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Research article

Grapevines and trees: A biodiversity study of microbiomes in an established temperate agroforestry system

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ABSTRACT

Biodiversity is threatened particularly in perennial crop cultivation such as fruit trees or grapevines. If established, agroforestry has the potential to increase biodiversity by providing a higher habitat heterogeneity at the example of grapevine (*Vitis vinifera* L. cv. Riesling) cultivated together with oak or poplar trees for 12 years. Together with the rhizosphere microbiome, the root metabolome was quantified as an indicator of root exudation. Since the root metabolome does not fully align with the exudate metabolome, we are using the root metabolome as a proxy for the exudate metabolome. The results reveal that co-cultivation of grapevine with trees reduces the nutrient availability in the soil and changes the root metabolome of both, grapevine and trees with a more distinct effect of trees on grapevine than *vice versa*, particularly for oak. Apparently, root-to-root signalling takes place between trees and grapevine. Co-cultivation of grapevine and oak trees also enhanced the alpha diversity of the microbiome. Correlation analysis revealed strong positive correlations between distinct microbial families and metabolites enriched in the roots of Riesling. Thus, microbiome analyses support the view that root-to-root interaction in mixed cultivation of grapevine with trees is mediated by root exudation.

1. Introduction

Agroforestry is a multifunctional land-use type where woody perennials are planted alongside agricultural crops in the same field, combining the cultivation of trees and crops in terms of space and time. This cultivation practice has been part of historic agriculture in Europe (Nerlich et al., 2013). As a result, none of these systems remained in production agriculture in Germany by the turn of the millennium, which lead to profound transformations in European agriculture during the past century, driven by both pragmatic and economic considerations. Nevertheless, traditional agroforestry systems persist in several Mediterranean regions, such as the Po Valley in Italy and selected areas of southern Europe. Trees were also used as growth support for vines or diversification of production. In southern France 'Le Hautain' was a common system for growth support of grapevines directly nearby trees as well as 'La Joualle', a system that combined grapevines with slow growing walnut, peach or olive trees and intercropping between rows (Eichhorn et al., 2006). Today, agroforestry is experiencing a revival, being discussed as a potential alternative or complement to a conventional agriculture. Like the broader agricultural sector, viticulture faced contemporary challenges, including declining biodiversity, reduced ecosystem services, and climate change-related issues, all of which may threaten the future production of quality wine (Gary et al., 2017). In historic systems grapevine, with its liana-like growth, relied on trees as natural support structures for their development (Zurowietz et al., 2022). In contrast, modern forms of agroforestry in vineyards allow for mechanization by integrating trees within row trellising systems or through alley-cropping practices (Trambouze and Goma-Fortin, 2013).

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Both traditional and modern agroforestry systems influence microclimate, water and nutrient dynamics, and biodiversity, offering both competitive and synergistic effects (Jose, 2009; Lang et al., 2019, Bayala and Prieto, 2020). For example, tree shading can be either advantageous (by reducing UV exposure) or disadvantageous (by limiting sunlight), depending on environmental conditions. In addition, trees and grapevines may compete for water and nutrients, but hydraulic lift from deep-rooted trees can provide benefits under mild water stress (Lang et al., 2019; Geilfus et al., 2024). These interactions are complex and depend on site-specific conditions and management. Furthermore, the additional pruning of trees, which is not a task typically favoured by winemakers, presents another challenge.

Silvoarable systems with grapevines and trees may offer advantages, especially in a temperate climate. Trees can serve as physical barriers against weeds and pests, improve soil fertility, and contribute to better air and water quality. In addition, silvoarable systems may increase biodiversity in herbaceous plant species, insects, and birds, leading to higher species richness and density. Furthermore, the biodiversity of the soil microbiome, particularly the rhizobiome, including different species of bacteria and mycorrhizal fungi, may be influenced by the presence of trees, especially through root exudates in the rhizosphere. However, the potential impact of these systems on soil biodiversity and wine quality remains underexplored. In this context, the long establishment period for silvoarable vineyards, which can take 5–7 years before grapevines become productive and trees reach a sufficient size, is a challenge.

This study was aimed to identify effects of grapevine-trees co-cultivation on *i*) the biodiversity of rhizosphere soil microorganisms, and *ii*) the diversity of the root metabolome as proxy for root exudation and signalling. The dynamics of root exudation and its impact on the root-associated microbiome can be hypothesized to have a relatively close relationship. Recent reports provide insights into the connections between the root metabolome, exudation dynamics, and the root microbiome (McLaughlin et al., 2023). We hypothesized that co-cultivation of grapevine and trees (i) supports the diversity of the rhizosphere microbiome, (ii) that this support is reflected by changes of the root metabolome, and (iii) that these changes also indicate signalling processes between grapevine and trees. To test our hypotheses, we established a unique silvoarable system through a randomized field experiment including Riesling monoculture, oak monoculture, poplar monoculture, and mixed cropping system with Riesling and oak or Riesling and poplar. We analyzed both the soil microbiome using metabarcoding approaches and the root metabolites of the grapevines using GC-MS metabolic profiling.

2. Materials and methods

2.1. Plant material and experimental design

The agroforestry system analyzed in the present was the so called 'Arbustum' vineyard at Ayl, Rhineland-Palatinate, Germany (Long. 49°37'N, Lat. 006°32'E). The field site is characterized by a mean annual temperature of 9.9 °C and a total precipitation annual of 717 mm. It was established in 2007 by co cultivation of 1 y old *Vitis vinifera* L. cv. Riesling grafted on rootstock Selection Oppenheim 4, and 3 y old oak (*Quercus petraea*) and poplar trees (*P. tremula x P. alba*). The soil at the field site is a hortic-anthrosol with a skeleton fraction of 20–30% and 15% clay with 26.6% inclination. The individual plots were arranged as Riesling monoculture (R), oak monocultures (O), poplar monocultures (P) and as a mixed cropping system with Riesling and oak (RO) or Riesling and poplar (RP) in 4 replicates, each (n = 4). All plot had a size of 12 m × 10 m with 15 trees and 25 vines per plot. Rows had a width of 2.20 m and a total length of 75 m (Fig. 1; for further details see Lang et al., 2019). The planting distance between trees was 4 m, and the distance between grapevines was 2 m (Fig. 1). Within each plot, vines and trees were planted in the same planting hole, with an additional vine planted between them (Fig. 1). Samples were taken from these vines or trees, which grew closely associated within the same root zone. Grapevines were trained with a Sylvoz training system with high fruiting wire and a cordon (Zurowietz et al., 2022). In autumn 2020 about 2 tons per hectare of lime were applicated to the Arbustum to reduce soil acidity. No other fertilization was conducted in 2019–2021. To maintain the agroforestry system, tree pruning to a height of 4 m was performed every third year. Over the past decade, spontaneous ground vegetation has been allowed to establish. In early May and again in early June, ground vegetation was mulched. Beneath the vine rows, vegetation was cleared by tilling.

2.2. Root and rhizosphere soil sampling

Two rhizosphere soil- and root samples were taken and pooled from each of the four plots (n = 4). The root and rhizosphere samples were collected from grapevines and trees that had grown in the same planting hole. The sampling areas were chosen randomly. The samples were taken from the soil area beneath the grapevines and trees. The topsoil was removed, and then a hole was carefully dug along the trunk until a primary root was reached, which was excavated at a depth of approximately 40 cm. The soil directly in contact with the roots was sieved into a glass bowl, using a sterile metal sieve of 1.5 mm pore size and an ethanol-sterilized spoon. The obtained rhizosphere soil was stored in

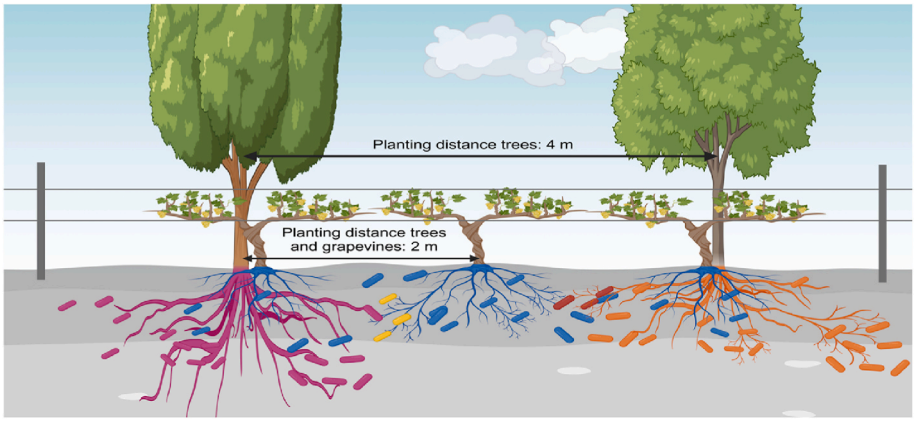

Fig. 1. Schematic view of the 'Arbustum' agroforestry system of Riesling cultivated with poplar and oak. Section of a vine row and trees. The planting distance between trees was 4 m, and the distance between grapevines was 2 m. Each plot consisted of 15 trees and 25 vines. Each plot type (e.g., Riesling with oak) was replicated four times (n = 4). Within each plot, vines and trees were planted in the same planting hole, with an additional vine planted between them. Samples were taken from these vines or trees, which grew closely associated within the same root zone. Soil microorganisms in the rhizosphere depicted as coloured rods.

sterile plastic centrifuge tubes at −80 °C (according to Quiroga et al., 2024). Moreover, at each plot three bulk soil samples of the corresponding rhizosphere sampling area were taken with soil auger at 1–30 cm depth for the assessment of soil nutrients and were pooled. The sampling was done on July 25th' 2021. Root and soil sample were directly frozen at −20 °C on site, and stored at −80 °C at University Hohenheim until further processing.

2.3. Bulk soil pH and nutrient analysis

Potassium, phosphorus, and sulfur were analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent 5110). Soil samples were mixed (soil/solution ratio 1:16, m/V) with HCl 36% and HNO_3 65% (v/v, 3.6:1) incubated for 15 h and boiled for 2 h. After cooling, the solution was filtered and analyzed by ICP-OES. Nitrate and ammonium were extracted using a 12.5 mM $CaCl_2$ solution (soil/solution ratio 1:4, m/V). Nitrate was determined by reduction with hydrazine sulfate/copper after dialysis, followed by diazotization with sulfanilamide and α-naphthylethylenediamine. Absorbance was measured at 540 nm. Ammonium was determined photometrically via the indophenol blue method, with citrate used as a complexing agent. Absorbance was measured at 660 nm using a continuous flow analyzer (Evolution II, Alliance Instruments, Salzburg, Austria). Soil pH was determined in a suspension of soil and 10 mM $CaCl_2$ solution (soil/solution ratio 1:2.5).

-5 0 5

log_2

Class	Metabolite	Riesling compared to …		Influence of … on Riesling		Influence of Riesling on …	
		Quercus (Riesling monoculture/ *quercus* monoculture)	***Poplar*** (Riesling monoculture/ *poplar* monoculture)	***Quercus*** (Riesling plus *quercus* / Riesling monoculture)	***Poplar*** (Riesling plus *poplar* / Riesling monoculture)	***Quercus*** *Quercus* plus Riesling/ *quercus* monoculture	***Poplar*** *Poplar* plus Riesling/ *poplar* monoculture
1	Alanine	4	5	0	-2	-2	4
1	Dihydroxyphenylalanine	5	6	-1	-1		4
1	Glutamic acid	4	6	-4	-2	2	-1
1	Glycine	2	2	-2	-1	0	0
1	Homoserine	1	2	-1	-1	0	1
1	Ornithine	4	4	-5	-1	0	0
1	Tryptophan	0	0	-1	-3	0	-1
2	Benzoic acid, 4-hydroxy-	-3	-5	2	3		-1
2	Tartaric acid	6	5	-2	-1	0	2
3	beta-D-Allose	-1	-3	3	3	1	0
3	beta-D-Fructofuranosyl-(2,1)-beta-D-Fructofuranose	-1	-3	3	3	1	-1
3	Digitoxose	0	-3	1	0	-3	-4
3	Fructose	1	2	0	-1	0	1
3	Galactose	0	-2	2	4	1	0
3	Glucose	1	2	0	-1	0	1
3	Lactulose	-6	-9	0	8	-4	2
3	Mannose	1	2	0	-1	0	1
3	Raffinose	2	0	-1	-2	1	0
3	Sorbose	1	2	0	-1	0	1
4	Inositol, myo-	3	0	0	-1	0	1
4	Salicin	-8	-10	1	8	-7	0
4	Salicylic acid-glucopyranoside	-1	-3	1	3	-2	-1
4	Viburnitol	-10	-7	5	3	1	-7
5	1-O-Benzoyl-beta-D-glucoside	-5	-6	0	5	-7	0
5	4-Methylcatechol	9	7	-1	-1	-1	4
5	Catechin	3	2	-1	-1	-1	1
5	Epigallocatechin	0	4	0	-2	2	2
5	Gallic acid	5	15	1	0	2	12
5	Hesperetin	6	6	1	-1	-4	4
5	Taxifolin	2	3	0	-1	-1	2
6	Benzoic acid, 3,4-dihydroxy-	9	6	0	0	2	4
6	Phenylpyruvic acid	-2	-3	2	2	-1	1
6	Prephenic acid	-4	-4	1	3	-5	0
7	Butanoic acid, 2,4-dihydroxy-	3	6	-2	-1	0	5
7	Butanoic acid, 4-amino-	2	3	-2	-2	-1	2
7	Hexadecanoic acid methyl ester	4	1	-2	1	3	1
7	Isocaproic acid, 2-oxo-	1	2	-2	-2	-1	0
7	Nonanoic acid	2	1	-3	-2	0	0
7	Octadecanoic acid	1	0	0	0	1	2
7	Octadecatrienoic acid methylester	-9	-9	-1	7	-11	0
8	Butane, 1,2,4-trihydroxy-	3	-1	1	-1	-3	-3
8	Carbodiimide	2	2	-2	-1	0	1
	Nicotinic acid, 6-hydroxy-	3	4	-1	-2		2
	Phosphoric acid	1	2	2	1	0	0
	Sitosterol, beta-	1	1	0	-8	-1	

Fig. 2. GC-MS analysis of low molecular substances of Riesling, oak and poplar roots in combined cultivations and as monocultures. Grey colour, missing values. Class (1) amino acids; (2) organic acids; (3) sugars; (4) sugar alcohols; (5) phenolic compounds; (6) carboxylic acids; (7) lipids and derivates; (8) steroids. Differences of the metabolome of Riesling roots compared to the metabolome of quercus roots (Riesling/quercus monoculture). Differences of the metabolome of Riesling roots compared to the metabolome of poplar roots (Riesling/poplar monoculture). Influence of quercus on Riesling root metabolome (Riesling mixed culture with quercus/Riesling monoculture). Influence of poplar on the Riesling root metabolome (Riesling mixed culture with poplar/Riesling monoculture). Influence of Riesling on the quercus root metabolome (quercus mixed culture with Riesling/quercus monoculture). Influence of Riesling on the poplar root metabolome (poplar mixed culture with Riesling/poplar monoculture).

2.4. Metabolome analysis of root samples by gas chromatography-mass spectrometry (GC-MS)

Water-soluble metabolites were extracted, derivatized and separated according to the method previously described in Du et al. (2021). Briefly, 50 mg of homogenised root tissue was extracted in 600 µl of 100% methanol with constant shaking at 1200 *g*, 70 °C for 10 min. Aliquots of 500 µl supernatant were mixed with equal volumes of double-distilled H_2O and chloroform. After centrifugation, 100 µL aliquots of the methanol phase were dried using a freeze dryer. The dried extracts were methoximated in 20 µL of methoxyamine hydrochloride solution (20 mg mL^{-1} in anhydrous pyridine) with incubation at 30 °C for 90 min. For trimethylsilylation, 50 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide was added, and the mixtures were incubated at 37 °C for 30 min with shaking at 1400 *g*. Samples were analyzed together with a mixture of n-alkanes (C8-C20, saturated alkane mixture; Sigma-Aldrich, Steinheim, Germany) for retention index calibration using a GC-MS system (Agilent GC 6820A coupled to a 5975 quadrupole MS detector; Agilent Technologies, Palo Alto, CA, USA) equipped with a HP-5ms capillary column (Agilent, 30 m × 250 µm × 0.25 µm). The settings of the GC-MS system were as described by Du et al. (2016). The data acquired from GC-MS analysis were pre-processed using Agilent MassHunter quantitative data analysis software (Version B.07.00) for peak picking and mass spectral deconvolution. Identification of compounds was performed using spectral matching by comparing the mass spectra of all detected compounds with the Golm metabolome database (Hummel et al., 2010). Peak areas were normalised using the peak area of the internal standard ribitol (Sigma-Aldrich) and the initial fresh weight of the frozen samples. Abundance of metabolites was indicated by normalised peak areas. Artefact peaks and common contaminants were identified by analysis of 'blank' samples (chemicals without root material) prepared in the same manner as biological samples; signals corresponding to these artefacts were omitted from peaks interpretation.

The individual metabolite values were averaged for each biological replicate. These mean values were then compared, and factors (quotients) were calculated to determine differences between the cropping systems. The heatmap (Fig. 2) visualizes the relative changes between the cropping systems, presented as log2-transformed factors.

2.5. DNA extraction and library preparation for microbiome analysis

DNA was extracted from 0.2 g of rhizosphere soil using the Nucleospin Soil Kit (Macherey Nagel) with buffer SL1 and SX according to the manufacturers' instructions. Amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene was performed on a MiSeq Illumina instrument (MiSeq Reagent Kit v3 (600 Cycle); Illumina, San Diego, CA, USA) using the universal eubacterial primers 515F (Parada et al., 2016) and 806R (Apprill et al., 2015). To identify potential contamination during DNA extraction and amplification, both extraction and PCR of no template control samples were performed. Sequencing library preparation was conducted using NEBNext high fidelity polymerase (New England Biolabs, Ipswich, USA) and Nextera XT Index Kit v2 (Illumina, Inc. San Diego, CA, US) as described previously (Kublik et al., 2022). The sequence data obtained in this study are deposited in the short read archive of NCBI under BioProject number PRJNA1181150.

2.6. Data processing and statistical analysis of microbiome sequencing data

Sequences were analyzed on the Galaxy web platform (https://usegalaxy.org). FASTQ files were trimmed with a minimum read length of 50 using Cutadapt (Martin, 2011). Quality control was performed via FastQC (Andrews, 2010). For subsequent data analysis, the DADA2 pipeline (Galaxy Version 1.20) (Callahan et al., 2016) was used with the following trimming and filtering parameters: 20 bp were removed n-terminally and reads were truncated at position 240 (forward) and 200 (reverse), respectively, with an expected error of 4 (forward) and 6 (reverse). With respect to rare variants the pseudo-pooled sample processing approach was chosen (Celis et al., 2022), which includes two rounds of independent sample processing, whereby the second round is trained by "prior" sequence variants from the first round (sequences which could be expected). This approach increases sensitivity to rare variants without an increase in spurious sequences. The resulting unique amplicon sequence variants (ASV) were assigned to the SILVA v138.1 (release 99%) reference database. To exclude potential contamination, ASV occurring in no template controls, as well as unassigned, mitochondrial and chloroplast reads, and singletons (ASV represented by only one read) were removed from the dataset. Moreover, only ASV occurring in at least three of five replicates per experimental group (oak, poplar, Riesling in mono and mixed culture) were considered for further analysis.

Downstream analysis was performed in R version 4.2.2. (https://www.R-project.org). Microbial count data were normalised via the Trimmed Mean of M-values method (Robinson et al., 2010). Alpha diversity was calculated using species richness based on ASV number, Pielou evenness and Shannon diversity index. Beta diversity was analyzed via Bray-Curtis and Jaccard distance matrix. For statistical purpose, Kruskal Wallis test, Wilcoxon-rank sum test and PERMANOVA with Benjamini- Hochberg p value correction for multiple comparison was used. To identify biomarker taxa, ANCOM-BC2 (Analysis of Compositions of Microbiomes with Bias Correction 2) was performed. Multiple test correction was performed by p value adjustment via Benjamini-Hochberg method. All plots were created in R using ggplot2, ggpubr and microViz (Wickham, 2016; https://rpkgs.datanovia.com/ggpubr/; https://doi.org/10.21105/joss.03201). Principle component analysis (PCA) was conducted by 'prcomp' R software (version 4.3.1) (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Co-cultivation reduces soil nutrient availability

Bulk soil samples of the plots were analyzed for pH and nutrient content. Soil pH ranged from 6.0 to 6.4, with the highest value in the Riesling monoculture. Plant-available nitrogen, measured as N_{min}, ranged from 5 to 16 kg ha^{-1} in all plots representing medium N supply for grapevines. Soil potassium (K) contents of all plots was approximately 200 mg K_2O kg^{-1} soil dry matter (DM) representing a good/high level for grapevines. The soil potassium (P) content ranged from approximately 130-160 mg P_2O_5 kg^{-1} soil DM, which is considered an optimal supply level for grapevines. The sulfur (S) content in the soil was around 200 mg kg^{-1} soil DM in all plots, representing a good supply. Compared to the mixed systems, the Riesling monoculture had higher bulk soil contents of N, P, K and S.

3.2. Riesling root metabolome altered by co-cultivation

Root metabolite profiles were analyzed as proxy for root exudation (Maurer et al., 2021; McLaughlin). The analysis reveals the interaction between the grapevine metabolome and the tree metabolome indicating a root-to-root signalling. Riesling exhibits higher concentrations of amino acids (class 1) and phenolics (class 5) compared to trees (Fig. 2 left two columns). Sugar alcohols (class 4) are found in lower concentrations in Riesling. Trees influence grapevine amino acids (class 1) and lipid (class 7) with both decrease in association with trees, while sugar alcohols (class 4) tend to increase (Fig. 2, middle two columns). However, the influence of oak differs from that of poplar on the grapevine root metabolome. As a general trend (Fig. 3), the impact of the grapevine on the trees is relatively lower compared to the trees' impact on the grapevine as indicated by PCA analysis.

The root metabolome of Riesling, oak and poplar in monocultures

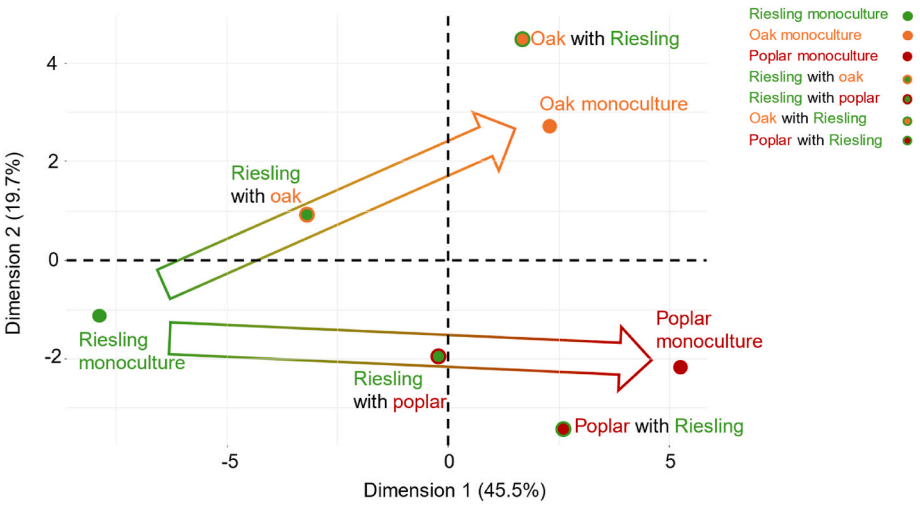

Fig. 3. PCA of root metabolites of Riesling (green) with oak (brown) or poplar (red) in combination and as monoculture, (average values). Colour gradient of the arrow indicates gradient of metabolites from grapevine (green) to trees (brown/red).

each show distinct differences (Fig. 3). The metabolome of Riesling with oak is positioned between the metabolomes of Riesling monoculture and oak monoculture. Thus, the metabolome of Riesling monoculture (green part of the arrow) gradually approaches that of the oak co-culture (brown arrowhead). The same phenomenon is observed starting with Riesling monoculture toward poplar with Riesling. Thus, the root metabolome of Riesling is influenced by the co-culture with poplar and oak. However, the metabolome of the trees is hardly changed.

3.3. *Rhizosphere microbiome diversity varies across cropping systems*

Alpha diversity of the rhizosphere microbiome showed significant differences between the monocultures with highest microbial diversity for poplar (Fig. 4A). In comparison to the respective monocultures, oak grown in combination with Riesling (OR) showed significantly higher diversity, whereas the diversity of poplar in mixed culture with Riesling (PR) was clearly reduced. Although not significant, Riesling mixed with oak or poplar (RO, RP) showed an increasing trend similar to OR compared to Riesling in monoculture. Consequently, all mixed cropping systems resulted in a comparable diversity. Despite this, every cropping system revealed a highly distinct microbial community (Fig. 4B). Whereas Riesling (R) and Riesling mixed with oak (RO) was clearly separated, the microbial community of oak showed highest similarity to oak grown with Riesling (OR). Monoculture of poplar formed a distinct cluster, while polar mixed cultures (PR, RP) showed the greatest similarity to each other. These results were confirmed when looking at the community composition: although most taxa occurred independently of plant species, their relative contribution to the microbial community was very different (Fig. 4C). In comparison to both tree species, Riesling showed a higher relative abundance of members of Proteobacteria (*Steroidobacter*, unclassified R7C24) and Actinobacteriota (unclassified *Micromonosporaceae*) that were also found for RO and, less pronounced, for RP. Overall, the microbial community composition of RO was more comparable to Riesling grown in monoculture than RP, which showed higher similarity to PR (Fig. 4C, S2). Accordingly, correlation analysis revealed strong positive correlations between *Steroidobacter, Cryptosporangium* and unclassified Micromonosporaceae of the rhizosphere microbiome and metabolites enriched in the roots of Riesling, mainly belonging to amino acids (class 1) and phenolics (class 5) (Fig. 4D).

4. Discussion

Silvoarable systems are thought to support higher biodiversity than monoculture croplands. A meta-analysis investigating modern agroforestry systems in temperate regions found a positive effect on biological control and pollination services compared to arable cropping systems (Staton et al., 2019). Biodiversity trends in plants, fungi, and insects also showed positive tendencies, although with considerable variation across different agroforestry systems and regions (Torralba et al., 2016). The results varied depending on the type of agroforestry system and analyzed ecosystem services (Bourgade et al., 2020). Results for provisioning services were inconsistent, while the types, regions, or composition of agroforestry systems had no clear influence on positive effects (Kay et al., 2018; Dufourcq et al., 2017). Therefore, it is crucial to identify region-specific patterns of biodiversity response to agroforestry. We investigated the effect of high-value system of grapevines and trees in the viticultural area of southwest Germany, a temperate region, where no data is currently available. To address the challenge of dynamics related to the transition from crop-based to agroforestry-based agriculture (rather than the sustainable effects of the silvoarable system itself), we establish tree crowns and grapevines 13 years prior to the analyses, with the understanding that they also need 4–5 years to produce a considerable harvest.

The integration of trees into viticulture has a notable impact on the microclimate, a dimension that warrants further investigation. In this context, tree shading can have a beneficial effect by preventing sunburn on grapes excessively exposed to sunlight, reducing grapevine transpiration through shading, and possibly influencing competition for nutrients and water resources. Conversely, the 'hydraulic lift' phenomenon facilitated by deeper tree roots may also yield positive effects. The analysis of plant nutrients in soil solution (Table 1) reveals that Riesling monoculture has highest concentrations of N, P, K and S compared to the mixed systems with trees. This suggests that co-cultivation may require greater attention to nutrient balance in mixed systems, as trees – and potentially different in vegetation and microbiomes – might induce a (slightly) greater demand for these nutrients over time. However, the concentrations of these plant nutrients in mixed cultures remained within a non-critical range.

The present study provides the first insights into the interactions between trees and grapevines in a cool-climate viticulture, specifically in terms of rhizosphere biodiversity. There is clear evidence that the direct cultivation of grapevines, here *cv.* Riesling, with trees alter the rhizosphere microbiome. The interaction between roots and microorganisms was distinct, even when Riesling is in co-cultivation with either oak or poplar. Notably, it is possible that roots of oak may sequester different exudate signatures than poplar, which could influence the interaction of grapevine and tree rhizosphere through root-to-root signalling. It has been clearly demonstrated that the metabolite signature in

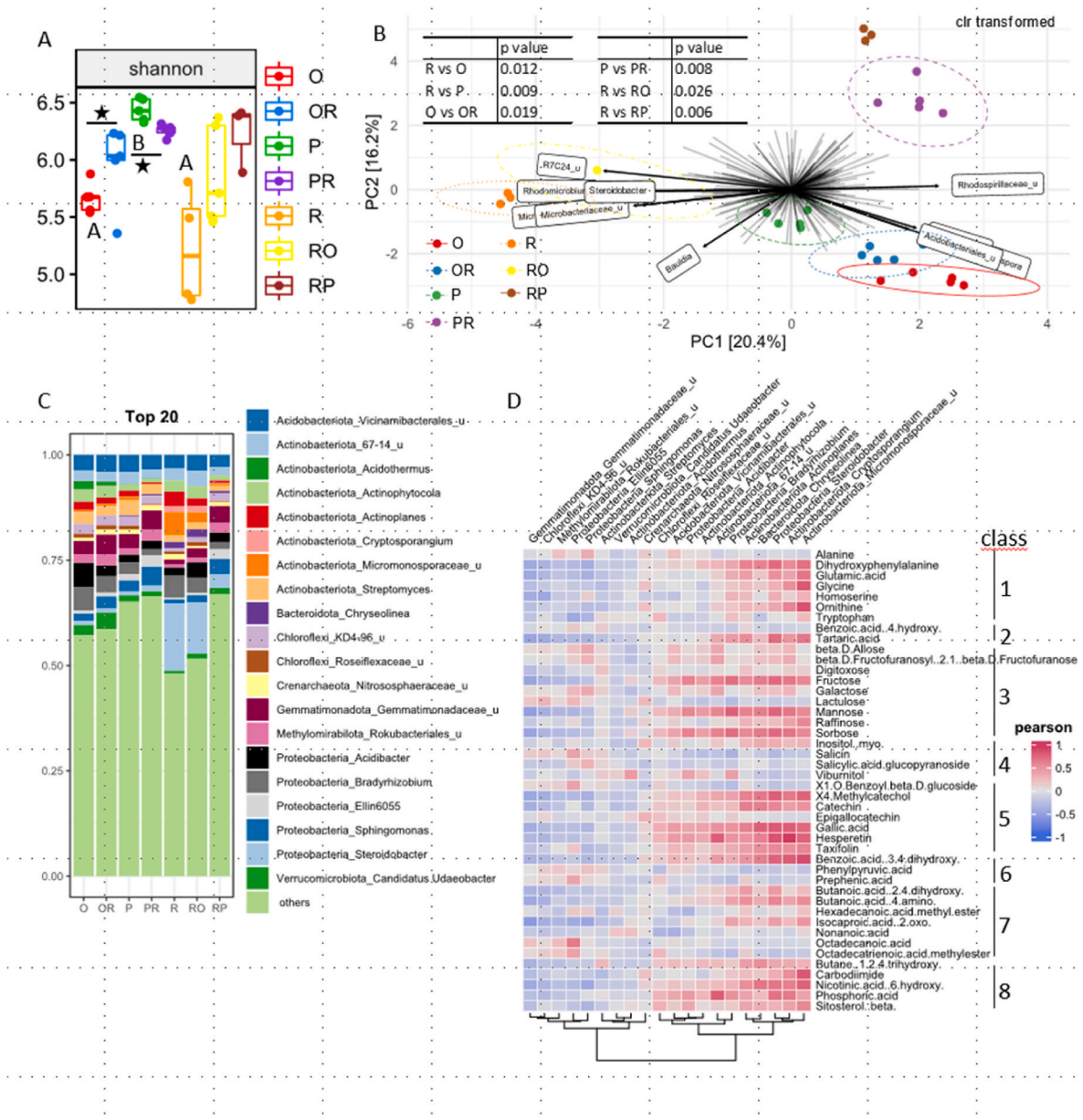

Fig. 4. Analysis of rhizosphere microbiome of Riesling (R) with oak (O) and poplar (P) in combination and monoculture. **A**: Boxplots of Sannon diversity index. Letters indicate significant difference between monocultures (O, P, R), asterisks indicate significant difference between the respective mono and mix culture. OR, rhizosphere of oak root grown in combination with Riesling; RO, rhizosphere of Riesling grown in combination with oak. PR, rhizosphere of poplar root grown in combination with Riesling; RP, rhizosphere of Riesling grown in combination with poplar. Statistical analysis was performed using Wilcoxon Rank-Sum test with Benjamini-Hochberg correction for multiple comparisons. **B:** PCA plot of clr-transformed data. Statistical analysis was performed using PERMANOVA with Benjamini-Hochberg correction for multiple comparisons. **C:** Relative abundance of top 20 genera. **D:** Heatmap showing Pearson correlation between top 20 genera and metabolites detected in rhizosphere. Classes according to Fig. 2.

Table 1
Mean bulk soil pH and nutrient contents. Riesling monoculture (R), oak monoculture (O), poplar monoculture (P), mixed cropping system with Riesling and oak (RO), and Riesling and poplar (RP). Significant differences between treatments (Tukey-Test, p = 0.05) are indicated by different letters.

	N_{min} (kg/ha)	pH	Potassium (K_2O mg/kg soil DM)	Phosphorous (P_2P_5 mg/kg soil DM)	Sulfur (mg/kg soil DM)
R	16.1 ± 0.8a	6.4 ± 0.1a	221.7 ± 13a	168.3 ± 12a	262 ± 23a
O	5.1 ± 2.5d	6.0 ± 0.1a	181.9 ± 9.6b	159.2 ± 21a	208 ± 39bc
P	13.4 ± 1.0b	6.2 ± 0.1a	175.1 ± 5.3b	133.6 ± 7.5b	220 ± 18b
RO	9.6 ± 2.1c	6.1 ± 0.1a	197.1 ± 10b	161.7 ± 13a	198 ± 20c
RP	7.7 ± 1.5c	6.2 ± 0.1a	184.1 ± 5.0b	149.6 ± 10b	201 ± 16c

the roots of Riesling is influenced by the association with either oak or poplar indication root-to-root signalling and leaving behind a distinct metabolite signature (see Fig. 3). Consequently, it is also expected that the rhizosphere microbiome will change specifically according to the type of tree associated. This was also demonstrated in the present study (see Fig. 4).

The most pronounced difference in the community composition between Riesling and the trees was the high abundance of members of the genus Steroidobacter in the rhizosphere of Riesling. Steroidobacter species are commonly found in soil environments and are versatile bacteria with several metabolic functions, particularly in the context of nitrogen turnover and organic compound degradation (Ikenaga et al., 2021; Xun et al., 2021). This enrichment most likely indicates a special

adaptation to the root exudation pattern of Riesling, which is further confirmed by the strong correlation found with metabolites that are increased in the Riesling rhizosphere. However, growing Riesling in mixed cropping systems affected microbial diversity and significantly shaped the microbial community, with an effect depending on the co-cultivated tree species. While Riesling in combination with oak showed highest similarity to the respective monocultures, a mixed cropping system with poplar appeared to establish a highly specialized microbial community, clearly differing from both Riesling and poplar, with unknown consequences for soil and plant health as well as grapevine yield and quality. This result clearly indicates that the choice of co-cultivated tree species matters, and further investigation into its role is necessary. Moreover, to complete the picture it would be valuable to include fungal communities in future analyses.

Another benefit of agroforestry involving trees and grapevines is of an economic nature. We have learned that the price of a bottle of wine from such a modern and fashionable system is rising due to its unique characteristics. The ability to tell an interesting story adds to its marketing appeal, driving the introduction of more silvoarable systems.

5. Conclusion

The present results demonstrate that co-cultivation of grapevine and trees modifies the root metabolome of the co-cultivation partners through root-to-root signalling in a species-specific manner, accompanied by an increase in biodiversity driven by specific microbial families. These processes can be attributed to changes in root exudation that are indicated by the observed changes in the root metabolome. However, it cannot be ruled out that signalling processes via volatile metabolites also contribute to the observed changes in the root metabolome of the co-cultivation partners and the microbiome. This aspect requires attention in future studies.

CRediT authorship contribution statement

Patrick Pascal Lehr: Writing – original draft, Software, Methodology. **Silvia Gschwendtner:** Writing – original draft, Software, Methodology, Data curation. **Baoguo Du:** Validation, Software, Methodology, Data curation. **Heinz Rennenberg:** Supervision, Investigation, Conceptualization. **Michael Schloter:** Supervision, Conceptualization. **Christian Zörb:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2025.124882.

Data availability

Data will be made available on request.

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