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Brief Report

Herbivory shapes the rhizosphere bacterial microbiota in potato plants

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Summary

Plant-associated microbiomes assist their host in a variety of activities, spanning from nutrition to defence against herbivores and diseases. Previous research showed that plant-associated microbiomes shift their composition when plants are exposed to stressors, including herbivory. However, existing studies explored only single herbivore-plant combinations, whereas plants are often attacked by several different herbivores, but the effects of multiple herbivore types on the plant microbiome remain to be determined. Here, we first tested whether feeding by different herbivores (aphids, nematodes and slugs) produces a shift in the rhizosphere bacterial microbiota associated with potato plants. Then, we expanded this question asking whether the identity of the herbivore produces different effects on the rhizosphere microbial community. While we found shifts in microbial diversity and structure due to herbivory, we observed that the herbivore identity does not influence the diversity or community structure of bacteria thriving in the rhizosphere. However, a deeper analysis revealed that the herbivores differentially affected the structure of the network of microbial co-occurrences. Our results have the potential to increase our ability to predict how plant microbiomes assemble and aid our understanding of the role of plant microbiome in plant responses to biotic stress.

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Introduction

Two decades of microbiome research have underlined the importance of microbial communities for plant growth, survival and reproduction (Turner *et al*., 2013; Schlaeppi and Bulgarelli, 2015; Cordovez and Dini-Andreote, 2019 Saikkonen *et al*., 2020). Microbes are major drivers of plant evolution (Rosenberg and Zilber-Rosenberg, 2016), and the manipulation of microbiomes may lead the next green revolution (Cordovez and Dini-Andreote, 2019). Prior to microbiome research, studies mostly focused on one or a few microbes at a time (Cordovez and Dini-Andreote, 2019). However, we know that the community of microbes, as a whole, has an important functional role for plants (Pineda *et al*., 2017). While several studies have described the plant microbiome, we still do not understand the rules governing plant microbiome assembly (Cordovez and Dini-Andreote, 2019; Saikkonen *et al*., 2020).

Microbiome composition and function differs between above- and below-ground plant compartments (Berendsen *et al*., 2012; Bulgarelli *et al*., 2013; Schlaeppi and Bulgarelli, 2015; Stone *et al*., 2018). The rhizosphere is considered one of the most dynamic interfaces in terms of plant–microbiome interactions (Philippot *et al*., 2013). While microorganisms living in this compartment help the plant to perform a variety of functions, they are under the direct influence of their host (Berendsen *et al*., 2012; Sasse *et al*., 2018). There is indeed growing evidence that plants can modulate the microbiome in the rhizosphere when exposed to stress episodes (Berendsen *et al*., 2012; Turner *et al*., 2013; Rolfe *et al*., 2019).

Currently, few studies have investigated the effects of herbivory on the rhizosphere microbial community. Early reports focused on single or a few members of the community. Infestation of whitefly on pepper plants has been linked to an increased abundance of Gram-positive bacteria in the rhizosphere (Yang *et al*., 2011). Similarly, Lee

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et al. (2012) studied the effects of aphid herbivory on the rhizosphere of pepper plant, observing an increase in abundance of *Bacillus subtilis* and a decreased abundance of the pathogen *Ralstonia solanacearum*. More recent studies used high-throughput methods to characterize the whole rhizosphere community. Kong *et al*. (2016) investigated a whitefly-pepper plant system similar to the one studied by Yang *et al*. (2011), reporting a shift of the rhizosphere microbiome composition due to herbivory. Similarly, our previous study reports an effect of aphid herbivory on the structure of rhizosphere microbiota in *Solanum tuberosum* and *Solanum vernei* (Malacrinò *et al*., 2021). Conversely, another study focusing on the interaction between *Myzus persicae* and its host *Brassica oleracea var. capitata* found no effect of aphid infestation on the rhizosphere microbiota (O'Brien *et al*., 2018). These previous reports focused on a single herbivore-plant combination, thus whether different herbivores with different feeding strategies can have a differential impact on the rhizosphere microbiome still needs to be tested.

In this study, we expanded the current knowledge by testing whether herbivory more generally alters the rhizosphere microbiota in *S. tuberosum*. According to our previous study (Malacrinò *et al*., 2021) and further supporting literature (Yang *et al*., 2011; Lee *et al*., 2012; Kong *et al*., 2016) we hypothesize that herbivory would produce a shift in the structure of the rhizosphere microbial community in *S. tuberosum*. In addition, literature suggests that plants exert a different response according to the identity of the herbivore attacking them (Wurst and Putten, 2007; Kafle *et al*., 2017). For example, it is well established that chewing herbivores primarily activate a defence pathway triggered by jasmonic acid, while sucking herbivores mainly induce a response driven by salicylic acid (Bari and Jones, 2009). The impact of plant defence signalling extends also to the root and their associated microbial community (Doornbos *et al*., 2012; Rolfe *et al*., 2019). Thus, here we also test the hypothesis that herbivores with different feeding strategies have contrasting effects on the microbial community living in the rhizosphere soil.

Results

To answer our questions, we characterized the rhizosphere bacterial communities of potato plants (*S. tuberosum*) exposed to herbivory by aphids (*Macrosiphum euphorbiae*), nematodes (*Meloidogyne* sp.), or slugs (*Arion* sp.), or were not exposed to herbivores (control). Bacterial microbiota was reconstructed using high-throughput 16S rRNA gene amplicon sequencing. The analysis of the rhizosphere bacterial community of our plants recovered 1624 Operational Taxonomic Units (OTUs). Most of the sequences were assigned to the phyla Proteobacteria, Verrucomicrobia, Bacteroidetes, Firmicutes and Acidobacteria (Supporting Information Fig. S1). We did not observe differences in relative abundance of bacterial phyla across treatments ($P > 0.05$).

In this study, we first tested the hypothesis that herbivory in general produces changes in the rhizosphere microbial community. While we did not find any difference when comparing the microbiota phylogenetic diversity (Faith's phylogenetic diversity index) of the different treatments ($\chi^2 = 3.54$, df $= 3$, $P = 0.31$; Fig. 1A), we found differences in the structure of these microbial communities (PERMANOVA $F_{1,16} = 1.402$, $P = 0.003$; Fig. 1B). Pairwise contrasts revealed that the rhizosphere microbiome of all plants exposed to herbivory differed from the microbiome of control plants ($P < 0.05$ for all pairwise contrasts).

The second hypothesis we tested is that different herbivores would produce a differential change in the

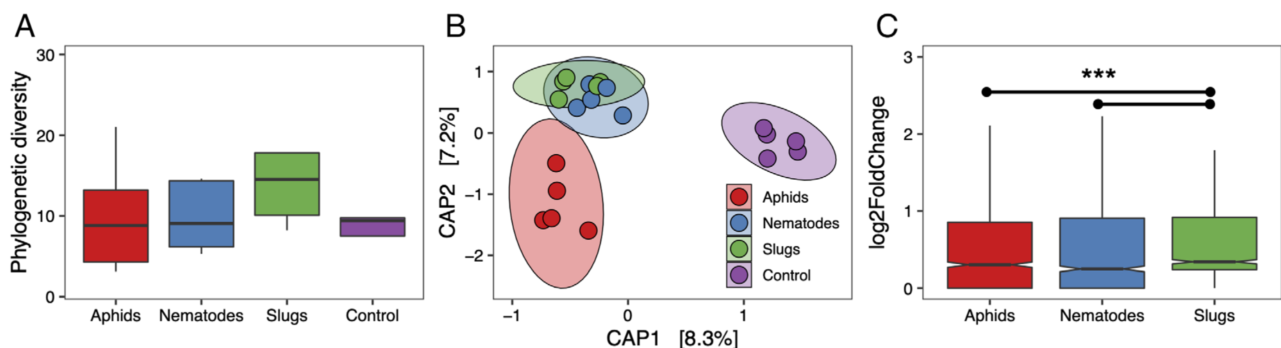

Fig. 1. A. Comparison of Faith's phylogenetic diversity index across treatments.B. Canonical analysis of principal (CAP) coordinates ordination using a Bray–Curtis distance matrix of samples. Percentages in parentheses report the variance explained by the respective axis.C. Magnitude of changes in abundance for each OTU (absolute log2 fold changes). For each treatment, we investigated the response of single OTUs to the treatment compared with the control. Comparisons were tested using a linear mixed-effects model, and contrasts were extracted using the function *emmeans* (FDR corrected). *** $P < 0.001$.

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rhizosphere microbiome. We tested this hypothesis in two ways. First, through pairwise PERMANOVA contrasts between our treatments, where we found no differences between herbivore treatments ($P > 0.05$ for all pairwise contrasts). Second, we investigated in more detail which treatment had a stronger influence on rhizosphere microbiota composition by examining the magnitude of change in abundance for each OTU (absolute log2 fold changes) contrasting each herbivory treatment against the control. We found differences across treatments ($F = 20.67$, $P < 0.001$; Fig. 1C), and post hoc contrasts revealed a greater impact of the treatment with slugs compared to aphids ($P < 0.001$) and nematodes ($P < 0.001$), and no differences between aphid and nematode treatments ($P = 0.98$).

We observed changes in below-ground biomass as an effect of herbivory ($\chi^2 = 18.9$, df = 3, $P < 0.001$; Supporting Information Fig. S2A), with increased biomass in plants exposed to aphids and slugs compared to control ($P = 0.031$ and $P = 0.006$, respectively) but not in those exposed to nematodes ($P > 0.05$). We also observed an effect driven by herbivory on aboveground biomass ($\chi^2 = 8.44$, df = 3; $P = 0.03$, Supporting Information Fig. S2B), although it was driven by a marginal increase in biomass in plants exposed to slugs compared to control plants ($P = 0.06$).

The fact that we did not observe differences in the diversity and structure between herbivore-exposed and control plants, but we did observe differences in the magnitude of response of OTUs as a consequence of each herbivore treatment, suggests that different herbivores may have an impact on the interactions between OTUs rather than on their structure. We tested this hypothesis by building a co-occurrence network of OTUs for each treatment and performing a pairwise comparison between networks (Table 1). When comparing each herbivory treatment against control plants, we found that aphids altered the relationship between OTUs (degree, $P < 0.001$) and the structure of hub OTUs ($P = 0.03$).

Table 1. Pairwise comparison of treatment groups using co-occurrence network analysis. For each pair, we tested differences in degree centrality, betweenness centrality and hub taxa.

	Degree	Betweenness centrality	Hub taxa
Aphids/Control	**<0.001**	0.08	**0.03**
Nematodes/Control	0.14	1	0.44
Slugs/Control	**0.002**	**0.03**	0.23
Aphids/Nematodes	**<0.001**	**<0.001**	0.05
Aphids/Slugs	**<0.001**	**0.001**	**0.001**
Nematodes/Slugs	**0.006**	**<0.001**	**0.02**

P-values in bold are <0.05.

While nematodes did not have any effect, slugs altered both the degree and betweenness centrality of the network but not the structure of hub taxa (Table 1), suggesting that herbivory by slugs alters the network interactions between OTUs downstream of hub taxa. When comparing the different herbivores between them, we found that degree and betweenness centrality always differed between the herbivore types. In addition, slugs also altered the hub taxa composition when compared with aphids and nematodes ($P = 0.001$ and $P = 0.02$, respectively; Table 1).

Discussion

While several studies focused on the description of plant-associated microbiomes, we are still in the process of understanding how microbial communities assemble and respond to external factors. Few previous studies reported the influence of herbivory on the rhizosphere microbial community. Most of them exposed pepper plants to a sap-feeding insect (aphids or whiteflies) (Yang *et al*., 2011; Lee *et al*., 2012; Kong *et al*., 2016) reporting that herbivory influenced few microbes or the entire community of the rhizosphere. In contrast, O'Brien *et al*. (2018) reported no effects of aphid herbivory on cabbage plants. Here, we test and compare the effects of three herbivore types feeding on potato plants and report novel findings about their differential effects on the rhizosphere microbiome.

Our results show that herbivory influenced the interactions of rhizosphere microbiota in *S. tuberosum* by changing the structure of the microbial communities and the network of interactions. These results support our previous study (Malacrinò *et al*., 2021), where we observed an effect driven by *M. euphorbiae* herbivory on the structure of rhizosphere microbiota in *S. tuberosum* and *S. vernei*. The mechanism behind the influence of herbivory on rhizosphere microorganisms might be explained by the composition of root exudates. We know that herbivory impacts the composition of root exudates (Rasmann and Turlings, 2016; Hoysted *et al*., 2018), which is a strategy that plants use to recruit beneficial organisms in an effort to alleviate the negative effects of herbivory (Rolfe *et al*., 2019). The activation of the jasmonic acid pathway (which is activated by many herbivore types) has been linked to changes in the structure of the rhizosphere microbial community (Carvalhais *et al*., 2013). Indeed, the activation of plant defences and the consequent change in root exudate composition have been proposed as the mechanism to explain herbivore-mediated changes in the root microbiome (Carvalhais *et al*., 2017). While our results agree with those of Kong *et al*. (2016), they do not support the findings of O'Brien *et al*. (2018). Since our model plant is phylogenetically

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close to pepper plants (Kong *et al*., 2016) but distant from cabbage plants (O'Brien *et al*., 2018), we can speculate that plant phylogeny plays an important role, and that the shift of rhizosphere microbiota as a response to herbivory is not consistent across plant species (or genotypes). The fact that plant genotype can modulate the herbivore-driven effect on the rhizosphere microbial communities can also explain why in this study, we observed an effect of herbivory (regardless the identity of the herbivore) on the structure but not on the diversity of the rhizosphere microbiota, which partially contrasts with our previous findings (Malacrinò *et al*., 2021).

To the best of our knowledge, no previous studies have investigated the effect of herbivores other than sap feeding Hemiptera on the rhizosphere microbiota. Here, we tested the effects of three herbivores, with different feeding strategies, on the rhizosphere microbiota. While we did not observe an influence of herbivore identity on the overall structure and diversity of rhizosphere microbial community, we did find an herbivore-specific response in the microbial co-occurrence network. Previous studies reported that different herbivore types induce differential responses in plant chemistry and defence pathways (Gutbrodt *et al*., 2012; Xiao *et al*., 2019). However, we are not aware of previous reports linking the herbivore identity with a specific blend of root exudates. While we expected to observe a different influence of each herbivore on the diversity and structure of rhizosphere microbiota, an unanticipated finding was that they differentially alter the network of interactions between microorganisms. This is an interesting result, which might be also explained by a direct action of the host plant through a targeted root exudation, but further tests are needed to clarify this aspect.

Overall, our results suggest an herbivore-specific effect on the rhizosphere microbiome. The number of previous studies testing similar hypotheses is quite limited and report contradictory results, thus we need further research to be able to draw general conclusions. One aspect that needs attention is whether the identity of the plant modifies herbivore effects on the microbiome, and further research can expand our observations to different species spanning plant phylogeny. Also, we still need to prove the link between activation of plant defences, changes in the blend of root exudates and changes in the structure of the rhizosphere microbiome. Future research can focus on integrated multi-omics approaches, linking the plant defence response to changes in rhizosphere metabolome and microbiome. This will allow us to test this model and to gain understanding on the factors regulating the plant-associated microbiomes, which are thought to be the basis of the next green revolution.

Experimental procedures

Study system

Potato seeds (*Solanum tuberosum* genotype ADG-3690) were obtained from the Commonwealth Potato Collection at The James Hutton Institute (Dundee, Scotland, UK). Seeds (non-sterilized) were germinated on steam-sterilized coir and then transplanted to the experimental pots after 3 weeks. The sterile background soil was prepared by mixing Steam Sterilized Loam (Keith Singleton, Cumbria, UK) and sand (ratio 1:1), and autoclaving this mixture at 121°C for 3 h, allowing it to cool for 24 h and then autoclaving it again at 121°C for a further 3 h. Soil inocula was collected from an uncultivated field at the James Hutton Institute (56.457 N, 3.065 W), sieved to 3 cm to remove rocks and large debris, homogenized and stored at 4°C.

We selected three herbivores for our treatments: aphids (*Macrosiphum euphorbiae*), slugs (*Arion* sp.) and nematodes (*Meloidogyne* sp.). These herbivores are all pests of potato plants, they have two different feeding types (sap-feeding and chewing) and thrive in two different compartments (aboveground and belowground). Aphids (*M. euphorbiae*, clonal line AK13/08) were previously collected from a potato field in Scotland in 2008 (The James Hutton Institute, Dundee, UK – 56.457 N, 3.065 W) and reared for several generations on excised leaves of *S. tuberosum* cv. Desirée in ventilated cups at 20°C and 16:8 h (light:dark). Slugs and nematodes were both collected from a field under a long-term potato cultivation (The James Hutton Institute). Slugs were added to the assigned treatment pots immediately after collection. Nematodes were extracted following the sugar centrifugation method (Freckman *et al*., 1977), checked under the microscope to ensure successful extraction, and were inoculated into the appropriate pots using a pipette.

Experimental setup

To evaluate the effects of three herbivores on rhizosphere microbiota, we did or did not expose potato (*S. tuberosum*) plants to three herbivores (aphids, slugs and nematodes). Each treatment was replicated five times producing 20 plants in total. We used 16S rRNA gene amplicon sequencing on rhizosphere soil to reconstruct the bacterial communities thriving in this compartment.

Experimental pots (1 l) were assembled as follows. We added 100 ml of sterile background soil to the bottom and top of each pot to reduce the risk of microbial contamination between pots. Between the layers of sterile background soil, we added a mix of 800 ml soil (90% sterile background soil and 10% inoculum soil from a

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potato field). One potato seedling was transplanted into each pot, and plants were left to grow in an insect-screened greenhouse with an average temperature of 25°C and 16:8 h (light:dark) photoperiod.

Five weeks after transplanting, plants were exposed to herbivory for 3 weeks by aphids (two apterous adults), slugs (two specimens), or nematodes (~10–15 specimens), or experienced no herbivory. All plants were screened with a microperforated plastic bag that allowed transpiration while preventing the escape of aphids or slugs. Three weeks following infestation, we collected ~500 mg of rhizosphere soil from each pot by gently removing the plant roots from the bulk soil, and then vigorously shaking the root system to release the rhizosphere soil. Samples were stored at −80°C until further processing for 16S rRNA gene metabarcoding (Abdelfattah *et al*., 2018).

DNA extraction, Illumina MiSeq libraries preparation and sequencing

Rhizosphere soil samples were crushed in an extraction buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) using three 1 mm Ø stainless steel beads per tube, with the aid of a bead mill homogenizer set at 30 Hz for 5 min (TissueLyzer II, Qiagen, UK). Total DNA was extracted using a phenol/chloroform method, and subsequently checked for quantity and quality with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). We conducted a metabarcoding analysis of bacterial communities by targeting the 16S rRNA gene with primers 515f/806rB (Caporaso *et al*., 2012). Amplifications were also carried out on non-template controls where the sample was replaced with nuclease-free water in order to account for possible contamination of instruments, reagents and consumables used for DNA extraction.

PCR reactions were performed in a total volume of 25 μl, containing about 50 ng of DNA, 0.5 μM of each primer, 1X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, USA) and nuclease-free water. Amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Germany) set at 95°C for 3 min, 98°C for 30 s, 55°C for 30 s and 72°C for 30 s, repeated 35 times, and ended with 10 min of extension at 72°C. Reactions were carried out in technical triplicate, in order to reduce the stochastic variability during amplification (Schmidt *et al*., 2013), and a non-template control in which nuclease-free water replaced target DNA was utilized in all PCR reactions. Libraries were checked on agarose gel for successful amplification and purified with Agencourt AMPure XP kit (Beckman and Coulter, CA, USA) using the supplier's instructions. Although no amplification was observed in non-template control samples, PCR products were processed and sequenced anyway. A second short-run PCR was performed in order to ligate the Illumina i7 and i5 barcodes and adaptors following the supplier's protocol (Illumina, CA, USA), and amplicons were purified again with Agencourt AMPure XP kit. Libraries were then quantified through Qubit spectrophotometer (Thermo Fisher Scientific), normalized using nuclease-free water, pooled together and sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 300PE following the supplier's protocol (Illumina).

Raw reads processing and data analysis

De-multiplexed forward and reverse reads were merged using the PEAR 0.9.1 algorithm with default parameters (Zhang *et al*., 2014). Data QC, OTU clustering and chimera removal were carried out using VSEARCH 2.14.2 (Rognes *et al*. 2016). The few sequences obtained from non-template control samples failed QC and were thus discarded. Taxonomy was assigned to each OTU using VSEARCH by querying the SILVA database (v. 132) (Quast *et al*., 2012). A phylogeny was obtained by aligning representative sequences using MAFFT v7.464 and reconstructing a phylogenetic tree using FastTree (Price *et al*., 2009). Singletons and OTUs coming from amplification of chloroplast DNA were discarded from the downstream analyses.

Data analysis was performed using R statistical software 3.5 (R Core Team, 2020) with the packages phyloseq (McMurdie and Holmes, 2013), vegan (Dixon, 2003) and picante (Kembel *et al*., 2010). Faith's phylogenetic diversity (Faith, 1992) was fit to a linear model using the *lmer()* function (Bates *et al*., 2014) specifying herbivore as a fixed factor and block as random variable. The package *emmeans* was used to infer pairwise contrasts (corrected using false discovery rate [FDR]). Furthermore, we studied the effects of herbivory on the structure of the microbial communities using a multivariate approach. Distances between pairs of samples, in terms of community composition, were calculated using a Bray–Curtis distance matrix, and then visualized using canonical analysis of principal coordinates (CAP) procedure (Anderson and Willis, 2003). Differences between sample groups were inferred through PERMANOVA multivariate analysis (999 permutations, stratified at block level). Pairwise contrasts from PERMANOVA were subjected to FDR correction.

We assessed the impact of each different herbivore (aphids, nematodes, slugs) compared with control using the R package DESeq2 (Love *et al*., 2014). We first built a model using herbivore type as a factor and then we extracted the appropriate contrasts (herbivore/control) for each herbivore. From each contrast, we used the

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absolute log2 fold change values for each OTU to quantify the impact of herbivore type on the microbiota. Comparisons of absolute log2 fold change values were performed by fitting a linear mixed-effects model, specifying the herbivore type (aphids, nematodes, slugs) as fixed factors and OTU identity as a random effect, and using the package *emmeans* to infer contrasts (FDR corrected).

The network analysis was performed using the R package *NetCoMi* (Peschel *et al*., 2020), testing differences in network metrics between pairs of network using the function *netCompare()*.

Data availability

Raw sequencing data are available at NCBI SRA under the BioProject PRJNA733674.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information

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