Reciprocal influence of soil, phyllosphere and aphid microbiomes

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Abstract

Background

The effect of soil on the plant microbiome is well-studied. However, less is known about the impact of soil microbiome in multitrophic systems. Here we examined the effect of soil on plant and aphid microbiomes, and the reciprocal effect of aphid herbivory on the plant and soil microbiomes. We designed microcosms, which separate below and aboveground compartments, to grow oak seedlings with and without aphid herbivory in soils with three different microbiomes. We used amplicon sequencing and qPCR to characterize the bacterial and fungal communities in soils, phyllospheres, and aphids.

Results

Soil microbiomes significantly affected the microbial communities of phyllospheres and, to a lesser extent, aphid microbiome, indicating plant-mediated assembly processes from soil to aphids via the plant endosphere. While aphid herbivory significantly decreased microbial diversity in phyllospheres independent of soil microbiomes, the effect of aphid herbivory on the community composition in soil varied among the three soils.

Conclusions

This study provides experimental evidence for reciprocal influence of soil, plant and aphid microbiomes, with potential for the development of new microbiome-based pest management strategies.

Background

Soil microbiomes influence the microbiome associated with plants, which are known to have major implications for plant resilience, growth and vigor (Hardoim et al., 2015; Vandenkoornhuyse et al., 2015; Berg et al., 2017). Plants further interact with various invertebrate animals during their lifespan, for instance soil-inhabiting, pollinating or herbivorous arthropods. Insect herbivores often depend on their associated microbiome including microbial symbionts, providing pivotal nutrients, or for detoxifying secondary plant metabolites (Baumann, 2005). Interaction between multicellular organisms like plants and aphids consequently lead to a concurrence and interaction of two host-associated microbiomes as well (van der Heijden & Hartmann, 2016; Brinker et al., 2019). While we know from literature that soil microbiome influences phyllosphere (Bergna et al., 2018; Grady et al., 2019; Malacrinò et al., 2021a) and aphid microbiomes (Malacrinò et al., 2021a), it is yet unclear whether these effects are direct or plant-mediated, or whether reciprocal effects of aphid herbivory to phyllospheres and soil microbiomes are direct or plant-mediated. Investigating only plant-mediated interactions between soil, plant and aphid microbiomes would indicate to what extent plants themselves are able to modulate and shape soil- and
aphid-associated microbiota in their environment. This would have major implications for future pest biocontrol options and our general understanding of plant microbiome assembly under biotic stress.

Phyllosphere microbiome assembly starts during seed germination through microbial inheritance (Bergna et al., 2018; Wolfgang et al., 2020; Abdelfattah et al., 2021b; Fort et al., 2021). During seed germination, a specific set of microorganisms migrate from seed to phyllosphere (Abdelfattah et al., 2021). Subsequent phyllosphere colonizers are then recruited from the surrounding environment, especially from soil, through horizontal acquisition (Bergna et al., 2018; Grady et al., 2019; Dastogeer et al., 2020; Trivedi et al., 2020; Malacrinò et al., 2021a; Massoni et al., 2021), but also dust, air, and water (Berg et al., 2014; Trivedi et al., 2020). It is yet unclear whether the observed effect of soil on the plant, especially the phyllosphere, microbiome is due to a direct transmission of microorganisms from the environment, or mediated through the plant. Soil physicochemical properties have a substantial effect on the soil microbiome, which can subsequently influence phyllosphere microbiomes (Thapa et al., 2018; Mittelstrass et al., 2021). Therefore, understanding direct and plant-mediated effects on plant microbiomes will reveal to what extent plants use present soil microbial diversity for microbiome assembly, and to what extent plant anatomy and physiology are able to influence the respective plant microbiome.

The aphid microbiome can be divided into primary endosymbionts such as Buchnera aphidicola, secondary symbionts, and transient bacteria (Zytynska et al., 2021). While the presence of primary symbionts is guaranteed by vertical transmission (Baumann, 2005; Bennett & Moran, 2015), the mechanism by which the remaining members of the aphid microbiome are assembled or maintained is yet not fully known (Zytynska & Weisser, 2016). At the current state of knowledge, factors including aphid species identity, plant host species identity, geographical location, aphid predator and aphid parasitoid frequency are known to influence the composition of the aphid microbiome (Zytynska & Weisser, 2016). Although soil microbial diversity was shown to influence aphid bacterial community (Malacrinò et al., 2021a), aphid fungal communities were not investigated using culture-independent methods so far. Furthermore, it is difficult to determine whether in vivo observed effects of soil microbiome on aphid microbiomes are due to plant-mediated mechanism (plant assembly of soil microbiome and subsequent transmission to aphids) or due to environmental contamination, particularly from soil. The effect of soil microbes on aphids, with plants connecting below- and above-ground microbiomes, may have important consequences for understanding effects on herbivore performance as well as biocontrol approaches.

Information regarding the effect of aphid infestation on phyllosphere microbiomes is limited, but often attributed to the production and deposition of honeydew to phyllosphere surfaces. Honeydew is known to favor sooty mold species (Dhami et al., 2013) and aphids were found to increase abundance of culturable epiphytic fungi and bacteria on leaves and shoots of several forest tree species (Stadler & Müller, 1996, 2000; Mühlenberg & Stadler, 2005). Moreover, aphids are known to deposit associated microbes in and on leaves and induce stress responses in plants (De Vos & Jander, 2009; Chaudhary et al., 2014; Furch et al., 2015; Whitfield et al., 2015; Luna et al., 2018). Plant stress responses affect phyllosphere microbiomes as well (Liu et al., 2020a). For instance, woolly beech aphid (Phyllaphis fagi) infestation leads to a bacterial community shift in beech (Fagus sylvatica, L.) (Potthast et al., 2022).
However, to what extent phyllosphere microbiome response upon aphid herbivory depends on the soil microbiome remains elusive.

Reports on the effect of aphid herbivory on the soil microbiome are relatively scarce and partially contradictory. Some studies have shown that aphid herbivory can change the composition of the microbial communities in the rhizosphere (Liu et al., 2020b; Malacrinò et al., 2021b), while others reported no observable effects (O'Brien et al., 2018). In nature, soil microbiome shifts upon aphid herbivory may be caused either by throughfall of honeydew, influencing carbon and nitrogen fluxes (Michalzik & Stadler, 2005; Potthast et al., 2022), or by changes in root exudation patterns, known to shape soil microbiomes (Liu et al., 2020a). While honeydew throughfall promotes microbial activity in soil (Seeger & Filser, 2008), the effect of changed root exudates are less clear and probably depend on the biotic or abiotic soil characteristics.

The objective of this study was to examine the effect of the soil microbiome on the assembly of oak phyllosphere and aphid microbiomes, as well as the effects of aphid herbivory on phyllosphere and soil microbiomes. We used the pedunculate oak (Quercus robur L.), the common oak aphid (Tuberculatus annulatus, HARTIG) and three soil microbial communities with the same physicochemical background as test system to answer the following questions:

1. Do different microbial soil communities lead to differences in phyllosphere communities? *(Fig. 1a: Q1)*
2. Do different microbial soil communities lead to differences in aphid communities? *(Fig. 1a: Q2)*
3. Does aphid feeding alter the phyllosphere microbial communities? *(Fig. 1a: Q3)*
4. Does aphid feeding alter soil microbial communities? *(Fig. 1a: Q4)*

By investigating these questions in a controlled setting, we aim to reveal which formerly observed effects on microbiomes in a plant-herbivore system are truly plant-mediated, and which effects are potentially influenced or influenceable by the soil microbiota in a given soil.

**Materials And Methods**

**Raw material**

Three different types of soil were collected on March 29, 2019 including a loamy sandy soil from Stockholm University campus (henceforth called ‘mixed’ soil), a sandy soil and a clayey soil from Tovetorp Zoological Research Center, situated 60 km southwest of Stockholm. Each soil type was divided into two parts. The first part, was autoclaved twice at 120 °C for 20 min with 24 hours interval at room temperature. The second part, was used later as inoculum. To minimize the effects of soil physicochemical properties, 1.25 l of all soil types were mixed in same proportions (v/v) across treatments, together with 7.5 l of commercial sterilized potting soil (Så och pluggjord, SW Horto, Hammenhög, Sweden) *(Supporting Information Table S1)*. All soil types were sterile except the soil of
interest, which acted as inoculum for the otherwise identical soil mixture (Fig. 1b). These soil mixtures are further denoted according to their corresponding non-autoclaved inoculum ('clayey', 'mixed', and 'sandy'). Two liters of sterilized MilliQ water was added to each soil mix.

Pedunculate oak (*Quercus robur*) acorns were collected from a single oak tree located on Stockholm University campus (Tree # 000369) to minimize the effect of genotype. Acorns were surface-sterilized to minimize contamination with environment-derived microbes using 5% NaOCl for 30 minutes, followed by three rinses in sterile MilliQ water each for 10 minutes. Surface-sterilized acorns were stored in sterile sand at 4 C until use. Before the start of the experiment, acorns were surface sterilized again for 5 min in 5% NaOCl and rinsed as previously mentioned. Common oak aphid (*Tuberculatus annulatus*) was originally collected from natural populations in Stockholm (2018) and reared on oak saplings in a climate chamber (10 h light at 20°C light, 14 h dark at 18°C) for several generations prior to the experiment.

**Experimental Setup and sample collection**

To capture solely plant-mediated microbiome assembly processes, we used microcosms that physically separate above- and belowground plant compartments to grow seedlings under aseptic conditions (Abdelfattah, 2021; Abdelfattah *et al.*, 2021b). Microcosms included openings with filters in the upper compartments to allow gas exchange, but prevent microbial contaminants from the surrounding (Fig. 1b). To separate microbiome shifts in soil due to experimental settings and general plant-mediated effects (e.g. normal root exudation) from herbivory-mediated effects, ten soil samples per soil type, each consisting of 500mg collected before planting acorns from microcosms with and without aphids. These samples are further denoted as “inoculum”, despite being the readily prepared soil mixtures at the beginning of the experiment (Fig. 1b). A total of 45 microcosms per soil type were prepared, making a total of 135 microcosms. The lower compartment of the microcosms was filled with 250 ml of soil and left for 10 days in a growth chamber at 20°C for acclimatization. One surface-sterilized acorn per microcosm was planted under aseptic conditions. Once germinated, a seal was applied to encapsulate the acorn, limiting cross-contamination between below- and above-ground plant parts, preventing neither aphid nor honeydew to come in direct contact with soil, or soil to come in direct contact with neither seedling phyllospheres nor aphids. Seedlings were kept in growth chambers (10 h light at 20°C, 14 h dark at 18°C, light intensity 110 µmol m⁻² s⁻¹, air humidity 65%) until they reached the three- to four-leaf stage. For 35 randomly selected seedlings per soil type, twenty aphids were added to the uppermost leaves using a sterile needle. Ten seedlings per soil type were grown without aphids, acting as a control group (Fig. 1b). Microcosms were randomly divided into 4 sampling groups in the course of processing. After seven days, soil, leaves without petiole which were thoroughly checked for aphid remains, and living aphids were collected for DNA extraction. Microcosms containing plants that showed symptoms of wilting or disease were removed from further analyses (Fig. 1b). For soil samples, 500mg was collected at the center of each microcosm. All samples were stored at -20°C until further processing. Leaves were lyophilized using ScanVac CoolSafe™ (LaboGene), and grounded using TissueLyser II (Qiagen). Leaf samples are further denoted as “phyllosphere
DNA extraction and library preparation

Inoculum and soil samples were extracted using DNEasy PowerSoil pro Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. For phyllosphere samples, 200mg of the lyophilized phyllosphere powder was extracted using DNEasy PowerSoil pro Kit (Qiagen, Hilden, Germany). Aphids were extracted using a modified protocol of the DNeasy® Blood&Tissue (QIAGEN GmbH, Hilden, Germany) standard procedure for insects (Supporting Information Methods S1). One extraction control sample was added per extraction procedure, which was further treated like additional samples to remove potential contaminants in silico.

Amplification of 16SrRNA and ITS sequences was performed using the primer pairs 515f/806r (Caporaso et al., 2011) and ITS1f/ITS2r (White et al., 1990) for bacteria and fungi, respectively. Primers included sample-specific barcodes and Illumina adaptors. For phyllosphere and soil samples, peptide nucleic acid (PNA) PCR clamps were added to block the amplification of plant plastid and mitochondrial DNA (Lundberg et al., 2013). PCR was performed in 30μl reactions, with 2μl template for soil and phyllosphere, and 5μl template for aphid samples (Supporting Information Methods S2). To identify and remove potential contaminants in silico, technical control samples (no-template PCR control samples and extraction control samples for aphid extraction) were also sequenced. In total, 333 and 311 samples for bacteria and fungi were successfully amplified, respectively. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Final DNA concentrations were estimated using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). Since the source of phyllosphere- and aphid-associated microorganisms is one of the main questions of this study, soil, bacterial phyllosphere, fungal phyllosphere, bacterial aphid and fungal aphid samples were separately pooled to equimolar concentrations to avoid index hopping (Costello et al., 2017; Ros-Freixedes et al., 2018). Amplicon sequencing was performed by Eurofins Genomics (Konstanz, Germany) on a MiSeq V3 (600-cycle) platform.

Quantification of fungal and bacterial communities

To quantify the gene copy number of 16S rRNA and ITS rDNA, we used a subset of 4 samples from each treatment for quantitative real time PCR (qPCR). Target genes were amplified using KAPA SYBR® Green 2X MM (KAPA Biosystems, Cape Town, South Africa) in 10 μl reaction mixtures (for details see Supporting Information Methods S2). PNA clamps (Lundberg et al., 2013) were used for soil and phyllosphere samples. Each measurement was performed in three independent runs on a Rotor-Gene 6000 device (Corbett Research, Mortlake, Australia). Mean fragment copy numbers were blank-corrected and extrapolated to copy numbers per g initial sample weight. We still observed mitochondrial, plastid DNA (16SrRNA dataset), unassigned and plant-assigned reads (ITS dataset) in our amplicon sample results. Therefore, the corresponding relative abundance in the amplicon dataset was used to remove non-target reads from qPCR data. Reads were log10-transformed and will be further denoted as “microbial abundance”.

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Data preprocessing and Bioinformatic analyses

Preprocessing of amplicon data was performed in QIIME2 v. 2019.10 (Bolyen et al., 2019). Raw amplicon sequences were demultiplexed using cutadapt (Martin, 2011). Sequences were truncated at 150bp and 170bp for bacteria and fungi respectively, and denoised using DADA2 (Callahan et al., 2016). Taxonomy assignment was performed using VSEARCH (Rognes et al., 2016) with SILVA v.132 (Quast et al., 2013) and UNITE v. 7 (Nilsson et al., 2019) as bacterial and fungal reference sequences, respectively. Amplicon sequencing variants (ASVs) table, taxonomy and metadata was imported to R v. 4.1.1 (R Core Team, 2018) and further processed using the 'phyloseq' package (McMurdie & Holmes, 2013). For the bacterial dataset, chloroplast, mitochondrial, and reads unassigned at the kingdom level were removed. Due to low remaining ASV read counts in phyllosphere samples, only forward reads were used for diversity analyses. For fungi, plant reads and reads unassigned at the kingdom level were removed. Bacterial and fungal contaminants were identified and removed with the prevalence-based method of the R package 'decontam' using PCR and extraction control samples (Davis et al., 2018).

Statistical analyses

Statistical analyses were performed in R v. 4.1.1 (R Core Team, 2018). To account for uneven sequencing depth, ASV tables were rarefied to an even depth of 3900 and 4000 for soil, 100 and 4000 for phyllosphere and 1500 and 4000 for aphid samples for bacteria and fungi, respectively. Species richness and Shannon diversity index were estimated using phyloseq package and checked for normal distribution using Shapiro-Wilks test. For community composition analysis, ASV tables were normalized using Cumulative Sum Scaling (CSS) which was used to calculate Bray-Curtis dissimilarities.

To test the effect of soils on phyllosphere (Q1) and aphid (Q2) on microbial community descriptors, we modelled fungal and bacterial richness, Shannon diversity, and abundance as a function of soil type using Kruskal-Wallis test with FDR-correction followed by Wilcoxon signed-rank test for pairwise comparisons. To test the effect of aphid infestation on fungal and bacterial diversity of phyllosphere (Q3) and soils (Q4), we modelled fungal and bacterial richness, Shannon diversity, and evenness as a function of aphid infestation using Wilcoxon signed-rank test for pairwise comparisons.

To investigate the effect of soil type on the microbial community composition of phyllosphere (Q1) and aphids (Q2), we modelled multivariate fungal and bacterial community composition as a function of soil type, using Bray Curtis distances and the adonis function in the vegan package (Oksanen et al., 2020). Pairwise Adonis (Martinez Arbizu, 2017) with subsequent Bonferroni correction was conducted separately for each soil type. To investigate which taxa differed in relative abundance between phyllosphere and aphids grown in different soils, we conducted a Linear discriminant analysis Effect Size (LEfSe) implemented in the 'microbial' package (Segata et al., 2011; Guo & Gao, 2021). Due to the dominance of aphid primary endosymbiont Buchnera aphidicola while displaying varying relative abundances between
samples, the analyses of aphid microbiomes were repeated with a *Buchnera*-filtered dataset, which was rarefied to 500 reads.

To investigate the effect of aphid infestation on the microbial community composition of phyllosphere (Q3) and soils (Q4), we modelled multivariate fungal and bacterial community composition as a function of aphid infestation, using Bray Curtis distances and the adonis function in the vegan package (Oksanen et al., 2020). Pairwise Adonis (Martinez Arbizu, 2017) with subsequent Bonferroni correction was conducted separately for each combination of soil type and herbivory. To ascertain that potential differences in microbial community composition in soil due to aphid infestation (Q4) do not arise from legacy effects of initial differences in soil communities, we modelled community composition as a function of aphid infestation in inoculum, comparing soil of control plants and soil of plants being later infested with aphids. To investigate which taxa differed in relative abundance between infested and not infested phyllosphere (Q3) and soils (Q4), we conducted a Linear discriminant analysis of effect size (LEfSe) implemented in the ‘microbial’ package (Segata *et al.*, 2011; Guo & Gao, 2021). Using the same method, we identified differential abundant taxa in inoculum and soil to discriminate between general trends in soil community composition due to normal root exudation or experimental settings, and effects mediated by aphid infestation.

**Results**

**Amplicon data overview**

A total of 6,437,014 bacterial and 13,822,677 fungal reads were retained after quality filtering, decontamination, removal of plastid DNA, contaminants and unassigned sequences. A total of 27,596 ASVs were identified in the bacterial amplicon library, in fungi 8,671. Maximum read count per sample was 180,761 with a mean of 20,765 reads per sample in bacterial amplicon samples. For fungal amplicon samples, maximum read counts of 542,484 with a mean of 47,338 reads per sample were retained. All three soil inocula differed significantly in community composition both in bacteria (PERMANOVA: $R^2 = 0.43; p = 0.001$) and fungi (PERMANOVA: $R^2 = 0.19; p = 0.001$). All inocula combined, bacterial community was dominated by *Proteobacteria, Firmicutes*, and *Actinobacteria*; fungal community was dominated by *Basidiomycota* (dominant genus: *Lyophyllum*) and *Ascomycota* (dominant genus: *Rasamsonia*) ([Supporting Information Notes S1](#)).

**Assembly from soil to phyllosphere**

Soil microbiome had a significant-effect on the bacterial species richness ([Fig. 2a](#)) and Shannon diversity ([Fig. 2b](#)) in the phyllosphere. Among the different soil types, phyllosphere of plants grown in sandy soil microbiome displayed the highest bacterial richness and Shannon diversity. Whereas phyllosphere of plants grown in mixed soil microbiome exhibited the lowest bacterial richness and Shannon diversity. Bacterial abundance in phyllosphere did not differ significantly according to soil microbiome ([Fig. 2c](#), but
bacterial community composition differed significantly (Fig. 2d). Fungal phyllosphere species richness (Fig. 2e) and Shannon diversity (Fig. 2f) were not affected by the soil microbiome, yet fungal abundance was significantly higher in phyllosphere grown in clayey than in mixed soil microbiome (Fig. 2g). Fungal phyllosphere community composition significantly differed among the three soil types (Fig. 2h) microbiomes. Pairwise PERMANOVA revealed phyllospheres grown in the three soil microbiomes to significantly differ from each other (Supporting information Table S2). Differential abundance analysis showed that *Burkholderia* s. lat is a biomarker for the phyllosphere grown in clayey, *Pseudomonas* for the phyllosphere in mixed, and *Streptomyces, Sphingomonas, Erwinia* and *Acinetobacter* for the phyllosphere microbiota. High microbial species richness, Shannon diversity and abundance in soil was rarely correlated to high species richness, Shannon diversity and abundance in phyllosphere (Table 1).

### Assembly from soil to aphid

Soil did not have an effect on bacterial species richness (Fig. 3a), diversity (Fig. 3b), or abundance (Fig. 3c) in bacterial aphid communities, but on community composition (Fig. 3d) in aphids. The same pattern was observed in fungal aphid community (Fig. 3e-h). The variance explained ($R^2$) by the factor soil in community composition was higher in fungal (Fig. 3h), than in bacterial aphid microbiomes (Fig. 3d). Pairwise PERMANOVA showed a significant difference between aphid’s bacterial and fungal communities, except for the bacterial community of aphid from clayey and sandy soil microbiomes (Supporting Information Table S3). Aphid microbiomes were dominated by *Burkholderia* s. lat., and *Pseudomonas*, while fungal microbiomes were dominated by *Cladosporium* and *Penicillium* (Supporting Information Notes S2). Differential abundance analyses using LefSE only showed *Micromonosporaceae* to be significantly higher in aphids reared on sandy soil microbiome (Supporting Information Table S4). In the fungal dataset, *Mortierella* spp. and *Parasola* spp. were higher abundant in aphids reared on clayey soil microbiome, while *Cladosporium* spp. was higher in aphids reared on mixed soil microbiome, yet not significant (Supporting Information Table S5). High microbial species richness, Shannon diversity and abundance in soil was rarely correlated to high species richness, Shannon diversity and abundance in aphids, but bacterial abundance and fungal Shannon diversity in aphids showed similar patterns when compared to bacterial abundance and fungal Shannon diversity in phyllosphere (Table 1).

Table 1: Dynamics in alpha diversity during soil microbe assembly. Comparison of bacterial and fungal species richness, Shannon diversity, and abundance between different soil microbiomes relative to each other for the three tested compartments soil, phyllosphere, and aphids. Abundance based on log10 transformed qPCR reads of 16S rRNA (bacteria) and ITS (fungi) gene read counts. Rank position (1 = high, 2 = medium, 3 = low) of richness, Shannon diversity and abundance values compared between corresponding compartments (rows). Compartments where phyllosphere and aphid samples show similar patterns are highlighted in bold.
### The effect of aphid herbivory on phyllosphere microbiota

Aphid herbivory had a significant decreasing effect on the phyllosphere bacterial species richness (Fig. 4a) and Shannon diversity (Fig. 4b). Bacterial abundance (Fig. 4c) and community composition (Fig. 4d) did not differ significantly. In fungal phyllosphere species richness (Fig. 4e), Shannon diversity (Fig. 4f) and abundance (Fig. 4g), microbiome response showed the same pattern than in bacteria, but community composition (Fig. 4h) significantly differed between infested and control phyllosphere. Pairwise comparisons of fungal community composition showed that aphid herbivory to was significantly affected in clayey ($R^2 = 0.045$, $p = 0.018$) and sandy soil microbiome ($R^2 = 0.060$, $p = 0.013$), but not in mixed soil. Lefse analysis showed that 72 fungal taxa were more abundant in the phyllosphere of non-infested compared to infested plants. Among those taxa *Russula* and *Preussia* were amongst the most affected taxa (Supporting Information Table S6). On the other hand, the phylum *Ascomycota* was significantly higher relative abundant in aphid-infested phyllosphere.
The effect of aphid herbivory on soil microbiota

Aphid herbivory did not have an effect on microbial soil species richness, diversity, abundance, and community composition (Supporting information Fig. S1), except for bacterial species community composition in sandy soil (Supporting information Fig. S2d). According to LefSe analyses results, effect of aphid infestation on relative abundance of sandy soil microbiota was not significant after p-value correction. Strongest decrease was observed in *Rhodanobacter* and -amongst others- *Bacillaceae*, while relative abundance of *Rhizobiaceae* (*Rhizobium* s. lat., *Mesorhizobium*) and *Xanthobacteraceae* was increased in sandy soil of aphid-infested plants. However, (Supporting information Table S7). Differential abundance of these taxa did not refer to general abundance shifts from inoculum to soil (data not shown), although a soil type-dependent community development was observed when comparing inoculum and soil microbiomes (Supporting information Fig. S2-S4). No specific fungal taxa showed significantly different relative abundances in soil microbiomes of aphid-infested plant.

Discussion

In the current study, we showed that manipulating soil microbiome changed the plant phyllosphere, and subsequently the aphid microbiome. We found aphid infestation to have significant effects on phyllosphere microbiomes and soil microbiome-plant interactions, interfering with plant-mediated assembly. The implications of aphid infestation for plant-associated microbiomes depend on microbial communities in soil. In this way, this study provides experimental evidence for reciprocal influence of soil, plant and aphid microbiomes.

Soil microbiome affected plant-mediated phyllosphere microbiome assembly. Microbial assembly in plants is regarded as a non-random process governed by selective pressures within the host plant itself (Grady et al., 2019; Xiong et al., 2021), and the fact that soil microbiome shapes phyllosphere microbiomes was observed before in other plant species (e.g., Bai et al., 2015a; Wagner et al., 2016; Grady et al., 2019; Tkacz et al., 2020; Malacrinò et al., 2021a). By investigating the effect of different microbial communities in soil of same physicochemical properties, we could show that phyllosphere microbiome assembly is plant-mediated and thus at least partially driven by biotic factors. Still, selective pressures on microbial endophytes in oak appeared to be generally high, since microbial phyllosphere diversity was low in comparison to other tree species (Laforest-Lapointe et al., 2016; Beckers et al., 2017) which was observed in oaks grown in microcosms (Abdelfattah et al., 2021b) and under field conditions (Faticov et al., 2021). The effect of soil microbiomes on phyllosphere microbiomes was most evident for plants grown in sandy soil microbiomes. This soil, was dominated by *Proteobacteria* which are known to be the most common phylum of the plant microbiomes (Levy et al., 2018; Trivedi et al., 2020). Thus, experiments conducted in *Proteobacteria*-rich soils may result in more apparent effects on above-ground microbiomes. Although we found taxa dominating endophytic acorn communities as dominant taxa in phyllosphere, namely *Pseudomonas, Burkholderia, Erwinia, Cladosporium* or *Penicillium* (Abdelfattah et al., 2021a), we further identified bacterial biomarkers arising from soil communities in phyllospheres (e.g., *Streptomyces, Acinetobacter*). This experimentally confirms soil microbiomes to shape oak phyllosphere
microbiomes via plant-mediated assembly processes and indirectly by modulating endophytic communities even without any direct physical contact between soil and phyllosphere.

We found that the soil microbiome had an effect on aphid microbiome assembly, even without any direct physical contact. Several studies reported effects of soil microbiome on aphid performance before (Hol et al., 2010; Blubaugh et al., 2018; Brock et al., 2018), and these effects were discussed to arise from indirect effects on plant defense systems (e.g. Pineda et al., 2012). A similar experiment in potato and potato aphids ( Macrosiphum euphorbiae Thomas) revealed soil diversity to affect bacterial aphid microbiomes (Malacrinò et al., 2021a), reporting a higher effect size of soil on aphid microbiomes compared to our study. Here, soil microbiome more evidently affected fungal communities in aphids in this experiment, although soil microbiome effect on phyllosphere was more evident for bacteria. To our knowledge, this is the first culture-independent study investigating aphid-associated fungal communities, and we found generalistic fungal taxa common to phyllosphere surfaces in aphids. Therefore, we hypothesize aphid-associated fungi to arise from direct exchanges between epifoliar and epicuticular fungi. Epifoliar fungi are considered to be generalists (Arnold, 2007), and such fungi may be enhanced by aphid honeydew deposition, increasing chances to re-associate with aphids. Interestingly, bacterial species richness is around elevenfold higher in aphids ($\bar{X} = 180.4 \pm 112.7$) than in phyllosphere ($\bar{X} = 7.3 \pm 3.7$). Due to the limited diversity in oak phyllospheres and additional selective pressures in the digestive tract of aphids, direct uptake of soil microbes via plant sap may only have minor effects on the autochthonous aphid microbiome.

We observed that aphid herbivory decreased the microbial diversity in phyllosphere, yet abundance was not affected. The statement that soil communities impair phyllosphere microbiomes more than aphid herbivory (Malacrinò et al., 2021a) could be confirmed in this pathosystem. Herbivore-associated microbiomes are known to interfere with plant metabolism and plant defense responses, thus acting as a hidden driving force of plant–herbivore coevolution (Zhu et al., 2014). For instance, bacteria in the saliva of Colorado potato beetle (Leptinotarsa decemlineata SAY) act as plant immunological decoy by triggering antimicrobial (SA-regulated) rather than antiherbivore (JA and ethylene-regulated) plant defenses (Chung et al., 2013). An increase in SA and decrease in JA response-related gene expression was also observed and discussed for bacteria in aphid honeydew (Schwartzberg & Tumlinson, 2014). Interestingly, we found an example of such a potentially "misled" plant immune response in phyllosphere grown in mixed soil microbiomes. Abundance of the entomopathogenic fungus Metarhizium brunneum PETCH, only found in mixed soil microbiomes and corresponding phyllospheres, was reduced in aphid-infested phyllospheres, despite being potentially beneficial to the plant by infecting and killing aphids (Reingold et al., 2021). These results indicate an untargeted, general decrease of several fungal taxa upon aphid herbivory, either arising directly via plant stress responses or indirectly by affecting soil microbiome assembly processes in the plant. The deposition of honeydew may further favor microbial generalists, masking the loss in abundance. Therefore, we hypothesize phyllosphere microbiome shifts upon aphid herbivory to be the consequence of an untargeted antifungal plant defense response, as well as honeydew deposition by the aphid.
Soil microbial community composition determines whether soil microbiome shifts upon above-ground herbivory. This explains why effects of sap-sucking insects on soil microbiomes were reported before (Yang et al., 2011; Kong et al., 2016; Malacrinò et al., 2021b), while some studies observed no effect (O’Brien et al., 2018). Similar to our results, soil microbiome-dependent responses upon aphid infestation was reported in a wild tomato (Solanum pimpinellifolium) -potato aphid (Macrosiphum euphorbiae) pathosystem (French et al., 2021). In contrast to our results, Bacillaceae were discussed as positive responders to aphid herbivory in soil. In our experiment, Bacillaceae responded negatively, while Xanthobacteraceae and Rhizobiaceae (ad Proteobacteria) responded positively in soil upon aphid herbivory. Soils used by French and colleagues (2021) did not significantly differ in relative abundance of Proteobacteria, therefore the exact cause for responsiveness of soil microbiomes to herbivory remains a matter of debate. However, responses are most likely driven by plant root exudation. Oak root exudate composition is known to shift towards a higher concentration of secondary metabolites under abiotic stress (Gargallo-Garriga et al., 2018). Given that exudate composition changes accordingly under biotic stress, soil microbiome responses upon herbivory may be indirectly driven by susceptibility of soil microbes towards such root exudates. For sandy soil microbiomes, the 'cry-for-help' hypothesis (Rolfe et al., 2019) cannot be excluded, but observations can be also explained with decreased amounts of metabolizable root exudates (Hoysted et al., 2018). Firstly, taxa increased in aphid-infested sandy soil are not known for antiherbivore or plant growth-promoting effects. Secondly, genera known for nitrogen fixation (Rhizobium, Mesorhizobium) are increased, indicating a more nutrient-depleted environment compared to control plants. Thirdly, bacterial species richness is higher in aphid-infested sandy soil, indicating lower selection pressure. Fourthly, bacterial abundance is generally lower in infested plant soil. Lastly, when combining all soil data, we do not find significantly responding biomarker taxa in differential abundance analyses. Altogether, this indicates a weaker selective force of root exudates on microbes in the root periphery of aphid-infested plants compared to control plants.

**Conclusion**

Soil, plant and aphid microbiomes are in a dynamic tripartite interaction, in which the strength of effects depends on the represented microbial communities and not the physicochemical properties of soil. While directly shaping phyllosphere and aphid microbiome using soil is possible to some extent, the effect size of soil microbiome gradually decreases from phyllosphere to aphids. Still, soil microbes being transmitted to aphids via the plant are of interest for biocontrol of pests, since soil or seed treatments are easier to handle, and have less mechanical impact on agricultural plants than spray applications (McQuilken et al., 1998). Herbivory has implications for phyllosphere and partially soil microbiomes, although the specific responses depend on yet unidentified soil microbiome specifics. To fully disentangle the role of soil microbiome from soil physicochemical properties in tritrophic systems, future studies could investigate the response of plant microbiomes on synthetic or otherwise defined microbial soil communities under different physicochemical soil conditions and stressors.

**Declarations**
Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The dataset supporting the conclusions of this article is available in the European Nucleotide Archive (ENA) repository, accession number PRJEB50358. The code used in this study will be made available on zenodo upon acceptance of the manuscript.

Competing interest

The authors declare that they have no competing interests.

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Author Contributions

AA and AT conceptualized the experiments; AA performed the microcosm experiment and sampling; AW prepared amplicon libraries, performed bioinformatics analyses, and wrote the first draft of the manuscript. GB contributed to the interpretation of the results. All authors approved and contributed to the final version of the manuscript.

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References


Figures

Figure 1
Main research questions (a) and experimental setup (b). a: Main research question of the current study. Q1: Do different microbial soil communities lead to differences in phyllosphere communities? Q2: Do different microbial soil communities lead to differences in aphid communities? Q3: Does aphid feeding alter phyllosphere microbial communities? Q4: Does aphid feeding alter soil microbial communities? Assembly processes are depicted as solid arrows, potential feedback effects depicted as dashed arrows.

b: Conceptual figure of experimental design, including soil community preparation. Substrate was standardized in physicochemical properties by combining all soils, but with all soils sterilized except for one providing soil community inoculum (bottom). Black arrows indicate DNA extraction for amplicon sequencing of the corresponding microhabitat. +: wilted seedlings and corresponding aphids were removed from sampling process; therefore “n” refers to the total number of successfully assessed metagenome samples (phyllosphere and aphid samples) or number of replicate samples per soil in clayey, mixed, and sandy soil microbiomes. *: Ten “inoculum” replicates were drawn from microcosms before planting, five replicates per soil type going to be the substrate for aphid-infested plants, and five replicates going to be in the control group.

Figure 2

Effect of soil microbiome on phyllosphere microbiomes. Bacterial (top, a - d) and fungal (bottom, e - h) species richness (a, e), Shannon diversity (b, f), abundance based on qPCR of 16S rRNA (c) and ITS read counts (g), and community composition (d, h). Box plots show the median (horizontal line), the lower and upper bounds of each box plot denote the first and third quartiles, and whiskers above and below the box plot show 1.5 times the interquartile range. Points located outside of the whiskers (grey) represent outliers. Ordination plots of bacterial (d) and fungal (h) community composition were based on Bray-Curtis dissimilarity index with corresponding colors (phyllospheres from clayey soil samples: olive circles;
mixed: medium green triangles; sandy: lime green squares). Results of global statistical analyses for the factor ‘soil community’ are displayed above each panel, FDR-corrected p-values of pairwise comparisons for alpha diversity differences added within the graph (*: p < 0.05; **, p < 0.01; ***: p < 0.001; ns: not significant).

**Figure 3**

**Effect of soil microbiome on pooled (n = 4) aphid microbiomes.** Bacterial (top, a - d) and fungal (bottom, e - h) species richness (a, e), Shannon diversity (b, f), abundance based on qPCR of 16S rRNA (c) and ITS read counts (g), and community composition (d, h). Box plots show the median (horizontal line), lower and upper bounds of each box plot denote the first and third quartiles, and whiskers above and below the box plot show 1.5 times the interquartile range. Points located outside of whiskers (grey) represent outliers. Ordination plots of bacterial (d) and fungal (h) community composition based on Bray-Curtis dissimilarity index with corresponding colors (aphids from clayey soil samples: dark blue circles; mixed: light blue triangles; sandy: pink squares). Bacterial community composition was calculated without aphid primary endosymbiont *Buchnera aphidicola*. Results of the global statistical analyses for the factor ‘soil community’ displayed above each panel, FDR-corrected p-values of pairwise comparisons for alpha diversity differences added within the graph (*: p < 0.05; **, p < 0.01; ***: p < 0.001; ns: not significant).
Figure 4

Effect of aphid infestation on phyllosphere-associated microbiomes established in three different soil microbiomes. Bacterial (top, a-d) and fungal (bottom, e-h) species richness (a, d), Shannon diversity (b, e), abundance based on qPCR of 16S rRNA (c) and ITS read counts (g), and community composition (d, h) of phyllosphere communities. Global p refers to differences if all phyllosphere samples were combined. Box plots show the median (horizontal line), the lower and upper bounds of each box plot denote the first and third quartiles, and whiskers above and below the box plot show 1.5 times the interquartile range. The points located outside of the whiskers of the box plot (grey) represent outliers. Results of the global statistical analyses for the factor 'aphid infestation' (WRT: Wilcoxon rank-sum test) are displayed above each panel, soil community-specific values within the graph.

Supplementary Files

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