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Artificially induced and precise small-scaled infection with *Plasmopara viticola* on living vines

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ABSTRACT

Grapevine downy mildew, caused by the oomycete *Plasmopara viticola*, affects most of the world's finest and widely planted grapevine (*Vitis vinifera* L.) cultivars and is typically controlled by fungicides, which are often applied in excess. Reducing fungicide use through early pathogen detection is therefore a major goal, which could be supported by emerging optical remote sensing techniques that detect subtle changes in leaf phenotype associated with disease. However, considerable variability in grapevine leaf structures, both within and across varieties, poses a major challenge in the application of optical remote sensing techniques for early downy mildew detection. To address this problem, we present a simple and effective method for the artificial and precise infection of *P. viticola* on living grapevines, enabling scheduled infections with an average incubation period of five days. This method is non-destructive, highly targeted, and differs from existing techniques by avoiding leaf removal and diffuse infection patterns, while being fast and easy to perform. Restricting infection to small, defined areas on individual leaves enables direct comparisons between healthy and infected tissue within the same leaf, minimising variability effects. This precise infection protocol offers significant advantages for training remote sensing applications, developing pathogen detection models, and supporting detailed analyses of *P. viticola* development, contributing to improved early detection through normal visualisation methods and emerging optical remote sensing techniques and a potential reduction in fungicide use.

KEYWORDS: artificial infection, pathogen detection, infection method, *Plasmopara viticola*, precision

INTRODUCTION

Most cultivars of the world's leading grapevine species, *Vitis vinifera* L., are highly susceptible to *Plasmopara viticola* (Berk. & Curt. ex de Bary), the causal agent of downy mildew. Infection severity is strongly influenced by local environmental conditions (Kassemeyer *et al.*, 2015). Under humid and moderate weather, *P. viticola* penetrates leaf stomata and initially colonises the intercellular space of the spongy mesophyll (Stoll *et al.*, 2008), causing substantial damage to green tissues and resulting in significant yield and quality losses. Effective grapevine protection typically requires multiple fungicide applications. Efforts to reduce fungicide use include replanting resistant cultivars and deploying infection forecasting systems such as VitiMeteo-Plasmopara (Bleyer *et al.*, 2011; Fouillet *et al.*, 2022). Nevertheless, targeted, needs-based applications remain essential for minimising environmental impact (Stoll *et al.*, 2008; Portela *et al.*, 2024).

Conventional disease monitoring in any crop relies on visual assessment, which is subjective, slow, and limited to the visible spectrum (Bock *et al.*, 2020). Advances in model-based remote sensing and optical sensor technologies now enable earlier detection of physiological changes in plants associated with biotic or abiotic stress (Mahlein *et al.*, 2012; Lowe *et al.*, 2017; Pantaleoni-Reluy *et al.*, 2022; Tanner *et al.*, 2022; Chin *et al.*, 2023). Spectral sensors enable early plant disease identification of biochemical and biophysical changes by detecting infection-related changes in leaf reflectance (Mahlein, 2016). However, validation in the field for grapevine diseases remains challenging as the architecture of grapevine canopies can obscure affected leaves, and abiotic and biotic stresses introduce signal variability (Portela *et al.*, 2024). Furthermore, studies using hyper- and multi-spectral data have highlighted major obstacles, including limited comparability between healthy and infected grapevine tissues (Bendel *et al.*, 2020) and low spatial resolution, which is reported in other crops (Tanner *et al.*, 2022). Leaf morphology, structure, and age variability within and across grapevine cultivars and other crop species, such as maize, further complicate both qualitative and quantitative analyses (Ambrosi *et al.*, 2011; Mertens *et al.*, 2023), necessitating extensive sampling. Additional approaches, such as thermal imaging, have shown that early pathogen activity can be detected based on altered grapevine leaf temperature dynamics (Zia-Khan *et al.*, 2022), though environmental variables like rainfall and solar radiation can distort results. For instance, Stoll *et al.* (2008) demonstrated that inoculation effects on leaf temperature differ between irrigated and water-stressed grapevines. Collectively, these findings underscore the need for refined methodologies to support machine learning approaches and modelling for the training of optical remote sensing technologies. Therefore, the objectives of this study were: i) to establish small-scale infection sites allowing precise referencing of healthy and infected tissues within the same leaf, ii) to induce isolated and predefined infections to enable high-resolution observations, and iii) to develop a simple, rapid infection protocol under controlled conditions to advance sensor-based disease detection and deepen the understanding of *P. viticola* pathogenesis.

MATERIALS AND METHODS

1. Grapevine material

Clonal plants of the *Vitis vinifera* cultivar Bacchus, known for its high susceptibility to *P. viticola*, were used. Grapevines were cultivated in pots under controlled, disease-free greenhouse conditions and ranged in age from two to four years. For both pre-infection and infection procedures, only the fourth and fifth fully expanded leaves were selected. During the post-infection incubation period, the pots were covered to prevent excessive soil moisture caused by elevated humidity levels.

2. Pathogen material

The field isolate 1136 of *P. viticola*, obtained from Gómez-Zeledón *et al.* (2013), and its derived clones were used exclusively. This isolate was chosen based on its high virulence against a broad range of grapevine genotypes. Sporangia were either freshly propagated or dry stored at –70 °C for later use.

3. Propagation solution

An *in vitro* propagation method for *P. viticola*, further developed based on Gómez-Zeledón *et al.* (2013), was used. Propagation was carried out in distilled water supplemented with bovine serum albumin (BSA) and calcium nitrate to improve pathogen stability and prolong the viability of detached leaves, thereby ensuring consistent sporangia formation. The propagation medium consisted of 2 µg/mL BSA (Merck Darmstadt, Germany, cat. nº 1.12018.0025) and 0.42 µg/mL $Ca(NO_3)_2$*4 H_2O (Carl Roth Karlsruhe, Germany, cat. nº P740.4). The solution was stored at 8 °C and remained stable for at least two weeks.

4. Environmental conditions

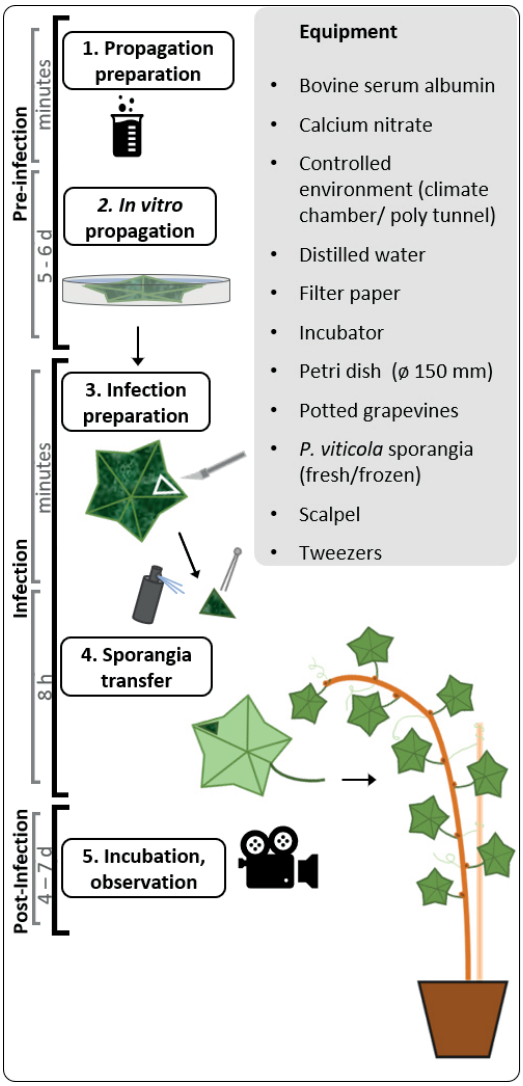
Propagation, infection, and incubation were conducted under controlled environmental conditions as follows:

- Incubator: 16-hour photoperiod (long-day conditions) at 18 °C and 60 % relative humidity.
- Climate chamber/poly tunnel: 16-hour photoperiod at 22–24 °C, 40–60 % relative humidity during the light phase; 18 °C and 85–98 % relative humidity during the dark phase.

The detailed infection procedure is provided in supplementary data S1.

RESULTS

Following Spring *et al.* (1998), Gómez-Zeledón *et al.* (2013) developed a modified *in vitro* propagation method for *P. viticola*, which served as the foundation for the procedure presented in this study. Building on this approach, a new infection protocol (supplementary data S1) was developed to enable precise, localised symptom development on living grapevine leaves and to simplify the droplet inoculation method employed by Stoll *et al.* (2008), Cséfalvay *et al.* (2009), and Grünwald (2024). The method is structured into three phases (Figure 1).



Pre-infection involves *in vitro* propagation of *P. viticola* to generate fresh sporangia without requiring strict sterility. Cultivation was conducted in an incubator over five to six days, after which the inoculum was prepared for infection.

Infection refers to the transfer of spores to living grapevines by attaching an inoculated leaf fragment (~5 mm²) to the abaxial side of a target leaf. This process requires only a few minutes and was conducted under controlled environmental conditions in a climate chamber or polytunnel (see supplementary data Figure S1).

Post-infection describes the incubation period of inoculated leaves on potted plants, up to seven days after inoculation, during which symptom development was monitored by eye and image measures of chlorophyll fluorescence.

FIGURE 1. Simplified overview of the method for precise infection of grapevine leaves with *Plasmopora viticola* on a small scale. The flowchart illustrates the timeline and key steps of the procedure. The equipment required for the method is listed on the right.

1. Evaluation of *in vitro* propagation (pre-infection)

After an incubation period of four to five days, a whitish-grey coating appeared on the abaxial leaf surface (Figure 2). Sporulation typically covered the entire lower surface of the leaf. Sporangia and sporangiophores were examined microscopically, with particular attention paid to the typical branched structures and overall sporangia density. Sufficient presence and proper development of sporangia were considered more critical than the absolute quantity to ensure homogeneous infection results.

2. Symptom occurrence and success of the *Plasmopara viticola* infection procedure (infection and post-infection)

A total of 312 leaves (two leaves per plant) from 156 potted grapevines were inoculated with spores generated during the in-vitro-propagation process. Among these, 266 leaves (85.3 %) showed successful and precisely delimited infection sites by day 7 (Table 1).

First visible signs of *P. viticola* sporulation on the lower leaf surface of inoculated leaves of potted plants were observed between four and seven days post-infection, and more than 80 % of them were observed within five days (Table 2).

The *P. viticola* infection sites on the abaxial (lower) surface of inoculated leaves corresponded precisely to the size of the infected leaf fragment (~5 mm²) applied during inoculation. No further spread beyond the initial infection area was observed, as confirmed by chlorophyll fluorescence imaging (Cséfalvay *et al.*, 2009). Moreover, no visible discolouration appeared on the adaxial (upper) leaf surfaces within the first eight days post-infection (Figure 3).

DISCUSSION

As one of the most important pathogens in viticulture, *P. viticola* has been extensively studied to improve understanding of its behaviour and to develop effective disease management strategies (Gessler *et al.*, 2011; Hernández *et al.*, 2022). Current research is increasingly

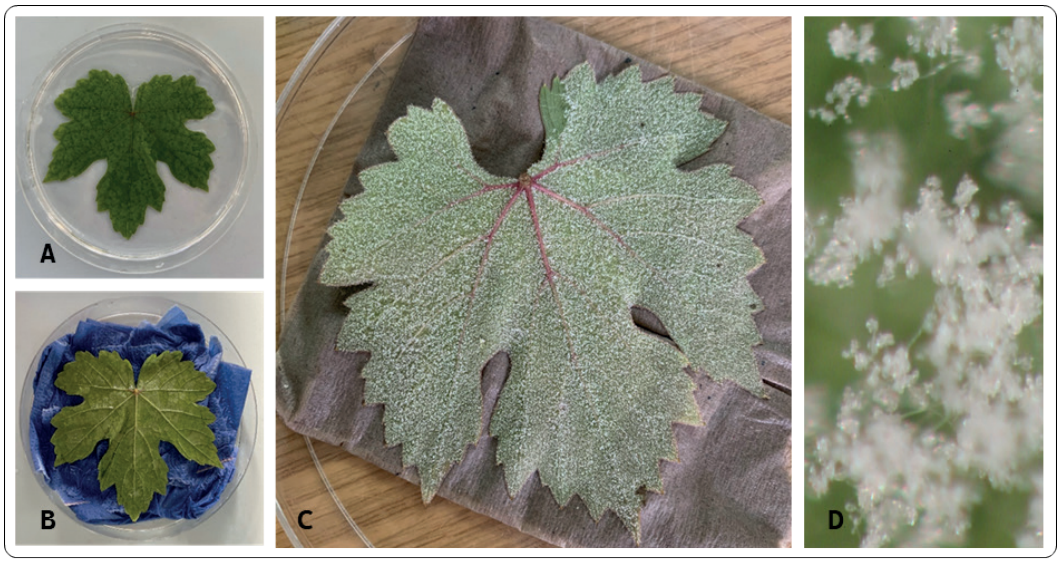


FIGURE 2. *In vitro* propagation of *Plasmopara viticola* on detached grapevine leaves: (A) leaf floating bottom-side down in sporangia suspension; (B) leaf positioned bottom-side up on moist filter paper after solution removal; (C) visible sporulation on the lower leaf surface after incubation; (D) close-up of sporangiophores under a light microscope.

TABLE 1. The number and proportion (%) of leaves of potted grapevine plants successfully infected by *Plasmopara viticola*.

	Total number of leaf samples	Number of successful *P. viticola* infections	The number of symptoms observed and the cause			
			Mechanical damage	Fragment fell off early	No obvious reason	In total
Quantity *(n)*	312	266	11	7	28	46
Proportion (%)	100	85.3	3.5	2.2	9	14.7

TABLE 2. The day post-inoculation that symptoms caused by *Plasmopara viticola* were observed on successfully infected grapevine leaves (*n* = 266).

Incubation time (days post-infection)	1	2	3	4	5	6	7
Number of leaves with first symptoms (sporulation)	0	0	0	93	124	40	9
Total number of leaves showing symptoms	0	0	0	93	217	257	266
Proportion of leaves showing symptoms (%)	0	0	0	35.0	81.6	96.6	100

focusing on modern sensor technologies for early pathogen detection to aid management and reduce the impact of disease in numerous crops, including grapevines (Farber *et al.*, 2019; Portela *et al.*, 2024). The method presented here is a model for training optical sensor applications for early detection of downy mildew to support this progress by enabling precise observation of early disease symptoms from the onset of *P. viticola* infection and by facilitating direct referencing of healthy and infected tissues within the same leaf. With a success rate for leaf infection exceeding 85 %, the method for creating the model is highly reliable, requires minimal equipment, and is simple and rapid to perform.

The model we have developed, which can be used for training optical sensors, simplifies the detection of signals of *P. viticola* infection at an early stage. Under natural conditions, the exact time of infection is often difficult to track, and healthy leaf material with an identical leaf structure is usually lacking for sensor-based referencing. Only a few studies suggest that early cellular modifications, induced by fungal toxins or plant defence responses, are detectable via hyperspectral imaging (Mahlein *et al.*, 2012). Additionally, RGB, thermal, and fluorescence sensors have shown promise for non-invasive detection of pathogen interactions by detecting discolouration, necrosis, and intercellular anomalies (Cséfalvay *et al.*, 2009; Farber *et al.*, 2019; Singh *et al.*, 2020). Nevertheless, sensory approaches must be continuously developed in parallel with advances in sensor technology. In the future, the *P. viticola* infection model developed in this study could be

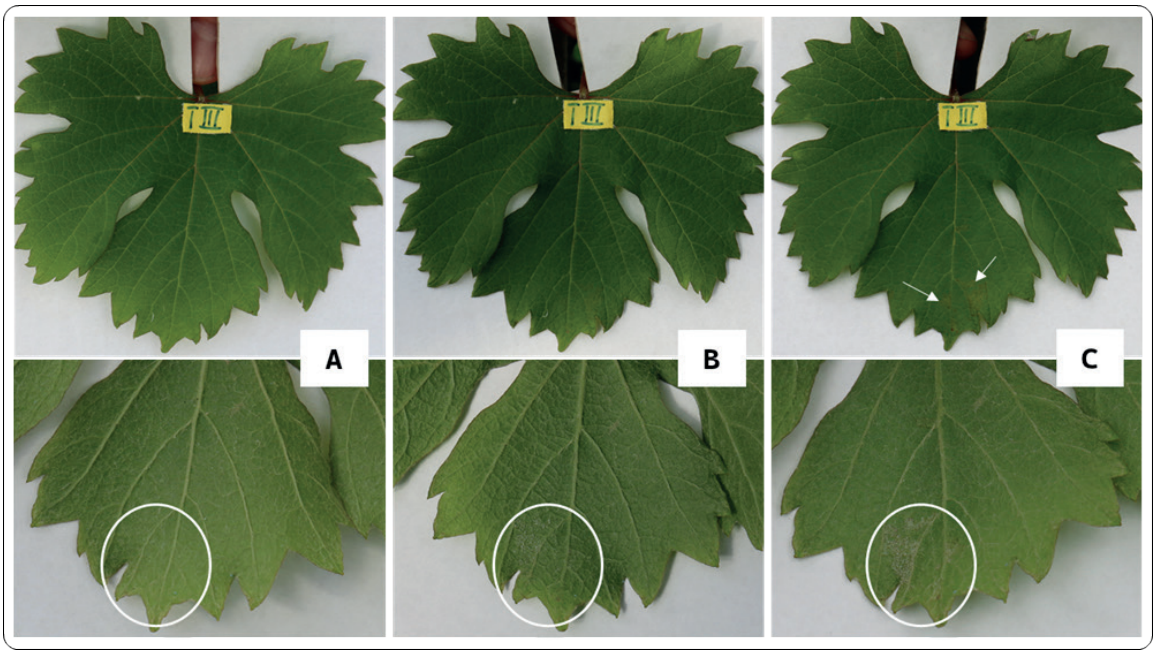


FIGURE 3. Development of spore formation by *Plasmopara viticola*: (A) one day after infection; (B) five days after infection; (C) eight days after infection. The upper side of the leaf is shown at the top, and the corresponding lower side at the bottom. White arrows indicate small discolouration spots visible on the upper leaf surface.

used to investigate more deeply the plant host interactions that can inform and train new technologies as they emerge. Controlled environments, such as those used in this study, offer significant advantages for disease monitoring, as plants can be assessed under optimal light conditions from multiple angles (Tanner *et al.*, 2022) and minimising confounding abiotic and biotic stresses.

Other inoculation methods have been used to evaluate optical imaging for early disease detection in grapevines, such as the drop-based *P. viticola* sporangia suspension used by Stoll *et al.* (2008) to evaluate thermal imaging technology in glasshouse conditions for early, pre-symptomatic, detection of downy mildew. However, these traditional approaches are more time-consuming, require complex preparation of sporangia suspensions—often involving adjustments to zoospore concentration—and demand greater practitioner expertise. In contrast, our method, which needs less expertise, streamlines the infection process, requiring only seconds to minutes per inoculation, while ensuring precise localisation without the need for central positioning between leaf veins or prolonged manipulation of leaves. The confined liquid film between the leaf fragment and the intact leaf further minimises uncontrolled pathogen spread, allowing only planned infection sites to occur. Currently, this method has not yet been used to test optical sensor methods, but it will be done in future experiments to test our infection model as a tool for evaluation.

Greenhouse findings provide essential foundations for advancing field applications for optical sensing of disease: often, the incubation times for disease development associated with pathogens such as *P. viticola* and the controlled environments can be comparable to field conditions. Therefore, traits observed under controlled conditions, such as lesion size or sporulation patterns, can, in some cases, be extrapolated to field settings (Simko *et al.*, 2017; Rebetzke *et al.*, 2019). Importantly, the *P. viticola* infection model presented in this study enables the induction of disease symptoms in clearly defined leaf areas, allowing precise differentiation between infected and healthy tissues. This facilitates the identification of early pathogen-induced signal changes across different grapevine varieties that might be detected by optical sensing technologies and supports the development of transferable approaches for field monitoring. Furthermore, close, early-stage monitoring may enable disease detection before visible symptoms appear, offering a valuable tool for timely intervention and contributing to reduced fungicide use.

CONCLUSION

The infection model developed in this study will enable the induction of precise, small-scale *P. viticola* infections on living grapevines by establishing individual, localised infection sites, allowing direct comparison between healthy and infected tissues on the same leaf. Improving understanding of pathogen–host interactions provides a valuable basis for the development and validation of optical sensor technologies for early disease detection. In the future, this could also contribute to improving our specific understanding of the physiological and biochemical changes associated with early infection by *P. viticola*, which in turn could further advance the application of new control strategies. Compared to existing *P. viticola* inoculation techniques, the method is simpler, faster, and requires less equipment and expertise. In the long term, it may contribute to more targeted disease management strategies and a significant reduction in fungicide applications.

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Not applicable.

DECLARATION OF AI USE

AI tools were used for refining the language of the manuscript.

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