Direct quantitative assessment using digital droplet PCR and field-scale spatial distribution of *Plasmopara viticola* oospores in vineyard soil

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Abstract

Grapevine downy mildew, caused by the oomycete *Plasmopara viticola*, is one of the most devastating diseases of grapevine worldwide. While primary inoculum plays a decisive role in disease epidemics, we still know very little about the abundance and the distribution of oospores in soil, which are the overwintering forms of the pathogen resulting from sexual reproduction. In this study, we present a ddPCR-based

approach to quantify *P. viticola* oospores in soil samples. We used this method to describe the spatial distribution of *P. viticola* primary inoculum in a vineyard soil at the onset of the growing season. We found *P. viticola* oospores in all soil samples (n=318) except one. The distribution of primary inoculum at the field scale was not random but characterized by 25 m-diameter patches of concentrically increasing oospore concentration. The results indicated that oospores accumulated 5 times more in the ridge of soil below the vine stocks than in the inter-row. We conducted a leaf disc bioassay to assess soil infectious potential on a subset of samples collected in the field and evaluated whether the ddPCR-based assessment of inoculum concentration had an epidemiological significance. Soil infectious potential estimated through infected leaf-disc area was positively correlated with our DNA-based quantification of oospores. This approach could be used to quantify soil oospores in vineyards, which would be useful to improve models for epidemic risk prediction and decision-making tools.

Keywords: oomycete, downy mildew, *sexual reproduction,* primary inoculum, geostatistics, ddPCR, bioassay, soil infectious potential

Introduction

Grapevine downy mildew, caused by the obligate biotrophic oomycete *Plasmopara viticola*, is one of the most devastating diseases of grapevine worldwide. Its management still relies mainly on intensive use of fungicides throughout the growing season, which leads to environmental pollution threatening both biodiversity (Beaumelle *et al*., 2023; Beketov *et al*., 2013) and human health (*e.g.* Dall'Agnol *et al*., 2021). Moreover, the efficacy of this fungicide-based strategy may decrease over time due to the emergence of pathogen resistant strains (Chen *et al*., 2007; Delmas

et al., 2017). As large spectrum multisite fungicides are increasingly banned in Europe, the substitutive use of unisite products will increase the risk for resistance apparition because fewer mutations in the pathogen genome will be necessary. In France, recent public policies have clearly encouraged the reduction of the use of chemical plant protection products (National Action Plan Ecophyto; Lamichhane *et al*., 2019). Accordingly, major efforts are currently underway to develop alternative methods to manage grapevine downy mildew epidemics, including genetically resistant grapevine varieties and biosolutions (*e.g.*, biocontrol or biostimulation products). Some studies have shown that the efficacy of biocontrol products or agents may be limited (e.g., only 36.5% of substance-dosage combinations were as effective as the copper standard in Dagostin *et al*., 2011) or even unsatisfactory in real conditions due to their sensitivity to environmental factors (*e.g.*, rain, UV radiation; Dagostin *et al*., 2011). On the other hand, the development of grapevine resistant varieties is a long process, their adoption is slowed down by protected designation of origin (DPO) regulation on authorized varieties, and resistance durability may be limited in time due to resistance breakdown by pathogens (*e.g.*, Peressotti *et al*., 2010). All of these methods affect the pathogen during its asexual cycle and reduce the progression of the disease during the grapevine growing season.

In temperate regions, the life cycle of *P. viticola* comprises a phase of asexual multiplication on its host tissues during the growing season and a phase of sexual reproduction, which occurs in infected grapevine leaves between thalli of sexually compatible strains (as *P. viticola* is heterothallic, Wong *et al*., 2001) leading to the formation of oospores. Leaves then fall on the ground and oospores overwinter in leaf debris or as free propagules in the soil. The stock of oospores in the soil

constitutes the primary inoculum for downy mildew epidemics in the next growing season. In contrast to the widely-held assumption that primary contaminations rarely occur after flowering, primary inoculum is increasingly considered to play a major role in grapevine downy mildew epidemics (Gessler *et al*., 2011) for several reasons. First, population genetic studies evidenced that grapevine downy mildew populations are panmictic across european vineyards (Fontaine *et al*., 2021, 2013) showing that genetic mixing resulting from sexual reproduction largely structures pathogen populations. Second, it has been shown that oospores are able to germinate and cause infections throughout the growing season (until late September in the northern hemisphere; Kennelly *et al*., 2007; Maddalena *et al*., 2021) and that a substantial amount of infections, including in the middle of the growing season, can arise from oospores (up to 50% of contaminations; Gobbin *et al*., 2003; Hong *et al*., 2020). Third, while the epidemic continues to progress after harvest (from what we observe in surveyed vineyards), the incidence of typical mosaic-like downy mildew symptoms on grapevine leaves was shown to be proportional to the amount of oospores formed in the leaves (Si Ammour *et al*., 2020). Finally, downy mildew incidence at the end of the season was found to explain the precocity and intensity of the disease the following season in Canadian vineyards (Carisse, 2016).

The notable contribution of *P. viticola* primary inoculum to epidemic dynamics suggests that sanitation measures based on primary inoculum management or interruption of the pathogen's life cycle from one season to the next have the potential to significantly reduce epidemic pressure. Sanitation measures are often used in annual crops, involving mostly cultural practices such as mechanical destruction or removal of crop residues containing disease inoculum, but rarely in perennial crops (but see Gomez *et al*., 2007). We believe that limiting inter-annual

inoculum accumulation could be particularly relevant for integrated disease management in perennial cropping systems, such as vineyards, where crop rotation is impossible and pathogens are well established, more importantly if pesticides are being phased out.

Although the stages of the sexual reproduction cycle of *P. viticola* are well known (see for example Gessler *et al*., 2011), their quantitative description remains incomplete. DNA extraction methods and ITS-based molecular approaches have been developed and used to quantify airborne inoculum and oospores in leaves (Carisse *et al*., 2021; Douillet *et al*., 2022; Si Ammour *et al*., 2020; Valsesia *et al*., 2005). In spite of this, the assessment of downy mildew epidemic risk mainly relies on meteorological indicators that control the development of *P. viticola*. Direct indicators of the pathogen abundance in the vineyards, such as spore concentration in the air, are progressively included in decision tools or regional monitoring programs (Brischetto *et al*., 2020; Douillet, 2023). However, indicators of primary inoculum reservoir in the soil compartment have never been used yet because quantitative data is scarce. Instead, predictive models assume a fictitious quantity of primary inoculum and calculates rates of change (associated with maturation, dispersal or germination; *e.g.* Rossi *et al*., 2008a). Recently, a molecular method based on real-time qPCR was used to quantify oospores in soil of vineyards in China (Yang *et al*., 2023). Quantitative data on oospores that could be gained from such methods could improve predictions of downy mildew epidemic risk. It will also be important to better understand the spatial variation in oospore abundance within a field in order to design optimized sampling protocols for research but also for practical management purposes. The study of disease inoculum abundance and spatial distribution in agricultural soils are generally restricted to soilborne

pathogens, including oomycetes (see for example Botero-Ramirez *et al*., 2021; Moussart *et al*., 2009). To our knowledge, these questions have rarely been addressed regarding airborne pathogens with a dormant telluric stage, since the emphasis is generally on the management of the pathogen during the growing season of the plants.

Here, we present a method for the direct quantification of *P.viticola* oospores in vineyard soil based on the quantification of *P. viticola* DNA by digital droplet polymerase chain reaction (ddPCR). We also provide an estimation of the number of copy of the ITS sequence used for amplification (ITS1/5.8S) in the genome of *Plasmopara viticola* that we used to convert the ddPCR output into a number of oospores per gram of soil. By means of geostatistics, we described the spatial distribution of downy mildew primary inoculum in a vineyard at the onset of the growing season. If *P. viticola* oospores can persist in the soil and remain infectious for several years (Caffi *et al*., 2011), the determinants of oospore germination rate are poorly understood in natural conditions and the effect of aging of oospores on germination probability remains unstudied to date. However, we hypothesized that the DNA-based quantification of primary inoculum in the soil is positively related to grapevine primary contaminations. Building on previous works (Hill, 1998; Pertot and Zulini, 2003), we finally conducted a bioassay to assess the infectious potential of soil samples and discuss the epidemiological significance of our DNA-based assessments of downy mildew primary inoculum.

Material & Methods

Soil sampling and preparation

We conducted our study in an experimental vineyard with a sandy-gravel soil located at the INRAE Bordeaux Nouvelle-Aquitaine research center (Villenave d'Ornon, France; 44.79194, -0.57702) within the Pessac-Léognan appellation area. The surveyed vineyard was planted in 2011 with 18 rows of 68 Merlot grapevine stocks over a 0.22 ha surface (70 x 32 m), corresponding to a density of 6600 vines per ha (*i.e.*, 1.6 m between rows and 0.95m between vines along the row). It was managed according to French organic farming specifications, meaning that Bordeaux mixture (copper) was the only chemical fungicide allowed to control downy mildew. Inter-rows were either sown with a mix of plant species selected for green manuring or covered by spontaneous vegetation in an alternating arrangement rotating every year. All inter-rows were tilled before grapevine budburst. For winter protection and weed management purposes, soil from both sides of the row was turned over on the lower part of the vines in the fall. The ridge formed under the row was then reopened in the spring. The surveyed vineyard was bordered on each of its long sides by a diversified shrubby hedgerow (*ca*. 1.5-2 m high) that separated it from other experimental vineyards.

Soil sampling was done in February 2022. We collected 198 soil samples at the nodes of a 2.85 x 3.2 m regular grid covering the entire surface of the vineyard; *i.e.* one sample every three vine stocks in the row, in one row out of two (Figure 1). To explore the spatial structure of primary inoculum of downy mildew in the soil at a finer scale, we delineated five plots in the experimental vineyard within which we collected soil samples following a nested sampling design with three distance levels. Each plot consisted of a 2.85 x 3.2 m rectangle containing 28 sampling points (Figure 1). The first distance level corresponded to the four corners of the plot (n_3=4), hence sampling points were separated by about 3 m (samples were the same as for

the regular grid sampling). The second distance level was set at 0.8 m (half the inter-row width), and sampling points were located in the corners of four 0.8 m squares located in each corner of the plot ($n_{0.8}$=16, including 4 points in common with the first distance level; see Figure 1). The third distance level was set at 0.2 m with sampling points corresponding to the corners of four 0.2 m squares located in the corners of one of the four 0.8 m squares ($n_{0.2}$=16, including 1 point in common with the first distance level and 4 points in common with the second distance level; see Figure 1). We also considered that the points from the regular grid were located in the row, while the points from the 2nd and 3rd levels of the nested sampling were located in the inter-row space. At each sampling point of both the regular grid and the nested sampling plots, we collected 500 g of soil from the top 15 cm using an auger. We ended with 318 soil samples, including 198 samples from the regular grid and 120 additional samples from the five nested sampling plots.

To investigate the vertical distribution of *P. viticola* primary inoculum in the soil, we dug a hole 40 cm deep and 30 cm in diameter at six points located in the inter-row space in the experimental vineyard (Figure 1). We divided the soil profile into four strata of 10 cm (0-10 excluding leaf debris on soil surface, 11-20, 21-30 and 31-40 cm) and sampled 500 g of soil from each stratum, starting at the bottom of the profile to avoid contaminating deeper layers with upper ones. This corresponded to 24 samples in total (4 samples per hole or sampling location). All soil samples were stored at 5°C before processing.

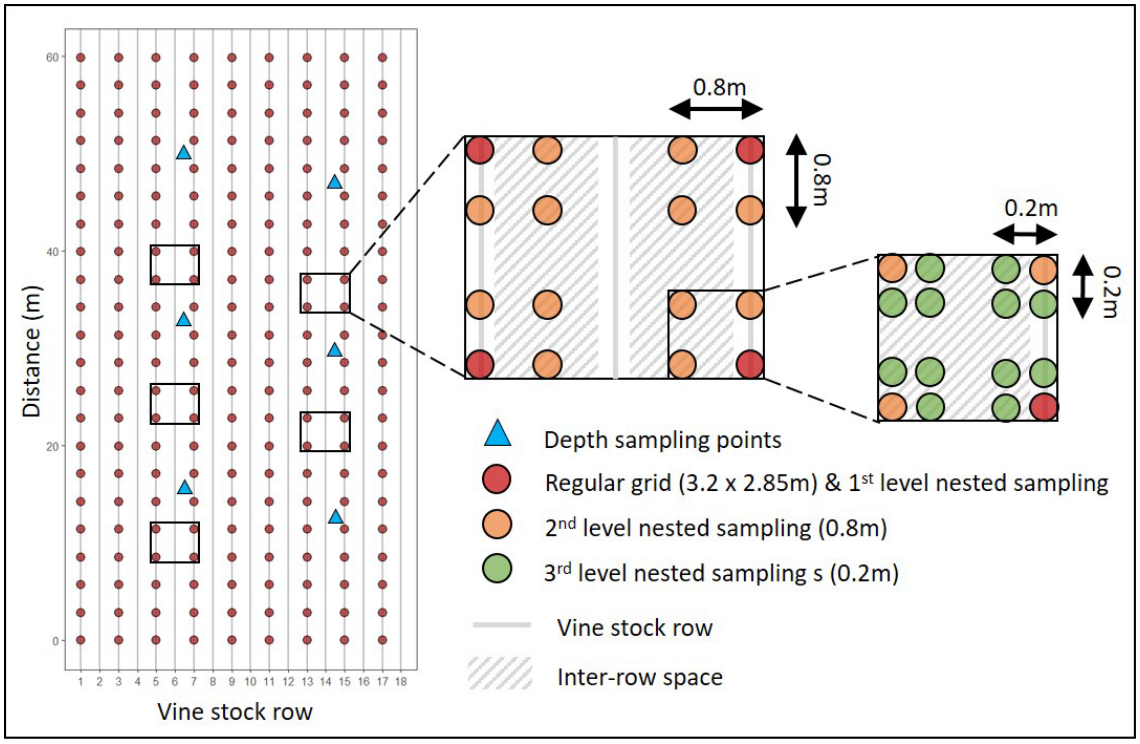


Figure 1. Map of the soil sampling points within the experimental vineyard.

Quantification of *Plasmopara viticola* primary inoculum in vineyard soil samples

DNA extraction

Each sample was passed through a 4 mm mesh sieve and thoroughly homogenized for 30 s using an electric mixer set at medium speed. Directly after homogenization, *ca*. 6 g of the sample was collected and placed in a 5 ml tube to be freeze-dried. Then, a 2 g subsample of dry soil was placed in a 15 ml Falcon tube containing 4 g of 0.1 mm diameter silica beads, 5 g of 1.4 mm diameter ceramic beads and eight 4 mm diameter glass beads (as recommended by MP-Biomedicals for TeenPrep™ Lysing Matrix E). For the grinding and lysis extraction step, a volume of 8mL of lysis buffer composed of 100 mM Tris-HCl (pH 8), 100 mM EDTA (pH 8), 100 mM NaCl, 20% SDS and ultra-pure water was added in each tube. Mechanical cell lysis was achieved placing the tubes in a FastPrep-24 classic instrument (MP-Biomedicals) set

for three runs of 30 s at 4 m.sec^{-1}. Tubes were then incubated for 30 min at 70°C to activate chemical lysis and centrifuged at 5000 g for 7 min at 20°C to eliminate particles in suspension. DNA extraction was carried out using 1 ml of supernatant obtained from the centrifugation, as follows: 1) *Deproteinization:* addition of 100 µl potassium acetate 3M pH5.5 to the sample, homogenization and incubation on ice for 15 min to precipitate proteins, centrifugation at 14000 g for 5 min at 4°C to eliminate precipitated proteins (supernatant recovery and pellet disposal); 2) *DNA precipitation:* addition of 900µl cold isopropanol (v/v) to the supernatant from the deproteinization, gentle agitation of the tubes that were then placed at -20°C for 30 min, centrifugation at 13000 rpm for 30 min at 4°C, elimination of the supernatant; 3) *DNA Washing:* rinsing of the pellet with 400µl 70° cold ethanol, centrifugation at 13000 rpm for 5 min at 4°C, elimination of the ethanol supernatant, drying of the pellet by placing the tubes in a drying oven at 60°C for 20 min; 4) *DNA Purification:* resuspension of the pellet in 500 µl of ultra-pure water. Raw DNA extracts were further purified using a NucleoSpin Soil kit (Macherey-Nagel) following the manufacturer's protocol from step 4 (addition of SL3 buffer). Finally, purified DNA extracts were eluted in 60 µL of buffer (TrisHCL, 5mM, pH 8.5) and stored at −20°C.

Digital droplet polymerase chain reaction (ddPCR)

To avoid common PCR inhibition problems with the soil matrix and to obtain direct absolute DNA quantification (no standard curve needed), samples were analyzed by droplet digital PCR (ddPCR) using the QX200 ddPCR system (Bio-Rad) composed of a droplet generator, a thermal cycler and a droplet reader. We used the specific PCR primers and TaqMan probe (Giop set) designed by Valsesia *et al*. (2005) that target the ITS1/5.8S to amplify *P. viticola* DNA in our samples.

For each sample, we added 4 µl of DNA extract diluted to the tenth to 18 µl of a PCR mix composed of 11 µL of 2x ddPCR Supermix for Probes (Bio-Rad), 2.2 µL of 7.5 µM Giop F, 2.2 µL of 7.5 µM Giop R, 2.2 µL of 5 µM Giop P-VIC and 0.4 µL of ultra-pure water. The samples (20 µl) were first placed in the droplet generator to proceed to the breakdown of the samples into 15000 to 20000 micro-droplets using oil. Samples were then carefully transferred to 96-well plates with a pipette, with one sample per well (one single analysis per sample). The sample plates were placed in the thermal cycler for DNA to be amplified according to the following program: 1) an initial phase at 95°C for 5 min; 2) 40 cycles of 30 s at 95°C then 1 min at 60°C then 30 s at 72°C; and 3) a final phase at 98°C for 10 min. Samples were cooled at 12°C before being transferred to the droplet reader for DNA quantification (ratio of positive *vs* negative droplets). Finally, we obtained one value per sample that corresponded to the number of ITS1 copies per µl of reaction mix.

Estimation of ITS copy number in Plasmopara viticola genome

In order to estimate the ITS1/5.8S copy number in the genome of *P. viticola*, we compared the ddPCR signals obtained with the ITS primer pairs (Valsesia *et al*., 2005) *vs* primers targeting the single copy β-tubulin gene (Weerakoon *et al*., 1998). First, sporangia of seven *P. viticola* strains frequently used in laboratory for genetic applications were collected with a paintbrush from grapevine (*Vitis vinifera* cv. Cabernet Sauvignon) leaves infected beforehand on purpose and placed into separate 2 mL microcentrifuge tubes with 1 mL Tris-EDTA buffer. Second, freeze-dried samples were extracted following a SDS based method (adapted from Möller *et al*., 1992) and DNA quantity and quality were checked by dosage with either Qubit 4 or Denovix-11. About 35 sequences from NCBI (Fontaine *et al*., 2021; Rouxel *et*

al., 2013) were aligned with *P. viticola* genome using Geneious Prime. Primers (Forward: ACAGCCAATTTTCGTAAGTCC, Reverse: GCCTTGTACGACATTTGCTTT) and a TaqMan probe (5' FAM-TGTCCGGGAAATCGAAGGCA-MGB 3') were designed using the consensus sequence with 100% similarity. Specificity was tested using NCBI Blast (blast.ncbi.nlm.nih.gov) and by running quantitative PCRs on either pure *P. viticola* strain, *P. viticola* with grapevine leaf or grapevine leaf only (see Figure S1 in Supplementary Materials) using a QuantStudio 5 (Life Technologies). Reaction mix was composed of 10 µL of 2X PCR Buffer (Takyon Low Rox Probe MasterMix - Eurogentec), 1,6 µL of each primer at 10 µM, 0,4 µL of probe at 10 µM, 3,9 µL of molecular biology water (Sigma) and 2,5 µL of DNA template for a 20 µL final volume. Due to a large copy number difference between both genes and ddPCR sensitivity, we run the reactions for each marker in separate reaction wells. In consequence, each sample were normalized with a final quantity of 5 pg for ITS samples and 5 ng for β-tubulin samples. All reactions were run with a cycling program starting with 3 min at 95°C followed by 40 cycles of 10 s at 94°C alternating with 60 s at 60°C. For each strain, the number of ITS1/5.8S copies was estimated as the ratio of β-tubulin to ITS copy number per ng.

Spatial structure of *Plasmopara viticola* primary inoculum in a vineyard

The spatial structure of *P. viticola* primary inoculum in the experimental vineyard was investigated at both field-scale and plot-scale by analyzing the regular grid data and the nested sampling plots data, respectively, using a geostatistical approach. Hereafter, we describe the field-scale analysis only, but the same methodology

(except model fitting and kriging) was applied for the plot-scale analysis (see Table S1 in Supplementary Materials for details).

Plasmopara viticola DNA in soil samples, expressed as the number of ITS copies per µl of reaction mix, was log-transformed before analysis to reduce the skewness of the distribution and limit variance inflation and variogram distortion (Oliver and Webster, 2014). First, all pairs of data (N=19503) were segregated into distance classes. The maximal distance between two points in our dataset was 65.09 m. In our analysis, however, we considered a maximal separation distance of 60 m to exclude highest distance classes with a number of point pairs below the empirical threshold for semivariance estimates accuracy (at least 30 to 50 pairs required; Chiles and Delfiner, 1999; Journel and Huijbregts, 1976). We considered a spatial lag of 2 m, which led to a total of 29 distance classes, with 79 to 1498 pairs each. The sampling points in the first distance class were separated by a median distance of 3.02 m. We then computed the semivariance for each distance class using the Matheron estimator (Matheron, 1963), defined as follows:

$$\gamma(h) = \frac{1}{2n(h)} \sum_{i=1}^{n(h)} [z(x_i) - z(x_{i+h})]^2,$$

where $z(xi)$ is the primary inoculum at the first point of the ith pair, $z(xi + h)$ is the primary inoculum at the second point of the ith pair, h is the median of the class of distance considered and n is the number of point pairs in the distance class considered. We represented the omnidirectional experimental semivariogram of the primary inoculum of *P. viticola* in the vineyard by plotting the semivariance against the sampling point separation distance h. A semivariogram shows how the similarity between samples changes with increasing separating distance *i.e.* spatial

dependence. It can take various forms, and semi-variance often increases with distance until it reaches a plateau at which point it remains stable. The variogram is flat in the absence of spatial structure.

A visual inspection of the experimental semivariogram allows one to determine its general shape. We fitted a spherical model (linear-sill shape) to the experimental semivariogram using ordinary least square residuals method (Cressie, 1985). The spherical model is as follows:

$$\gamma(h) = C_0 + C\left(\frac{3h}{2a} - \frac{1}{2}\left(\frac{h}{a}\right)^3\right) \quad 0 < h \leq a$$

$$\gamma(h) = C_0 + C \quad h > a$$

The model parameter estimates were regarded as quantitative descriptors of the spatial pattern of the data. They included i) the spatial range a, or the distance beyond which variance is constant; ii) the nugget variance C_0, an estimate of error measurement plus error due to uncontrolled variability at distance lags shorter than the shortest lag considered; iii) the structural variance C; and iv) the sill $C_0 + C$, or sample variance.

We used the shape and parameter estimates of our semivariogram model to interpolate *P. viticola* primary inoculum values at unsampled locations over a 0.05 m × 0.05 m grid by ordinary kriging, which is an optimal interpolation procedure described in detail in various sources (*e.g.*, Goovaerts, 1998; Oliver and Webster, 2015). After back-transforming the resultant values, we produced a contour map of the spatial distribution of the primary inoculum in the field, as interpolated from our data (*i.e.*, given the local conditions and our sampling plan).

All geostatistical analyses were performed using the package gstat (Pebesma, 2004) in the R statistical software (v4.3.1, R Core Team, 2023). The contour map was designed using the ggplot2 package (Wickham, 2016).

Plasmopara viticola primary inoculum along depth and distance-to-row gradients

In the nested sampling plots, the sampling points were located either below the vine stocks in the row (10 samples per plot) or in the inter-row space at 20, 60 or 80 cm from the nearest row (respectively 4, 4 and 10 samples per plot). We used this data to test whether the amount of primary inoculum in the soil varied along a gradient of distance to the row. We fitted a linear mixed model with the log-transformed number of ITS copies per µl as a response variable, the distance to the row as a fixed effect and the plot as a random intercept effect (to account for the dependence of the data within one plot). Model parameters were estimated by restricted likelihood estimation and the significance ($\alpha = 0.05$) of the regression coefficients was tested with Student t tests and Satterthwaite's approximation for degrees of freedom, using the lmer function of the lmerTest package (Kuznetsova *et al*., 2017). We evaluated model fit by calculating the percentage of variance explained by fixed (R^2m) and by fixed plus random effects (R^2c) (Nakagawa and Schielzeth, 2013). Finally, we tested whether the amount of primary inoculum varied with the depth of sampling using a Kruskal-wallis test and a Dunn test of multiple comparison. Statistical analyses were conducted using the R statistical software (v4.3.1, R Core Team, 2023).

A bioassay to assess soil infectious potential

The principle of the bioassay is to place soil samples in optimal temperature and humidity conditions for oospore germination, to trap the released zoospores using susceptible grapevine leaf discs, and finally to approximate the infectious potential by evaluating the extent to which leaf discs are infected. The method described below was adapted from previous works (Hill, 1998; Rossi *et al*., 2008b).

We performed the bioassay on one eighth of our 318 soil samples (n=40) using a 1 g portion per sample. Each sample was placed in the bottom of a 10 cm diameter circular plastic box half-filled with osmosed water and incubated at 20°C in the dark to stimulate oospore germination (day 0). The sample was held at the bottom of the box using a piece of blotting cloth (80µm mesh). We collected leaves from untreated *Vitis vinifera* cv. Cabernet Sauvignon grafted plants (SO4 rootstock) grown in greenhouses. We specifically picked the third or fourth leaves from the apex, as leaves at this stage of development are particularly susceptible to downy mildew infection (Isabelle Demeaux, personal communication). We cut 10 mm diameter discs in the leaf blade with a manual round cutter. On the first day of the incubation (day 0), we placed 10 discs from 10 different leaves (to avoid biases due to differences in downy mildew susceptibility between leaves) on the surface of the water (abaxial face down) in each box. The leaf discs were used to trap the zoospores released into the water following germination of oospores present in the soil sample, *i.e.* the zoospores swam to the leaf discs and infected them via the stomata. The blotting cloth allowed the zoospores to pass through but prevented the discs coming into direct contact with the soil and limited contamination of the discs by saprophytic microorganisms. A separation structure was used to prevent the discs in a box from overlapping and maintain the same trapping surface in each box (see Figure S2 in Supplementary Materials). After 48 h, we placed the 10 discs abaxial

face up on a moist Whatman® filter paper sheet in a transparent box sealed with plastic film (to maintain high humidity) and incubated at 20°C with a 12h-12h day-night light cycle for 6 days. After 6 days of incubation, we visually inspected the discs and evaluated the percentage of the area covered with *P. viticola* sporangiophores for each disc. The germination of a cohort of oospores under optimal conditions (immersed in water, in the dark and at 20°C) extend over 6 to 8 days, with a peak around the second and third days of incubation (Isabelle Demeaux, personal communication). To account for germination dynamics, we repeated zoospore trapping using leaf discs on day 2, 4 and 6 from incubation start (day 0). We used the cumulative number of infected discs and the mean percentage of leaf area infected as indicators of the infectious potential of the soil.

We assessed the relationship between the abundance of primary inoculum in the soil (as estimated with the ddPCR approach) and soil infectious potential by fitting one generalized linear model per indicator (response variable following Poisson distribution in both cases) with the number of *P. viticola* ITS copies per µl as a predictor. In both cases, model parameters were estimated by maximum likelihood and the significance ($\alpha = 0.05$) of the regression coefficients was tested with z tests. We computed the McFadden's pseudo R^2 of the two models to determine the indicator best fitting the primary inoculum data. Statistical analyses were conducted using the R statistical software (v4.3.1, R Core Team, 2023).

Results

Quantification of *Plasmopara viticola* primary inoculum in vineyard soil samples

Considering the regular grid and nested sampling plot data, we found *P. viticola* DNA in all samples except one, with 6.62 ± 6.68 ITS copies per µl on average. The value for positive samples varied from 0.05 to 40.4 ITS copies per µl. A map of the raw data is available in Supplementary materials (Figure S3).

By comparing the ITS1/5.8S to the β-tubulin signal obtained by ddPCR for seven *P. viticola* strains, we found that the genome of *P. viticola* contains on average 215 ± 37 copies of the ITS sequence, but varied from 175 to 281 copies depending on the strain (see Table S2 in Supplementary Materials for details). Using the average estimate for the number of ITS1 copies, we estimated that the number of oospores per gram of soil ranged from 0 to 1858 and that there was on average 303 ± 308oospores per gram of soil in the experimental vineyard (see Figure S4 in Supplementary materials for computation details). Using the minimal and maximal estimates for the number of ITS1 copy, the average number of oospores per gram of soil was 372 ± 378 or 232 ± 235, respectively.

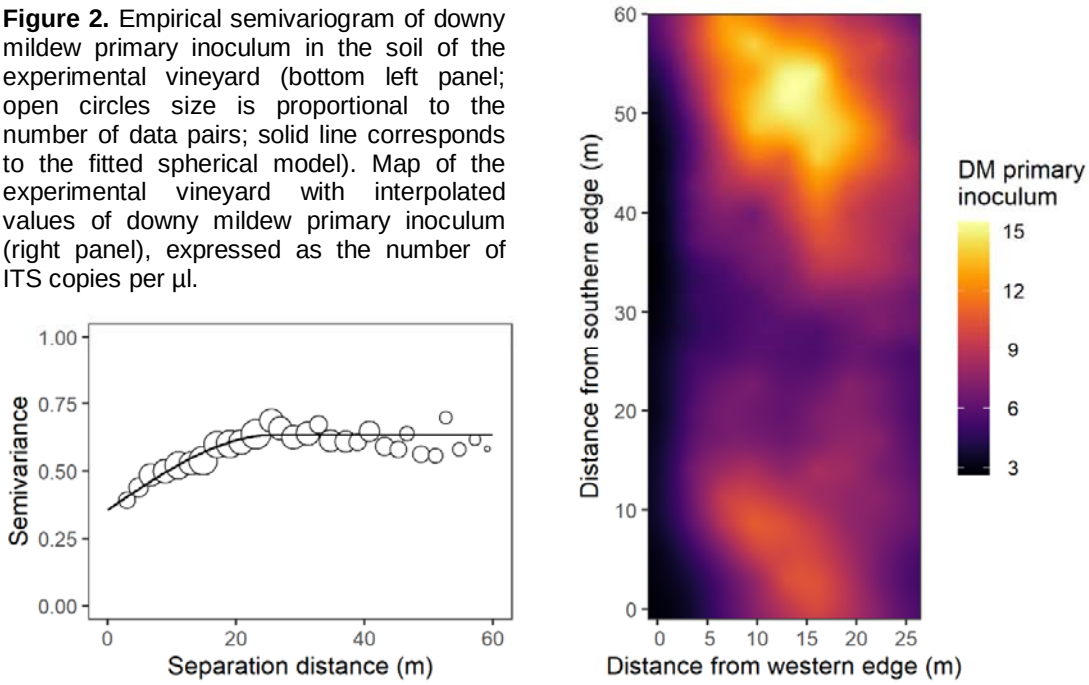
Spatial structure of *Plasmopara viticola* primary inoculum in a vineyard

After log-transformation, the skewness of the distribution of *P. viticola* DNA data was reduced from 1.69 to -0.35 for the regular grid, and from 2.40 to 0.42 for the nested sampling plots. The shape of the fitted spherical model (regular grid data) revealed that the downy mildew primary inoculum was spatially structured at the field scale (Figure 2). The range was 25.8m. The nugget variance, the structural variance and the sill were 0.36, 0.27 and 0.61 respectively. The high nugget value indicated that very close points could be quite dissimilar in terms of primary inoculum, suggesting the existence of a source of variation operating at a scale finer than that of the

regular grid. The map of the values interpolated by kriging using the fitted model is shown in Figure 2.

The pattern of the plot-scale experimental semivariogram was not typical, with semivariance alternatively increasing and decreasing along the gradient of separation distance (Figure S5 in Supplementary Materials). Semivariance peaks corresponded to distance classes with a higher proportion of heterogeneous pairs of points, *i.e.* pairs consisting of one sampling point in a row and one sampling point in an inter-row. On the other hand, lowest semivariance values corresponded to distance classes with a higher proportion of homogeneous pairs of points, *i.e.* pairs with two sampling points in a row or pairs with two sampling points in an inter-row (Figure S5 in Supplementary Materials). We consistently found that semivariance was higher for heterogeneous pairs of points than for homogeneous ones, on average (anova P <0.0001, Figure S5 and Table S3 in Supplementary Materials).

Figure 2. Empirical semivariogram of downy mildew primary inoculum in the soil of the experimental vineyard (bottom left panel; open circles size is proportional to the number of data pairs; solid line corresponds to the fitted spherical model). Map of the experimental vineyard with interpolated values of downy mildew primary inoculum (right panel), expressed as the number of ITS copies per µl.



Plasmopara viticola primary inoculum along depth and distance-to-row gradients

Our results clearly indicated that there was a decreasing gradient of primary inoculum from the row to the middle of the inter-row (Figure 3; $\beta = -0.0263 \pm 0.0023$; df = 133; t value = -11.54; $P < 0.001$; $R^2m = 0.48$; $R^2c = 0.50$). Downy mildew primary inoculum was on average five to six times more concentrated in the ridge of soil below the vine stocks (in the "cavaillon"; 9.32 ± 6.32 ITS copies per µl) than in the inter-row space (1.72 ± 1.66 ITS copies per µl). These results were in line with the results from the geostatistical analysis of nested sampling plot data (see above). We also found that the primary inoculum concentration decreased with soil depth (Figure 3; Kruskal-Wallis test's statistic = 12.1; df = 3; $P < 0.01$). In particular, it was significantly lower between 21 and 30 cm and between 31 and 40 cm than in the first 10 cm from the soil surface (see results of Dunn's post-hoc test in Table S4 in Supplementary Materials).

Although we were not seeking to address this point, we also found that primary inoculum concentration in the soil varied between rows, with significantly less *P. viticola* DNA on average in the westernmost row compared to others in the vineyard (Figure S6 Supplementary Materials; F = 6.522; $P < 0.001$; see Table S5 in Supplementary Materials for results of Tukey's multiple comparison test). This unexpected result was consistent with the distribution of primary inoculum as predicted by kriging of observed data (Figure 2).

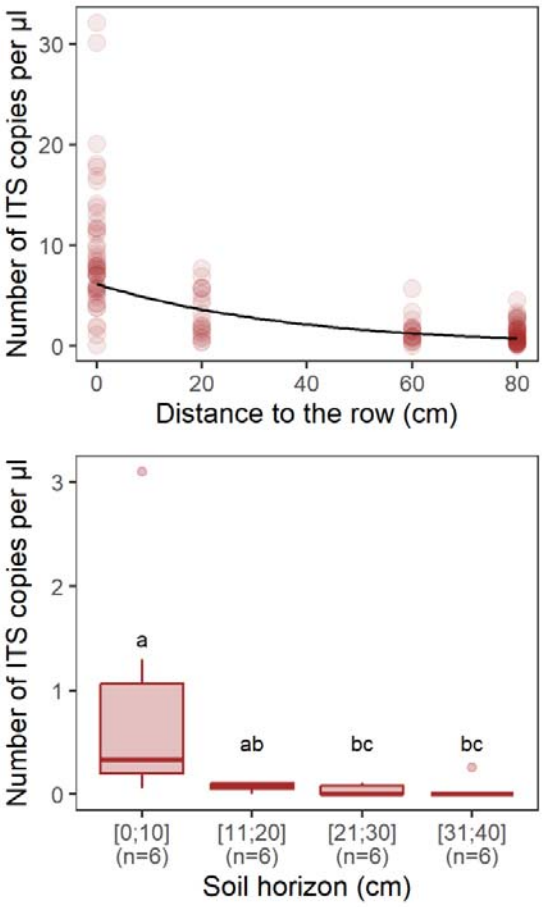


Figure 3. Abundance of downy mildew primary inoculum (as number of ITS copies per µl estimated by ddPCR) along gradients of distance to the row (upper panel) and soil depth (lower panel). Solid line in the upper panel corresponds to model predictions. Different letters between depths in the lower panel indicate a significant difference.

A bioassay to assess soil infectious potential

The cumulated number of leaf discs infected by *Plasmopara viticola* from 1g of soil sample over the duration of the assay varied from 0 to 12 discs (out of 40). The average proportion of disc area covered with *P. viticola* sporulation varied from 0 to 10.3%. The cumulated number of infected discs significantly increased with the number of ITS copies per µl (Figure 4; □ = 0.029 ± 0.008; P < 0.0001; McFadden's

pseudo R^2 = 0.05). In addition, the average infected area significantly increased with the number of ITS copies per µl (Figure 4; □ = 0.06 ± 0.01; P < 0.0001; McFadden's pseudo R^2 = 0.16).

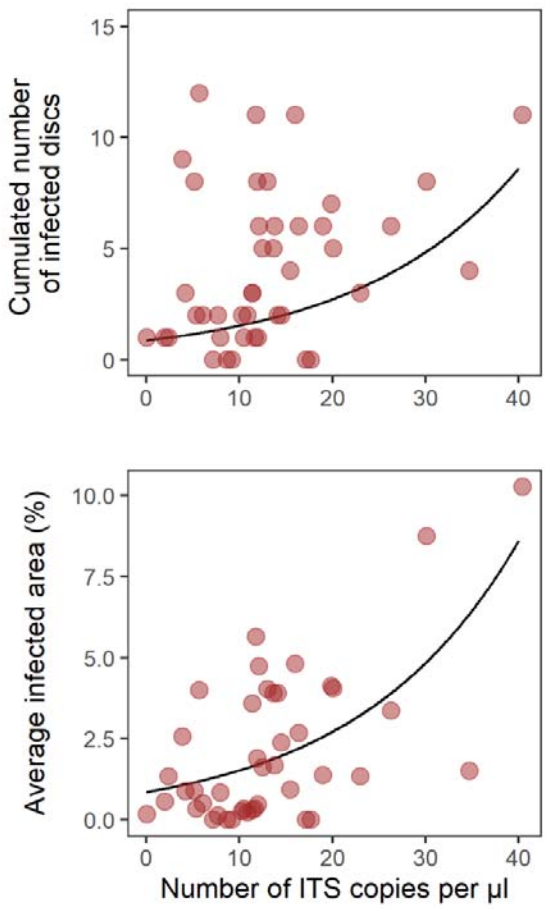


Figure 4. Comparison of bioassay and digital droplet PCR. Relationships between the abundance of DM primary inoculum in the soil (measured as number of ITS copies per µl by ddPCR) and the infectious potential of the soil measured by a bioassay, as either the cumulated number of infected discs (upper panel) or the average trap disc infected area (lower panel). Solid lines represent fitted model predictions.

Discussion

We present a sensitive and specific method for the quantification of *P. viticola* oospores in vineyard soil samples based on a ddPCR approach. We used this method to describe the spatial distribution of downy mildew primary inoculum (oospores) at different spatial scales in one vineyard. We also evaluated whether the ddPCR-based assessment of inoculum concentration was related to an indicator with

an epidemiological significance, *i.e.* the soil infectious potential as assessed using a bioassay. The method presented here could be used to survey primary inoculum in vineyards and obtain quantitative data on soil oospore content, which would be useful to improve models for epidemic risk prediction.

The method used for inoculum assessment in soil is based on the ddPCR technique, which provides an absolute quantification of DNA and is particularly well suited for rare targets in complex matrices. We combined these advantages to the use of an ITS sequence occurring in numerous copies which increases the probability of detection of *P. viticola* DNA in small quantities in the sample. The partition of the samples into thousands of droplets improves the yield of amplification and the sensitivity of the analysis as compared to qPCR, including in the case of oomycete quantification in soil samples (Gossen *et al*., 2019; Rački *et al*., 2014; Wen *et al*., 2020). Using ddPCR and the average estimate for the number of ITS1 copy in the genome of *P. viticola* (215 copies), we found that the number of oospores per gram of soil ranged from 0 to 1858 in the experimental vineyard and averaged 303 oospores per gram of soil. This result falls within the range of values (*i.e.* from a few dozen to 4000 oospores per gram of soil approximately) previously estimated by Yang *et al*. (2023) in Chinese vineyards using qPCR.

A potential drawback of the oospore estimate from a multicopy DNA sequence lies in the accuracy of the assessment of the number of copy of the sequence in the genome of the target species. Variability in ITS copy numbers among isolates from the same oomycete species has been poorly studied but can potentially vary among strains (McLeod *et al*. 2024). In our case, we found that the number of ITS1/5.8S copies in *P. viticola* genome varied from 175 to 281 copies depending on the strain

(n=7), which caused the primary inoculum concentration estimates to vary from 232 to 372 oospores per gram of soil on average. However, the number of ITS copies found in our study is consistent with what was found for other oomycetes (95 copies per haploid genome on average; Gangneux *et al*., 2014). Further analyses should be undertaken to better assess the occurrence of the ITS1/5.8S on a population level. Another possible drawback for the quantification of oospores may result from the assessment of the extraction yield, as oospore concentration could be underestimated due to suboptimal DNA extraction and amplification efficiency. This bias need to be further addressed for a more accurate estimate of oospores concentration in future studies. However, DNA was extracted from 2g of soil in our protocol, which is eight times greater than with extraction kits (*e.g.* 250 mg for the DNeasy PowerSoil Pro kit from QIAGEN). Together with the sample homogenization step, we believe that this may have two important advantages: i) limit estimation bias due to small-scale spatial variability in oospore abundance and improve the repeatability of the method (which we assessed; see Supplementary Materials) and ii) allows increasing the sensitivity of the method because higher amount of oospores is present initially in the sample.

In our study, *P. viticola* was encountered at all sampled locations in the field contrary to soilborne pathogens whose presence is generally restricted to some areas (Botero-Ramirez *et al*., 2021; Moussart *et al*., 2009). This difference may be explained by the greater dispersal ability of the airborne form of *P. viticola* (as compared to strictly soilborne pathogens) resulting in a more even distribution of the disease in the field. Still, we showed that the distribution of downy mildew oospores was not random or regular on a field scale, but that it was characterized by 25 m-diameter patches of concentrically increasing oospore concentration. This pattern

could be related to the distribution of the disease in the vineyard at the end of the growing season, which we unfortunately did not assess the year before our soil survey (but see Supplementary materials for comparisons of the spatial distribution of primary inoculum against soil electrical resistivity and grapevine NDVI distributions, as indirect proxies for disease incidence). Alternatively, environmental factors could affect grapevine leaf litter (containing the oospores) distribution or the survival of the inoculum during autumn and winter. Future studies should investigate the relationship between the spatial distribution of the symptoms at the end of the growing season and the distribution of the inoculum in the soil the following year.

We found that, on average, oospores accumulates preferentially in the ridge of soil below the vine stocks (or “row location”) at a rate of 4 to 5 times the inter-row space, and in the first 10 cm of soil. Although oospore concentration varied on a wider spatial scale within the field (25m patches), this row *vs* inter-row contrast was observed at all locations in the field. Moreover, oospore concentration was more similar at two row locations, whatever the distance between the two points, than when comparing row *vs* inter-row locations. This suggests that the distribution of oospores is primarily determined by the distribution of grapevine leaf litter that carry them, which first falls and accumulates at the foot of the grapevines. However, the distribution of grapevine leaf litter may change during autumn and winter under the influence of the wind and of cultural practices. For instance, tillage was found to modify the spatio-temporal distribution of crop residues in annual cropping systems, and in particular the distribution between ridges and furrows (Grigera *et al*., 2007; Guérif *et al*., 2001; Schneider *et al*., 2006). Here, we hypothesize that soil ridging at the foot of the vines contributes to “trap” the fallen leaves and could prevent their dispersion by the wind (see Figure S7 in Supplementary Materials for an illustration),

resulting in a higher concentration of inoculum locally. Future research should study the dispersion of grapevine leaf litter (or crop residues) during winter and how it is affected by cultural practices to better understand oospores distribution in vineyards. This could help identify cultural practices that prevent inoculum from accumulating in the vineyard below the grapevines, where it is more likely to reach the host tissue in the event of rain splashes. Finally, data on oospore distribution within a vineyard could be useful to determine an optimal sampling strategy (number and distribution of soil sampling points within a field) for the design of cost-effective field-scale indicators of primary inoculum reservoir. This could be done using methods such as hierarchical variance analysis based on mixed models with nested random effects (Webster *et al*., 2006) for example. Nevertheless, data from various vineyards should be gathered for such an indicator to be as generic as possible.

Our study also revealed that soil oospore concentration can vary between rows within a field and be subjected to border effects. Indeed, we found that the primary inoculum was 7 to 8 times less concentrated in the westernmost row compared to the rest. This difference might arise from various mechanisms mediated by the presence of a tree hedgerow alongside the experimental field including : i) wind erosion (Campi *et al*., 2009; Jacobs *et al*., 2022; Weninger *et al*., 2021) that may modify leaf litter dispersion on the ground during autumn and winter; ii) microclimate modification in the vicinity of the hedgerow (Campi *et al*., 2009; Jacobs *et al*., 2022) that may modulate disease incidence locally; iii) modulation of grapevine vigor and susceptibility to downy mildew potentially due to competition for resources (Favor and Udawatta, 2021; but see Bourgade *et al*., 2020). In our case, the absence of a border effect on the east side of the field (also bordered by a hedgerow) suggests that mechanisms at play operate asymmetrically, which restricts the choice to

mechanisms associated to wind erosion and microclimate modification. In any case, these results raise questions regarding the effects of agroecological infrastructures on downy mildew epidemiology, as well as their possible contribution to preventive disease management strategies.

We further demonstrated that the concentration of *P. viticola* oospores in the soil is related to soil infectious potential as estimated in controlled conditions using a leaf disc bioassay. In particular, we found that primary inoculum abundance is the soil was positively related to the proportion of leaf discs infected during the bioassay. Consistently, pea root rot inoculum potential estimated from soil samples using trap seedlings was positively related to disease severity observed on peas during the cropping season (Moussart *et al*., 2009). Similar results were found for canola clubroot disease, which is caused by another soilborne pathogen (Hwang *et al*., 2011), but we found no study that addressed the inoculum density-disease severity relationship for soil-to-aerial plant parts infection pathway, such as in the case of downy mildew primary contaminations. Contrary to DNA-based methods, the bioassay discriminate between viable and unviable cells, as only viable oospores will be able to cause leaf disc infections. However, considering that the oospore germination period in a population extends over several months, our bioassay (carried out on a single date) “captured” only part of the oospore population present in the samples, which may explain that the relationship was not strong (McFadden’s $R^2 = 0.16$). Future works should establish whether this method for soil infectious potential assessment is a good proxy for the “true” infectious potential, *i.e.* accounting for the entire oospore population. Nevertheless, our result suggests that DNA-based quantification of primary inoculum have an epidemiological significance and could be used in models to improve epidemic risk predictions.

The relationship found between primary inoculum concentration in the soil and our indicator of infectious potential suggests that any measure reducing the accumulation of primary inoculum in a field could result in lower epidemic pressure. Such measures could include the removal of infected leaves (*i.e.*, inoculum reservoir) from one season to the next (in autumn). A study of prophylactic disease management in apple orchards showed that the sweeping and plowing of apple leaf litter affected by apple scab resulted in significant reduction of the primary inoculum (*i.e.*, *Venturia inaequalis* ascospores released from pseudothecia) and disease severity the next year (Gomez *et al*., 2007). It is worth noting that *V. inaequalis* is necrotroph and has no conservation structure that allows it to survive in the absence of the host (living or dead). In the case of *P. viticola* which survives without its host thanks to conservation structures (namely oospores), leaf litter should instead be exported from the vineyards. Sanitation measures aimed at limiting primary inoculum accumulation in the vineyards will have synergistic effects with other disease management strategies. By lowering the epidemic pressure, they could make the use of biocontrol or biostimulation products more efficient. The probability of a resistance breakdown in a pathosystem depends on the evolutionary potential of the pathogen which is affected by many factors including the size of the pathogen population (Aubertot *et al*., 2006). By removing the products of sexual reproduction (*i.e.*, oospores), prophylactic practices could also slow down resistance breakdown and improve the sustainability of resistant varieties, which was previously suggested (Bitencourt *et al*., 2021). This was experimentally demonstrated in apple orchards where it was found that the removal of leaf litter in autumn together with spraying of fungicides in the case of moderate or high risks of scab infection delayed the breakdown of the major resistance Rvi6 of Ariane by virulent isolates (Didelot et al.

2016). Future studies should evaluate the efficacy of sanitation measures aiming at reducing levels of initial inoculum in controlling diseases in vineyards.

As agriculture moves away from chemical biocides, the design of sustainable, adaptative and cost-effective crop disease management strategies will rely more and more on the prevention of epidemic risks and a predictive evaluation of pathogen population dynamics. In this context, the quantitative and spatially-explicit survey of inoculum reservoirs is crucial. The insights gained into downy mildew primary inoculum from our methods could contribute to the design of monitoring and decision-making tools to accompany the implementation of sanitation measures preventing primary inoculum accumulation in the vineyard from one season to the next.

Acknowledgements

The authors thank the Experimental Viticultural Unit of Bordeaux 1442, INRAE, F-33883 Villenave d'Ornon, for the maintenance of the experimental vineyard that was used in this study, and the GenoSol platform (DOI 10.15454/L7QN45) for sharing its expertise and helping with methodological development for DNA extraction from soil samples. Part of the experiments (ddPCR analysis) were performed at the Genome Transcriptome Platform of Bordeaux (PGTB; doi:10.15454/1.5572396583599417E12) with the help of Adline Delcamp and Emilie Chancerel. Finally, the authors are grateful to the sponsors of the Chaire Alexis Millardet for their support to research activities, namely Château Ausone, Château Cheval Blanc, Petrus and Château Montrose.

Fundings

This work was initiated thanks to the Ministries for an Ecological Transition, for Agriculture and Food, for Solidarity and Health and of Higher Education, Research and Innovation, with the financial support of the French Office for Biodiversity, as part of the call for research projects “Overall approaches to limit the use of phytosanitary products : coupling preventive and curative solutions within agricultural sectors from farmers to consumers”, with the fees for diffuse pollution coming from the Ecophyto II+ plan (PROFIL project 2020-2024). This work was also financially supported by the ISVV Fund of the Fondation Bordeaux Université, through the Chaire Alexis Millardet program. The authors acknowledge the financial support of the French National Research Agency (ANR) under the grant 20-PCPA-0010 (Vitae project from the PPR Cultiver et Protéger Autrement). The GenoSol platform (DOI 10.15454/L7QN45) of the infrastructure ANAEE-Services received a grant from the French state through the National Agency for Research under the program “Investments for the Future” (reference ANR-11-INBS-0001), as well as a grant from the Regional Council of Bourgogne Franche-Comté. The BRC GenoSol is a part of BRC4Env (10.15454/TRBJTB), the pillar « Environmental Resources » of the Research Infrastructure AgroBRC-RARe 10.15454/b4ec-tf49.

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