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## Do we need to consider grape phyllosphere microbiome in breeding schemes?

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## Summary

The aerial surface of the plant (phyllosphere) is the habitat of complex microbial communities. These communities may have profound effects on host plant health and its performance traits.

When breeding new cultivars, *i.e.* the aerial component of a grape plant, one can simply ignore the phyllosphere in breeding schemes if its composition is mainly dependent on the environment. It is considered an important component if the genotype is the main driver of the phyllosphere composition. In order to answer this question, we have analysed several factors influencing the structure of the phyllosphere microbial community. Using amplicon sequencing of the 16S rRNA gene and of the internal transcribed spacer (ITS), we explored the microbial diversity at genus level for both bacteria and fungi present in the phyllosphere of leaves and grape berries. We analysed it on different grape taxonomic level (between five *Vitis* species or a set of *Vitis vinifera* cultivars chosen to represent the three genetic pools of the species), for different years and on five commercially important varieties of *Vitis vinifera* that were sampled from three different French terroirs. Our results indicated the presence of complex microbial diversity and assemblages in the phyllosphere. A significant effect of several factors (organ, grape species, growing year and terroir) on taxa abundance was observed with varying degrees of effect. At a given location, genotypes have an impact on microbial assemblage in the phyllosphere of leaf and berries, most pronounced on fruits but the effect of terroir was much stronger than the cultivar identity when the leaf phyllosphere of five grapevine varieties grown in different agro-climatic zones was compared. Limitations of the study as well as implied consequences of this work will be discussed.

## Keywords

**Biotic interactions, Phyllosphere microbiome, amplicon sequencing, extended ideotype**

## Introduction

Microbiome is the collective name for the ecological communities that live on, in or near an organism, including pathogenic, commensal and symbiotic partners. The 'Plant Microbiome' for its part, consists of microbial communities in three compartments: the rhizosphere, corresponding to the zone of soil immediately surrounding the roots, the endosphere composed of the micro-organisms present inside the plant tissues and the phyllosphere corresponding to the aerial surface of a plant (stem, leaf, flower, fruit), and by extension the microorganisms present on the surface of the plant.

The plant microbiome, which mostly consists of bacteria and fungi, are involved in major functions such as nitrogen fixation (Jones 1970), carbon sequestration (Bringel and Couée, 2015), degradation of pesticides and organic pollutants (Brandl *et al.*, 2001; Bulgarelli *et al.*, 2013) and plant resistance to biotic and abiotic stresses (Vandenkoornhuys *et al.*, 2015), but also diseases.

In grape, the microbiome on berries may also have an impact on wine fermentation (Bokulich *et al.*, 2013). It could provide solutions in the future, in order to maintain grapevine cultivation in a context of climate change (Gambetta *et al.*, 2020) and pesticide-free agriculture (Jacquet *et al.*, 2022; Pertot *et al.*, 2017). The grape microbiome evolves during the lifetime of the vine, from the grafting stage in the nursery through to decline in the vineyard, and also on a larger scale, through the domestication (Fournier *et al.*, 2022).

The phyllosphere is rather less extensively studied as compared to the rhizosphere and endosphere (Vorholt, 2012). Due to the limited nutrient availability and because the phyllosphere is directly in contact with the atmosphere and fluctuating climatic conditions, it is a dynamic and stressful habitat for its microbial colonizers.

Until now, the microbiome has not been considered as an important component in breeding schemes. We could in particular take into account the capabilities of the genotype to



recruit specific microbial communities as an important trait to improve. But does the grape genotype have an important role as driver of microbiome composition?

Several work in grape have suggested that microbiome differs from one organ to the other (Zarraonaindia *et al.*, 2015), that the environmental conditions at different geographic locations (Bokulich *et al.*, 2013; Gao *et al.* 2019), the growing seasons (Guzzon *et al.*, 2021; Marzano *et al.*, 2016) and the soil composition (Zarraonaindia *et al.*, 2015) are important drivers of the microbiome. Similarly, farming system (Castrillo *et al.*, 2019; Vega-Avila *et al.*, 2015) and more specifically copper treatments (Martins *et al.*, 2012), watering and tillage (Vink *et al.* 2021), the age of the plant or the organ (Berlanas *et al.*, 2019; Ji *et al.*, 2019; Martins *et al.*, 2012) and post-harvest treatments (Salveti *et al.*, 2016) influence microbiome composition. In a given location, several studies have also identified an effect of the varieties (Awad *et al.*, 2020; Bao *et al.* 2022; Marasco *et al.*, 2018; Zhang *et al.*, 2019; Zhang *et al.*, 2020) but on different components of the microbiome. Analyses considering different levels of variation and enabling comparison of different drivers, on the same microbiome components were however sorely needed.

We thus analysed grape phyllosphere, both bacterial and fungal communities, through a metabarcoding approach, taking into account several levels of variation: the genetic identity of the scion (analysing diversity at different botanical levels), the organ (analysing both leaf and berry phyllosphere) and the environment (analysing phyllosphere during 2 years, at spring and harvest and for five cultivars analysing leaves harvested in 3 agro-climatic zones in France).

## Material and Methods

### Samples

Twenty fully developed asymptomatic leaves from *Vitis vinifera*, *V. riparia*, *V. pentagona*, *Muscadinia rotundifolia* and *Parthenocissus* were collected from the repository of Institut Agro Montpellier in southern France (Mediterranean) in the Spring season (mid of May 2017 and 2018, before fungicide spraying) by Singh *et al.* (2019). These species represent quite well separated branches on a phylogenetic tree (Fig. 1).

Similarly, leaf samples from five commercially important varieties (Cabernet-Sauvignon, Chardonnay, Syrah, Grenache, Sauvignon blanc) were taken from three different French geographic locations, (INRA field stations from Bordeaux, Montpellier, and Colmar) representing the three agro-climate zones in the mid of spring season (before spraying of fungicides) by Singh *et al.* (2018b).

Finally, five cultivars from each of the three main genetic pools, which were selected (Table 1) to maximize the distance between genetic pools were analysed (Singh *et al.* 2018b). Leaf samples were taken at Vassal INRAE Experimental Unit (Marseillan-Plage, France near Montpellier) from four to five plants of each cultivar in the spring season (mid of May 2017, before spraying of fungicides) and harvesting season (September 2017). Berries were also collected from eleven of these varieties during the harvest season.

### Processing of the samples and data analysis

Microbial communities from the surfaces of the organs were washed and DNA was extracted according to the procedures of Singh *et al.* (2018b).



Unrooted tree of 15 species of vitacea based on sequenes at 229 genes (Wen et al, 2013)

Fig. 1: Phylogenetic unrooted tree of 15 species of *Vitaceae* based on sequences at 229 genes (Wen *et al.*, 2013) and position of the species sampled in the study (photo credit: INRAE domain of Vassal)

Table 1: Identity of the 15 grapevine cultivars sampled grouped in the three genetic pools

	Genetic Pool		
	WW	WE	TE
	Donzelinho	Basicata	Ichkimar
<b>Cultivars of <i>Vitis</i></b>	Petit Verdot	Alba Imputotato	Khoussainé blanc
<b><i>vinifera</i></b>	Camaraou Noir	Gros Bourgogne	Sourkhak Biley
	Courbu	Koilliniatico	Abouhu
	Savagnin Blanc	Negru Vertos	Dabouki

To access bacterial communities, the V4 region of the 16S ribosomal gene was amplified using primers 515F and 806R (Caporaso *et al.* 2011). Fungal community diversity and abundance were accessed using modified ITS9 and ITS4 primers targeting the ITS2 region (Lundberg *et al.* 2013). PCR products were then analysed by sequencing using 2 × 250 bp MiSeq v2 sequencing (Illumina Inc., San Diego, CA, USA).

Paired-end sequence reads from 16S and ITS sequences were filtered, trimmed and processed with the dada2 v1.8 (R Bioconductor package). A core Divisive Amplicon Denoising Algorithm (DADA) was performed on these filtered files and amplicon sequence variants (or OTUs) were inferred after chimeras removal. Taxonomy was assigned to bacterial and fungal

OTU sequences using the RDP classifier and UNITE data base. Alpha and  $\beta$ -diversity estimates were obtained using phyloseq package. PCoA ordination was performed on variance stabilised log-transformed data using the Bray-Curtis dissimilarity matrix and visualised by using their base functions in the phyloseq package. For more details on the data analysis, see Singh *et al.* (2018b and 2019).

## Results

### Effect of the organs

Comparison of the phyllosphere of leaves and berries (Fig. 2), revealed significantly different Chao1 estimates of  $\alpha$ -diversity for both bacterial and fungal communities (ANOVA, for 16S-data: Chao1,  $P = 0.007$ ; for ITS data: Chao1,  $P = 4.53e-08$ ). The distinction between leaf and berry phyllosphere was also clearly observed on the PCoA analysis (Figure 2 C and D): total bacterial and fungal communities for leaves were well separated from those of berries (PERMANOVA; for 16S data: at  $F = 45.384$ ,  $R^2 = 4.121$ ,  $P = 0.001$ ; for ITS data: at  $F = 48.306$ ,  $R^2 = 2.539$ ,  $P = 0.001$ )

### Effects of year and genetics

In a second experiment, we analysed the phyllosphere of leaves for the five species *Vitis vinifera*, *V. riparia*, *V. pentag-*

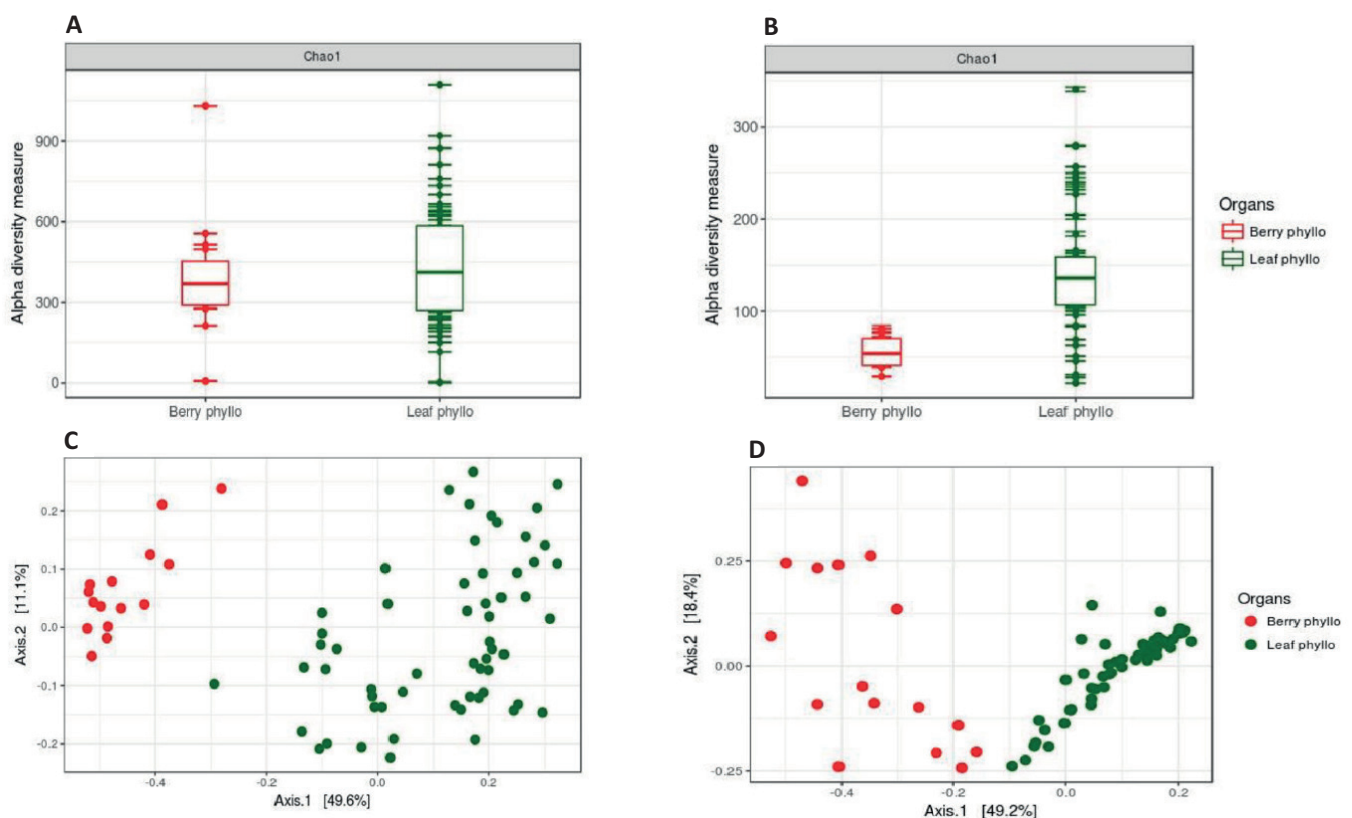


Fig. 2: Chao1 estimates of  $\alpha$ -diversity for (A) bacterial and (B) fungal data-sets for both the organ types. PCoA plots using Bray-Curtis distance between samples for (C) bacterial and (D) fungal data-sets as per leaf and berry samples based on Bray-Curtis distance matrices, explaining >60% variations with first two axes (taxa with variance < 1e-05 were trimmed). From Singh *et al.*, 2018b.

ona, *Muscadinia rotundifolia* and *Parthenocissus* during two consecutive years (2017 and 2018).

Samples for fungal communities from the five species were clearly differentiated by the PCoA analysis on microbial abundance data according to the year (Fig. 3 B). This differentiation was not as clear for bacterial communities (Fig. 3 A). In a given year, a lower but significant impact of grape species in shaping phyllosphere microbiome, especially the fungal microbiome, could also be observed, even if distance of the PCoA did not coincide with genetic distances.

### Effects of environment and genetics

In a third experiment, five commercially important varieties, ‘Cabernet-Sauvignon’, ‘Chardonnay’, ‘Syrah’, ‘Grenache’, ‘Sauvignon blanc’, were sampled in three different French agro-climate zones: Oceanic, Continental and Mediterranean.

The analysis of the leaf phyllosphere from each cultivar in the 3 zones were then compared: the PCoA on the Bray-Curtis dissimilarity matrix (Figs. 4 A and B) revealed a clear differentiation of the samples according to the agro-climatic zones rather than the identity of the cultivars (PERMANOVA for 16S data:  $F = 12.98$ ,  $p = 0.001$ ; for ITS data:  $F = 6.094$ ,  $p = 0.001$ ). The diversity estimates also indicated very significant differences in OTU richness (Figs 4 C and D) between the three regions (ANOVA for 16S data:  $F = 25.73$ ,  $p = 3.11 \cdot 10^{-7}$ ; for ITS data: at  $F = 26.329$ ,  $p = 2.5 \cdot 10^{-7}$ ).

In addition, different relative abundance of few genera were observed among the three agro-climate zones (Figs. 4 E and F).

### Conclusion

Many published papers identified the effect of varieties on microbiome composition (Awad et al., 2020; Bao et al. 2022; Marasco et al., 2018; Zhang et al., 2019; Zhang et al., 2020). Many papers also detected a greater effect of environment on

microbiome than genotype (Bagheri et al., 2019; Belessi et al., 2022; Berlanas et al., 2019; Bokulich et al., 2013; Kioroglou et al., 2019; Mezzasalma et al., 2018; Portillo et al., 2016). These papers however did not necessarily compare the same component of the plant microbiome, nor the same level of genetic diversity. For these reasons, we designed several experiments analysing the phyllosphere using the same metabarcoding methods and comparing different botanical levels.

The experiments described in the presentation clearly demonstrated on one hand that the organ has a strong effect on the microbial communities that inhabit its surface. It is probably due to difference in nutrient availability but also of microclimate around the fruits differing from the harsher climatic condition of leaves.

On the other hand, the comparison of the effect of both year and environment versus genetic identity of the scion revealed that the main driver of phyllosphere microbiome composition is the environment. Comparison of *V. vinifera* cultivars in the three different agro-climatic zones suggested that there is not only a difference in taxonomic compositions, but each agro-climate zone has a unique microbial signature. Comparing a larger diversity of *V. vinifera* cultivars, Singh et al. (2018a) confirmed the small effect of genotype and found only two bacterial taxons (genus *Gemmatimonas* and *Hymenobacter*), and one fungal taxon (genus *Penicillium*) differentially abundant between *V. vinifera* cultivars from different genetic pools.

At a given year, a lower but significant impact of more distant genetic material could however be observed.

In conclusion, since the grape genotype does not have an important role as driver of microbiome composition, particularly in *V. vinifera*, one can consider that breeding cultivars that may be used in very different environments may not need to take into account the global composition of the phyllosphere in breeding schemes. It does not mean that microbiome may not be important, in particular in order to maintain grapevine

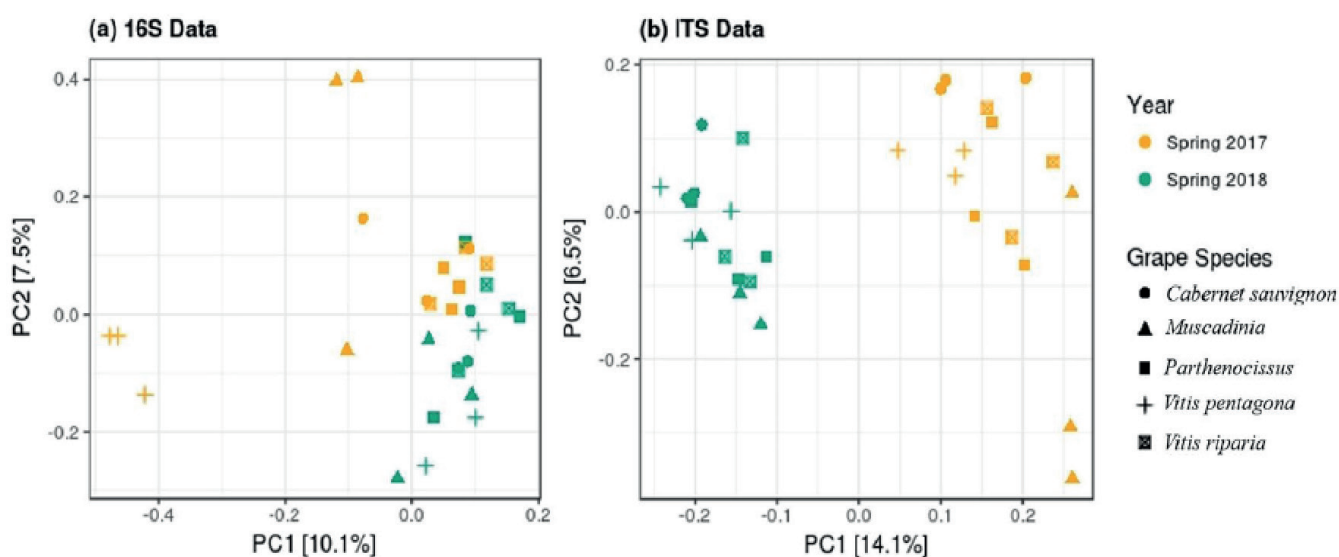


Fig. 3: PCoA ordinations of (a) bacterial and (b) fungal communities derived from leaf phyllosphere of 5 different species at two growing years, using Bray-Curtis distance matrix. Both the axis explains ~20% of variations. The shape represents grape species (N = 30). From Singh et al., 2019.



Fig. 4: PCoA plots displaying strong (A) bacterial and (B) fungal compositional dissimilarity among agro-climate zones and observed (C) bacterial and (D) fungal  $\alpha$ -diversity measures of each variety (X-axis) grouped in three agro-climate zones and relative abundance plot for (E) bacterial and (F) fungal genera displaying differential abundance of few genera among three agro-climate zones (or region).  $n = 45$ . From Singh *et al.*, 2018b.

cultivation in a context of climate change and pesticide-free agriculture, and it would be interesting to investigate more deeply to identify specific taxa differentiating the microbial communities on specific cultivars. Similar analysis should be done for the other components of the plant microbiome, the endosphere and the rhizosphere.

### Conflicts of interest

The authors declare that they do not have any conflicts of interest.

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