Advances in molecular and optical detection strategies for grape downy mildew

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1 Introduction

Within vineyards, downy mildew (DM) of grapes, caused by *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, is an aggressive polycyclic disease. This hemi-biotrophic oomycete pathogen overwinters in leaf litter as oospores and causes infections throughout the season as zoospore secondary inoculum spread by way of water splash or wind turbulence (Gessler, *et al.* 2011). Control of grapevine downy mildew is accomplished using chemical control as well as cultural practices, but resistance to numerous fungicides has developed over the years due to poor chemical rotation and excessive use.

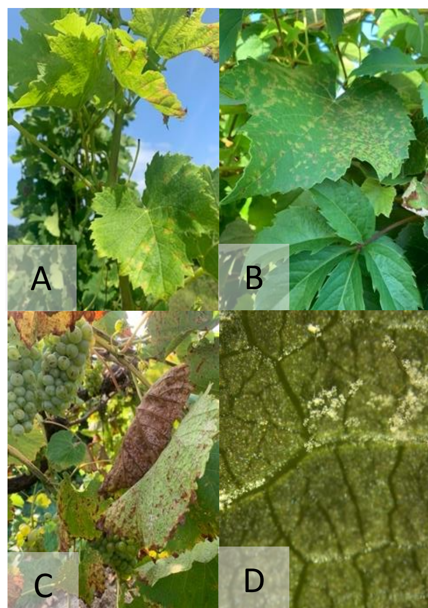

Figure 1: A-C, Grape leaf tissue infected with P. viticola showing oil spot symptom; D, P. viticola sporangiophore with sporangia on leaf tissue.

To understand inoculum load and mutations in a field found throughout a growing season, molecular detection methods using quantitative polymerase chain reaction (qPCR) and TaqMan probes are used. These tools allow for pathogen quantification as well as low-inoculum detection within fields for asymptomatic diagnosis (Salcedo *et al.* 2021). Diagnostic tools are often paired with a method of disease sampling, like air or water collection, to understand pathogen levels across a site.

2 Spore trapping technologies and advances

Across numerous mildew pathosystems, spore trapping technologies have been implemented to monitor pathogen inoculum load, fungicide resistance, and pathogen genotype (Klosterman *et al.* 2016; Kunjeti *et al*. 2016; Thiessen *et al*. 2016). The ability of the spores to travel great distances contributes to high diversity within pathogen populations spread from one field, or state, to another (Aylor 2017). Trapping and quantifying spores to measure disease pressure throughout a season, within a field or vineyard, allows growers to better control disease levels while also understanding changes in population genetics. Coupling spore trapping devices with molecular techniques designed with the ability to detect mutated isolates provides growers with rapid identification of resistant or sensitive isolates within their fields that then can influence season-long fungicide applications. Spore trapping is, and has been, a common method for monitoring disease, with recent trends indicating an increased use in these methods. More recently, trap deployment is used for purposes beyond pathogen detection, such as identifying fungicide resistance and understanding associated shifting population structures.

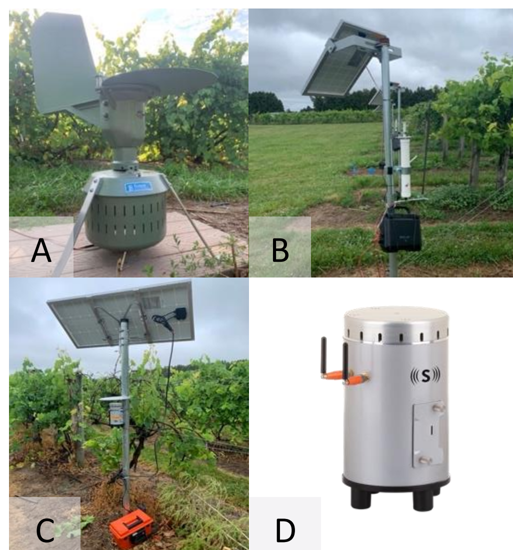

Figure 2: Types of spore traps deployed. A: Burkard spore trap; B: impaction spore trap; C: ScanIt SporeCam™ field installation; D: ScanIt

The most widely used sampling traps include impaction traps and volumetric Burkard traps (Crandall *et al* 2018). Both Burkard and impaction traps fall under the category of active samplers using a vacuum-powered adhesive drum and motor-powered sampling arm, respectively. Burkard 7-day volumetric traps (Fig. 2 A) are common, yet costly, tools used in crop disease detection and management that can be used for both visual and molecular downstream analysis to quantify disease. The traps require large batteries and are

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difficult to manipulate in the field due to size and function. In contrast, impaction spore traps (Fig 2 B) are highly affordable, easily adapted, traps that are intended for molecular downstream analysis. While the impaction traps, designed by Dr. Walt Mahaffee (United States Department of Agriculture–Agricultural Research Service [USDA-ARS], Corvallis, OR), are very versatile in design and affordability, they require more seasonal maintenance. Trapping systems are chosen based on the crop being monitored, the location, cost, and the purpose for trapping. Due to agricultural requirements, such as location and canopy management, modifications to the trapping systems

are often necessary. To accommodate these changes as well as improve ease of access to data, spore monitoring devices have been recently redesigned to optically observe aerial pathogens, requiring less molecular involvement for detection. The *Scanit Technologies* SporeCam™ (Fig. 2 C-D) uses autonomous technology to actively capture airborne particles onto a cassette to then be processed and analyzed by machine learning. The SporeCam™ device is a more expensive option, likely limiting its accessibility to larger growing operations. The collected data, including weather data, is then reported back to the scientist or grower managing the site using Wi-Fi or 4G wireless to provide quick and accurate information pertaining to the pathogen type and level found in a field (*Scanit Technologies)*.

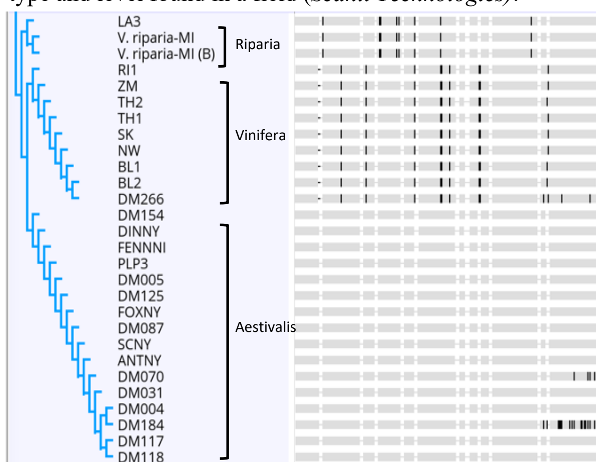

Figure 3: Pylogenetic tree construction of 2019-2020 collected isolates of the ITS1 region using maximum likelihood and a bootstrap of 70% in Geneious. This data depicts the occurrence of the previously described P. viticola clades across collected isolates.

The SporeCam™ can be taught specific disease signatures to recognize pathogens in a field, such as grapevine downy mildew. Spore classification can be carried out with spores as small as 6µm and as large as 1000µm, creating a range of pathogen morphology that can be captured, analyzed, and confirmed. When using the SporeCam™, downstream analysis is limited to optical analysis using machine learning and classification algorithms, leading to possible concerns in misidentification of pathogens with similar morphologies, like *P. cubensis* and *P. humuli.*

In this study, three spore trapping technologies were deployed at the Michigan State University Clarksville Research Station (Clarksville, MI): Burkard volumetric trap, impaction spore traps, and the *Scanit* SporeCam™. The objective of this study was to uncover the benefits and pitfalls of these individual spore trapping technologies and the subsequent molecular techniques as pertains to grapevine downy mildew and other grape pathogens.

A SporeCam™ training module device was taught the disease signature for *P. viticola* using a controlled environment at Michigan State University and pure cultures of numerous *P. viticola* isolates over a combined 6-week period. After completing training and data processing through *Scanit*, a SporeCam™ field device was installed and deployed in a vineyard site to be used alongside the Burkard and 12-impaction spore traps in an unsprayed 'Vignoles' block. The impaction spore trap custom design was acquired from Dr. Walt Mahaffee and deployed in similar manner to those used in *Erysiphe necator* studies (Thiessen *et al.* 2016). The impaction spore trap rods were collected twice a week, sampling every 3 and 4 days. This field experiment extended over the course of 13-weeks in 2021 and will be repeated in 2022. Upon collection, DNA was extracted from impaction rod samples to be used for downstream analysis using qPCR (Klosterman *et al.* 2014).

3 *Plasmopara viticola:* a species complex impacting diagnostic tools

Rouxel *et al*., 2014 describes *P. viticola* as a unique species (*P. viticola sensu lato)* complex infecting eight *Vitis* (grape) species. This characterization gives insight into the pathogen's infectivity and aggressiveness towards its various hosts. Currently, three *P. viticola* clades have been reported on cultivated grape: clades *aestivalis, riparia, and vinifera* (Fig. 3). This research describes *P. viticola* clade *aestivalis* as the predominant pathogen species in Eastern North America, with *P. viticola* clade *vinifera* and clade *riparia* as the other identified clades in the region. Currently, there are no methods for differentiating these key cryptic species of *P. viticola sensu lato*, thus making further analysis of the predominant species challenging without using expensive sequencing technology. Methods for rapid differentiation would provide better understanding of *P. viticola* clade distribution that could impact disease management decisions and strategies (Rouxel *et al.* 2014).

The current marker system for *P. viticola* is designed using the internal transcribed spacer 1 (ITS1) and -5.8S rDNA to detect *P. viticola* as a general species (Valsesia *et al.* 2005).

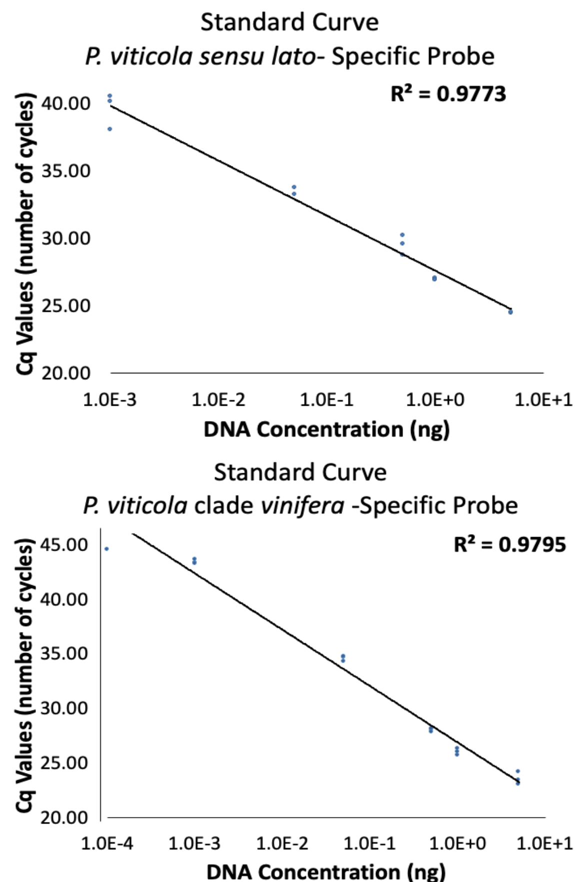

Figure 4: A. Standard curve plot of sensitivity detected using the Plasmopara- viticola sensu lato-specific probe. B. Standard curve plot of sensitivity detected using the Plasmopara viticola clade vinifera probe.

The ITS region in downy mildew species known to be variable and often contain significant heterogeneity and insertions, making amplification challenging (Salcedo *et al.* 2021). Within this study, new conserved amplification primers were developed along with a *P. viticola sensu lato* probe capable of detecting all pathogen types. Probes specific for *P. viticola* clade *vinifera, riparia,* and *aestivalis* were also developed to provide rapid detection of grapevine downy mildew, and identify the cryptic species present as well as the prevalence of each clade throughout the course of the growing season (Fig. 5). The locus used in this system is a *Plasmopara*-unique mitochondrial gene order, allowing for improved specificity and sensitivity (Crandall *et al.* 2018). Genus- and species-specific primers and probes were studied and developed to optimally detect *P. viticola* within vineyards from impaction style spore traps and Burkard Melinex-style tapes. The developed system was compared with the ITS system designed by Valsesia *et al.* 2005 to determine sensitivity and specificity when used both in pure and field samples. The *sensu lato* TaqMan probe and clade *vinifera* TaqMan probe were determined to be able to detect at approximately 1pg and 0.1pg respectively (Fig. 4). The clade *aestivalis* and *riparia* probes are still in the process of optimization. Primers and probes were validated against other *Plasmopara* spp. as well as species within the Peronosporales. To confirm clade identification, the ITS 1region was sequenced in the tested isolates.

4 Future directions and conclusions

Future work for this project includes the continued optimization of the *P. viticola sensu-lato* and clade TaqMan marker systems to detect each clade accurately, particularly at low sample volumes. These marker systems, and the ITS system designed by Valsesia *et al.* 2005, will be tested using DNA samples from Melinex-type tapes from a Burkard, spore rod samples from impaction traps, Tough Spot samples, and infected leaf tissue.

This work provides foundational data to encourage the focus of detection to shift from Burkard volumetric sampling to the cheaper, easily replicable option of impaction spore traps, which can be modified as needed. Impaction traps can be paired with the autonomous optical detection method using the *Scanit* SporeCam™, thus alleviating the need for long term rod sampling to identify initial inoculum levels. Upon initial SporeCam™ detection, the impaction traps can be deployed to identify molecular differences in a vineyard, or other field.

This study aims to better understand which methods of sampling and detecting are optimal for downy mildew of grape. The research performed in this study will provide the foundational knowledge needed to further analyze downy mildew populations regarding fungicide resistance and population diversity and structure. The development of diagnostic tools and technology used to monitor pathogen prevalence plays a critical role in integrative disease management for increased control of recurrent and resistant populations of downy mildew in vineyards.

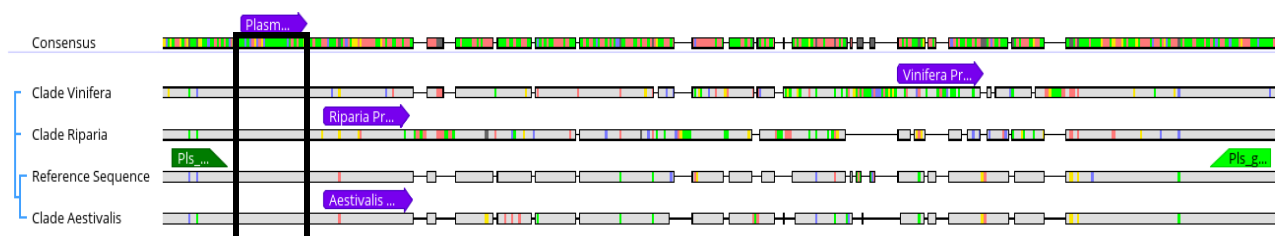

Figure 5: Annotated alignment for primer and probe design for *Plasmopara viticola* sensu-lato and *P. viticola* clade vinifera using a *Plasmopara* unique locus. Probes are indicated by purple arrows; qPCR primers are indicated by green arrows.

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