Evaluation of spore collection methods for detection and quantification of fungicide resistance in powdery mildew

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Sample collection methods for powdery mildew were evaluated in vineyards as part of a Wine Australia-funded project. Seventy samples were collected using spinning rod spore traps, mini-vacuums, washing infected leaves and cotton bud swabs. Using a molecular detection system, based on next generation sequencing (NGS), all sampling methods were able to collect *E. necator* spores from the air and leaves. Two mutants Y136F and G143A (linked to resistance to C14-demethylase inhibitors and quinone outside inhibitors, respectively) were identified in the three locations monitored, however H242R/Y (linked to resistance to succinate dehydrogenase inhibitors) was not detected.

INTRODUCTION

Erysiphe necator, an obligate parasite that causes grapevine powdery mildew, is one of the most widespread pathogens in Australian vineyards and worldwide. The ubiquitous nature of this pathogen puts pressure on fungicides used for control, particularly the quinone outside inhibitors (QoIs), the sterol C14-demethylase inhibitors (DMIs) and the succinate dehydrogenase inhibitors (SDHIs). Intensive use of these fungicides has led to resistance, particularly to QoIs and DMIs, in many countries (Vielba-Fernández *et al*. 2020) including Australia (Hall *et al*. 2017, McKay *et al*. 2020, Savocchia *et al*. 2004).

Several mutations have been identified in *E. necator* populations that are responsible for imparting resistance to these fungicides, including G143A (linked to QoI resistance), Y136F (linked to DMI resistance) and H242R/Y (linked to SDHI resistance) (Kunova et al. 2021). Resistance can be monitored using either laboratory-based phenotypic bioassays and/or genetic tests to detect the associated mutations. Culture maintenance and bioassays with *E. necator* require live plant tissue and, as a result, these processes are time-consuming and laborious (Corio-Costet 2015). If the genetic mechanisms of resistance are known, molecular detection for resistance is faster, more sensitive, accurate and cost effective than bioassays, (Falacy *et al*. 2007, Kunova *et al*. 2021).

Collecting samples from vineyards that are suitable for molecular resistance tests has been a focus of research in recent years, particularly using spore traps. Spore traps have been used for more than 60 years to detect and/or quantify fungal pathogens in the field (West and Kimber 2015), including airborne powdery mildew spores (Mahaffee and Stoll 2016). To date, detection of *E. necator* spores using spore traps has been primarily used for disease forecasting and decision making with respect to spray applications. There are broadly two types of traps: 1) passive traps which rely on airflow or wind to deposit spores onto the trap surface, and 2) active or volumetric traps that require a power source and fan to actively draw the air containing spores into the trap.

Spores of *E. necator* move most readily within the vineyard in air currents between rows and vines (Gonzalez-Fernandez *et al*. 2019). Several designs of spore traps are commercially available, with spinning rod impaction-style passive traps being the cheapest and most portable. A large research project led by Dr. Walt Mahaffee (Oregon State University) is currently evaluating a network of these traps across viticultural regions of Oregon, Washington and California in the USA to detect and quantify fungicide resistance. *E. necator* has been successfully detected (Thiessen *et al*. 2016) as well as the G143A mutant (Miles *et al*. 2021). Dr Mahafee has kindly provided three spinning rod spore traps to Australian researchers who have built a further nine traps based on his design.

IN BRIEF

- *Erysiphe necator*, an obligate parasite that causes grapevine powdery mildew, is one of the most widespread pathogens in Australian vineyards and worldwide
- Intensive use of the fungicides used to control *E. necator*, has led to resistance in many countries including Australia.
- Several mutations have been identified as being responsible for imparting resistance; laboratory-based phenotypic bioassays and/or genetic tests can be used to detect these mutations.
- Researchers evaluated spinning rod spore traps and other methods to obtain vineyard samples suitable for the molecular detection of *E. necator* and its fungicide resistance mutants.

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The aim of this research was to evaluate the spinning rod spore traps along with other methods of sample collection, including a mini-vacuum spore collector, washing infected leaves and cotton bud swabs to obtain spore material suitable for molecular detection of *E. necator* and the fungicide resistance mutants G143A, Y136F and H242R/Y.

METHODS

Spore collection methods

Spinning rod spore trap

Nine spinning rod spore traps were constructed based on a prototype and instructions provided by Dr Walt Mahafee. The spore traps comprised 4cm welding rods attached to horizontal sampling arms, connected to a 6-volt motor powered by a 12-volt battery, which is charged by a 10-watt solar panel (Figure 1). The traps were fixed to a vineyard post so that the rods were approximately 10cm above the canopy to maximise the likelihood of trapping *E. necator* spores (Mahaffee and Stoll 2016). Prior to each sampling period Molykote® grease was smeared onto the collection rods to ensure that the spores stuck to the rods. Once each collection period finished, rods were placed into tubes, transported at ambient temperature to the laboratory and stored in a freezer at -20°C until required for DNA analyses.

Mini-vacuum spore collector

A mini electric cordless vacuum pump (4.2V, Kogan) was modified to manually collect spores from infected leaves in the vineyard. The device was modified by attaching a plastic spout and tube to connect a cyclone separator and Eppendorf tube to collect spores (Figure 2A). The Eppendorf tube containing spores

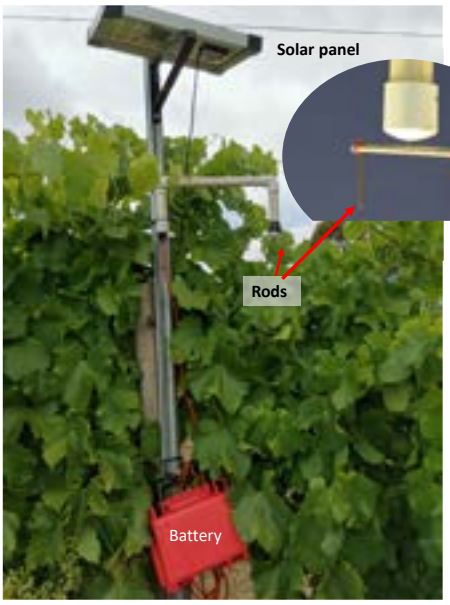

Figure 1. Spore trap set up in the vineyard consisting of battery, spinning rods and solar panel.

were removed, transported at ambient temperature to the laboratory and stored in a freezer at -20°C.

Leaf washing

Spores were collected by adding 10-15mL of water to a zip-lock plastic bag containing infected leaves (Figure 2B) and shaking vigorously for 30 seconds. The spore suspension was transferred to a tube, transported at 4°C to the laboratory and stored at -20°C.

Cotton bud swabs

Sterilised cotton buds were rubbed across the surface of infected leaves to collect spores (Figure 2C). Swabs were placed in sterile tubes, transported at ambient temperature to the laboratory and stored at -20°C.

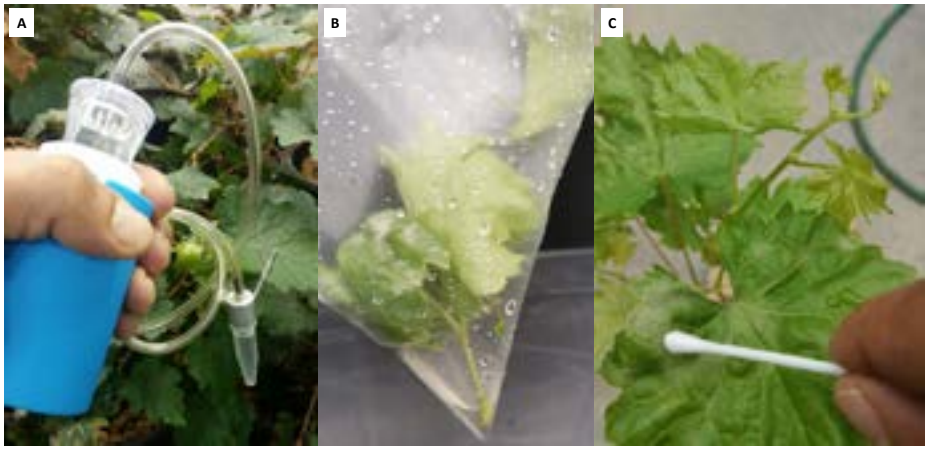

Figure 2. Sample collection methods: (A) assembled mini-vacuum ready to collect spores from leaves, (B) leaves washed to collect spores, (C) cotton bud swab collecting spores.

VINEYARD EVALUATION

To evaluate the sampling methods for detection of *E. necator* spores, spinning rod spore traps were installed in January 2020 in a Chardonnay vineyard at the Nuriootpa Research Centre in the Barossa Valley, South Australia (SA) and a Grenache vineyard at the Waite Campus, Urrbrae, SA. There were seven sampling periods where rods were collected then replaced approximately every four days over a four-week period during January and February 2020. In addition, the mini-vacuum and leaf washing methods were also used on the last collection day at the Urrbrae site on 6 February.

In the 2020-21 season, to evaluate the sampling methods for identification of the mutations G143A, Y136F and H242R/Y, four spore traps were installed in the same two vineyards in the Barossa Valley and Urrbrae, as well as another in Renmark in the Riverland, SA. The traps were run continuously over four days for five separate time periods from December 2020 to February 2021. For each sampling period, rods were collected from traps that had been running for one, two, three and four days. In total, 20 samples were collected each from the Barossa Valley and Urrbrae spore traps and 12 samples from the Riverland trap. In addition, the mini-vacuum, leaf washing and cotton bud swabs were used to collect spores at the Barossa Valley site on 15 January 2021.

DNA ANALYSIS

DNA was extracted from samples using the Mag-Bind Environmental DNA 96 Kit (Omega Biotek). To detect *E. necator*, a next generation sequencing (NGS)-based fungal profiling methodology was applied (Sternes *et al.* 2017). This uses a DNA-barcoding strategy based on the fungal internal transcribed spacer (ITS) region to determine the proportions of species present in mixed environmental samples.

For resistance allele testing regions of the *cytB, cyp51 and sdhB* genes that potentially contained resistance, mutations were amplified by polymerase chain reaction (PCR). Proportions of resistant and sensitive alleles were determined from analysis of NGS data (Ramaciotti Centre for Genomics, UNSW Sydney, Australia) produced from the pooled the PCR products.

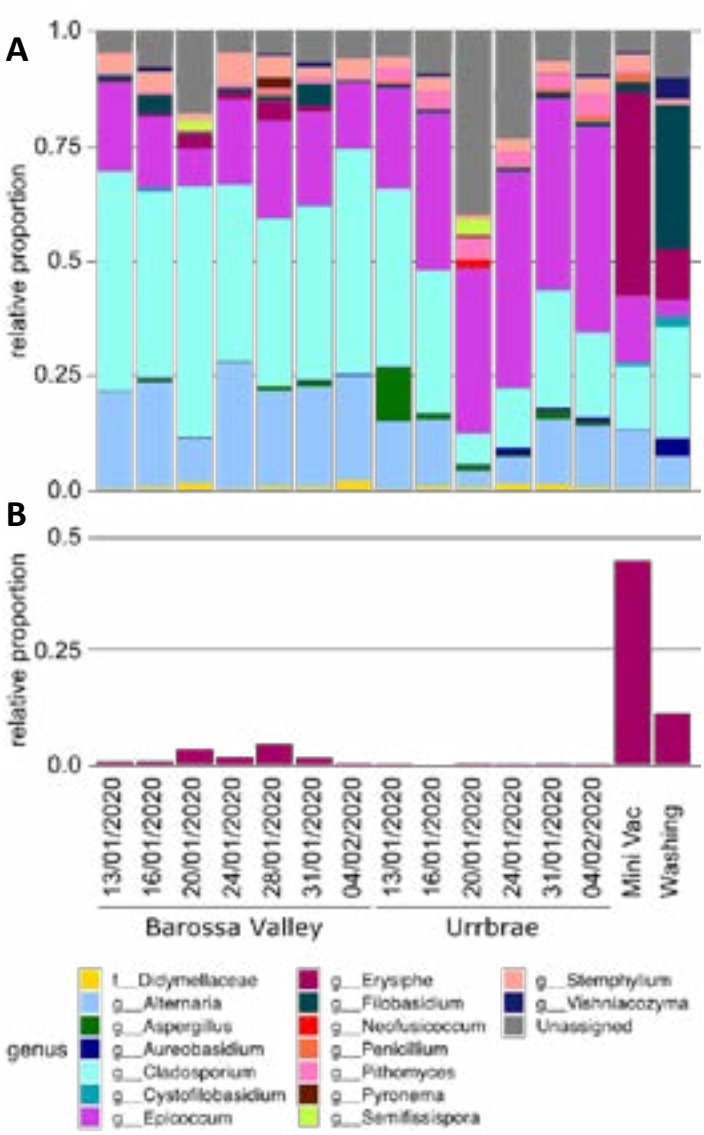

Figure 3. Fungal profiling of spore trap rods located in Barossa Valley and Urrbrae, mini-vacuum and leaf washing in 2020. (A) Population results, species were consolidated by genus and are presented as relative proportions within the population (B) *E. necator* detection results.

RESULTS AND DISCUSSION

Spinning rod spore trap

Fungal ITS profiling detected *E. necator* at low relative quantities from within a broad fungal population in most spore trap samples collected in January-February 2020 (Figure 3, see page 50). Greater quantities were detected in samples collected from leaves with powdery mildew symptoms using the mini-vacuum and direct leaf washing. Previous studies reported that *E. necator* can be detected using spore traps (Gonzalez-Fernandez *et al*. 2019, Mahaffee and Stoll 2016) and that spore traps can be used to monitor disease in vineyards (Thiessen *et al*. 2016). The advantage of spore trapping is that detection of low spore numbers provides early warning so that preventative management decisions can be made. When combined with untargeted monitoring, such as that provided by ITS profiling, multiple species (and pathogens) can be tracked without the need for species-specific assays.

In the 2020-21 season, the G143A (associated with QoI resistance) and Y136F (associated with DMI resistance) mutations were detected in 18 of 20 samples collected at the Barossa Valley site (Figure 4, see page 52). The frequency of G143A and Y136F mutants ranged from 54-77% and 53-100%, respectively. At Urrbrae, six of 20 samples were detected with G143A and frequency ranged from 8-42%, but no Y136F was detected. In the Riverland, seven samples were detected with G143A with frequency ranging from 2-32%, however only two samples were detected with Y136F, both with 100% frequency. The low frequencies of G143A detected in the Urrbrae and Riverland vineyards indicates potential development of fungicide resistance in the future. The mutation H242R/Y, associated with resistance to the SDHIs, was not detected in any sample. The results also revealed that running the spore trap for one day was sufficient to detect the mutations. The other collection methods — mini-vacuum, leaf washing and cotton bud swabs — also enabled detection of the mutations in the Barossa vineyard (Figure 5, see page 52).

E. necator and two mutations associated with resistance were detected using spinning rod spore traps at particularly high frequency in the Barossa Valley, even when there was no visible powdery mildew symptoms in the other locations (Urrbrae and Riverland). However, visible powdery mildew infection was required for the other three collection methods — mini-vacuum, cotton bud swabbing and leaf washing. The spore rods, mini-vacuum device and cotton swabs can be easily transported to a diagnostic laboratory at ambient temperature, whereas the spore suspension from leaves requires refrigeration to prevent DNA degradation. Spore traps have an advantage in that they can detect spores early in the disease cycle, before visible symptoms appear. If fungicide resistance is detected, once symptoms appear, more comprehensive sampling can be undertaken using the other methods. Detection of fungicide resistance at low levels, before it can be detected in bioassays and/or manifests as field failure, may enable appropriate anti-resistance strategies to be applied in a timely manner, prolonging the effective life of fungicides.

In this study, the spinning rod spore trap, mini-vacuum, leaf washing and cotton bud swabs all proved to be valid and practical methods for collecting spores of powdery mildew in the vineyard for subsequent molecular detection of the pathogen. Thiessen *et al*. (2016) demonstrated that growers were able to effectively manage grape powdery mildew using spore trapping and molecular detection to initiate fungicide applications. In the USA, a spore trapping study conducted by Miles *et al*. (2021) reported that G143A had been detected in 58 out of 132 samples from California and Oregon. In this study, two mutations (Y136F and G143A) were detected, indicating that multiple mutations can be detected from the same sample. As G143A is strongly associated with QoI resistance (Rallos *et al*. 2014) and Y136F is linked to DMI resistance (Kunova *et al*. 2021) the

use of molecular methods makes detection of resistance faster, targeted and more accurate and can complement or replace the conventional bioassay phenotyping. Molecular detection for fungicide resistance has been adopted widely in the USA and a rapid DNA test for QoI resistance has been developed by the Fungicide Resistance Assessment, Mitigation and Extension Network (FRAME) and is being applied and reported across the major grapegrowing regions of California, Michigan, Washington, Oregon and Ohio (Oliver *et al*. 2021; https://framenetworks.wsu.edu/).

CONCLUSION

Detection of powdery mildew fungicide resistance in grapevines is a key element in resistance management. The use of spore traps and other simple collection methods followed by testing with molecular technologies to detect mutations associated with resistance is a powerful tool in monitoring the development of resistance. The spinning rod spore trap was most sensitive and practical, particularly during the early stage of infection when there were no visible symptoms. The other methods will be valuable for more intensive sampling across an affected vineyard to determine the extent of fungicide resistance, or for in-field sampling and detection. Further research is now required to develop high-throughput and in-field molecular analysis methods to achieve greater volumes of sample testing with a quicker turnaround time for the Australian industry.

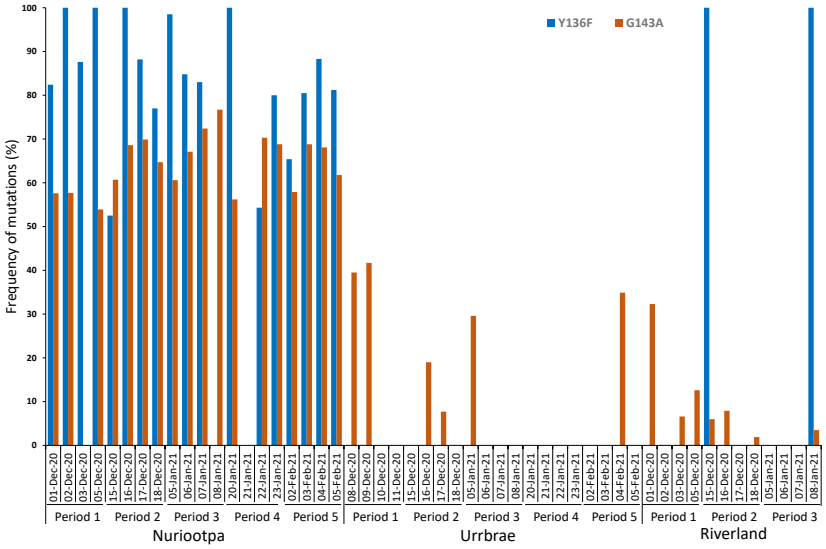

Figure 4. Detection of Y136F and G143A by next generation sequencing in Erysiphe necator samples collected over 4-day sampling periods from the Barossa Valley, Urrbrae and Riverland vineyards using the spinning rod spore traps in the 2020/21 season.

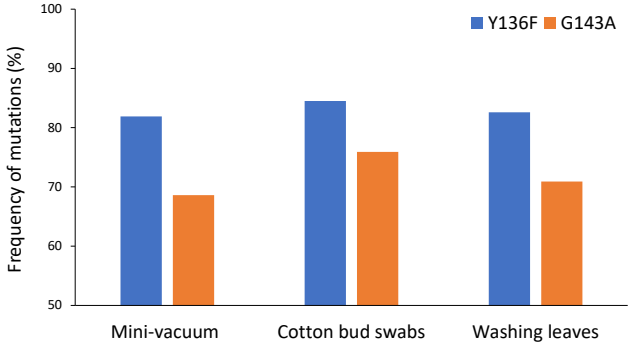

Figure 5. Detection of Y136F and G143A by next generation sequencing in Erysiphe necator samples collected from the Barossa Valley vineyard using the mini-vacuum, cotton bud swabs and washing leaves in the 2020/21 season.

ACKNOWLEDGEMENTS

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