Quantification and management of *Plasmopara viticola* primary inoculum in soil – Towards prophylactic control of grapevine downy mildew

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1 Introduction

Downy mildew (DM) caused by the oomycete *Plasmopara viticola* is one of the most devastating disease of grapevine (*Vitis vinifera*) worldwide. Its control mainly relies on intensive use of fungicides to control powdery and downy mildew throughout the growing season, which leads to environmental pollution threatening both biodiversity and human health. Moreover, the efficacy of this fungicide-based strategy may decrease over time due to the emergence of pathogen resistant strains. There is a need to develop alternative disease management strategies to shift from a curative approach to an agroecological approach based on prevention and improved agroecosystem resistance. In France, this transition is encouraged by public institutions through policies and research funding, and a strong social incentive.

Agroecological alternatives to fungicides in wine growing generally have partial effects and must be combined to attain acceptable pathogen control levels. They include for instance the cultivation of disease-resistant varieties, the use of biocontrol products, the stimulation of plant defenses, or plant diversification of the vineyard to promote ecological pathogen control. Most of these disease management options target the plant (*e.g.* growth or defense stimulation) or the asexual reproduction phase of the pathogen life cycle, *i.e.* mycelial growth and formation of asexual spores responsible for secondary contaminations. They require cultural interventions during the grapevine growing season, when workload is already high. A complementary - rather neglected - strategy would consist in reducing pathogen primary inoculum in the vineyard to lower epidemic pressure year after year. Such prophylactic measures have been successfully developed in other crop-pathogen systems. For example, the sweeping and ploughing of apple leaf litter previously attacked by *Venturia inaequalis* in apple orchards resulted in significant reduction of apple scab primary inoculum (*i.e. V. inaequalis* ascopsores released from pseudothecia) and severity the next year (Gomez *et al*., 2007). To our knowledge, no equivalent disease management approach has been developed for oomycete-caused crop diseases.

Primary inoculum plays a major role in grapevine DM epidemics (Gessler *et al*., 2011). Therefore, there is a major challenge to develop prophylactic methods based on the management of the primary inoculum of grapevine DM. In autumn, sexual reproduction occurs in infected grapevine leaves between thalli of compatible mating type (*P. viticola* is heterothallic) leading to the formation of diploid oospores (Figure 1). Leaves will then fall on the ground and decompose, liberating the oospores that can survive in the soil during winter for up to 5 years. The stock of oospores in the soil constitute the primary inoculum of DM in the spring and determine the initial epidemic pressure.

At spring when temperatures rise up (≥ 11°C), the oospores of *P. viticola* present in the soil germinate and release the zoospores that cause primary DM contaminations on sprouting grapevines. This first generation of zoospores, which are biflagellated and mobile in water, reach the foliage via rain splashes and then encyst on the leaf surface before entering the leaves through stomata. Germination assays have shown that primary contaminations can occur throughout the entire growing season (Maddalena *et al.*, 2021), highlighting the importance of the primary inoculum in DM epidemics. Few days after infection and mycelial growth inside the leaves, sporangiophores form on the abaxial face. Zoospores released from the sporangiophores will disseminate and cause secondary contaminations. This asexual reproduction phase repeats many times during the growing season, depending on how favorable climatic conditions are.

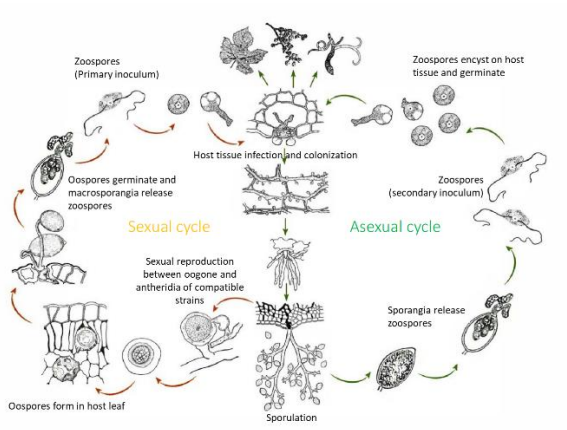

Figure 1. Life cycle of *Plasmopara viticola*, the causal agent of grapevine downy mildew.

Managing primary inoculum of grapevine DM requires developing tools to detect and quantify oospores in the soil. Canadian researchers have recently developed such molecular tools for the quantification of another plant pathogen oomycete in soil samples (*Peronospora destructor* causing onion DM) oospores in soil samples (Van der Heyden et al., 2020). However, to our knowledge, this has never been done for *P. viticola*.

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Here, we present a method to detect and quantify the primary inoculum of *P. viticola* in vineyard soils. Our objectives are i) to provide a quantitative assessment of grapevine DM primary inoculum in the field, and ii) to characterize its spatial heterogeneity at field scale. We also aim at describing the relationship between the amount of primary inoculum in the soil and epidemic initiations, in order to determine whether inoculum reduction (through prophylactic measures) could lead to reduced epidemic initiations. Our goal is to bring new insights on *P. viticola* primary inoculum to open new perspectives for the prophylactic management of grapevine DM.

2 Molecular detection and quantification of *P. viticola* oospores in inoculated soil samples

To validate the DNA extraction protocol and establish a standard curve for the quantification of *P. viticola* oospore concentration in environmental soil samples, we inoculated 250mg-soil samples with a suspension of mature oospores at different concentrations: 10^1, 10^2, 10^3, 10^4 and 10^5 oospores per gram of dry soil. We prepared three replicates of each oospore concentration. Oospores used for inoculation were derived from crosses between *P. viticola* strains of compatible mating types produced in controlled conditions on Cabernet Sauvignon leaf discs. At the time of the experiment, oospores were mature and had artificially overwintered for 8 months at 5°C in saturated humidity conditions.

Total DNA was extracted from soil samples using the DNEasy PowerSoil Pro kit (QIAGEN). Each replicate was amplified three times in separate wells. The reaction mixes contained LAMP specific primers targeting the ITS2 sequence of *P. viticola* (Kong *et al*., 2016), MasterMix ISO-004, PCR-grade water, and DNA extraction for a final volume of 25µl (Douillet *et al*., submitted). In negative control, DNA template was replaced by PCR-grade water. Reaction was conducted at 65°C for 45 minutes using Genie® HT thermocycler (OptiGene Limited). The reaction time (Rt in minutes) was defined as the point on the amplification curve at which the linear phase of matrix amplification was effective (point of inflection of the fluorescent sigmoid with respect to time).

3 Quantification of soil primary inoculum in a vineyard

In the spring, we collected samples of sandy gravel soil from an experimental organic vineyard in Villenave d'Ornon (SW France, 44°47'32.15''N, 0°34'36.92''O). This plot was planted in 2011 on a 0,2 ha area (70 x 32 m) and consisted of 18 rows of 68 Merlot vine stocks managed according to the French organic farming standards. Half of the inter-rows were untilled and covered with spontaneous vegetation and half were tilled and sowed with a mix of plant species used for green manuring. In 2009, soil resistivity was measured in the plot using a geolocated electronic probe system according to a 2 m²-grid.

We collected 117 soil samples in the rows and 117 soil samples in the inter-rows according to a regular grid pattern that covered the whole field (*i.e.* one sampling point every 5 vine stocks along the row, one row out of two). At each sampling point, we collected 250g of soil from the top 10 cm. Each sample was homogenized, passed through a 4 mm sieve and air dried at room temperature for 24 to 48h. We assessed oospore concentration in each sample using the molecular approach described above.

Figure 2. Experimental vineyard in Villenave d'Ornon (SW France) and a map of the regular grid pattern used for soil sampling.

From this data, we describe the range of oospore concentration encountered in the field and characterized its spatial variability. We further used linear models to evaluate whether variability in DM primary inoculum could be explained by the row vs the inter-row position, by the type of inter-row (tilled or untilled) or by soil resistivity.

4 Relationship between the amount of primary inoculum and epidemic initiations

To assess the effect of primary inoculum concentration on DM epidemic initiations, we conducted a study consisting of grapevine phytometers exposed to varying quantity of primary inoculum. Briefly, we prepared four 100 x 100 x 5cm sandy gravel soil mesocosms with contrasted amount of primary inoculum (*i.e.* none, low, intermediate and high). Primary inoculum (oospores) was incorporated to the soil in the form of grapevine leaves with DM mosaic symptoms (collected in October 2021 and overwintered in natural conditions) that were roughly grounded. Assuming that oospore abundance in the leaves was proportional to leaf biomass, we generated low, intermediary and high primary inoculum levels by incorporating low, intermediate and high leaf biomass, respectively. The four mesocosms were installed outside on a previously weeded location

Every 10 days from the beginning of April to the end of May, we placed 10 rooted grafts Cabernet Sauvignon grapevine plants (*i.e.* phytometers) at the 6-leaf stage on each mesocosm. Each time, we simulated a rain event using a rotative spray boom during two days in order to allow primary contaminations of the grapevine leaves through rain splashes. We then moved the phytometers to a climatic chamber with conditions favorable to *P. viticola*

development (*i.e.* 20°C and 90% relative humidity). After 5 days (enough time to observe sporulation while avoiding secondary contaminations), we carefully checked all phytometer leaves and counted the number of *P. viticola* sporulation spot, or primary contaminations per plant.

5 Conclusions

We aim at promoting new ways of controlling grapevine downy mildew that are based on prevention and better prediction of epidemic risks. This approach requires addressing cutting-edge scientific issue that have been little explored until now. For grapevine downy mildew, this translates into a better understanding of the sexual phase of the pathogen's life cycle. Indeed, a rather neglected strategy consists in reducing DM primary inoculum with the aim to lower the epidemic pressure of the following year. The two experiments described here will increase our understanding of the distribution of the primary inoculum in the vineyard and of the impact of the quantity of inoculum on the epidemics. Beyond these experiments, this research program should lead to the development of new control methods based on breaking the cycle and reducing the primary inoculum of the pathogen.

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