TOWARDS MICROBIOTA-BASED DISEASE MANAGEMENT: ANALYSIS OF GRAPEVINE MICROBIOTA IN PLOTS WITH CONTRASTED LEVELS OF DOWNY MILDEW INFECTION

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Abstract:

Context and purpose of the study

Vineyards harbor a myriad of microorganisms that interact with each other and with the grapevines. Some microorganisms are plant pathogens, such as the oomycete *Plasmopara viticola* that causes grapevine downy mildew. Others, such as plant growth promoting bacteria and disease biocontrol agents, have a positive influence on vine health. The present study aims to (1) investigate whether vine-based culture media increase the cultivability of the grapevine microbiota, in comparison to standard culture media and (2) identify and isolate bacterial taxa naturally present in grapevine leaves and significantly more abundant in plots showing low susceptibility to downy mildew.

Material and methods

Seven pairs of vineyard plots differing significantly in downy mildew symptoms frequency and intensity were selected based on a long-term epidemiological survey conducted in France by the *Institut Français de la Vigne et du Vin* (IFV). In each plot, we sampled young leaves before the first fungicide treatments. Leaves were shredded and washed in a buffer to isolate and cultivate foliar bacteria, yeasts and filamentous fungi. We assessed the abundance (CFU/ml) of cultivable bacterial and fungal cells for several culture media, including a vine-based culture medium. Bacterial isolates were identified using MALDI-TOF MS.

Results

Yeasts, filamentous fungi, and bacteria were significantly less abundant on the vine-based culture medium than on the other media. Out of 965 total bacterial isolates analyzed, 597 were identified at least to the genus level by MALDI-TOF MS, and of these, 23% to the species level. Two bacterial genera, *Gordonia* and *Rahnella,* were isolated exclusively on the vine-based culture medium. On the other hand, 10 genera, *Rahnella*, *Paracoccus*, *Acinetobacter*, *Nocardia*, *Achromobacter*, *Peribacillus*, *Solibacillu*, *Xanthomona*, *Budvicia* and *Paenibacillus* were isolated exclusively from samples collected from plots with low infection levels.

Keywords: Grapevine, downy mildew, microbial biodiversity, culturomics, phyllosphere, biocontrol

1. Introduction

The oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni is a biotrophic and obligate parasite causing downy mildew, a major disease of cultivated grapevines reported in most wine-growing regions of the world (Bois et al., 2017; Fontaine et al., 2021; Koledenkova et al., 2022). *P. viticola* is a polycyclic pathogen that causes both primary and secondary infection cycles through the release of sexual and asexual spores, respectively (Gessler et al., 2011). The sexual spores differentiate in the fall on infected tissues and overwinter as oospores in soil (Rossi et al., 2009). The epidemic stage consists of a succession of asexual generations on green and young tissues during grapevine vegetative period. Due to the high susceptibility of *Vitis vinifera* L. to *P. viticola*, chemical fungicides are, to date, the major means of disease control (Gessler et al., 2011; Koledenkova et al., 2022). Research on alternative means of control, including microbiota management, represents a priority because of the proven negative consequences of fungicides on environmental and human health (Busby et al., 2017; Jacquet et al., 2022).

In the present study, we attempted to cultivate the myriad of microorganisms that colonize grapevine leaves (Bettenfeld et al., 2021; Fournier et al., 2022) to assemble microbial consortia that could protect vines against the asexual stage of *P. viticola*. To increase the probability of isolating microorganisms of interest for future biocontrol solutions, we developed a new plant-based culture medium and investigated whether it increases the cultivable microbial diversity in comparison to standard culture media.

2. Material and methods

Selection of study sites based on epidemiological records

Vineyard plots were selected based on the epidemiological data provided by the *Institut Français de la Vigne et du Vin* (IFV). The IFV performs weekly visual assessments of the intensity of downy mildew symptoms on leaves, the frequency of symptoms on leaves, the intensity of symptoms on bunches and the frequency of symptoms on bunches. The symptoms are visually assessed on vine rows that do not receive any fungicide treatment. These Untreated Controls (UC) are located within cultivated plots. Since 2002, the IFV has monitored 1200 UC, representing 4 major wine-producing regions in France. Based on these epidemiological records, we identified pairs of plots that differed in the frequency and intensity of downy mildew attacks (Fig. 1), while being geographically close (< 10 km), planted with the same grape variety and managed with the same farming practices. We considered that plots were contrasted in terms of downy mildew attacks if the area under the disease progression curve (AUDPC) of one of the two plots was higher for the 4 variables, for at least 80% of the years of common monitoring. We only kept plot pairs that had been monitored jointly for at least 4 years, including 2020 and/or 2021.

Sampling design

The sampling campaign occurred from April 18 to May 2 2022, corresponding to the phenological stage of 2-3 spread leaves. In each plot, we sampled leaves in 2 areas: the untreated controls used by IFV to monitor disease in 2021 (UC1) and the center of the plot (CEN). In each area, leaves were collected from four adjacent vines representative of the age and general condition of the plot. About 20 leaves were collected in the UC1 and CEN areas for analysis of the cultivable diversity (Fig. 1).

Isolation of cultivable microorganisms from leaf samples

Isolation and enumeration – On the day of sampling, leaves were shredded and then washed in a sterile buffer composed of 9g/L NaCl and 100µL/L Tween 20 in deionised water to recover and cultivate epiphytic and endophytic microorganisms indiscriminately. Three types of media were used for bacteria: Tryptic Soy Agar (TSA), Luria Bertani Broth diluted 1:10 and supplemented with 20g/L agar (LBA), and a solid medium consisting of 50mL/L grapevine (Cabernet-Sauvignon) leaf extract and 20g/L agar (GLA). All three media had their pH adjusted to 7 and were supplemented with 150mg/L biphenyl and 100mg/L pimaricin. Two types of solid media were used for yeasts and filamentous fungi: malt extract agar (MEA) and the GLA medium described above supplemented with 100mg/L chloramphenicol. Cell suspensions were serially diluted and plated in triplicate on

the solid media. Bacterial plates were incubated at 30°C for 2-5 days while fungal and yeast plates were incubated at 25°C for 5-12 days. We assessed the abundance of cultivable microorganisms (CFU/ml) at the end of the incubation period.

Taxonomic identification - For each sample, a single plate containing between 30 and 300 colonies was selected among the replicates of dilution and approximately 30 colonies were subcultured for identification and collection. The bacteria were identified by MALDI-TOF MS (Fig. 1).

Statistics

We used linear mixed-effects models to explain variation in CFU/ml for bacteria and fungi. The models had culture media type as fixed effect, and plot pair and susceptibility to downy mildew as random effects.

3. Results and discussion

3.1. *Seven pairs of vineyard plots (with low vs high susceptibility to downy mildew) were selected according to epidemiological records*

Fourteen vineyard plots, forming 7 pairs contrasted in terms of frequency and intensity of downy mildew attacks on leaves and bunches, passed the criteria of selection. We managed to sample ten plots, forming 5 pairs, at the chosen phenological stage and before the first fungicide treatments. The experimental design was therefore composed of 5 pairs of plots.

3.2. *Vine-based medium isolated a lower abundance of bacteria, yeasts and filamentous fungi compared to standard culture media*

At the end of the incubation period, the abundance (CFU/mL) of cultivable yeast and filamentous fungi was significantly lower on GLA compared to MEA ($n = 118$; $p < 0.05$). Similarly, the abundance of cultivable bacteria was significantly lower on GLA compared to LBA and TSA ($n = 168$; $p < 0.05$). These results are likely a consequence of the GLA medium not being as nutrient rich as the other media.

3.3. *62% of bacterial isolates were identified at the genus or species level using MALDI-TOF MS*

A total of 965 bacterial isolates were analyzed with MALDI-TOF MS. Of these, 597 (62%) were identified at the genus level, including 23% identified at the species level. Among the 30 bacterial genera identified across the three culture media, 22 were found on LBA, 18 on TSA and 13 on GLA. Eight genera - *Bacillus, Curtobacterium, Erwinia, Microbacterium, Pantoea, Pseudarthrobacter, Pseudomonas,* and *Streptomyces* - were found on all three media (Fig. 2), with *Pseudomonas* being most abundant on LBA and GLA, and *Bacillus* on TSA.

3.4. *Plot susceptibility to downy mildew may influence community composition of cultivable bacteria*

Of the bacterial genera identified at the genus level, 10 were exclusively isolated from samples collected from plots with lower infection levels. These genera were *Rahnella, Paracoccus, Acinetobacter, Nocardia, Achromobacter, Peribacillus, Solibacillu, Xanthomona* and *Budvicia*.

4. Perspectives

We will use metabarcoding approaches to analyze bacterial and fungal communities in leaves and soil in the same plots (Fig. 1). Statistical analysis of metabarcoding data will allow us to identify microbial taxa that are significantly more abundant in the vineyard plots that have a low susceptibility to downy mildew. Those microorganisms will be retrieved from our microbial collection (Fig. 1), or from international microbial repositories if they are not present in our collection. Their effect on *P. viticola* will be tested *in vitro* and *in planta*, as single strains and as consortia. Modes of action will be elucidated for the most promising consortia. Moreover, after completing the sequencing of the microbial isolates, we will analyze thoroughly the complementarities between the vine-based culture medium (GLA) and other culture media (Fig. 2). We will test

the hypothesis that the vine-based medium selects for microbial assemblages that are different from those selected on standard media and/or poorly referenced in the current version of the MALDI-TOF MS database. These analyses will allow us to provide recommendations for future studies requiring culturomics of grapevine microbiota.

5. Acknowledgments

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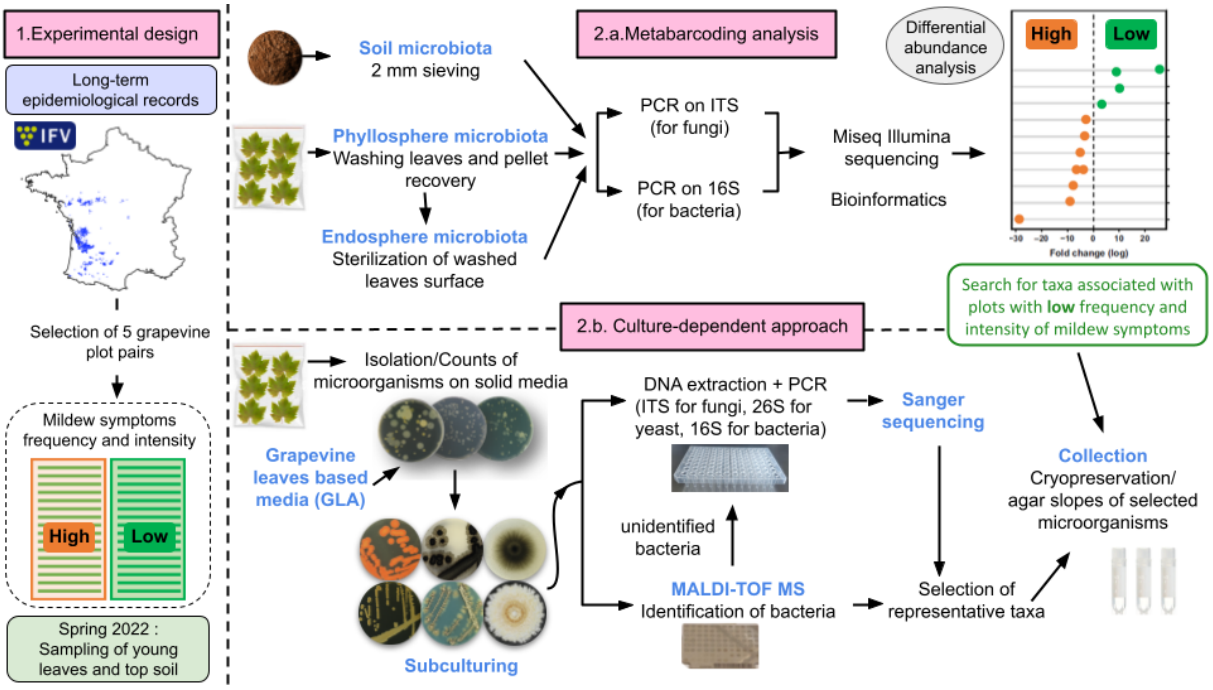

Figure 1: General workflow used in the present study to characterize and isolate bacterial and fungal communities naturally colonizing vineyard soil and leaves in plots with low vs high susceptibility to downy mildew. Results presented here belong to work packages 1 and 2.b.

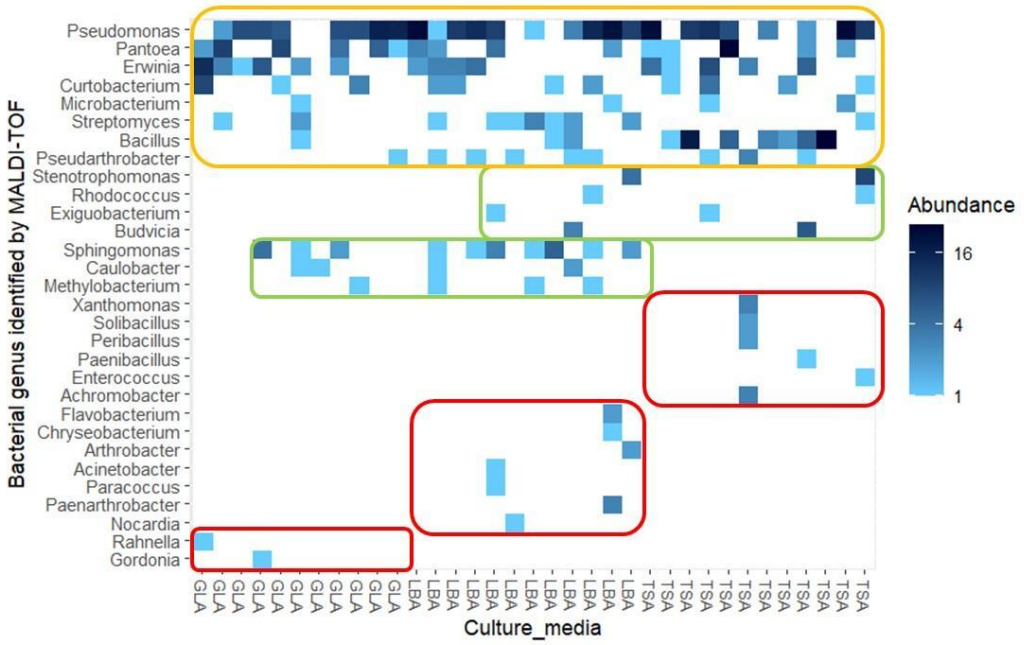

Figure 2: Bacterial genera identified using MALDI-TOF MS in grapevine leaf samples, depending on the culture media (GLA, LB and TSA). Abundance is the number of bacterial isolates belonging to each genera. Bacterial genera identified in only one, two or three culture media are circled in red, green and orange, respectively.