicortin	41.40 ± 1.67	40.84 ± 1.90	0.04	0.828
Homaloside D	21.65 ± 0.95	21.39 ± 1.34	0.63	0.426
Nigracin	0.98 ± 0.06	0.96 ± 0.08	0.05	0.811
6'-o-benzoylsalicortin	0.84 ± 0.14	0.79 ± 0.10	0.01	0.894
PAB1	0.32 ± 0.01	0.30 ± 0.01	3.17	0.070
Catechin	0.41 ± 0.01	0.42 ± 0.02	0.18	0.675

Abbreviation: "=" p values from Kruskal Wallis test

Table 5: Statistical results of One-way ANOVA or Kruskal Wallis test of the phenolic compounds from the leaves harvested from control (c+h) and endophyte (e+h) inoculated black poplar trees that were fed *to L. dispar* (i.e., trees with herbivory). The leaves were harvested when the trees received 70% of the leaf damage. Mean ± SEM (n =6), where n is the number of trees per treatment in the case of *L. dispar.*

Phenolic compounds (mg/g DW)	**control**	**endophyte**	**F/X^2- value**	**p-value**
Salicin	4.19 ± 0.15	4.12 ± 0.24	0.05	0.815
Salicortin	62.44 ± 4.44	69.99 ± 5.06	0.67	=0.410
Homaloside D	32.36 ± 2.10	37.28 ± 2.76	1.99	0.166
Nigracin	1.37 ± 0.10	1.33 ± 0.11	0.02	=0.874
6'-o-benzoylsalicortin	9.48 ± 2.23	13.95 ± 3.14	1.22	=0.268
PAB1	0.49 ± 0.02	0.53 ± 0.03	1.22	0.276
Catechin	0.67 ± 0.05	0.66 ± 0.08	0.00	0.923

Abbreviation: "=" p values from Kruskal Wallis test

Although not significant the quantity of both the flavan-3-ols showed an increased trend upon herbivory by *C. tremulae*, specifically in trees treated with endophyte (e+h). For *L dispar*, the levels of catechin did not differ in (c+h) and (e+h) treated trees respectively.

Table 6: Statistical results of One-way ANOVA or Kruskal Wallis test of the phenolic compounds from the leaves harvested from control (c+h) and endophyte (e+h) inoculated black poplar trees that were fed *to C. tremulae* (i.e., trees with herbivory). The leaves were harvested when the trees received 70% of the leaf damage. Mean ± SEM (n =3), where n is the number of trees per treatment in the case of *C. tremulae.*

Phenolic compounds (mg/g DW)	**control**	**endophyte**	**F/X^2- value**	**p-value**
Salicin	3.58 ± 0.13	4.05 ± 0.18	4.19	**0.057**
Salicortin	64.42 ± 4.46	63.39 ± 3.52	0.03	0.858
Homalosid D	32.86 ± 2.54	33.31 ± 1.97	0.01	0.891
Nigracin	1.41 ± 0.13	1.83 ± 0.45	0.28	=0.596
6'-o-benzoylsalicortin	10.95 ± 3.03	9.31 ± 2.00	0.01	=0.894
PAB1	0.65 ± 0.02	0.72 ± 0.05	1.50	0.237
Catechin	0.68 ± 0.05	0.71 ± 0.04	0.16	0.685

Abbreviation: "=" p values from Kruskal Wallis test

The sum of salicinoids and flavonoids did not differ significantly among the trees with and without herbivory. The data was evaluated using the One-Way ANOVA or Kruskal-Wallis test to determine the difference in the loads of phenolic compounds in the treatments **(Fig 10)**

5 Discussion

Studies focusing on herbivores' performance in association with fungi have been often done with mycorrhizal fungi (Koricheva et al., 2009), or in the grass-endophyte system (Sieber, 2007). The present study focused on the tree-endophyte interaction and how the performance of the herbivores *L. dispar* and *C. tremulae* is impacted in the presence of the fungal endophyte *C. cladosporioides* when inoculated in the young Black poplar trees.

Endophytes are known to live inside the host without causing any symptoms of disease (Crawford et al., 2010; Eberl et al., 2019). To achieve successful colonization, the endophytes have developed a technique by which they avoid reorganization from the plant's immune system (Collinge et al., 2022).

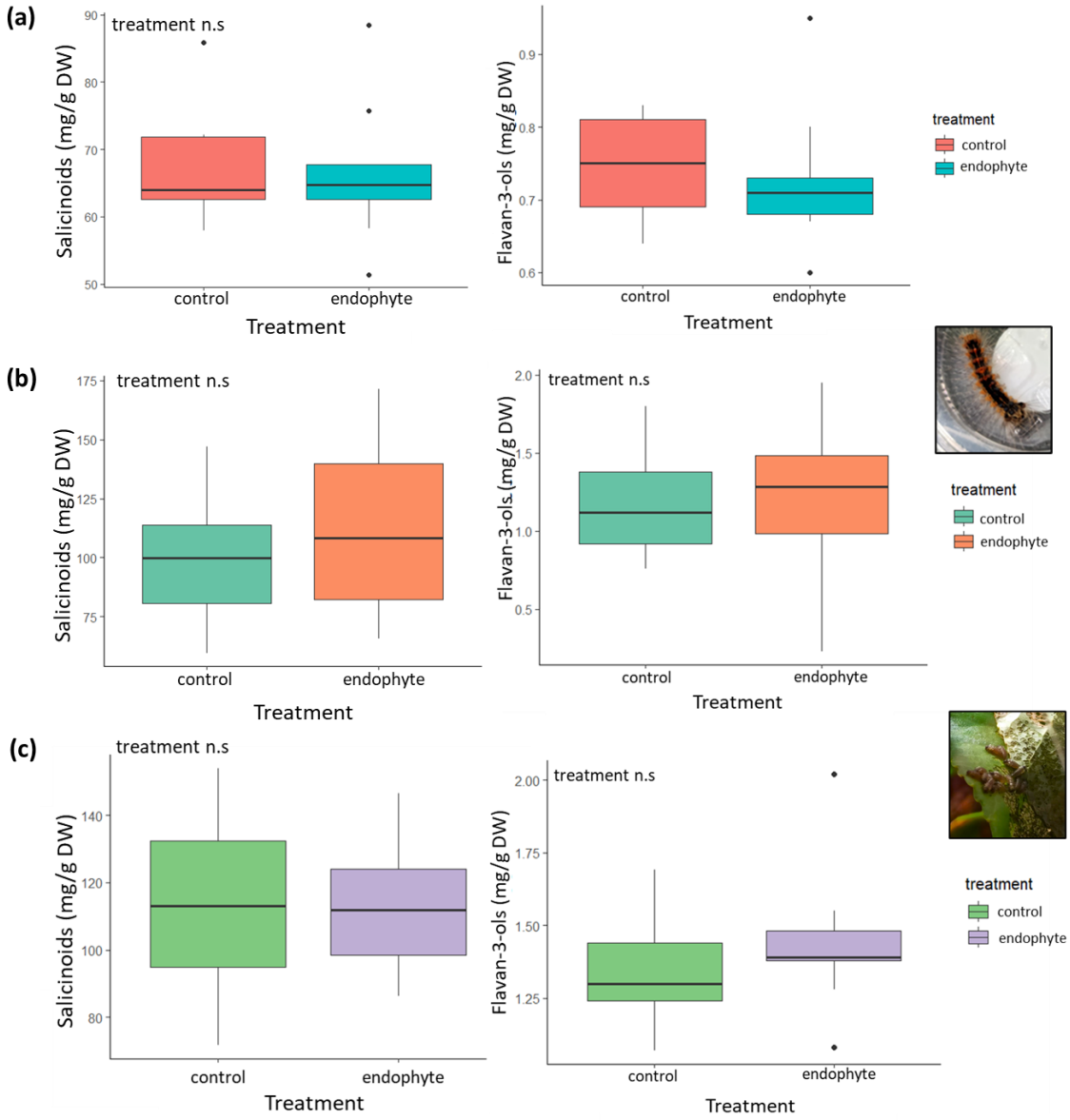

Figure 10: The difference between the sum of salicinoids (salicin, salicortin, homaloside D, nigracin and 6'-o-benzoylsalicortin) and flavan-3-ols (proanthocyanidin B1 and catechin) in the leaves of black poplar in all the treatments. The quantification was done on HPLC-UV **(a)** the sum of salicinoids and flavan-3-ols in the plants without herbivory, mean ±SEM (n =3), n = number pf trees per treatment. **(b)** the sum of salicinoids and flavan-3-ols for *L. dispar* in plants treated with (c+h) and (e+h), mean ± SEM (n =6), n = number pf trees per treatment. **(c)** the sum of salicinoids and flavan-3-ols for *C. tremulae* in plants treated with (c+h) and (e+h), mean ±SEM (n =3), n = number pf trees per treatment. One-way ANOVA or Kruskal-Wallis test was performed to determine the difference between the treatments. The significance is marked at the top left corner (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s = non-significant).

In the present study, I was able to observe the abundance of fungi in trees inoculated with endophytes with or without herbivory. In the trees that were subjected to herbivory, although the quantity of fungi showed variation without a specific direction as the days increased post-endophyte inoculation, the amount of fungal endophyte was still detectable in the trees that

were fed to both the herbivores **(Fig 7).** This indicates that the leaf spray method utilized for fungal endophyte inoculation was successful.

The presence of fungal endophyte affects the herbivore's performance

The endophytes affecting the performance of phloem feeders have been demonstrated more often when compared to the performance of leaf chewers (Hartley & Gange, 2009b) The findings of this project demonstrate that an endophytic fungus can affect the growth of the leaf-chewing herbivores. The caterpillar of *L dispar* and the larvae of the beetle *C tremulae*, gained more weight when feeding on the control trees compared to the individuals feeding on the endophyte-inoculated trees **(Fig 8)**. In addition, the caterpillars feeding on the control trees took less time to enter the pupation stage, while those feeding on the endophyte-inoculated plants took more time. This can be related to the fact that most of the caterpillars feeding on the control trees, emerged as males while those that fed on the endophyte trees emerged as females. The unbalanced sex ratio might explain the time the caterpillars take to enter the pupation stage. The nutritional stress received by the insects in their early developing stages hinders their development, fitness and reproduction (Kang et al., 2022). These effects of nutritional stress are often sex-specific and observed to affect females more than males, leading to female mortality (Teder & Kaasik, 2023). Therefore, in the present study, the unbalanced sex ratio raises a potential question of whether the presence and absence of the fungal endophyte might also influence the gender of the herbivores. However, the larvae of the specialist herbivore *C. tremulae* took almost similar days (30 ± 39 days after endophyte inoculation) to enter the pupation stage in control and endophyte-inoculated trees.

The sex ratio (male: female) and body mass of folivorous insects are often considered parameters that can be easily measured (Lukowski et al., 2015). For the generalist in my study, the pupal and adult weights did not differ across the two treatments. It is observed that females are generally heavier than males (Lukowski et al., 2015). This observation was also noticed in the present study. For the caterpillars, the pupae with heavier weights emerged as females, while those with comparatively lower weights emerged as males. The explainable reason for females to be heavier is that the females in the caterpillar state undergo six instar stages while the males undergo five instar stages (Orgel et al., 2020). Therefore, I can conclude that in the present study, the weights of the pupae and adults in the case of *L. dispar* are influenced by gender and not by the treatments **(Fig 9).** A similar observation was noticed in the case of specialist herbivore, where the larvae of *C. tremulae* gained less weight in their early developing stages. Still, the presence of *C. cladosporioides* did not impact late-stage development like the pupal

weight of the specialist herbivore. A detailed study is essential to examine whether the endophyte will impact the sex ratio and the oviposition in the case of the beetles. Similarly, a better methodology is needed to determine the gender of the beetles, as the colour coding of the beetles did not work quite efficiently in my study.

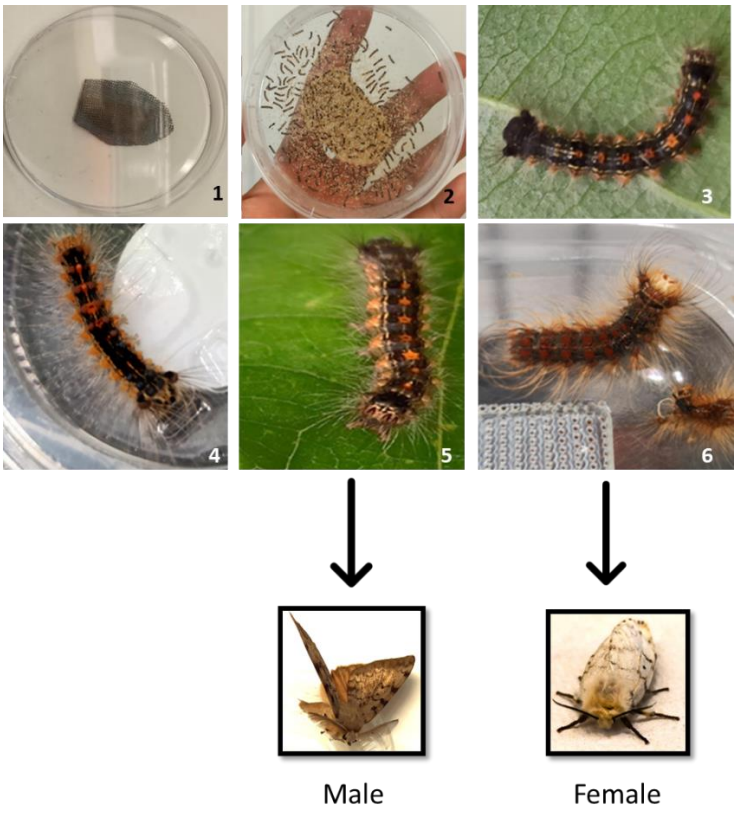

Figure 11: Different life stages of *L. dispar* **(1-6)**. **(1)** The egg batch of *L. dispar* in a petri dish. **(2)** Freshly hatched caterpillars from the egg batch. **(3)** Instar three caterpillar on the black poplar leaf. **(4)** Instar four caterpillar with orange colour hair. **(5)** Instar five caterpillar feeding on the black poplar leaf which emerges into the male (brown) and **(6)** Instar six caterpillar along with the exuviae (in the clip cage), with yellow head colour, along with yellow-orange hair on its body which emerges into the female (white).

The production and the role of Stachydrine

Colonization by endophytes can be beneficial to the plants, as the endophytes might offer protection to hosts against pathogen attack. One of the ways the endophytes protect their host is by producing naturally occurring bioactive compounds that can be toxic to herbivores (Vinale et al., 2017). The anti-herbivory role of secondary metabolites like alkaloids is often studied in the grass-endophyte system (Bastias et al., 2017; Faeth, 2002). In the present study, I quantified the amount of stachydrine, which was produced in comparatively larger amounts in trees inoculated with fungal endophytes. Apart from isolating the alkaloid from *C. cladosporioides*, it is also known to be isolated from plants like *Citrus*, *Chrysanthemum*,

Stachys and alfalfa (Murata et al., 2011). Stachydrine also has various medicinal applications, especially in the treatment of cardiovascular diseases and cancer (Cheng et al., 2020). From an ecological point of view, this alkaloid is known to act as an oviposition stimulant of *Papilio Xuthus* (Murata et al., 2011). The information on the role of stachydrine in plant-herbivore interaction is not yet known. However, in the current study, the presence of alkaloid stachydrine is seen to have a positive relation with the abundance of the endophyte *C. cladosporioides*. This indicates that the alkaloid is produced only by the fungal endophyte and not by the black poplar **(Fig 6).** Furthermore, if stachydrine is solely produced by the endophyte then we can speculate whether the presence or absence of this alkaloid is the reason why the generalist and the specialist herbivore gained less weight on the endophyte-inoculated black poplar trees.

Phenolic compounds of Black poplar do not increase significantly upon endophyte inoculation

In woody plants, Phenolics are considered one of the largest plant defence compounds (Boeckler et al., 2016). The main purpose of the phenolics production by members of Salicaceae is for anti-herbivore defence (Boeckler et al., 2013). Among *Salicaceae*, there are two main groups of phenolics: salicinoids (also known as phenolic glycosides) and flavan-3-ol ols (also known as condensed tannins, Boeckler et al., 2013). Studies investigating the role of tree-endophytic fungi acting in the indirect defence against herbivores are inconsistent (Eberl et al., 2019). In the present study, the overall salicinoids and flavonoids in Black poplar did not differ greatly upon endophyte inoculation. However, upon individual evaluation of the phenolic compounds, I could see an increase or decrease trend of certain compounds upon herbivory and also in the presence of fungal endophyte.

The production of salicinoids is known to deter the generalist herbivores, it has also been noticed to affect the larval performance (Boeckler et al., 2011; Philippe & Bohlmann, 2007). In the case of *L. dispar*, although not significantly, there was a trend noticed in the concentrations of salicortin, homaloside D, and the 6'-o-benzoylsalicortin were observed to be slightly higher in endophyte-treated plants after herbivory (e+h). It has been studied that, specialist herbivores such as leaf beetles are capable of converting salicin from their host into a salicylaldehyde, which they use for their defence against predators (Boeckler et al., 2011). In my study, I observed a marginal increase of salicin in the presence of endophyte coupled with herbivory rather than herbivory alone. While not significant, the nigracin concentrations also showed an increased trend in the presence of herbivory and endophyte (e+h). This finding indicates that certain salicinoids respond to herbivory and also the presence of endophytes like *C. cladosporioides* is of an advantage to black poplar.

The flavan-3-ols are known to deter the generalist herbivore and are also known to act as an anti-microbial agent (Philippe & Bohlmann, 2007; Ullah, Unsicker, et al., 2019). In the case of *L. dispar* and for the beetles *C. tremulae*., the flavan-3-ols, especially proanthocyanidin B1 (PAB1) showed an increased trend in the concentration in endophyte-treated plants upon herbivory, while the levels of catechin did not respond to the endophyte's presence. This indicates that not all phenolic compounds respond similarly to endophyte's presence and herbivory attack. In addition, the variation in the individual salicinoids observed in my findings can be because the salicinoids are differentially induced upon herbivory (Fabisch et al., 2019). It has been also studied that the abundance of phenolic compounds is also influenced by other factors like nutrients, water availability, genotype, season and plant ontogeny (Boeckler et al., 2011). Hence, further investigation is required to have a detailed account of how the phenolic compounds of black poplar are influenced by the presence of endophytes, and whether is there an involvement of biotic or abiotic factors is to be understood.

Conclusion and Perspective

In the present study, I was able to demonstrate that endophyte *C. cladosporioides* negatively affects the performance of the caterpillar and the beetle larvae. I also observed that in the presence of endophytes, the pupation period (time until pupation) of the generalist herbivore was delayed, while the presence of the endophyte did not affect the same in the case of specialist herbivore. I was also able to confirm that the endophyte produces the alkaloid stachydrine. However, the endophyte does not significantly change the phenolic compounds of black poplar but shows a slight variation in individual compounds along with the presence of herbivory. Although the sex ratio of *L. dispar* was unbalanced, it is important to verify if the endophyte plays a role in shaping the sex ratio in the case of the generalist herbivores. A better understanding of the alkaloid stachydrine's role is important to understand how the herbivore performance was affected by the endophyte. Furthermore, the exact effects of endophytes on the later-stage development parameters like the adult weight in the case of beetles, the number of eggs released by the females, and mortality rates in adults of both herbivores still need to be examined further for a better understanding of the fungal endophyte's role in tree-herbivore interaction. The results of this study could help to expand our knowledge that endophyte fungi can deter herbivores in a species-specific manner (e.g.: the time until pupation and pupal weight variation in the case of both the herbivore). From an ecological point of view, the study also highlights that the presence of this endophyte can benefit the trees, thus shedding light on the role of microbes as a biocontrol agent in the forest ecosystem.

6 Summary

Plants interact with various organisms in nature, during which they also have to deal with the herbivores. Plants have a variety of defence compounds to protect against herbivores, they also have mutual relationships with microorganisms that help them directly or indirectly to tackle herbivory. Microorganisms that live inside the plants without causing symptoms of disease are called as endophytes. Studies focusing on the grass-endophyte association and its influence on herbivory have been well documented. In contrast to this, the interaction of trees and their endophytes is less understood. To have a better understanding of the role the fungal endophyte plays in tree-herbivore interaction, the present study focused on determining the performance of a generalist *Lymantria dispar* and a specialist herbivore *Chrysomela tremulae* under the influence of common endophyte fungi *Cladosporium cladosporioides* in young black poplar trees *Populus nigra*. The experimental design consisted of control and endophyte treatment, with herbivory and without herbivory. A spore solution was prepared and inoculated on the black poplar trees, these plants were considered the endophyte-treated trees while a spore-free solution was inoculated on the remaining poplar trees, making them control-treated trees. Few trees from both treatments were utilized to rear the insect herbivores. The abundance of endophyte was quantified using quantitative real-time PCR. The quantification of alkaloid stachydrine was done using the HPLC/MS system, while the phenolic compounds were quantified with the help of HPLC-UV. The caterpillars and the beetle larvae feeding on the endophyte-treated trees gained significantly less weight as compared to those feeding on control trees. Additionally, the caterpillar also took more time to enter the pupation stage while feeding on endophyte-inoculated trees, resulting in an unbalanced sex ratio. The specialist herbivores showed no variation in the later-stage development across the two treatments. The results of regression analysis showed a positive effect of the abundance of fungi on the production of alkaloid stachydrine. The presence of endophyte in black poplars also showed an increased trend of certain phenolic compounds upon herbivory. These observations suggest that the endophyte produces alkaloid stachydrine, protects the plants by deterring the herbivore performance, and not significantly but causes minor changes in the phytochemistry upon herbivory attack. These results can further help to accept the role of endophytes as a biocontrol in forest ecosystems. However, a detailed study is essential to know the species-specific effect of this endophyte on herbivores and how its presence can help in elevating the defence compounds of the plants.

7 Zusammenfassung

Pflanzen interagieren in der Natur mit verschiedenen Organismen, wobei sie auch mit Pflanzenfressern zu tun haben. Pflanzen verfügen über eine Vielzahl von Abwehrstoffen, um sich gegen Pflanzenfresser zu schützen. Außerdem unterhalten sie wechselseitige Beziehungen zu Mikroorganismen, die sie direkt oder indirekt bei der Bekämpfung von Pflanzenfressern unterstützen. Mikroorganismen, die im Inneren der Pflanzen leben, ohne Krankheitssymptome zu verursachen, werden als Endophyten bezeichnet. Studien, die sich mit der Assoziation von Gräsern und Endophyten und deren Einfluss auf die Herbivorie befassen, sind gut dokumentiert. Im Gegensatz dazu ist die Interaktion zwischen Bäumen und ihren Endophyten weniger bekannt. Um die Rolle des Pilzendophyten bei der Interaktion zwischen Bäumen und Pflanzenfressern besser zu verstehen, konzentrierte sich die vorliegende Studie auf die Bestimmung der Leistung des Generalisten *Lymantria dispar* und des spezialisierten Pflanzenfressers *Chrysomela tremulae* unter dem Einfluss des weit verbreiteten Endophytenpilzes *Cladosporium cladosporioides* in jungen Schwarzpappelpflanzen *Populus nigra*. Die Versuchsanordnung bestand aus einer Kontroll- und einer Endophytenbehandlung, mit und ohne Herbivorie. Eine Sporenlösung wurde zubereitet und auf die Schwarzpappelpflanzen geimpft; diese Pflanzen wurden als endophytenbehandelte Pflanzen betrachtet, während die übrigen Pappelpflanzen mit einer sporenfreien Lösung geimpft wurden, was sie zu kontrollbehandelten Pflanzen machte. Nur wenige Pflanzen aus beiden Behandlungen wurden zur Aufzucht der Insektenherbivoren verwendet. Die Häufigkeit der Endophyten wurde mittels quantitativer Echtzeit-PCR quantifiziert. Die Quantifizierung des Alkaloids Stachydrin erfolgte mit dem HPLC/MS-System, während die phenolischen Verbindungen mit Hilfe von HPLC-UV quantifiziert wurden. Die Raupen und Käferlarven, die sich von den mit Endophyten behandelten Pflanzen ernährten, nahmen im Vergleich zu denen, die sich von den Kontrollpflanzen ernährten, deutlich weniger Gewicht zu. Darüber hinaus brauchte die Raupe auch mehr Zeit, um in das Verpuppungsstadium einzutreten, während sie sich von mit Endophyten geimpften Pflanzen ernährte, was zu einem unausgewogenen Geschlechterverhältnis führte. Bei den spezialisierten Pflanzenfressern gab es keine Unterschiede in der Entwicklung der späteren Stadien zwischen den beiden Behandlungen. Die Regressionsanalyse ergab eine positive Auswirkung der Fülle der Pilze auf die Produktion des Alkaloids Stachydrin. Das Vorhandensein von Endophyten in Schwarzpappelpflanzen zeigte auch einen erhöhten Trend bestimmter phenolischer Verbindungen bei Herbivorie. Diese Beobachtungen deuten darauf hin, dass der Endophyt das Alkaloid Stachydrin produziert, die

Pflanzen durch Abschreckung der Herbivoren schützt und bei Herbivorenangriffen nur geringfügige Veränderungen in der Phytochemie verursacht. Diese Ergebnisse können dazu beitragen, die Rolle von Endophyten als Biokontrollmittel in Waldökosystemen zu akzeptieren. Eine detaillierte Studie ist jedoch unerlässlich, um die artenspezifische Wirkung dieses Endophyten auf Pflanzenfresser zu ermitteln und um herauszufinden, wie seine Anwesenheit dazu beitragen kann, die Abwehrstoffe der Pflanzen zu erhöhen.

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Appendix

Table A1: Spore germination test of C. *cladosporioides* was done to ensure the successful preparation of the endophyte spore solution and to determine the germination rate of the spores. Therefore, the remaining endophyte spore solution was diluted to 100 spores/ mL and poured into the four Petri dishes containing autoclaved PDA media. These Petri dishes were kept in the incubator at 25° C.

Number of replicates	Germinated spores
1	100
2	97
3	107
4	102

Table A2: Primers used in the current study

Name	fw sequence	rev sequence	Reference
Actin2	CCCATTGAGCAGGTATTGT	TACGACCACTGGCATACAGG	Walther 2021 and the references therein "in press." Walther et al., (2024)
CladoITS	TGTTCGAGCGTCATTTCACC	CGCTTAGGGGACAGAAGACC	"In press." Walther et al., (2024)

Abbreviation: fw= forward, rev= reverse

Figure A1: Growth of the endophyte (Brownish mycelium marked by white circle) on the leaves of black poplar noticed 10±12 days after endophyte inoculation.

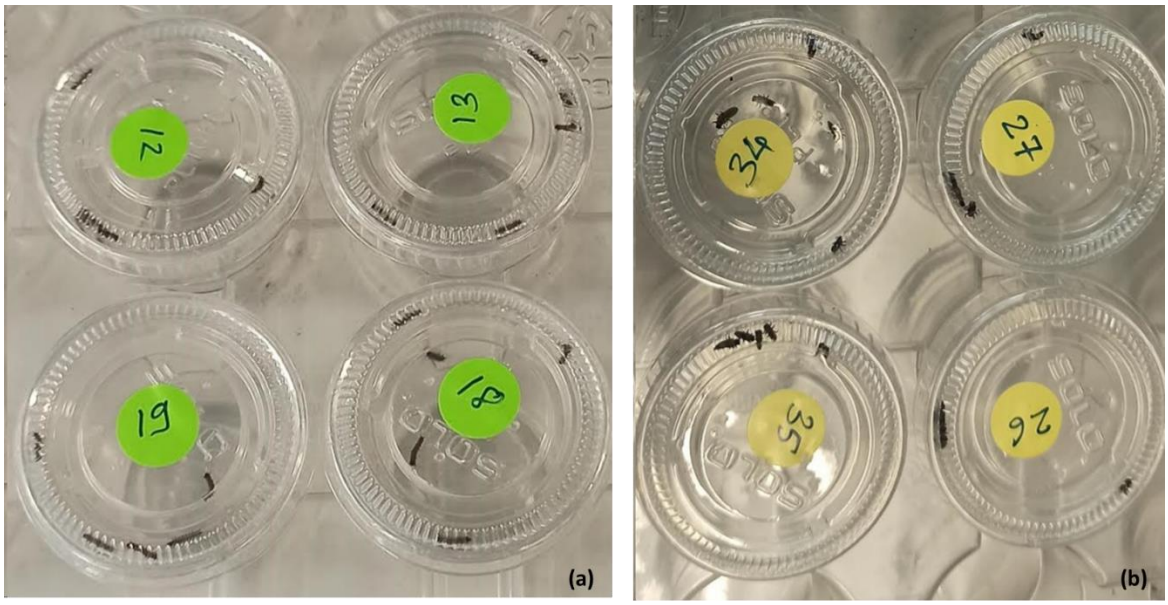


Figure A2: The herbivores in the labelled solo cups (five in each) before being placed on the black poplar trees inoculated with control or endophyte solution. **(a)** Two-day-old caterpillars in the solo cups and **(b)** Newly hatched beetles in the solo cups. Both the herbivores were carefully transferred into the solo cups by using a size zero paint brush or with entomological tweezers.

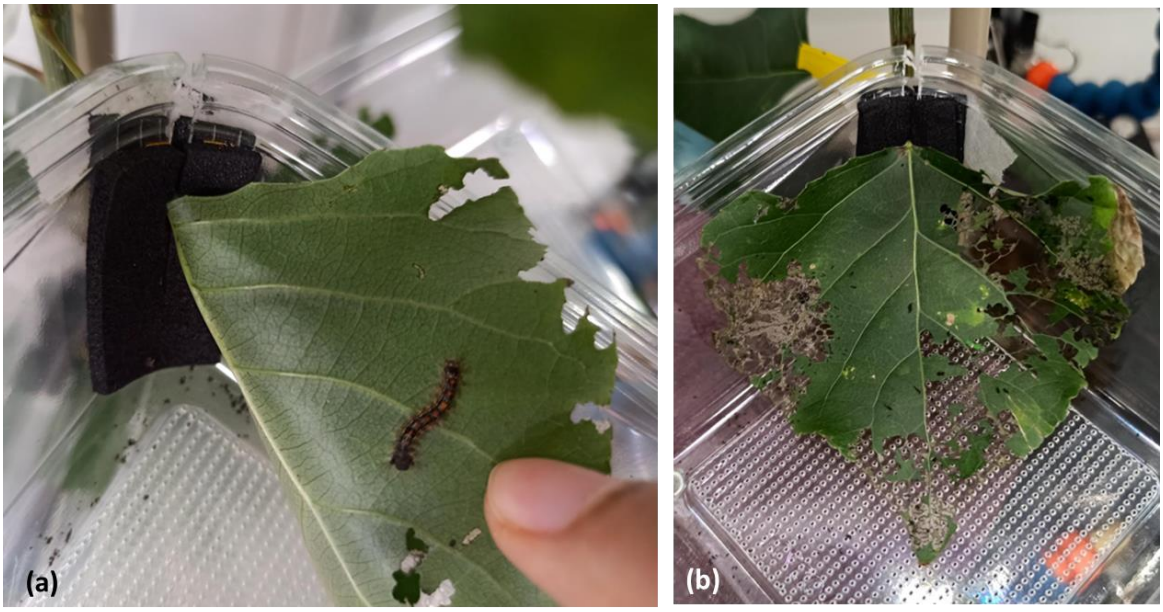


Figure A3: The damaged leaf in the clip cage while still being attached to the black poplar trees. **(a)** Chewing/feeding pattern of the (instar three) caterpillar. **(b)** Chewing/feeding pattern of the beetles.

Table A3: Weights (mg/ind) of pupae and adults (males and females) of *L. dispar* feeding on control and endophyte-inoculated black poplar trees. Data presented as mean± SEM (n =7-9), where seven is the number of caterpillars feeding on the endophyte-inoculated trees and nine is the number of caterpillars feeding on the control trees.

Life stage	Males feeding on control trees	Males feeding on endophyte-inoculated trees	Females feeding on control trees	Females feeding on endophyte-inoculated trees
pupae	548.09 ± 120.73	453.04	1391.49 ± 91	1256.12 ± 188.80
adult	140.26 ± 21.65	109.09	809.88 ± 88.06	773.38 ± 93.60

Table A4: Weights (mg/ind) of pupae beetles *C.tremulae* feeding on control and endophyte-inoculated black poplar trees. Data presented as mean± SEM (n =10), where ten is the number of pupae on control and endophyte-inoculated black poplar trees.

Pupal weight of beetles (mg/ind)	Control trees	Endophyte inoculated trees
	58.02 ± 1.65	55.55 ± 1.66

Table A6: Parameters used in HPLC-UV analysis of phenolic compounds quantified from the leaves harvested control and endophyte inoculated black poplar trees with and without herbivory. Using the relative peak area of the internal standard phenyl-β-glucopyranoside, the analytes were quantified according to the equation (1).

Analyte	RT (in min)	Wavelength	RRF
IS	6.9	200	
Salicin	5.4	200	0.448501
Salicortin	10.59	200	0.870295
Homalosid D	15.229	200	0.647017
Nigracin	12.5	200	0.390564
6'-o-benzoylsalicortin	19.47	200	0.870295
PAB1	6.6	200	0.178358
Catechin	7.7	200	0.258559

Abbreviation: "RT" = Retention time, "RRF" = Relative Response factor, IS= Internal standard

Table A5: Statistical results of One-way ANOVA or Kruskal Wallis test of the sum of salicinoids and flavan-3-ols from the leaves harvested from trees inoculated with control (c) solution, endophyte (e) solution, control +herbivory (c+h) and endophyte +herbivory (e+h). Data presented as mean ± SEM (n =3), where n is the number of trees per treatment in the case of trees without herbivory (c & e) and in the case of *C. tremulae.* Mean ± SEM (n =6), where n is the number of trees per treatment in the case of *L. dispar.*

Trees without herbivory	**control**	**endophyte**	**F/X^2-value**	**p-value**
Sum of salicinoids (mg/g)	67.66 ± 2.83	66.75 ± 3.51	0.04	0.841
Sum of flavan-3-ols (mg/g)	0.744 ± 0.02	0.727 ± 0.03	0.164	0.690
Trees with herbivory				
1) *L. dispar*				
Sum of salicinoids (mg/g)	100.37 ± 6.38	112.73 ± 7.85	1.489	=0.230
Sum of flavan-3-ols (mg/g)	1.16 ± 0.07	1.20 ± 0.11	0.069	0.794
2) *C. tremulae*				
Sum of salicinoids (mg/g)	113.2 ± 9.48	111.9 ± 6.722	0.012	0.910
Sum of flavan-3-ols (mg/g)	1.34 ± 0.067	1.44 ± 0.08	0.930	0.349

Abbreviation: "=" p values from Kruskal Wallis test

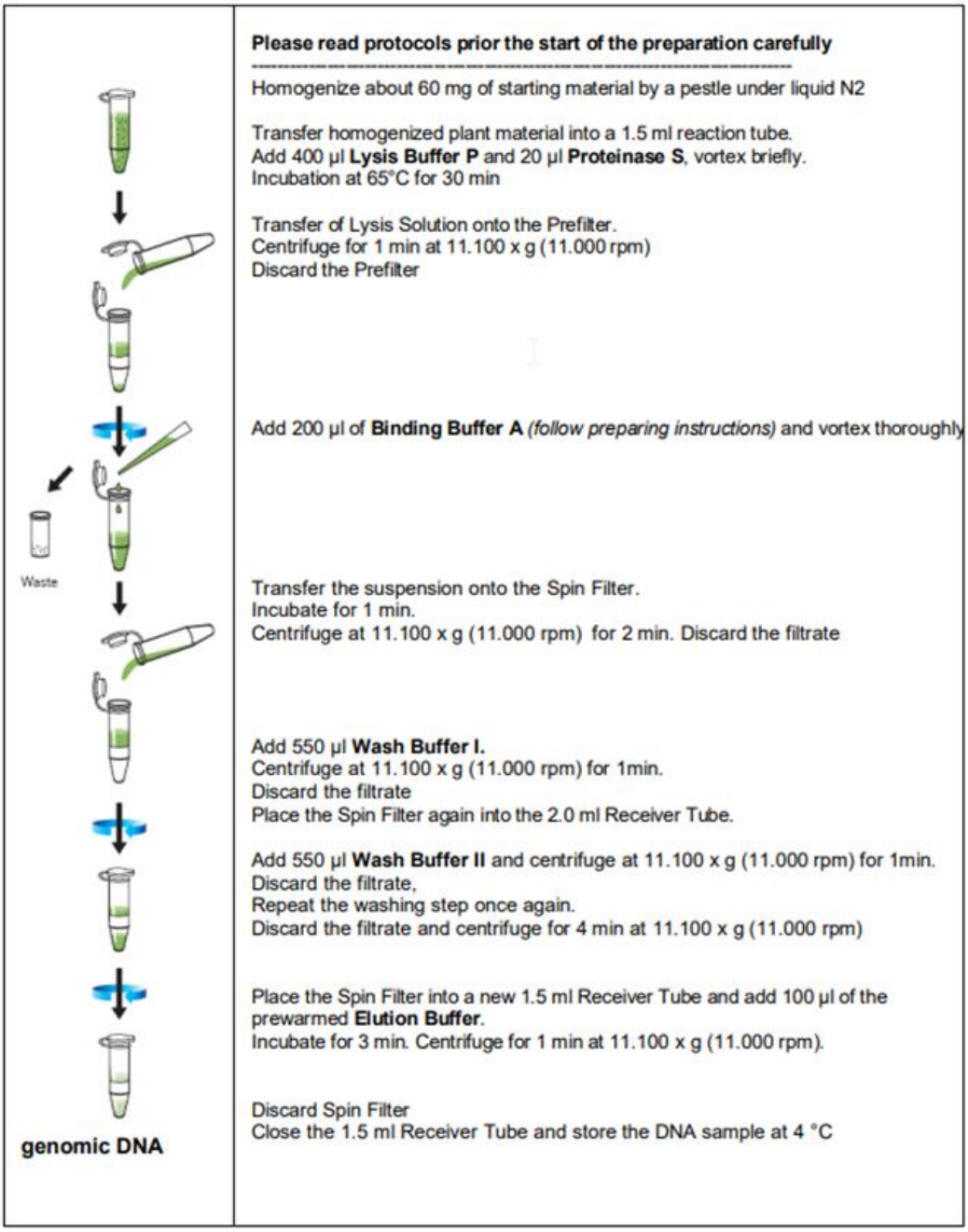
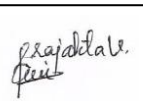


Figure A4: The Protocol *(*Instructions for Use InviSorb ® Spin Plant Mini Kit Instruction InviSorb ® Spin Plant Mini Kit InviSorb ® Spin Plant Mini Kit 2*, 2023)* used for the DNA extraction from the leaves harvested from trees with and without herbivory inoculated with control or endophyte solution respectively. The leaves from trees without herbivory were harvested 42 days after endophyte inoculation. The leaves from trees with herbivory were harvested when the tree received 70% of the leaf damage. The leaves were lyophilized and ground into fine powder. 30 mg freeze-dried leaf sample powder was weighed and then subjected to the extraction process.

Declaration of Self-Dependence

Herewith I declare that I prepared this thesis on my own, that I did not use any other sources and resources than those that are specified, that all arguments and ideas that were literally or analogously taken from other sources are sufficiently identified, and that the thesis in identical or similar form has not been use as part of an earlier course achievement or examination procedure.

Stuttgart, Germany 13.02.2024



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Place, Date Signature