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Charles Sturt University

Determining thresholds for bunch rot tolerance in wine and detection of unwanted fungal aromas



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Project CSU 1702-02 Determining thresholds for bunch rot tolerance in wine and detection of unwanted fungal aromas

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Abstract

Grapevines are susceptible to several fungal diseases, including grey mould, caused by the fungus *Botrytis cinerea*. This rot attacks grape bunches close to harvest resulting in a loss of yield and grape quality. Different techniques for grey mould estimation were evaluated for their applicability and compared with visual estimations with the naked eye. Measuring the fungal sterol ergosterol allowed for the quantification of fungal biomass present in a grape sample in a space of two days. Detection of Botrytis antigens was a more rapid and specific method for detecting grey mould contamination in a matter of minutes.

Executive summary

Botrytis cinerea is a filamentous fungal organism that infects grape tissues, most notably grape berries after the onset of ripening. The fungus is responsible for the disease grey mould of grapes. In some vineyards and some growing seasons grey mould infection can be so severe that it can result in total crop failure when none of the vineyard is economically worth harvesting. Disease severity increases with increasing Baume as grape berries ripen. Early detection is crucial so that a vineyard can be harvested before the level of grey mould reaches a point where it is no longer economically viable to harvest the crop. Detection and ideally quantification of grey mould contamination is not a clear-cut process especially as the disease is less obvious on dark skinned grape varieties.

Grape varieties that produce tightly packed clusters of grapes are more susceptible to grey mould particularly as a result of berry splitting as the individual berries expand. Consequently grey mould is often found within the interior of the grape bunch hidden from view. On first observation an apparently disease-free bunch can subsequently be found to be affected by grey mould once the bunch is harvested and pulled apart. Grey mould that is hidden from view within the bunch is difficult to detect using visual estimation with the naked eye. To overcome these issues, a number of experimental techniques to detect and ideally quantify grey mould in grapes were investigated. The methodologies investigated encompassed imaging techniques, chemical analysis and molecular biology techniques. To investigate the different ways in which grey mould contamination could be evaluated, a series of experiments involving field samples of grape bunches affected with grey mould and grape berries and bunches inoculated in the laboratory were utilised. The varieties examined included Chardonnay, Cabernet Sauvignon, Semillon and Shiraz. Grapes were sourced from vineyards in the Riverina (NSW) and also for one experiment, from the Tamar Valley (Tas). The amount of apparent grey mould contamination was estimated by visual observation using the naked eye. Depending upon the experiment, the grapes were analysed for gluconic acid, the fungal sterol ergosterol, amount of Botrytis antigens, the presence of Botrytis by loop mediated isothermal amplification (LAMP) and quantification of Botrytis by the polymerase chain reaction (qPCR). In one experiment grey mould affected grape bunches were analysed by hyperspectral imaging. All of the techniques investigated for grey mould detection were of some value. Yet all had some form of limitation in terms of applicability for a wine industry setting.

As is the case with estimations of grey mould contamination with the naked eye, hyperspectral imaging does not provide information on the presence or absence of grey mould growth within the interior of a tightly packed cluster. Compared to visual estimations, hyperspectral imaging is more accurate for grey mould estimation when the fugal growth is largely on the exterior as opposed to the interior of the bunch. This is particularly the case with dark skinned grape varieties which are harder to assess with the naked eye.

Loop-mediated isothermal amplification (LAMP) for the molecular detection of Botrytis DNA was found to be a sensitive assay but lacked quantification. In some experiments false negative results were obtained with the LAMP assay. The LAMP assay is of value in detecting Botrytis rather than quantifying Botrytis although some inconsistencies were noted in some of the data with regards to false negatives. Unlike the LAMP assay, qPCR was quantitative, but variable results were obtained in some experiments that looked at whole bunches infected with grey mould. There was a weak

correlation between the amount of Botrytis detected by qPCR and the perceived amount as determined either by visual estimation or by hyperspectral imaging of Cabernet Sauvignon infected bunches. For individual grape berries that were inoculated and mixed with healthy berries, variability in the qPCR data was evident at infection levels of greater than 6 % on a per berry weight basis. The variability in the qPCR data can be explained by the nature of the fungus Botrytis, which is multinuclear and highly variable. This limits the usefulness of qPCR for Botrytis quantification as opposed to Botrytis detection.

Three commercially available enzyme-based kits for gluconic acid were evaluated for Botrytis quantification and the results for all three kits were comparable. The kits can be used in a basic laboratory setting and results can be obtained in a matter of hours. Data for all of the kits examined were comparable. Gluconic acid was detected in all un-inoculated grape samples, and in some experiments it was not possible to differentiate between healthy grapes and grapes with low levels of grey mould contamination. Gluconic acid is also not specific for Botrytis – some of the other fungi associated with the rotting of grapes also produce gluconic acid. This limitation has to be weighed up against the fact that gluconic acid assays are relatively simple to use and the time frame to conduct the assays is of the order of minutes or hours as opposed to days.

The amount of ergosterol detected in grape samples correlated with the amount of grey mould as determined by either visual estimations or hyperspectral imaging in a linear manner. The amount of ergosterol present was used to calculate the amount of fungal biomass present on a dry weight basis which can be an advantage in some circumstances. Ergosterol is not unique to Botrytis – yeast species naturally residing on the surface of the grape berry also contain ergosterol and so for accurate estimates of Botrytis, the background ergosterol level on infected grapes should be subtracted. While ergosterol measurements provide an accurate estimate of the amount of grey mould present, the applicability to a field situation during vintage is limited by the time required for analysis, which is typically two days.

Taking less than one hour, detection of grey mould by measuring Botrytis antigens is one of the quickest and genus-specific methods for grey mould estimation. A commercially available kit provided by Mologic Ltd (Bedfordshire, UK), was evaluated and was found to be simple to use and require minimal access to laboratory consumables. The assay is specific for Botrytis and is not subjected to interference from other organisms. The amount of Botrytis antigens detected in experiments positively correlated with both ergosterol estimations and observations with the naked eye. The relationship between the amount of antigens detected and the actual amount of fungus present isn't strictly linear, and follows a curved relationship. This, however, can be overcome by diluting the sample. As a guide, a Signal Intensity (SI) reading using the Mologic Botrytis antigen lateral flow device (LFD) of 50 equated to approximately 0.1g dry mass of *Botrytis cinerea* per kg fresh weight of grape material when a 1/100 dilution of a grape homogenate was assayed.

In practice, wineries may consider a combination of the methods described in this report. It is anticipated that better detection and quantification of grey mould will allow for more informed decisions to be made around harvest dates and a prediction of likely grape and wine quality.

Helpful discussions with Drs Alison Wakeham and Andrew Bentham (Mologic Ltd, Bedfordshire, UK) and the generous gift of the Botrytis LFD cube reader and LFD test strips are gratefully acknowledged.

Background

The rotting of grapes due to fungal bunch rot pathogens is a common problem in vineyards worldwide. While a range of organisms are implicated (Steel, et al. 2013), the principal culprit is primarily *Botrytis cinerea*. This fungus causes grey mould not just in grapevines but also in a range of other edible and ornamental horticultural crops (Elad, et al. 2016). *B. cinerea* was first described over a century ago and it is now recognised as the second most important plant pathogen worldwide (Dean, et al. 2012) as well as a global threat to food security (Richards, et al. 2021). The grapevine is one of the principal hosts for *B. cinerea*. In some seasons and in some vineyards grey mould can results in total loss of the crop. The fungus is particularly adaptable to different environments and fungicide resistance is common (Korolev, et al. 2011,Latorre and Torres 2012,Shao, et al. 2021,Walker, et al. 2017). Grey mould frequently develops in the vineyard when rain falls late in the season close to harvest. Lack of fungicide choice due to the need for Australian winemakers to meet negligible residue tolerances set by some export markets means that many effective fungicides cannot be applied to wine grapes post veraison. A number of biocontrol agents are available for grey mould control (Jacometti, et al. 2010,Pertot, et al. 2017,Schilder 2013), however once the disease becomes established management options frequently fail. Grape growers and wine makers are then faced with making decisions as to when and if to harvest the fruit. Accurate determination of the amount of fungal rot present in a parcel of fruit and the potential impacts on wine quality remain imprecise. Visual observations are subjective (Hill, et al. 2014) and prone to errors particularly if different operators are scoring the vineyard. Further errors occur as a result of the very nature of *B. cinerea* and where it is typically found within the bunch and the fruiting zone (Figure 1). *B. cinerea* often infects split berries, particularly in cultivars that produce bunches with tightly packed clusters. Incidence of grey mould is also often associated with the activities of Light Brown Apple Moth (Braybrook 2013). Botrytis grey mould may be present in the interior of what appears to be a perfectly healthy uninfected grape bunch. When the vineyard is assessed for grey mould, this can lead to false negative results. Consequently there is a need to develop alternative methods of grey mould assessment that have greater accuracy than the existing methods. Any new techniques need to provide rapid results either in the vineyard or at the grape receivable area of the winery within a time frame of hours (ideally minutes) rather than days.

Previous research funded by Wine Australia (project CSU 1301) investigated the impacts three fungal organisms responsible for the rotting of grapes have on grape chemistry and wine quality. The aims of this previous work were to discriminate between bunch rot types and ultimately to allow quantification of the amount of contamination. Grapes infected with either *B. cinerea*, *Penicillium expansum*, *Aspergillus niger* or *A. carbonarius* were analysed as was wine made from these grapes. Aside from a number of compounds commonly associated with mouldy musty characters in fungal contaminated commodities, grapes affected by *B. cinerea* accumulated 1,5-dimethylnaphthalene and several unidentified sesquiterpenes. *A. niger* and *A. carbonarius* grapes accumulated 2-carboxymethyl-3-hexylmaleic acid anhydride, while *P. expansum* samples were higher in *γ*-nonalactone and *m*-cresol. Detection of these and other secondary metabolites was postulated as a way in which the different types of bunch rotting fungi could be discriminated in infected grapes (Schueuermann, et al. 2019). The procedures involved with the detection and quantification of these fungal metabolites requires access to sophisticated laboratory equipment involving gas chromatography / mass spectral analysis as well as the skill set to operate the instrumentation and

analyse the data obtained. Such methodology requires an analysis time of maybe two days rather than hours. This limits the applicability of the technique during the vintage period when rapid answers are needed.

The current project aimed to build on the achievements of WA project CSU 1301 (Steel 2018), to more accurately define thresholds for bunch rot contamination of wine grapes and explore alternative ways to detect and ideally quantify the amount of grey mould contamination in a batch of grapes.

Part of the activities of Wine Australia project CSU 1301 (Steel 2018) involved an investigation of the applicability of ergosterol as a tool to measure the amount of fungal biomass present in a batch of grapes affected with grey mould. Ergosterol is a fungal sterol not found in animal or plant tissues. As such it may serve as marker for fungal contamination of wine grapes. The current project investigated ergosterol as a measure of grey mould contamination further, refining the methodology and comparing this technique with other methods for bunch rot detection and quantification.



Figure 1. A tightly packed Chardonnay bunch affected with Botrytis grey mould in a vigorous canopy. Quantification of grey mould within the interior of a tightly packed cluster is hard to determine based on visual inspection. Arrows point to berries affected with grey mould that may be hidden from view.

Project Aims

The initial aims of this project were concerned with determining thresholds for Botrytis contamination and the impact grey mould has on grape and wine quality. As the project evolved the emphasis of the research shifted focus to detection and quantification of Botrytis. This shift in focus occurred as a result of feedback and direction from Wine Australia as part of the Strategic Partnership Agreement (SPA) with the NWGIC received mid-way through the project at the end of the first year. As a result of this feedback several techniques were compared for Botrytis bunch rot detection. The comparison was based on specificity for Botrytis, field applicability and the time commitment to conduct the analysis. The initial wine making aspects of the work, comparing bunch rot contamination with wine quality parameters were largely abandoned.

The initial and subsequently modified outputs are summarised below.

Initial Outputs up to December 2019

- Grape bunches affected with Botrytis collected and sorted into different levels of grey mould infection and subsequently vinified. Wine made from bunch rot affected fruit analysed.
- Impact of fungal physiology on ergosterol content evaluated so that an assay for fungal contamination based on the ergosterol content of a parcel of fruit could be developed.
- Recommendations for bunch rot thresholds and techniques for wine fault amelioration evaluated in a commercial winery setting.
- Dissemination of knowledge by at least two different extension mechanisms on the impacts that bunch rot diseases have on wine taints and quality, on objective measures of bunch rot in grapes and on likely bunch rot tolerance levels in relation to wine quality.

Modified Outputs from January 2020

- Thresholds established for botrytis contamination of Chardonnay grapes and at least one other wine grape variety.
- Impact of fungal physiology on ergosterol content evaluated so that an assay for fungal contamination based on the ergosterol content of a parcel of fruit could be developed.
- Techniques evaluated and compared for the rapid analysis of bunch rot contamination of grapes.
- Dissemination of knowledge by at least two different extension mechanisms.

Methods

Plant and fungal material

Fungal isolates

Isolates of *Botrytis cinerea* were sourced from the fungal culture collection held at the National Wine and Grape Industry Centre at Charles Sturt University. Additional isolates were obtained from Dr Fran Lopez Ruiz (Curtin University, WA). When required, isolates were cultured on Potato Dextrose Agar (PDA) plates (21°C). Once the cultures had sporulated, fungal spores were harvested in sterile distilled water by gently rubbing the surface of the PDA plates with a plastic spreader. The spore suspensions were filtered through two layers of cheese cloth and one layer of Miracloth (Sigma Aldrich, Australia). Spore concentrations for grape inoculation were adjusted to 0.5 x 10^6 spores per mL by dilution with sterile distilled water supplemented with Tween 20 (0.01 % v/v).

Inoculum Preparation

When field samples of Botrytis grey mould affected grapes were not available from commercial vineyards, detached grape berries and / or bunches were inoculated in the laboratory using a mixture of *B. cinerea* isolates obtained from a range of geographical regions. A mixture of the following *B. cinerea* isolates, (in equal proportions) BC332, TN162, BC663, BC701, TN162, and VRU0008 was used to prepare the spore suspension inoculum for laboratory inoculation.

Grape material

Preliminary experiments were conducted on table grapes purchased from a local supermarket (unidentified varieties). Further experiments involving wine grapes utilised material harvested from commercial vineyards in the Riverina, NSW and also from the Tamar Valley, TAS. Bunches were collected at maturity and the Baume recorded. A range of different grape varieties were examined during various phases of the study as discussed below.

Individual berry inoculation

Individual grape berries were harvested at E-L growth stage 38 (Coombe 1995) by cutting the pedicel to ensure that the brush remained intact. The berries were surface sterilised using sodium hypochlorite (1.0 % v/v, plus 0.05 % v/v Tween 80, 2 mins) followed by three successive washings in sterile distilled water. Berries were placed into the wells of a microtitre plate (24 well, flat-bottom, Iwaki Microplates), wounded once with a sterile needle and inoculated with 10 µL of a *B. cinerea* spore suspension (10^6 spores / mL) onto the apex of the berry surface. Control berries were wounded once with a sterile needle, and inoculated with 10 µL of sterile distilled water, prior to incubation. Sterile distilled water was added to the space between the wells to achieve a humid environment and the plates sealed. The berries were incubated at room temperature (~21°C) for ten days to achieve 100% fungal infection. Infected berries were then mixed with the control un-infected berries in different ratios to obtain different infection levels. Each treatment consisted of a total of 100 berries, (e.g. 10 infected and 90 un-infected) replicated three times. The actual weight of replicates of the infected and un-infected berries was recorded and from this the percentage infection of each replicate was expressed on a weight / weight berry basis. Each replicate was then stored at -20°C for subsequent analysis.

Inoculated whole bunches – comparison of varieties

Cabernet Sauvignon (14 °Bé), Chardonnay (12 °Bé), Semillon (10.2 °Bé) and Shiraz (13.2 °Bé) bunches with no apparent disease symptoms or damage were harvested from vineyards in the Riverina in 2020 and stored at 4°C overnight before use. The bunches were surface sterilized as described above for the experiment involving individual bunches and then damaged multiple times with a sterile needle prior to submersion in a *B. cinerea* spore suspension (10^6 spores / mL) in a large beaker. Bunches were incubated at room temperature (~21°C) in sealed plastic containers (volume =25 L, dimensions = 57 x 38 x 17 cm) until such time (typically 7 to 10 days) that the entire bunch was fully colonized with grey mould. Non-inoculated bunches incubated under the same conditions served as controls. The bunches were then destemmed and homogenized using an UltraTurrax T25 high speed grinder (IKA Dispersers, Staufen, Germany). The homogenate from the inoculated bunches was then mixed in different proportions with the homogenate from healthy un-inoculated bunches to obtain the following infection levels on a w/w basis; 0%, 2%, 4%, 6%, 9%, 12% and 15% with three replicates of each infection level. The homogenates were then stored at -20°C for subsequent analysis.

Inoculated whole bunches harvested at daily intervals post-inoculation 2020

Cabernet Sauvignon grapes were used for this study and the inoculation and incubation procedures were carried out as described above. Five inoculated and three un-inoculated bunches were randomly taken at daily intervals post-inoculation and stored at -20°C for later analysis. The experiment was conducted over a 7 day period, thus resulting in 35 inoculated and 21 un-inoculated bunches in total. Results were expressed as the mean values for the five inoculated or three un-inoculated bunches.

Inoculated bunches for winemaking in 2019

In 2019, Cabernet Sauvignon grape bunches were harvested from a commercial vineyard and assessed as being disease-free based on visual inspection. The bunches were surface sterilised with 70% ethanol (v/v) and exposure to UV light for 10 minutes. The bunches were then placed in a sterile plastic container (volume = 25 L, dimensions = 57 x 38 x 17 cm) on a steel wire mesh and randomly damaged with a sterile needle before a *B. cinerea* spore suspension was sprayed evenly on to the upper surfaces of the bunches.

The inoculated bunches were incubated at room temperature (~21°C) for five days. To maintain a high humidity to facilitate infection of bunches, moist sterile paper towel was placed underneath the steel mesh for the first 48 hours of incubation. This was then removed to facilitate growth of the fungus more typically seen in a vineyard. Un-inoculated grape bunches were incubated under the same conditions in a separate sterile plastic container. The fully infected bunches were then mixed with the un-inoculated bunches to obtain infection levels of 0%, 2%, 5% and 10% on a w/w basis in triplicate. The replicates of the bunch mixtures were then separately vinified, i.e. 12 ferments in total.

Inoculated bunches for winemaking in 2020

The preparation of inoculated grape material in 2020 for the purposes of wine making was performed in a slightly different way to the method used in 2019. Cabernet Sauvignon grapes were harvested at maturity from a commercial vineyard in the Riverina in March 2020. Approximately 3 kg of grape

berries were manually destemmed berries such that the brush was intentionally removed to facilitate Botrytis infection. The berries were then placed in a beaker containing 400 mL of a *B. cinerea* spore suspension (10^6 spores / mL) using a mixture of isolates for one minute with gentle agitation. The berries were then removed from the beaker and placed into a sealed plastic container (volume =25 L, dimensions = 57 x 38 x 17 cm) and incubated at room temperature (~21°C) for 10 days. The inoculated berries were mixed once a day by tossing the box gently.

After the incubation period, the fully infected berries were mixed with un-inoculated berries to form four infection levels (0%, 2%, 5% and 10% w/w) which were then vinified. There were three ferments for each of the four infection levels.

Wine making and sensory analysis of wine

Wine making

Laboratory-inoculated grapes were vinified using the Charles Sturt University winemaking procedure for red wine. Briefly, the four levels of grey mould contaminated grapes from each vintage were manually destemmed, crushed and divided into triplicates of approximately 3.5 kg each. Must samples were collected at this stage in 250 mL specimen jars and stored at -20 °C for subsequent analysis. Potassium metabisulfite (50 mg/L) was then added followed by addition of a pectolytic enzyme for colour extraction (Lafase Fruit, Laffort Australia, Sydney) after 2 hours. All ferments were inoculated with 250 mg/L of Lalvin *Saccharomyces cerevisiae* EC-1118 (Lallemand, Edwardstown, SA, Australia) after another 3 hours. All fermentations were maintained at room temperature (~20 °C) and temperature and total soluble solids (TSS, expressed as °Brix) were monitored daily with an Anton Paar DMA 35N portable density meter (Graz, Austria). The must was pressed up to 100 kPa using a small hydraulic basket press (Zambelli, Camisano Vicentino, Italy) and the resultant juice collected in a 3 L narrow neck glass bottle when TSS reached close to zero. Wines were racked from yeast lees after 24 hours settlement and inoculated with an *Oenococcus oeni* culture for malo-lactic fermentation (Alpha, Lallemand Australia Edwardstown, Australia). The wines were then left to settle for 2 weeks before addition of potassium metabisulfite (0.5mg/L) and kept at 4±1 °C for cold chilling for another three weeks before bottling. Crucial chemical analysis and quality assurance measures were carried out during the entire winemaking process, such as measurement of pH, TA, acetic acid, glucose, fructose, malic acid, free SO_2, residue sugar and alcohol concentrations.

Sensory analysis of wines

Experimental wines were examined by a trained sensory assessment panel. The panel consisted of 3 females and 9 males between the ages of 25 and 60. Participants were asked to freely choice profile the wines by writing a list of adjectives to describe the wines and to indicate a preference using a pivot profile (Pearson, et al. 2020). Wines were presented in randomised order in ISO tasting glasses. Each glass was marked with a random three digit code. The panellists were asked to describe each wine as either more or less than the reference Pivot wine. The data were then analysed using Correspondence Analysis (CA) (XLSTAT, Version19.6, Addinsoft, Boston, MA, USA) to give a biplot of the wines and the relative frequency of the attributes.

Botrytis detection and quantification methods

Hyperspectral imaging

Preliminary experiments conducted in 2018 involved table grape bunches that were purchased from a local supermarket outlet. Subsequent experiments used Cabernet Sauvignon bunches harvested from a commercial vineyard in the Riverina in February 2019. Whole bunches were inoculated with a *B. cinerea* spore suspension as described above, and they were then incubated for approximately five days to allow for the development of grey mould. Following the incubation period the bunches were scored for symptoms of grey mould by visual inspection with the naked eye. Images of the infected and un-inoculated bunches were then taken with a Specim FX10 hyperspectral camera (Specim, Finland) aided with halogen lighting. Hyperspectral images were collected across a wavelength range of 400 - 1000 nm. Images were taken of two opposite sides of each bunch. False colour images of infected berry material, healthy berry material, bunch peduncle and rachis as well as the background and shadows were created through a kernel fitting algorithm and support vector machines (SVM) in Scyven software (Habili and Oorloff 2015) The false colour images with the different identified materials were then imported into ImageJ Fiji (Schindelin, et al. 2012) for quantification by counting the pixels which constitute the individual identified materials. The percentage of infection (infection rate) of each individual bunch was calculated as:

$$Infection\ rate\ (\%) = \frac{Infected\ area}{Infected\ area + healthy\ area + shadow} * 100\%$$

A shadow category was created as material in shadows could not be identified. A high proportion of shadows may contain clean grapes so this was included in the calculation and assumed to be uninfected grape material. Data for the hyperspectral images was then compared with the data for the visual estimations based on the naked eye.

Ergosterol analysis and calculation of fungal biomass

The ergosterol content of *B. cinerea* growing in culture was determined prior to measurements using infected grape tissues. This was necessary to establish a conversion factor to determine the amount of fungal biomass present in a batch of grapes. To achieve this, twenty *B. cinerea* isolates from different grape cultivars and geographical locations were cultured in Potato Dextrose Broth (PDB) (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK). The procedure involved firstly obtaining a spore suspension from a culture growing on agar (PDA) which was adjusted to 10^6 spores / mL with sterile distilled water as detailed above, and then inoculation (10^5 spores in a 100 µL volume) into 100 mL of PDB contained in a 250 mL conical flask. There were five replicates for each isolate. The cultures were incubated without agitation at 25 °C for seven days, after which time the resulting mycelium was harvested by filtration (Whatman #1), washed with two successive volumes of sterile distilled water, frozen overnight at –20 °C and then freeze-dried. The dry weight of the freeze-dried mycelium was recorded, which was then ground in liquid nitrogen using a mortar & pestle. The dried mycelial powder was stored in a desiccator until analysis. To examine the effect of culture temperature on the ergosterol content of fungal mycelium, three of the *B. cinerea* isolates (TN080, TN162 and VRU0019)

were randomly selected from the NWGIC culture collection and cultured at one of six temperatures over the 15 to 27.5 °C range in 2.5 °C increments using five replicate flasks. After five days the mycelium was harvested by filtration, freeze-dried and stored as described above.

Ergosterol extraction

Ergosterol was extracted from fungal tissues using the method of (Porep, et al. 2014). Approximately 50 mg of dried fungal biomass was weighed, the weight recorded and the fungal material transferred to a 250 mL round bottom flask. Deionized water (50 mL), methanol (HPLC grade) (75 mL) and ethanol (HPLC grade) (50 mL) were added to the flask and the contents mixed. Once mixed 10 g of KOH pellets were added to the flask. The flask contents were refluxed for 30 mins using a Radley's Star Fish apparatus (In Vitro Technologies Pty Ltd, Lane Cove, NSW) at 120 °C, which allowed five flasks to be refluxed simultaneously. After cooling to ambient temperature the flask contents were filtered through Whatman #1 filter paper. The flask was rinsed twice with methanol (25 mL) to wash the filter paper. The filtrate was transferred to a 250 mL separating funnel and the contents extracted twice with *n*-hexane (50 mL). The upper organic phase (*n*-hexane layer) was collected and the extraction repeated with a further volume of *n*-hexane (50 mL). The *n*-hexane extracts were combined and dried using anhydrous sodium sulfate (Na_2SO_4) overnight. The extracts were filtered (Whatman #1) to remove any sodium sulfate, and the filter paper rinsed with *n*-hexane (5 mL). The *n*-hexane was removed under reduced pressure by rotary evaporation and taken to dryness. The extract was re-constituted in 5 mL methanol (HPLC grade) and dissolution assisted with ultra-sonication. The extracts were stored in a sealed container under a N_2 atmosphere at 4 °C for later high performance liquid chromatography (HPLC) analysis.

Ergosterol analysis by HPLC

The ergosterol content of extracts was quantified using a Waters 600 HPLC, using a C18 column (150 x 4.6 mm, 5 µm particle size) (Agilent Technologies Ltd., PA, USA) with a C18 guard column in place. The mobile phase consisted of 95% v/v HPLC grade methanol and 5% v/v reverse osmosis water that was pumped isocratically at a flow rate of 0.8 mL / min. Ergosterol was detected at 282 nm using a UV/Vis diode array detector. External calibration was achieved with a series of diluted ergosterol (Sigma- Aldrich, Castle Hill, NSW) standards (200, 20, 4, 1 and 0.04 mg / L) in HPLC grade methanol stored at -20 °C in the dark.

Estimation of fungal biomass in infected grape bunches

Homogenised grape must (10 g) was weighed into a 100 mL round bottom flask. Samples were saponified via a base hydrolysis reaction in a solution containing 37.5 mL methanol, 25 mL ethanol (absolute, 100 % v/v) and 5 g potassium hydroxide while under reflux with continuous stirring for 30 minutes using a Radley's StarFish apparatus (In Vitro Technologies Pty Ltd, Lane Cove, NSW) at 120 °C. After cooling, the mixtures were filtered through Whatman #1 paper and rinsed with methanol (12.5 mL). The filtrates were then transferred to a 250 mL separatory funnel. The funnel was rinsed with

12.5 mL ultrapure water and added to the separatory funnel. Ergosterol was extracted from the aqueous phase with 25 mL *n*-hexane twice. The *n*-hexane upper-layers were collected and combined. Anhydrous sodium sulphate (Na_2SO_4) was added to the combined extracts to remove residual water. The mixture was filtered (Whatman #1) and rinsed with 7 mL *n*-hexane. The *n*-hexane extracts were evaporated to dryness under reduced pressure using a rotary evaporator and re-constituted in 5 mL methanol. Methanol extracts were filtered through a 0.45 µm reconstituted cellulose membrane filter and kept at -20 °C until analysis. The extracts were then analysed by HPLC as described above for the freeze-dried mycelium. The fungal biomass in infected grape bunches was estimated using the equation, Fungal biomass (g/kg) = BCF x ERG, where BCF (fungal biomass conversion factor) = the reciprocal of the average ergosterol content in mg/g dry mycelia; and ERG = ergosterol concentration in mg / kg fresh weight of grapes (Porep, et al. 2014).

Gluconic acid

Approximately 10 mL of homogenized grape must was centrifuged at 6000 rpm for 10 minutes in an high speed centrifuge (Eppendorf 5810R, Germany) to obtain clear grape juice to determine the amount of gluconic acid present. D-gluconic acid was measured using three commercially available enzyme-based test kits. The Megazymne and Vinessential kits employed the use of a visible bench top spectrophotometer while the third kit examined supplied by Thermo Fisher Scientific (Waltham, MA, USA) employed an Arena Discrete Analyser (Thermo Fisher Scientific, Scoresby, Vic., Australia).

Isothermal Loop mediated Amplification (LAMP)

Total genomic DNA from approximately 1g of grape must homogenate (exact weigh recorded) was extracted using a rapid extraction kit from Geneworks (Adelaide, SA). The primer pairs were described by (Tomlinson, et al. 2010) as: F3: 5'-TCGGAGTGTCCTAGGAATGC-3'; B3: 5'-TGAGATGGCCAACTCTCAGA-3'; FIP: 5'-GCCTGCTCACCGGTAGTAGTGTGTGAGCCCTTGGTCTAA AGC-3'; BIP: 5'-GCAGAATCTGTCCCCGGTGAGCGGGAGCAACAATTAATCGC-3'; F-Loop: 5'-TGG GGTTAACTAGTCACCTATACG-3'; B-Loop: 5'-AGGTCACCTTGCAATGAGTGGA-3'. LAMP assays were carried out in a Genie II isothermal cycler (Optigene, UK). Each reaction contained 15 µL ISO-001 isothermal reaction mix, 0.8 µM of each FIP and BIP primers, 0.2 µM of each F3 and B3 primers, 0.4 µM of each F-Loop and B-Loop primers, 4 µL gDNA and Nuclease-free water to 25 µL. Amplification conditions involved a constant temperature of 65 °C for 40 minutes. Negative controls were included in each run.

Quantitative qPCR

Total genomic DNA (gDNA) was extracted from approximately 1 g of grape must homogenate (the exact weight was recorded) using the DNeasy Plant Maxi kit (Qiagen, Australia) following manufacturer's instruction. The primers and probes for TaqMan quantitative PCR were described by (Suarez, et al. 2005) and were as follows: forward primer Bc3F: 5'-CTGTAATTTCAATGTGCAGAATCC-3'; reverse primer Bc3R: 5'-GGAGCAACAATTAATCGCATTTC-3'; and TaqMan MGB probe Bc3P with 5' FAM label: 5'-TCACCTTGCAATGAGTGG-3'. Primers and probe were synthesized by Sigma-Aldrich (Australia). Triplicated reactions were carried out in a Roto-gene-6000 real time PCR thermocycler

(Corbett Research, Australia) with the QuantiNova Probe PCR kit (Qiagen, Australia) consisting of 10 µL 2x QuantiNova Probe PCR Master Mix, 1 µL 20x pre-made primer-probe mix (0.4 µM each primer and 0.2 µM probe), 2 µL gDNA and made to 20 µL final volume with Nuclease-free water. Cycling conditions were as follows: initial heat activation at 95 °C for 2 minutes followed by 40 cycles of denaturation at 95 °C for 5 seconds and combined annealing/extension at 60 °C for 15 seconds. Signals were acquired at the end of each cycle through the Green channel. A 480 bp gBlocks gene fragment based on the targeted sequence (Appendix 5) was synthesised (IDTDna, Singapore) for constituting an external calibration curve by amplifying a series of dilution from 10^{-1} to 10^{-8} ng /µL. Negative controls were included in each run.

Botrytis antigens

The level of Botrytis antigens present in grape tissue homogenates was determined using a commercially available kit, (Mologic Ltd, Thurleigh, Bedfordshire, UK) which uses the Botrytis monoclonal antibody, BC-12.CA4 (Dewey, et al. 2000,Dewey, et al. 2013) and lateral flow device (LFD).

For analysis, homogenates were defrosted and a 2 mL aliquot centrifuged for 10 mins at 3000*g*. The samples were then diluted 1/100 using PBS buffer (Sigma) supplemented with 0.1% (v/v) Tween 20 (Sigma). The amount of dilution required depended on the amount of *B. cinerea* present in the sample. Aliquots (200 µL) of the diluted homogenates were then placed in Eppendorf tubes at room temperature. A Botrytis test strip was then placed into each of the Eppendorf tubes (Figure 2) containing the diluted centrifuged-berry homogenate for 10 minutes, after which time the Botrytis test strip was read in a Cube reader (Figure 3) and the signal intensity (SI) recorded. Successive grape homogenate samples were processed at 1 minute intervals.

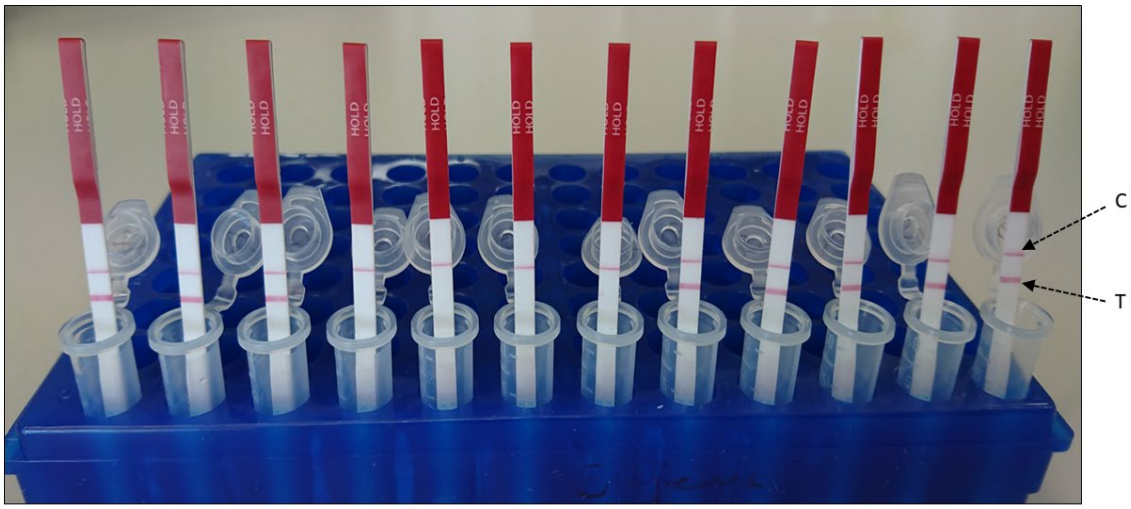


Figure 2. A series of Botrytis test strips in the process of development. C = Control line, T = Botrytis test line.



Figure 3. Botrytis test strip reading using a cube reader.

Estimation of B. cinerea *from field samples of examples of grey mould affected grapes using ergosterol analysis and a LFD kit for Botrytis antigen quantification in 2021*

The 2020/21 growing season was more conducive to grey mould occurrence in vineyards. Analysis of the ergosterol content of infected bunches and quantification of Botrytis antigens using the Mologic LFD was evaluated using field examples of grey mould collected from commercial vineyards located in the Riverina in 2021.

On 18th February 2021, 55 Shiraz grape bunches were randomly selected from a commercial vineyard in Griffith, NSW affected with Botrytis grey mould. In the laboratory each bunch was scored for grey mould contamination based on visual symptoms by the naked eye and each bunch was individually weighed and the data recorded. The berries were then detached from the bunch and the berry homogenate assayed for Botrytis antigens and the ergosterol content as described above. The ergosterol content was used to calculate the biomass of fungus present in each bunch. Homogenates were diluted 1/10, 1/50 and 1/100 for the determination of the Botrytis antigen levels.

Statistical analysis

Correlation of different sets of infection measurement data was obtained by plotting them against each other in Excel. Linear or logarithmic methods were used to find the best fitting equation. R-squared value representing the correlation coefficient were displayed on each graph.

Results and Discussion

Experiments conducted in the first year of study, 2019

Part of the experimental work conducted during the first year of this project involved developing the use of ergosterol measurements as a tool to quantify fungal contamination. Cultural factors that influence the amount of sterol that the fungus produces were investigated. Hyperspectral imaging was explored as a tool for bunch rot quantification in a red variety and the technique compared to molecular and chemical methods for Botrytis detection and estimation.

Four experiments were conducted during this phase of the project.

1. The ergosterol content of *Botrytis cinerea* mycelium growing in culture was investigated in a range of *B. cinerea* isolates and from this a bioconversion factor was established for the calculation of fungal biomass present in grey mould affected grapes.
2. Ergosterol was investigated as an indicator of grey mould contamination of Chardonnay grapes sourced from a commercial vineyard in the Tamar Valley.
3. Hyperspectral imaging, visual estimations, analysis of ergosterol and molecular methods involving qPCR and LAMP were compared for grey mould estimation in laboratory inoculated Cabernet Sauvignon grape bunches.
4. Wine was made from Cabernet Sauvignon grapes inoculated with *B. cinerea* was analysed.

The ergosterol content of Botrytis cinerea *mycelium growing in culture in Potato Dextrose Broth (PDB)*

Sterols are present in the cell membranes of all eukaryotic organisms (Nes 1974). The amount and sterol profile varies depending upon the organism. Fungi contain the sterol ergosterol that is absent in grapevines and other plant species (Mercer 1984). Measuring ergosterol has therefore been proposed as a potential indicator of fungal contamination in grapes (Porep, et al. 2015,Steel, et al. 2020).

Sterols function in cell membranes to regulate membrane fluidity. Compared to the phospholipids in the membrane, the sterol molecule is a bulky structure. Increasing the amount of sterol present in a membrane will thus reduce membrane fluidity, a mechanism that living organisms use to regulate membrane dynamics with changing temperature in the environment. Prior to investigating ergosterol as a potential marker of fungal contamination in grapes it was necessary to determine the ergosterol content of Botrytis mycelium growing under different conditions.

The ergosterol content of the three *B. cinerea* isolates examined increased with increasing culture temperatures and ranged from an average of 1.45 (at 15 °C) to 3.22 (at 27.5 °C) mg ergosterol per gram dry weight of fungus (Table 1). Variability in ergosterol content amongst the 20 isolates was then examined at a single temperature of 25 °C and ranged from 2.15 to 4.26 mg of ergosterol / g dry weight of fungus, depending on the isolate (Table 2). No apparent correlation between year of isolate collection, geographical origin or host plant tissue with the ergosterol content of the isolates was observed. The average ergosterol content of the twenty isolates used in this study was 2.97 mg of ergosterol / g dry weight of fungus which equated to a Botrytis conversion factor (BCF) of 0.337. This was then used to calculate the fungal biomass in the grape samples in subsequent experiments using the following formula:

Fungal biomass (g/kg) = BCF x Ergosterol

Table 1. Ergosterol content and biomass of three *Botrytis cinerea* isolates cultured in potato dextrose broth at a range of temperatures between 15 and 27.5 °C in 2.5 °C increments. Results are the means of three replicates after six days of growth in potato dextrose broth (PDB).

	Temperatures											
	15°C		17.5°C		20°C		22.5°C		25°C		27.5°C	
Isolate	ERG* (mg/g)	Biomass^	ERG (mg/g)	Biomass^	ERG (mg/g)	Biomass^	ERG (mg/g)	Biomass^	ERG (mg/g)	Biomass^	ERG (mg/g)	Biomass^
TN162	1.62 ± 0.07	104.5 ± 22.6	1.93 ± 0.18	200.1 ± 17.3	2.43 ± 0.23	243.7 ± 18.6	3.13 ± 0.78	299.7 ± 28.9	3.91 ± 0.35	309.9 ± 12.1	4.26 ± 0.26	300.0 ± 19.0
TN080	1.19 ± 0.10	161.4 ± 51.2	1.58 ± 0.31	183.0 ± 26.8	2.28 ± 0.09	205.3 ± 40.9	2.80 ± 0.41	184.0 ± 35.1	2.95 ± 0.17	174.4 ± 24.2	2.55 ± 0.04	86.7 ± 7.3
VRU0019	1.55 ± 0.09	113.4 ± 63.0	1.84 ± 0.12	162.8 ± 42.4	1.79 ± 0.09	183.3 ± 22.0	2.18 ± 0.31	186.1 ± 39.0	2.26 ± 0.22	254.7 ± 18.5	2.85 ± 0.57	160.1 ± 47.7
Average	1.45 ± 0.21	126.4 ± 49.8	1.78 ± 0.25	182.0 ± 31.1	2.17 ± 0.32	210.8 ± 36.4	2.70 ± 0.62	223.2 ± 64.7	3.04 ± 0.74	246.3 ± 60.2	3.22 ± 0.85	182.3 ± 97.4

*Erg= Ergosterol content (mg) per g freeze-dried fungal biomass. Number following the ± is the standard deviation.

^ Biomass= Total fungal material (mg) harvested from 50 ml of culture medium (PDB) over 6 days at set temperature. Number following the ± is the standard deviation.

Table 2. Ergosterol content of *Botrytis cinerea* isolates obtained from a range of grape growing regions when cultured on potato dextrose broth at 25 °C for six days. Results are the means of three replicates after six days of growth in the culture medium. Isolates arranged in order of increasing ergosterol content.

Isolate	Ergosterol (mg/g)*	Total Biomass^ (mg)	Host Cultivar	Vine structure	Geographical origin	Year collected
BC217	2.15 ± 0.41	323.77 ± 54.03	unknown	Flower	QLD	2014
VRU0019	2.24 ± 0.14	254.66 ± 16.54	Chardonnay	Berries	VIC	1994
VRU0030	2.35 ± 0.11	262.34 ± 31.90	unknown	Berries	VIC	1994
BC914	2.40 ± 0.67	38.25 ± 2.75	Petit Verdot	Flower	NSW	2017
BC896	2.53 ± 0.24	243.37 ± 33.46	Riesling	Flower	VIC	2017
BC924	2.65 ± 0.34	469.80 ± 54.79	Semillon	Flower	NSW	2017
VRU0008	2.66 ± 0.09	238.42 ± 60.42	Shiraz	Canes	VIC	1993
TN192	2.67 ± 0.15	273.82 ± 11.00	Chardonnay	Berries	NSW	1993
BC701	2.74 ± 0.27	265.97 ± 8.77	Chardonnay	Flower	WA	2016
TN080	2.95 ± 0.13	157.85 ± 29.03	Chardonnay	Berries	NSW	1990
VRU0027	3.09 ± 0.30	278.64 ± 26.05	Semillon	Berries	NSW	1994
BC663	3.18 ± 0.08	299.03 ± 13.11	Sauvignon Blanc	Flower	SA	2015
BC341	3.21 ± 0.29	310.05 ± 30.95	Riesling	Flower	TAS	2014
BC530	3.22 ± 0.33	311.23 ± 22.54	Grenache	Flower	SA	2015
VRU0009	3.29 ± 0.15	299.36 ± 5.23	Pinot Noir	Berries	TAS	1993
BC213	3.40 ± 0.65	307.23 ± 50.70	unknown	Flower	QLD	2014
BC773	3.46 ± 1.16	308.05 ± 8.95	Merlot	Flower	WA	2016
TN168	3.70 ± 0.26	232.42 ± 10.24	Chardonnay	Berries	NSW	1993
TN162	3.85 ± 0.30	309.94 ± 10.84	Riesling	Berries	NSW	1993
BC332	4.26 ± 0.60	229.53 ± 7.64	Riesling	Flower	TAS	2014
Average	2.97 ± 0.65	270.69 ± 78.64	-	-	-	-

* = Ergosterol content (mg) per g freeze-dried fungal material. Number following the ± is the standard deviation.

^Total Biomass= Total fungal material harvested from 50 mL culture medium (PDB) over 6 days at set temperature. Number following the ± is the standard deviation

Ergosterol as an indicator of grey mould contamination of Chardonnay grapes sourced from the Tamar Valley, Tasmania

Chardonnay grapes (12 °Be) affected with Botrytis grey mould were sourced from a vineyard in the Tamar Valley, Tasmania, on 13th March 2018. The grapes were sorted into one of the following three levels of infection based on visual inspection with the naked eye:

Clean = apparently healthy, clean fruit free of any visible signs of fungal rot.

Low infection = one to three berries per bunch infected with Botrytis grey mould

High infection = four to twenty berries per bunch infected with Botrytis grey mould.

A fourth category (medium infection) was obtained by mixing sub-samples of the low and high infection levels in equal proportions by weight.

Within two hours of harvest, the collected bunches were stored in a cool room located at the Tasmanian Institute of Agriculture Mount Pleasant Laboratories, Launceston, overnight at 4 °C. The following day bunches were shipped by air to the NWGIC in Wagga Wagga, NSW for analysis.

The ergosterol content of the four batches of fruit was determined by HPLC. The uninfected fruit had an ergosterol content of 0.232 mg / kg fresh weight of grapes, which can be attributed to the background yeast and other fungal organisms (Bloch 1983,Mercer 1984) naturally present on the surface of the fruit. The ergosterol content of the fruit increased with the increasing levels of infection, from 1.40 mg / kg fresh weight of grapes in the fruit with one to three berries per bunch infected with grey mould to 5.31 mg / kg fresh weight of grapes in the fruit with four to twenty berries per bunch infected with grey mould (Table 3).

Table 3. Ergosterol content of grape bunches affected with Botrytis grey mould harvested from a vineyard in the Tamar valley, Tasmania in March 2018.

Infection category *	Ergosterol (mg / Kg fresh wt of fruit)	Fungal biomass (g dry weight / kg fresh wt of fruit)
Clean	0.232 (± 0.112)	0.078
Low	1.401 (± 0.042)	0.472
Medium	3.574 (± 0.295)	1.204
High	5.307 (± 0.267)	1.788

* = Infection category based on the number of berries per bunch with visible symptoms of grey mould. Clean = 0 berries infected, Low = 1 to 3 berries infected, High = 4 to 20 berries infected. The Medium infection level was obtained by mixing equal weights of bunches from the Low and High infection categories.

The results demonstrated that ergosterol has the potential to be a quantitative marker of Botrytis grey mould contamination in wine grapes. The result support our earlier observations on Chardonnay grape bunches affected with grey mould harvested from the Hunter Valley, NSW in 2016 (Steel 2018,Steel, et al. 2020). The technique has some merit for the determination of fungal biomass present in plant tissues, although it should be noted that the technique is not specific to Botrytis. Any fungal organisms, pathogenic, saprophytic or otherwise such as those used in biocontrol agents and fungal organisms

present as endophytes will contribute to background levels of ergosterol present in grape tissues. Further work is warranted to determine background levels of ergosterol present in grape material from vineyards and vintages when grey mould is not an issue.

Comparison of methods to quantify Botrytis grey mould contamination in Cabernet Sauvignon grapes in 2019

The aim of this experiment, and subsequent experiments conducted in this research project, was to compare a number of different techniques for the detection and estimation of *B. cinerea* from grape tissues. The techniques investigated included hyperspectral imaging, ergosterol analysis, qPCR and LAMP. Sixty Cabernet Sauvignon grape bunches (12.7 °Bé) with no apparent disease symptoms were harvested from a vineyard in the Riverina (Oura), NSW at E-L growth stage 38 (Coombe 1995). Grape bunches were stored at 4°C overnight at the National Wine and Grape Centre, Wagga Wagga, NSW before use and then fifty of the sixty bunches were inoculated with a mixed spore suspension of five isolates of *B. cinerea.* The remaining ten bunches were not inoculated and served as controls.

Individual bunches were scored for visual symptoms using the naked eye and then analysed by hyperspectral imaging. An example of the hyperspectral images taken and the raw data recorded are included in Figure 4.

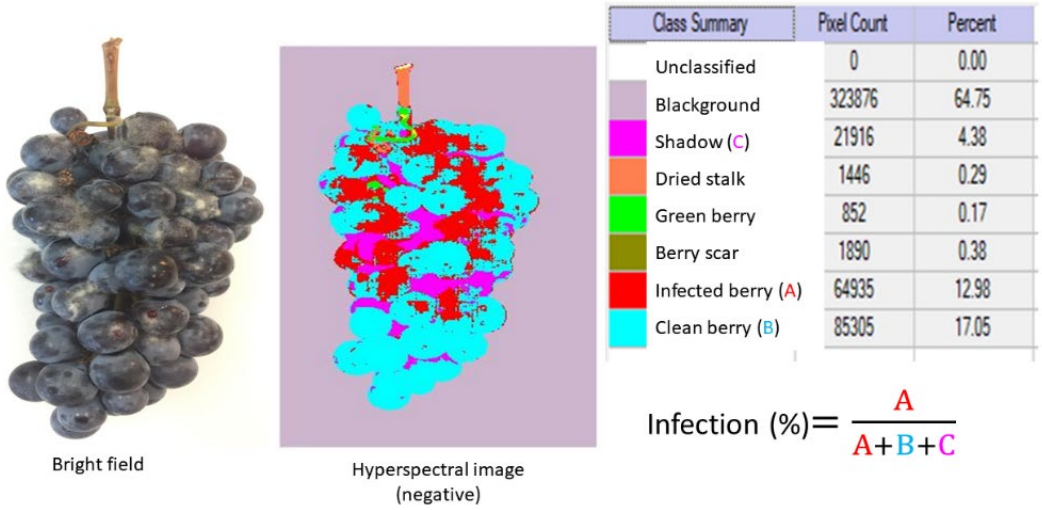
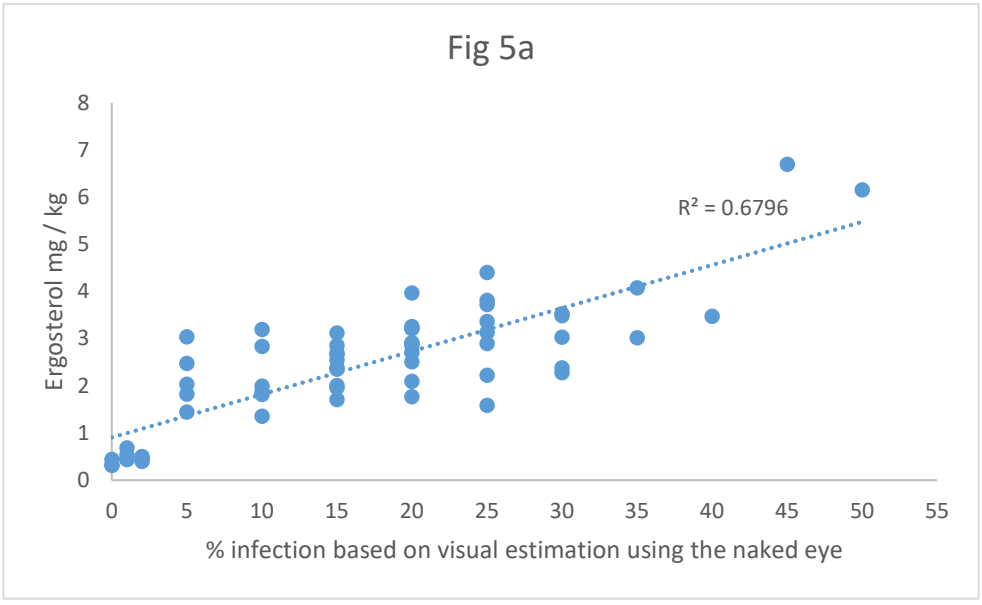


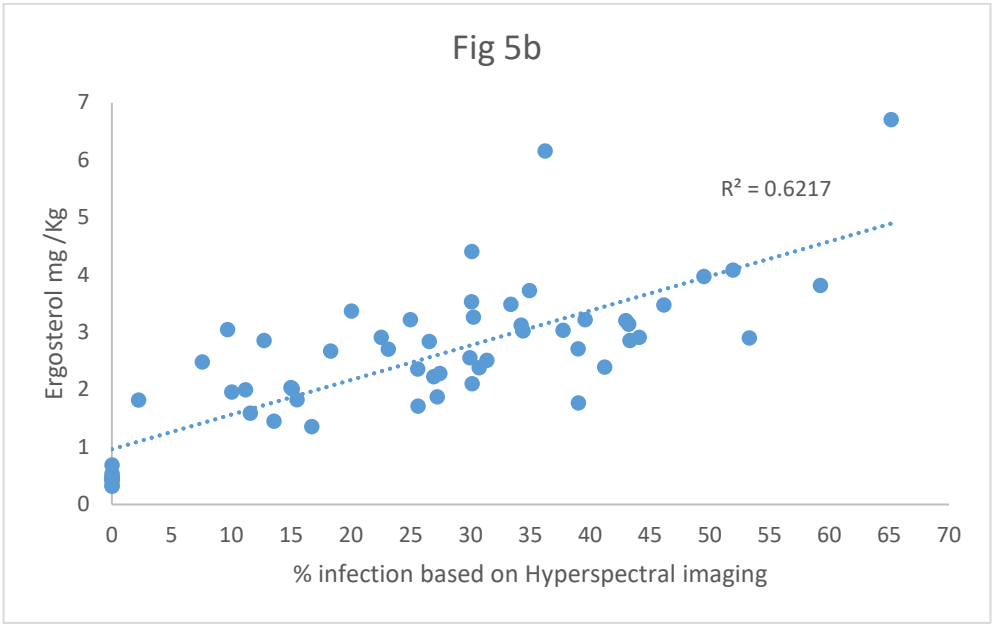
Figure 4. Analysis of a Cabernet Sauvignon bunch affected with grey mould and analysed by hyperspectral imaging.

These same bunches used for hyperspectral imaging were then homogenised and analysed for ergosterol and also for *B. cinerea* using qPCR and LAMP.

Data relating to the percentage infection, as assessed either with the naked eye or hyperspectral imaging with respect to the fungal biomass calculated using the ergosterol content, is presented in Figures 5 (a) and 5 (b) below. The linear correlation between the imaging method and the amount of ergosterol detected was comparable for both the naked eye and hyperspectral imaging.



(a)



(b)

Figure 5. Comparison of imaging techniques vs ergosterol content of laboratory-inoculated Cabernet Sauvignon grape bunches. (a) Visual estimation with the naked eye, (b) hyperspectral imaging.

However, the relationship between the hyperspectral and visual images score was not as strong (R^2 = 0.5881) (Figure 6).

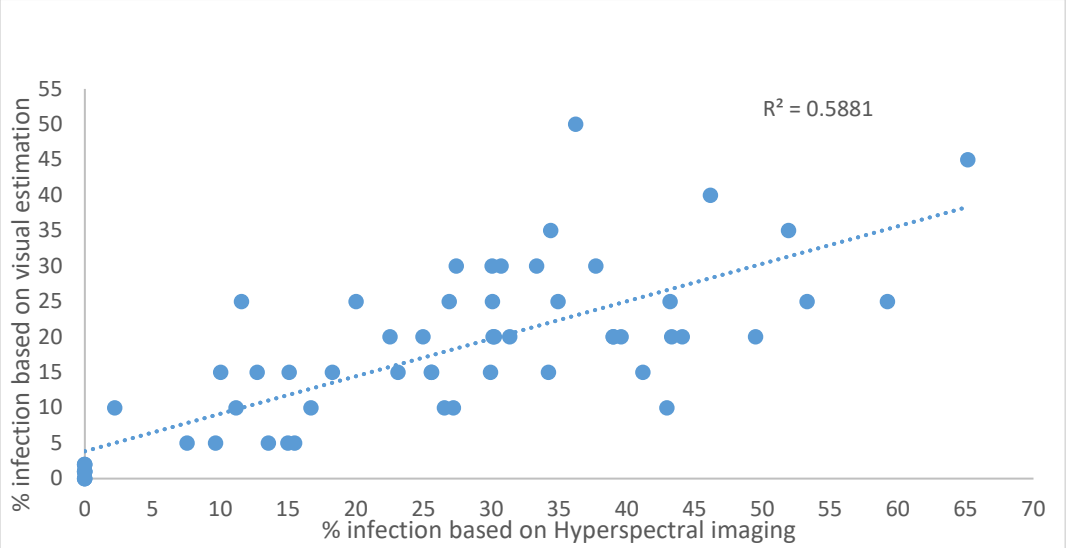
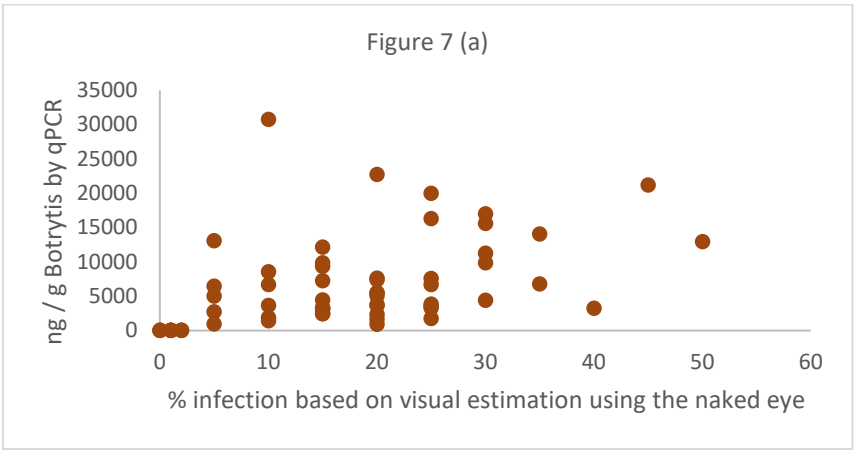


Figure 6. Comparison of hyperspectral imaging versus visual estimation using the naked eye of grey mould contamination of laboratory inoculated Cabernet Sauvignon grapes.

A large variation was observed when data relating to either imaging technique were compared with PCR for *B. cinerea* detection (Figures 7 (a) and (b)).



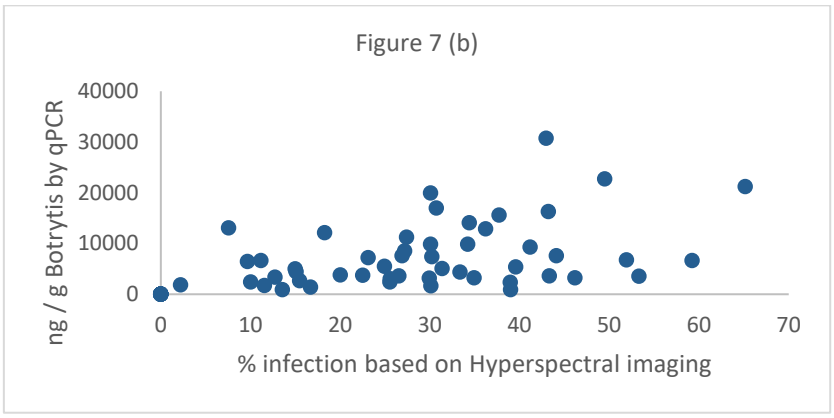
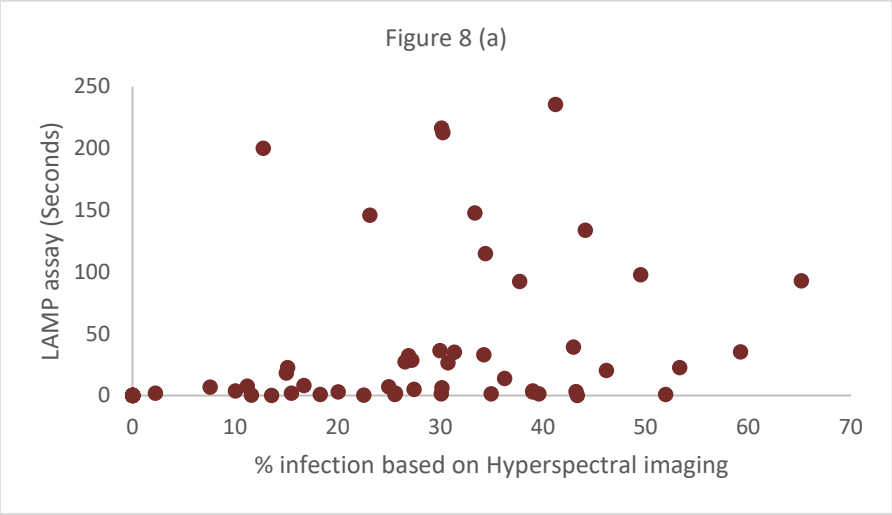


Figure 7. Comparison of imaging techniques vs qPCR for B. cinerea *of laboratory-inoculated Cabernet Sauvignon grape bunches. (a) Visual estimation with the naked eye, (b) hyperspectral imaging.*

The LAMP assay did not correlate well with the hyperspectral imaging data or with the ergosterol assay (Figures 8(a) and 8(b)). Bunches that were clearly infected with grey mould returned as false negatives in the LAMP assay. For these reasons the LAMP assay was not investigated further in this project.



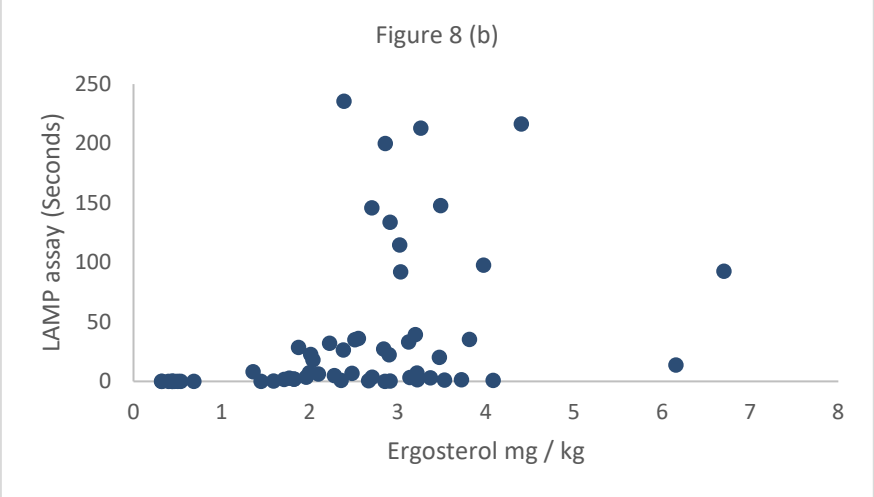


Figure 8. Comparison of LAMP assay with data for (a) % infection based on hyperspectral imaging and (b) ergosterol analysis

Of the techniques investigated in this experiment, analysis of the ergosterol content of the infected bunches appeared to be the most accurate with respect to either visual inspection or hyperspectral imaging. There is, however, a degree of imprecision in both of the imaging techniques in that neither is able to detect Botrytis grey mould contamination in the interior of the bunch that is hidden from view. This limitation isn't immediately noticeable in this experiment, since the bunches examined were inoculated in the laboratory by wounding berries. These wounds were made to berries that were on the exterior of the bunch rather than to those berries that were not accessible within the interior of the bunch.

Analysis of wine made from the Cabernet Sauvignon grapes harvested in 2019

A further batch of Cabernet Sauvignon grapes were hand harvested from the same vineyard as described above. The bunches were inoculated with a mixture of *B. cinerea* strains and after 4 days of incubation sorted into one of the four categories: clean, low, medium and high levels of grey mould contamination. These four levels of infection were approximately equivalent to 0, 2, 5 or 10 % of the bunch affected with grey mould. The four batches of grapes with the different levels of grey mould contamination were analysed for ergosterol, malic acid and also for *B. cinerea* using qPCR and LAMP (Table 4) after which they were vinified. Ergosterol and qPCR analysis were the best indicators of the level of grey mould infection in the four batches of grapes. The LAMP assay differentiated clean, healthy grapes from infected ones, but did not differentiate the level of infection.

Wines made with the uninfected grapes were described as having the typical descriptors associated with Cabernet Sauvignon, (e.g. confectionary, red fruit) while earthy, mouldy, acetic, and brown descriptors predominated with increasing levels of grey mould infection. A loss of wine quality was found at the lowest level of grey mould infection (i.e. 2%) and was more pronounced in the medium (5%) and high levels of grey mould contamination. The sensory analysis of the wines made from the uninfected grapes and the grapes with the four levels of grey mould infection are presented in Figure 9. The results of this experiment are discussed further under the analysis of wines made in the 2020 season.

Table 4: Ergosterol and Botrytis as detected by qPCR and LAMP in Cabernet Sauvignon grapes infected with different levels of *B. cinerea* and analysed prior to vinification in the 2019 vintage. Results are the means of three replicate determinations.

Botrytis infection [1]	**Ergosterol (mg / kg)**	**Fungal biomass (g/kg)**	**Corrected fungal biomass (g/kg) due to B. cinerea [2]**	**Malic Acid (g/L)**	**qPCR (ng/g)**	**LAMP (Secs)**
0	0.757 (± 0.084)	0.255	-	2.242 (± 0.069)	0 (±)	0
1	0.934 (± 0.176)	0.315	0.060	2.053 (± 0.026)	2836 (± 656)	766.5 (± 32)
2	1.274 (± 0.113)	0.429	0.174	2.080 (± 0.008)	5595 (± 378)	228.0 (± 51)
3	1.913 (± 0.222)	0.645	0.390	2.095(± 0.174)	9635 (± 446)	745.3 (± 99)

[1] Botrytis infection levels of 0, 1, 2 and 3 correspond to 0, 2, 5 and 10% infected bunches on a w/w basis.

[2] Corrected biomass calculated by deducting the calculated fungal biomass (due to the background yeast population) on the control un-inoculated fruit from the value determined for the infected fruit.

Numbers in parentheses are standard deviations.

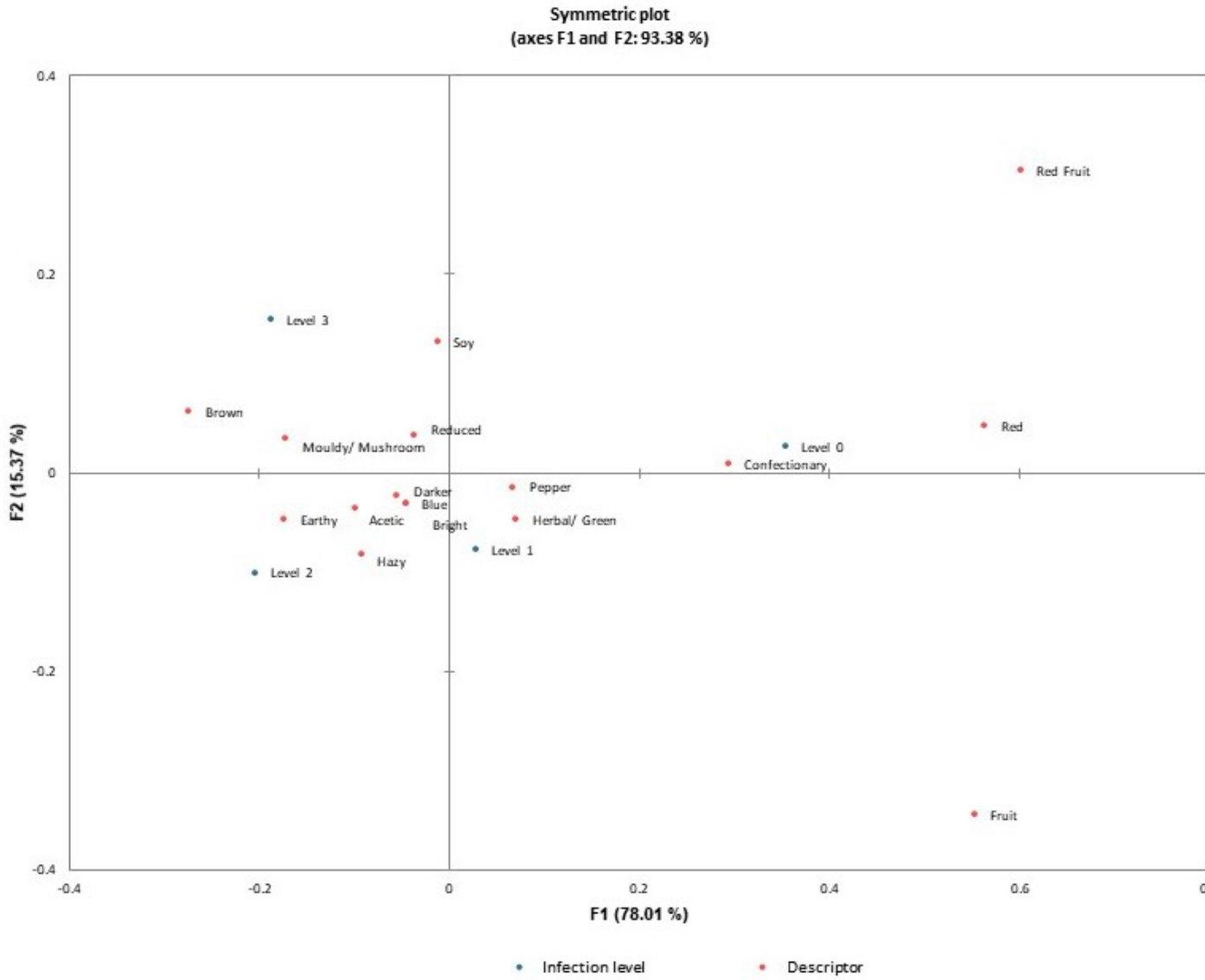


Figure 9. Sensory analysis of wines made in 2019 from grapes with different levels of grey mould infection. Level 0 = clean fruit, Levels 1, 2 and 3 respectively equal Low (2%), Medium (5%) and High (10%) grey mould infections.

Experiments conducted in the second year of study, 2020 and 2021

The 2020 vintage was an opportunity to refine some of the studies completed in 2019 and to further investigate techniques for grey mould detection and quantification. Additional work was conducted in early 2021 on field sourced examples of bunches affected with grey mould.

Research conducted during 2019 indicated that measuring ergosterol was a potentially reliable indicator of grey mould contamination of wine grapes. During the second year of the study, ergosterol was compared with other techniques that could conceivably be considered as measures of grey mould contamination. Detection of *B. cinerea* by measuring Botrytis antigens, using a commercially available Lateral Flow Device (LFD) kit was evaluated as was the assessment of gluconic acid. This was in addition to the other techniques investigated in 2019. The planned outputs were revised and refined in December 2019 as part of the Strategic Partnership Agreement (SPA) between Wine Australia and the NWGIC. These changes are reflected in this second half of the research.

Five experiments were conducted during this phase of the project.

1. Individual Chardonnay berries were inoculated with *B. cinerea* in the laboratory and mixed with un-inoculated berries to obtain different percentage of infection based on a berry weight basis.
2. Whole grape bunches from four different wine grape varieties were inoculated with *B. cinerea* in the laboratory and mixed with un-inoculated bunches to obtain different percentages of infection based on a bunch weight basis.
3. A time course experiment was conducted using Cabernet Sauvignon grape bunches inoculated with Botrytis and the bunches analysed at daily intervals post-inoculation.
4. Laboratory inoculated Cabernet Sauvignon grapes were analysed for grey mould contamination, the bunches were vinified and the wine analysed.
5. *B. cinerea* was estimated from field samples of grey mould affected bunches using ergosterol analysis and a LFD kit for Botrytis antigen quantification.

Individual Chardonnay berries inoculated with B. cinerea and mixed with un-inoculated berries to get different percentages of grey mould infection on a weight per weight basis in 2020

In this experiment, individual inoculated berries were mixed with un-inoculated berries to obtain different infection levels from 0% to 10% in 100 berry lots with three replicates. The actual weights of the infected and un-infected berries were recorded. The percentage infection was then expressed on a weight / weight basis and the berry homogenates analysed for ergosterol content, gluconic acid, glycerol, Botrytis antigens and for Botrytis using qPCR.

The ergosterol content of the berry mixtures correlated (R^2 = 0.9719) with the amount of grey mould present as a percentage of berry weight (Figure 10) indicative of the robustness of the method for predicting grey mould contamination.

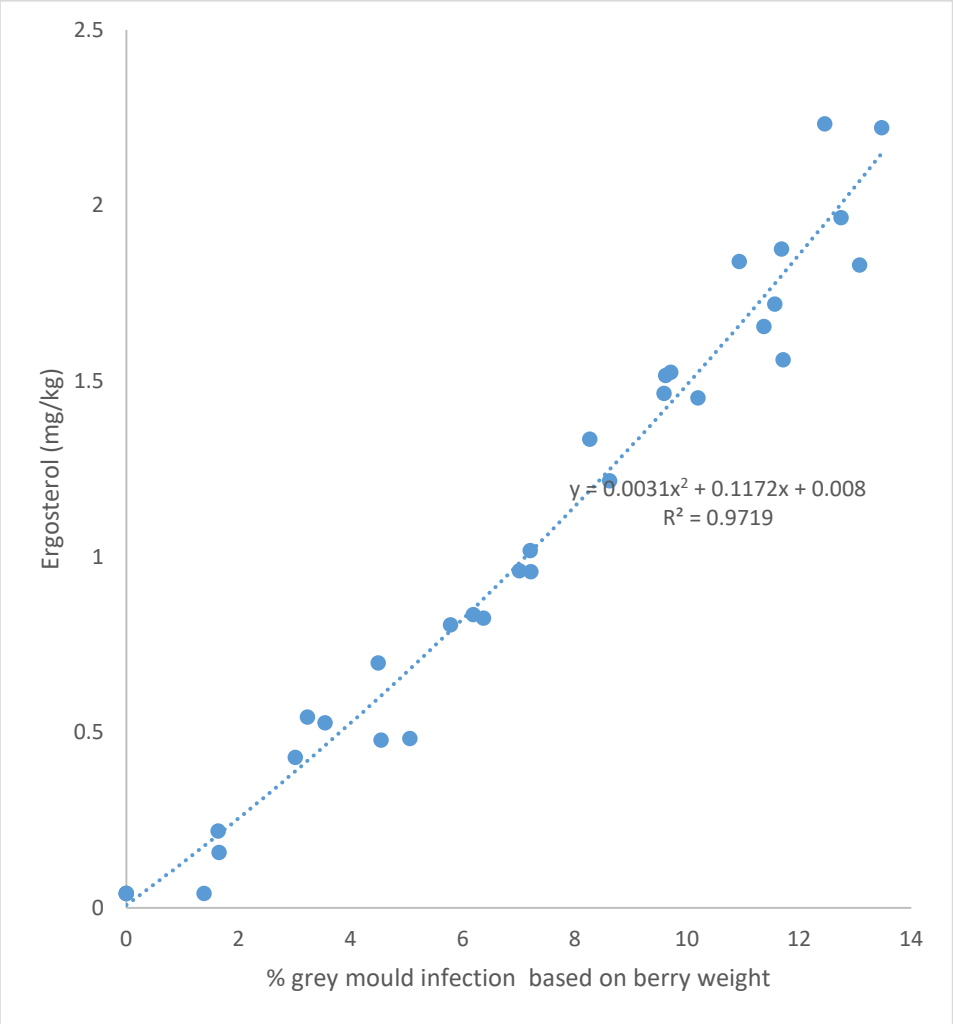


Figure 10. Ergosterol content of B. cinerea *infected Chardonnay grape berry mixtures*

The level of Botrytis antigens (Figure 11) correlated (R^2 = 0.9700) with the % of grey mould infection on a berry weight basis and the correlation was comparable with the data obtained for the ergosterol analysis, indicative that both methods quantification of Botrytis antigens and ergosterol are suitable techniques for estimating grey mould contamination.

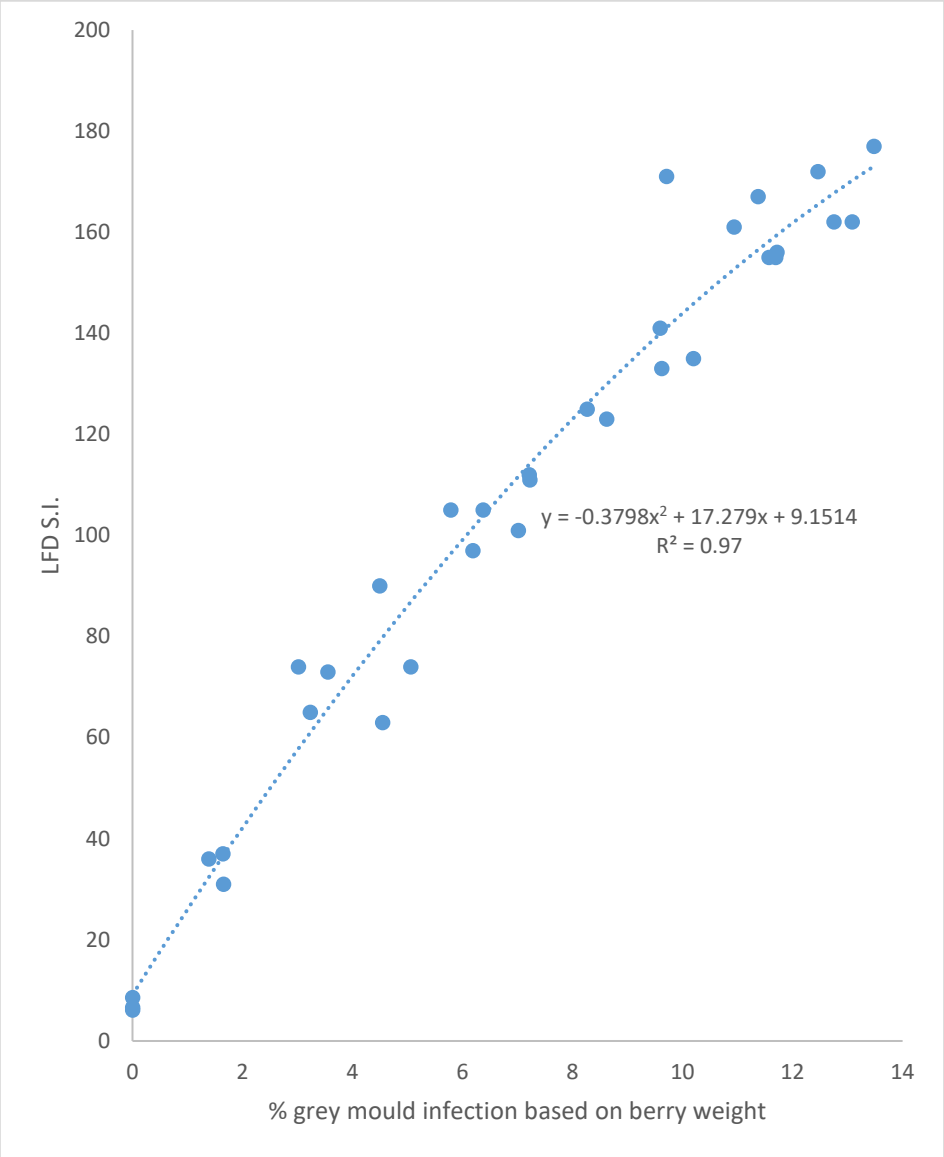


Figure 11. Botrytis antigens detected in B. cinerea *infected Chardonnay grape berry mixtures.*

A linear relationship (R^2 = 0.8980) also existed between the levels of gluconic acid detected in the Chardonnay grape berry mixtures and the amount of grey mould infection, although the healthy uninfected grape berries were also found to contain levels of gluconic acid ranging from 0.015 to 0.026 g/L (Figure 12).

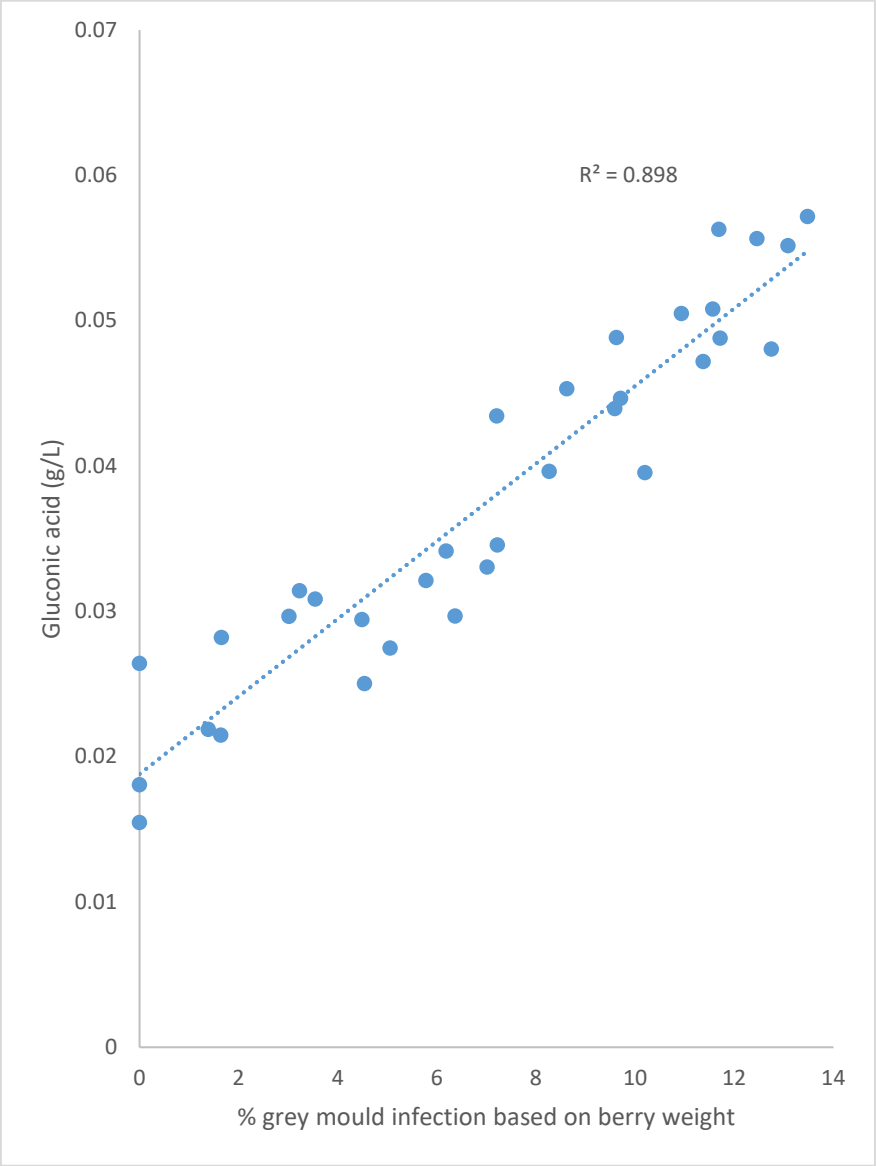


Figure 12. Gluconic acid content of B. cinerea *infected Chardonnay grape berry mixtures*

The grape berry mixtures were also subjected to analysis by qPCR for *B. cinerea* estimation. While a linear relationship existed between the amount of *B. cinerea* detected and the % of infection on a berry weight basis, the data was more variable and the relationship was not as strong. The variability in results was particularly noticable in samples with a percentage of infection > 6% (Figure 13).

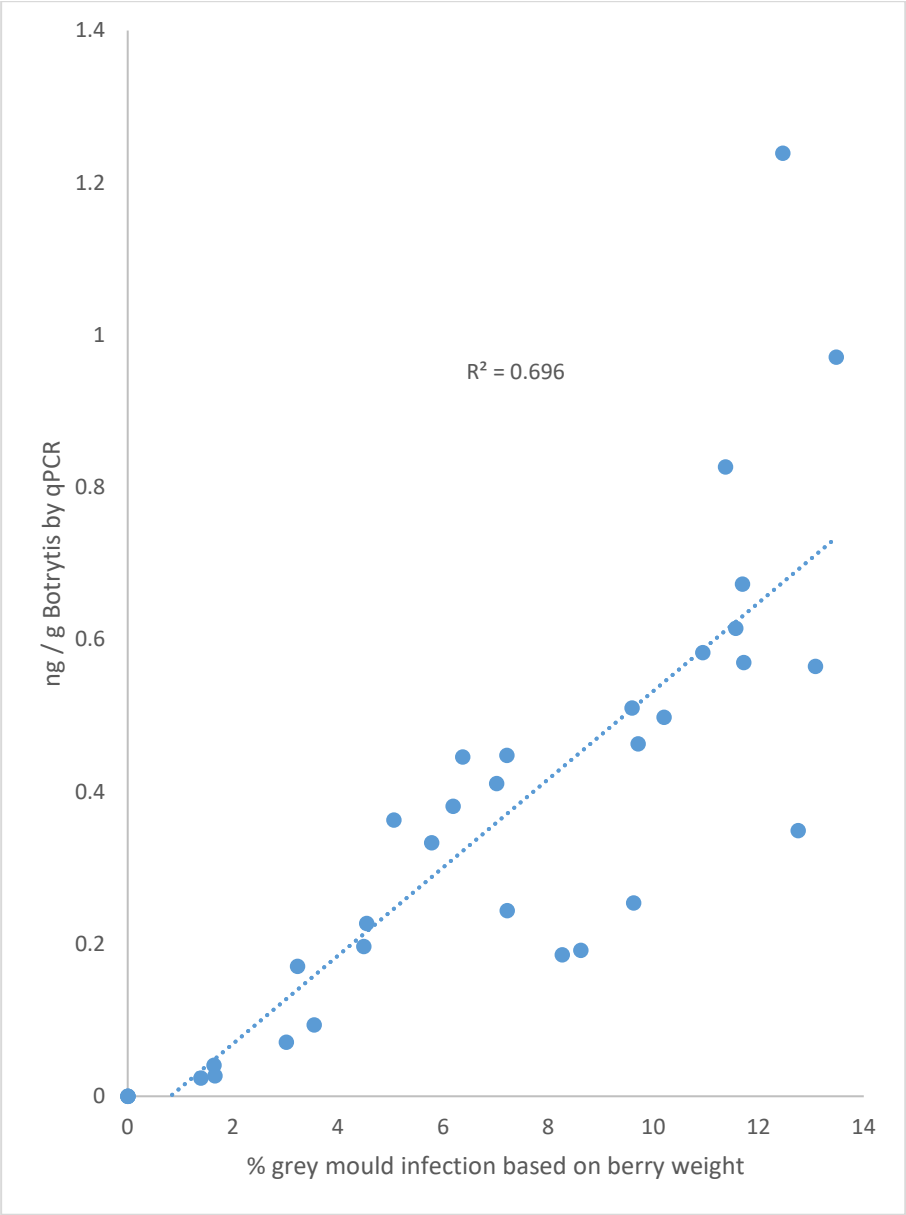


Figure 13. qPCR for B. cinerea *detection in* B. cinerea *infected Chardonnay grape berry mixtures*

From these experiements, it was concluded that of the methods evalauted, analysis of ergosterol and measurement of Botrytis antigens provided the most accurate determination of the level of grey mould contamination in individual Chardonnay grape berries inoculated with *B. cinerea*.

Infection matrix study with four varieties using grape bunches 2020

Having evaluated the various techniques for the estimation of grey mould contamination in individual Chardonnay grape berries inoculated in the laboratory, the work was extended to include additional grape cultivars using whole grape bunches rather than individual detached grape berries. Four wine grape varieties, Cabernet Sauvignon, Chardonnay, Semillon and Shiraz were utilised. Bunches were inoculated with a mixture of *B. cinerea* strains as described in the methods and incubated as described previously. Following incubation, bunches were destemmed and homogenised. The homogenates from infected bunches were mixed with homogenates from un-infected bunches in different ratios on a weight per weight basis to create seven infection levels: 0%, 2%, 4%, 6%, 9%,12% and 15% (w/w).

The amount of ergosterol found in uninfected grape bunches was deducted from amounts detected in infected bunch mixtures. The ergosterol detected in uninfected grape bunches can be attributed to the background yeast populations that naturally occur on the surface of the grape. The ergosterol contents of the uninfected Chardonnay, Semillon and Shiraz bunches were comparable (Table 5). All varieties used in this experiment were harvested from the one vineyard. The profile and amount of indigenous yeasts and other fungal species naturally residing on the surface of grape berries is likely to vary from vineyard to vineyard and also from season to season (Combina, et al. 2005,Rathnayake 2016,Rousseau and Doneche 2001).

Table 5. Ergosterol content of un-inoculated grape bunches. Results are the means of three replicates, each replicate consisting of 100 berries.

Grape Variety	**Ergosterol (mg/Kg) of clean un-inoculated berries**
Cabernet Sauvignon	n.d.
Chardonnay	0.040
Semillon	0.040
Shiraz	0.041

n.d. = not determined

The ergosterol content of the inoculated bunches increased with the level of *B. cinerea* infection in a linear manner. However, there were differences between the varieties which became more pronounced at the higher levels of infection examined. At a 15 % level of grey mould infection Chardonnay grapes contained 1.78 mg of ergosterol per kg fresh weight of grapes, while Shiraz bunches with the same level of grey mould infection contained 2.83 mg of ergosterol per kg fresh weight of grapes (Figure 14). This proportional difference in the amount of ergosterol detected in the four cultivars at the same infection level cannot be explained.

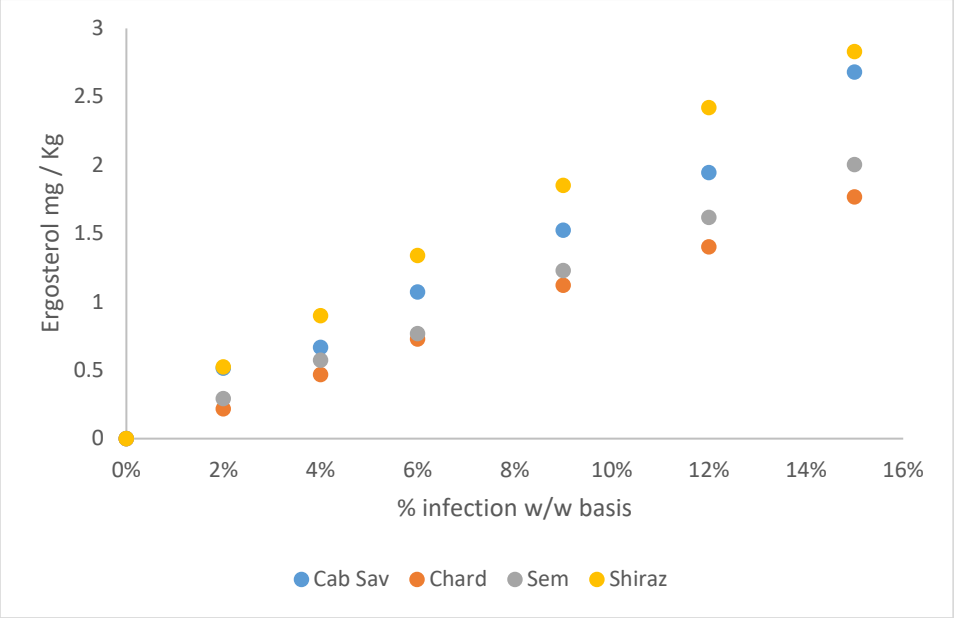


Figure 14. Ergosterol content of B. cinerea *infected grape bunches in four grapevine cultivars, Cabernet Sauvignon (Cab Sav), Chardonnay (Chard) Semillon (Sem) and Shiraz.*

More comparable results were obtained when the amount of Botrytis antigens was determined. There were still differences between the four varieties, but these were not as great. The signal intensity (SI) for the Botrytis antigens in the Chardonnay grapes with a 15% level of grey mould infection was 195, while the equivalent value for the Shiraz grapes was 190 (Figure 15). The relationship between the degree of grey mould infection and the amount of antigens detected is not a linear one, and at higher levels of infection the curve plateaus. Diluting the grape homogenate further overcomes this non-liner relationship.

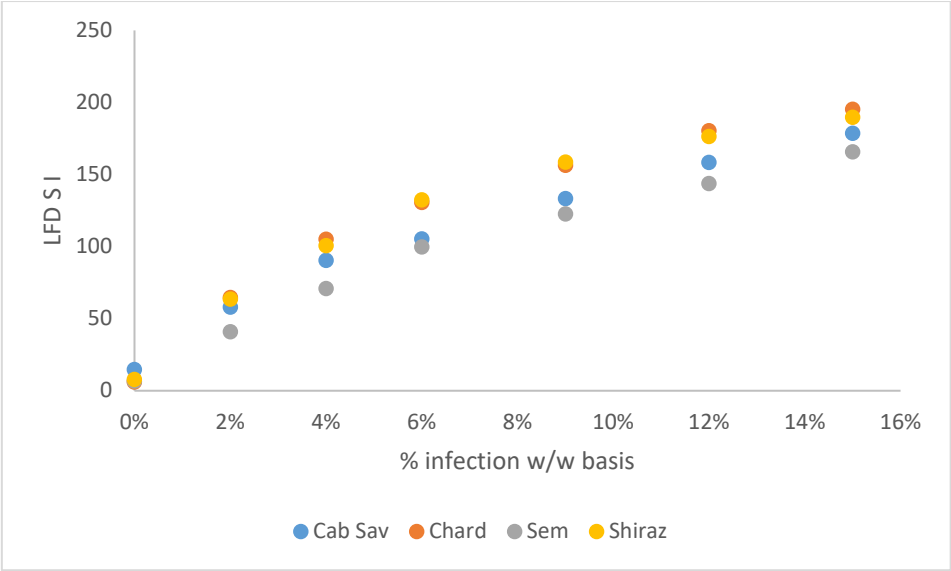


Figure 15. Levels of Botrytis antigens in B. cinerea *infected grape bunches in four grapevine cultivars, Cabernet Sauvignon (Cab sav), Chardonnay (Char) Semillon (Sem) and Shiraz (Shz).*

When the data for the ergosterol analysis was plotted against the SI data for the Botrytis antigens a curved relationship was observed. Figure 16 shows the combined data for all four grape varieties that were examined.

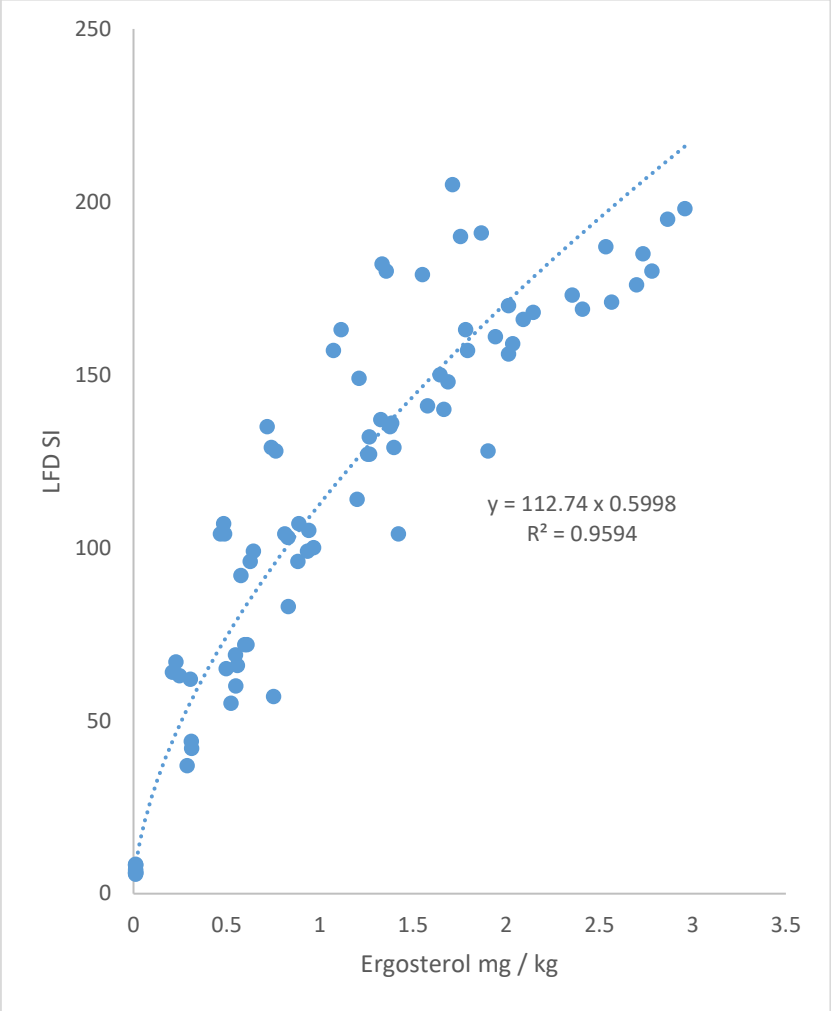


Figure 16. Relationship between Ergosterol analysis and Botrytis antigen detection for combined data for Chardonnay, Cabernet Sauvignon, Semillon and Shiraz grape bunches.

Using the BCF for ergosterol, the data was then expressed with respect to the amount fungal biomass present in the grape bunches on a g fungal dry mass per kg fresh mass of grape basis. Data for the four separate varieties are presented in Figure 17. LFD SI readings for the Chardonnay grapes were slightly higher than the readings obtained for the other three varieties examined. A fungal biomass of 0.2 g dry mass of fungus per kg wet weight of grapes equated to a LFD SI reading of 75, 100, 74 and 70 for the Cabernet Sauvignon, Chardonnay, Semillon and Shiraz grapes respectively. Similarly a LFD SI

reading of 50 approximately equated to 0.10, 0.08, 0.12 and 0.13 dry mass of fungus per kg wet weight of grapes, for the Cabernet Sauvignon, Chardonnay, Semillon and Shiraz grapes respectively.

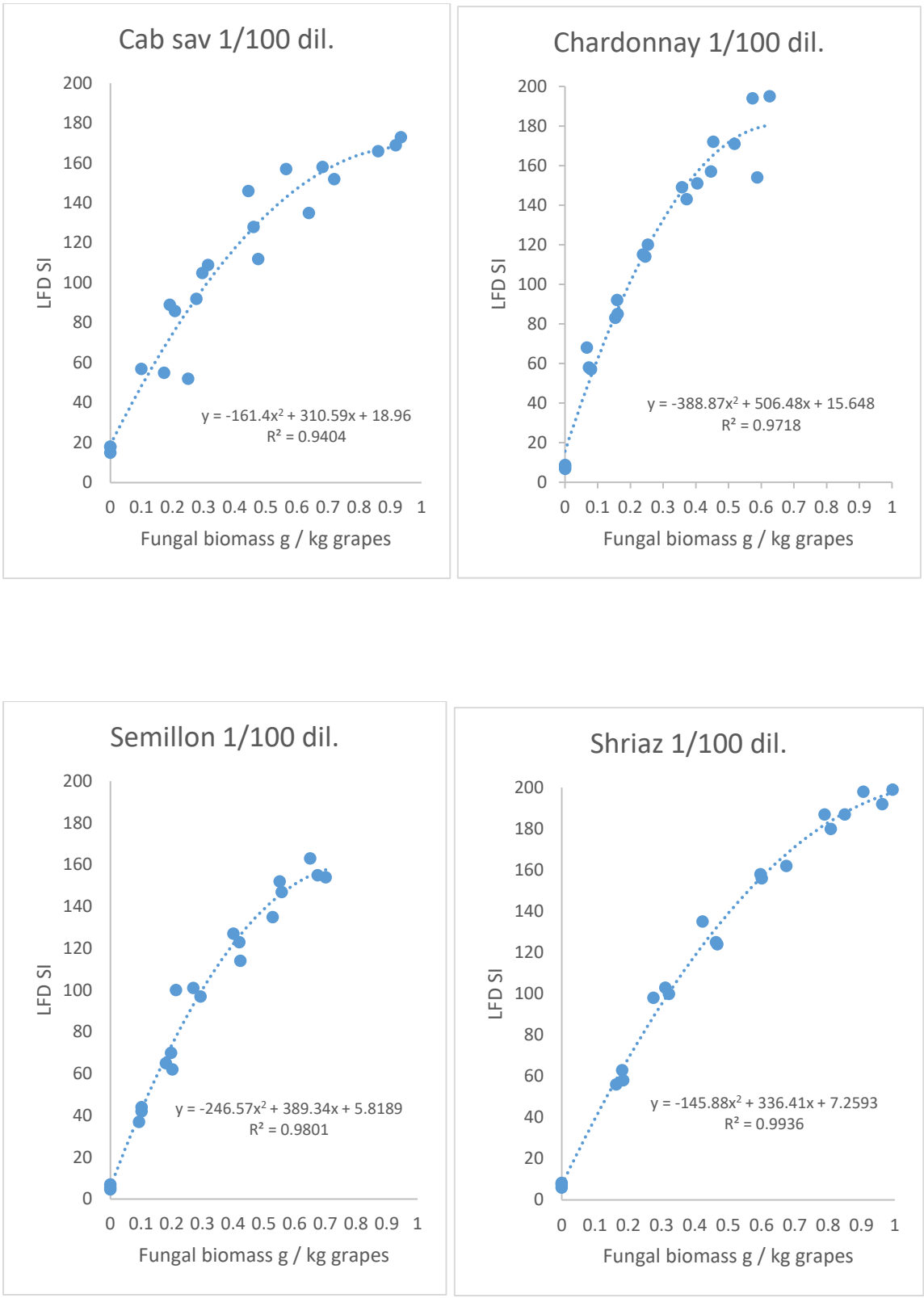


Figure 17. Relationship between Botrytis LFD SI and dry fungal biomass (g) per kg fresh mass of grapes for Vitis vinifera *cvs. Cabernet Sauvignon, Chardonnay, Semillon and Shiraz using 1/100 dilutions of homogenates from laboratory inoculated bunches.*

Results using qPCR to quantify Botrytis grey mould infection levels were more variable than that obtained for either ergosterol analysis or antigen detection (Figure 18). This variation could potentially be explained by the multi nuclear nature of the fungus, i.e. multiple gene copies are present per cell (Hansen and Smith 1932). However, the relative amounts of Botrytis detected by qPCR were somewhat consistent with the amount of ergosterol detected. For example, 0.750 and 0.313 ng of Botrytis per g tissue was detected using qPCR in the Shiraz and Chardonnay bunches respectively infected with 15 % grey mould. These same grape bunches were determined to contain 2.83 and 1.78 mg of ergosterol per kg fresh weight of grapes for Shiraz and Chardonnay grapes respectively.

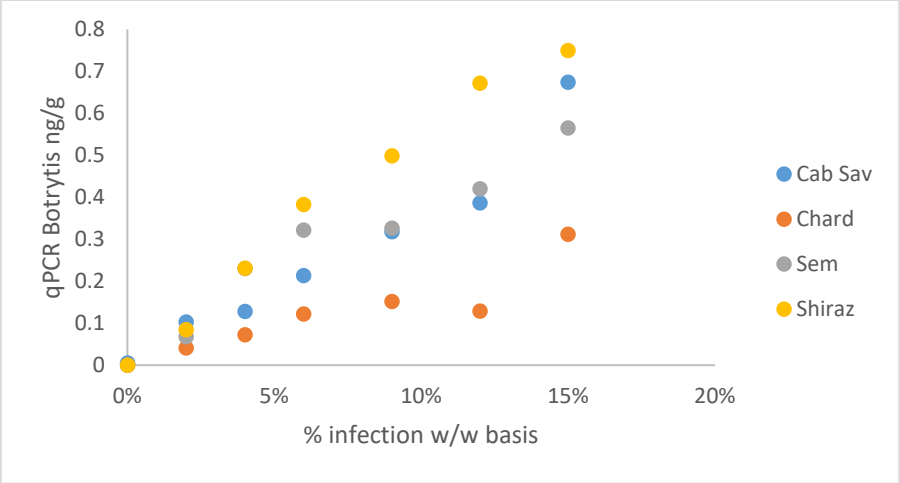


Figure 18. Correlation between qPCR for Botrytis detection and infection levels in B. cinerea *infected grape bunches in four grapevine cultivars, Cabernet Sauvignon (Cab Sav), Chardonnay (Chard) Semillon (Sem) and Shiraz.*

The concentration of gluconic acid in the inoculated grapes was measured using three different commercially available kits. The results for the Chardonnay berry mixtures are presented below in Figure 19. Comparable results were obtained for all four wine grape varieties examined.

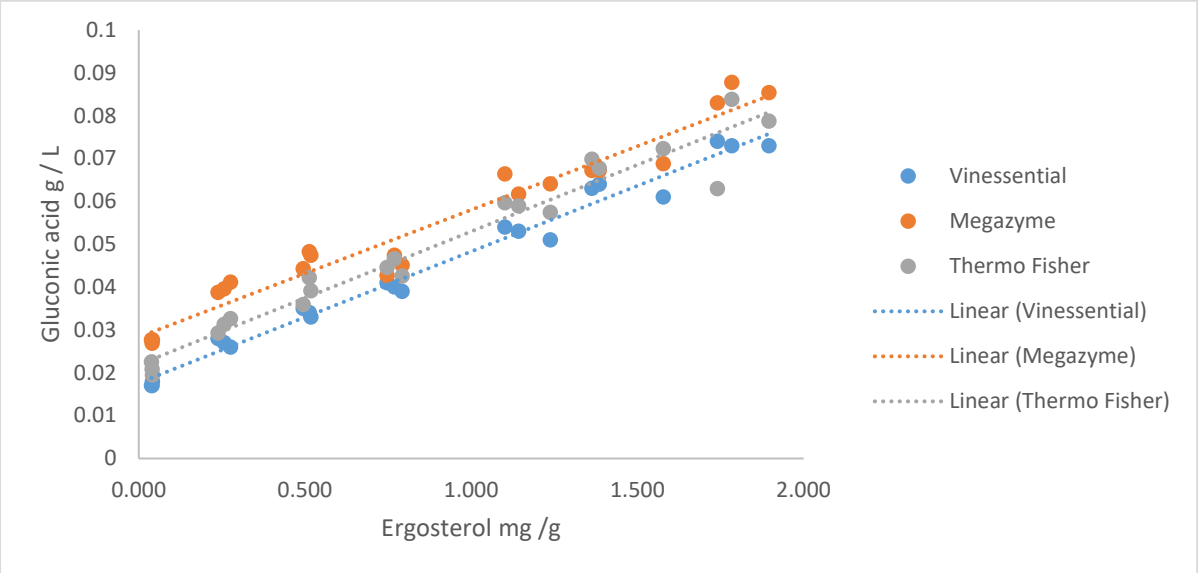


Figure 19. Gluconic acid in inoculated Chardonnay grape bunches assayed using three different commercially available enzymatic kits.

Cabernet Sauvignon individual whole bunches 2020

A time course experiment was conducted using Cabernet Sauvignon grape bunches inoculated with Botrytis. The formation of ergosterol, Botrytis antigens, and gluconic acid as well as qPCR were followed at daily intervals following inoculation over a 7-day period. For the purposes of this experiment, the background ergosterol content of clean un-inoculated bunches was not deducted from the data.

Data relating to the control un-inoculated bunches is shown in Table 6 and data for the infected bunches are shown in Table 7.

Table 6. Evolution of ergosterol, Botrytis antigens, gluconic acid, glycerol and Botrytis as detected by q PCR over a 7 day period in control un-inoculated Cabernet Sauvignon grape bunches. Homogenates for Botrytis antigen quantification were diluted 1/100 prior to analysis

	Days post inoculation							
	0	1	2	3	4	5	6	7
Ergosterol (mg / kg)	0.218±0.029	0.395±0.5	0.313±0.061	0.202±0.13	0.391±0.29	0.547±0.184	2.205±1.61	0.527±0.307
LFD S I	7.2±1.296	8.2±2.085	8.3±1.208	7±1.414	7±2.192	10±1.633	9.533±1.226	7.667±0.967
Gluconic acid (g /L)	0.035±0.002	0.048±0.024	0.064±0.004	0.069±0.008	0.053±0.006	0.077±0.012	0.124±0.074	0.085±0.036
Glycerol (g/L)	0.082±0.016	0.149±0.147	0.29±0.008	0.174±0.033	0.228±0.104	0.302±0.063	0.615±0.362	0.529±0.04
qPCR (ng/g)	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0

Table 7. Evolution of ergosterol, Botrytis antigens, gluconic acid, glycerol and Botrytis as detected by q PCR over a 7 day period post-inoculation in Cabernet Sauvignon grape bunches. Homogenates for Botrytis antigen quantification were diluted 1/100 prior to analysis

	Days post inoculation							
	0	1	2	3	4	5	6	7
Ergosterol (mg / kg)	0.218±0.029	0.311±0.371	0.357±0.098	0.993±0.718	1.512±0.627	3.352±0.342	6.688±2.048	7.775±1.476
LFD S I	7.2±1.296	11.32±5.934	9.58±0.913	34±4.817	85±17.967	189.4±16.693	217.2±18.454	236±5.621
Gluconic acid (g /L)	0.035±0.002	0.038±0.006	0.051±0.014	0.067±0.007	0.11±0.024	0.187±0.026	0.195±0.045	0.189±0.012
Glycerol (g/L)	0.082±0.016	2.271±0.222	2.029±0.312	1.842±0.256	2.216±0.39	2.526±0.245	2.917±0.097	2.982±0.059
qPCR (ng/g)	0±0	0.008±0.011	0.014±0.006	0.106±0.019	0.308±0.139	0.721±0.209	1.007±0.183	1.88±0.43

Analysis of the wine made with the Cabernet Sauvignon grapes in 2020

Lab-inoculated Cabernet Sauvignon grapes were analysed for grey mould contamination using the techniques described above, and the bunches were vinified. Data relating to the analysis of these bunches are presented in Table 8.

The sensory analysis of the wines made from the uninfected grapes and the grapes with the four levels of grey mould infection are presented in Figure 20.

The results of the sensory analysis of the 2020 wines were similar to that found for the 2019 wines. With increasing levels of grey mould infection and levels of the fungal sterol ergosterol, the participants recorded enhanced mouldy, earthy characters, and a loss of red wine colour and a depletion of the fruity characters. Wines made with grapes infected with Botrytis infection levels 2 and 3 were described as acetic and brown in colour. The uninfected grapes had an ergosterol content of 1.620 mg/kg fresh weight of grapes, due to the background yeast microflora on the berry surface. Results from the qPCR analysis clearly demonstrate that no Botrytis was present in the uninfected grape material confirming that the ergosterol detected was due to fungal organisms other than *B. cinerea*. The grapes with a botrytis infection level of 2 (equivalent to a 5% infection on a berry w/w basis) had an ergosterol content of 1.952 mg/kg fresh weight of grapes. This suggests that once the ergosterol content of the grapes increases by more than 0.3 mg/kg fresh weigh of grapes above the background levels then there is a noticeable loss of wine quality. For the wine made in this study, the threshold for a noticeable loss in wine quality due to grey mould is between 0.08 and 0.11 g of *B. cinerea* per kg fresh mass of grapes. With regards to the level of Botrytis antigens detected using the Mologic device, it would appear using a grape sample diluted 1/100 that the cut off for a loss of wine quality sits somewhere between a LFD signal intensity reading of 45 and 80.

Table 8. Ergosterol, Botrytis antigens, gluconic acid, glycerol and Botrytis as detected by qPCR in Cabernet Sauvignon grapes infected with different levels of *B. cinerea* and analysed prior to vinification in the 2020 vintage. Results are the means of three replicate determinations. Numbers in parentheses are standard deviations

Botrytis Infection [1]	**Ergosterol (mg/kg)**	**Fungal biomass (g/kg)**	**Corrected fungal biomass (g/kg) due to B. cinerea [2]**	**LFD SI**	**Gluconic Acid (g/L)**	**Glycerol (g/L)**	**qPCR (ng/g)**
0	1.620 ± (0.285)	0.5459	-	12 ± (2.2)	0.078 ± (0.002)	0.518 ± (0.101)	0
1	1.848 ± (0.301)	0.6228	0.0769	44.7 ± (2.5)	0.381 ± (0.028)	0.531 ± (0.03)	0.022 ± (0.009)
2	1.952 ± (0.165)	0.6578	0.1119	80.3 ± (3.1)	0.795 ± (0.096)	0.836 ± (0.051)	0.09 ± (0.033)
3	2.750 ± (0.348)	0.9267	0.3808	125 ± (9.6)	1.514 ± (0.07)	1.289 ± (0.028)	0.11 ± (0.007)

[1] Botrytis infection levels of 0, 1, 2 and 3 correspond to 0, 2, 5 and 10% infected berries on a w/w basis.

[2] Corrected biomass calculated by deducting the calculated fungal biomass (due to the background yeast population) on the control un-inoculated fruit from the value determined for the infected fruit.

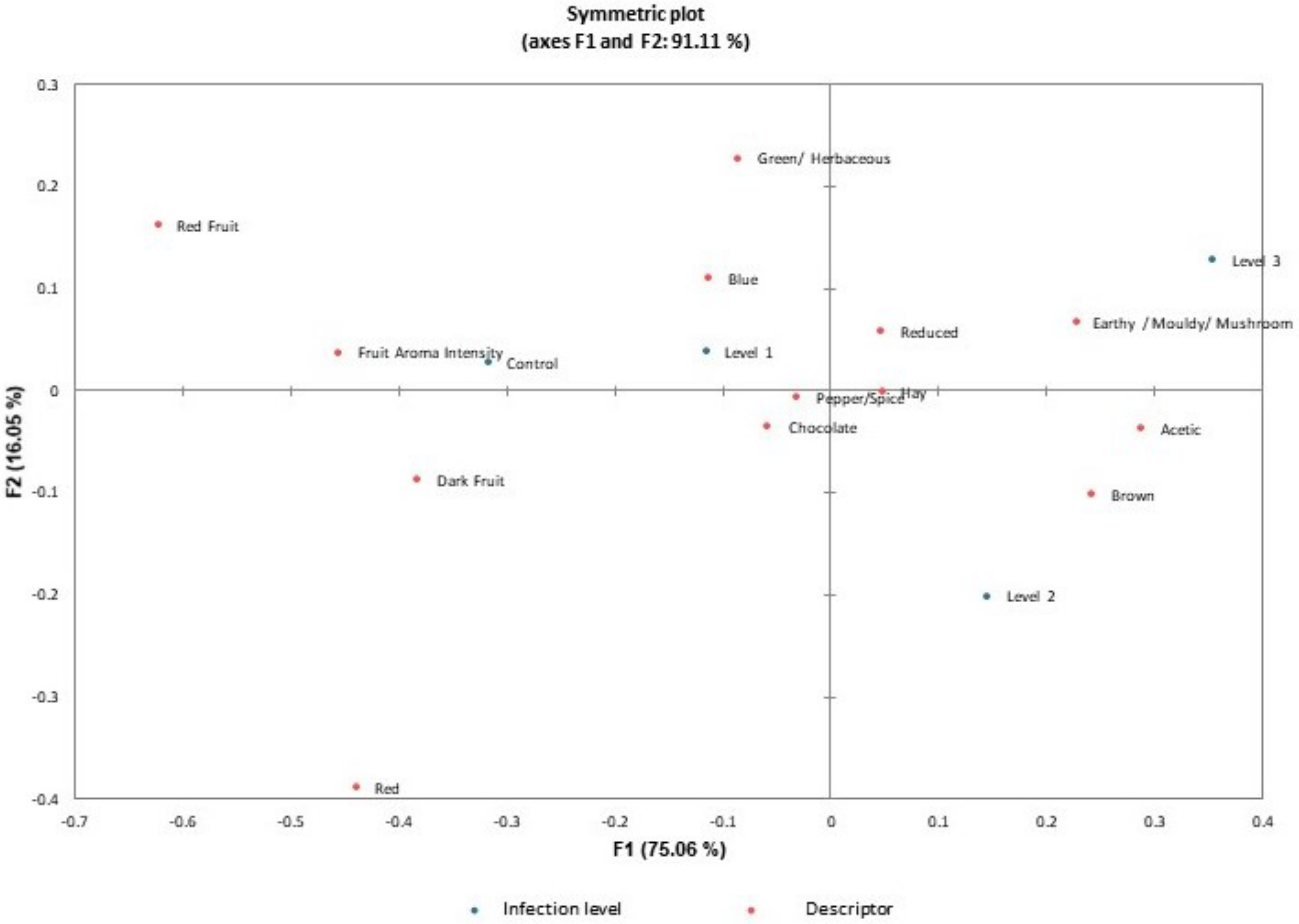


Figure 20. Sensory analysis of wines made in 2020 from grapes with different levels of grey mould infection. Botrytis infection levels of 0, 1, 2 and 3 correspond to 0, 2, 5 and 10% infected berries on a w/w basis.

Estimation of B. cinerea from field samples of examples of grey mould affected grapes using ergosterol analysis and a LFD kit for Botrytis antigen quantification in 2021

The fungal biomass present in each of the 55 Shiraz grape bunches collected as part of this experiment was determined based on the ergosterol content. The number of berries per bunch visibly affected with grey mould in this study ranged from 0 to 25. The actual number was recorded for each whole bunch, prior to the removal of the berries for homogenisation. This was done to reflect the type of observation that might be made in a field situation, where whole bunches are not dissected. Bunch weights ranged from 67.4 to 247.7 g.

There was a linear relationship (R^2 = 0.7219) between the amount of ergosterol detected per bunch and the number of berries per bunch that were observed with the naked eye to be affected with Botrytis grey mould (Figure 21). The relationship between visual observations and the actual amount of fungal biomass detected was not perfect. As a dark skinned variety, detecting grey mould on the surface of a Shiraz grape by the naked eye is difficult less than perfect, and additional errors were likely as the fruit displayed typical signs of Shiraz shrivel.

Of 13 bunches that were scored as having no berries with visible symptoms of Botrytis grey mould, nine returned a LFD SI of > 15 indicating the presence of Botrytis antigens. These bunches had between 0.45 and 2.33 mg ergosterol per kg fresh weight of grapes, highlighting the inaccuracy in visual assessments of grey mould. The four bunches that returned a LFD SI of < 15 were found to contain between 0.43 and 0.53 mg ergosterol per kg fresh weight of grapes.

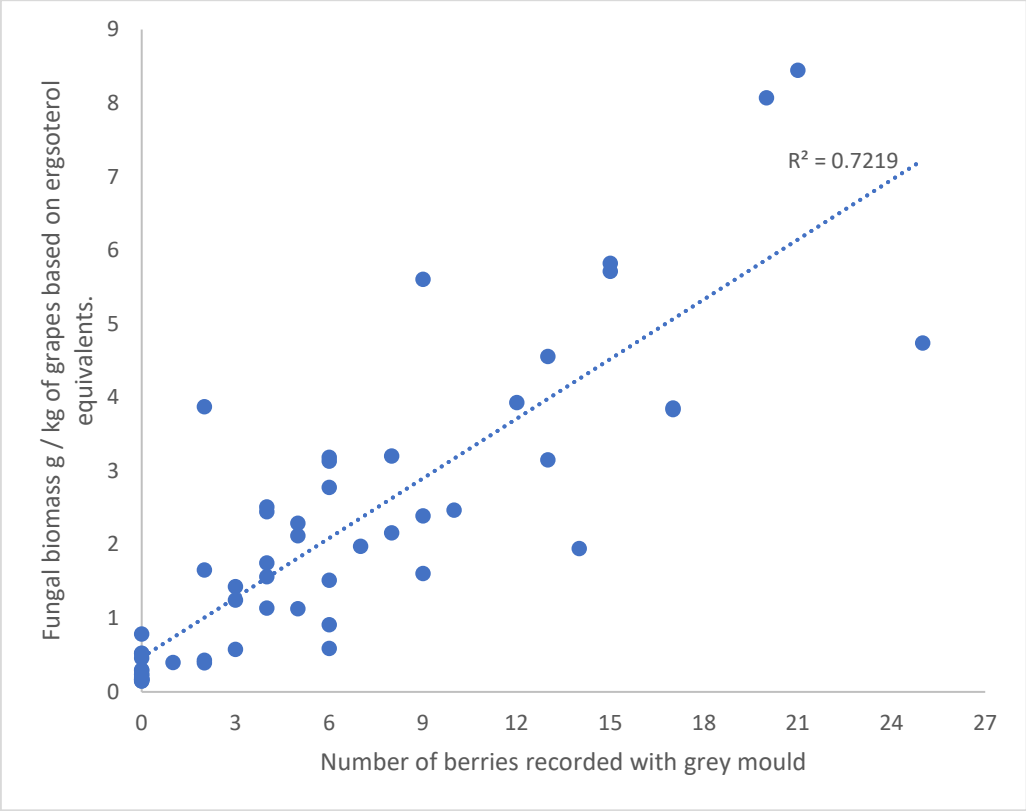


Figure 21. Fungal biomass present in grape bunches with various number of berries per bunch with visible signs of grey mould based on field observations.

Our earlier observations indicated that the relationship between the SI of the LFD used to measure the Botrytis antigens and the actual amount of Botrytis present in a sample was not a linear one and that the readings plateaued with higher amounts of Botrytis. Diluting the grape sample overcomes this effect and to demonstrate this, samples were diluted 1/10, 1/50 and 1/100 prior to analysis for Botrytis antigens. The data are presented with respect to the amount of fungal biomass detected by measuring ergosterol equivalents in Figures 22 to 24 below. The non-linear response to Botrytis antigens at the higher levels of grey mould contamination at the higher concentrations is evident.

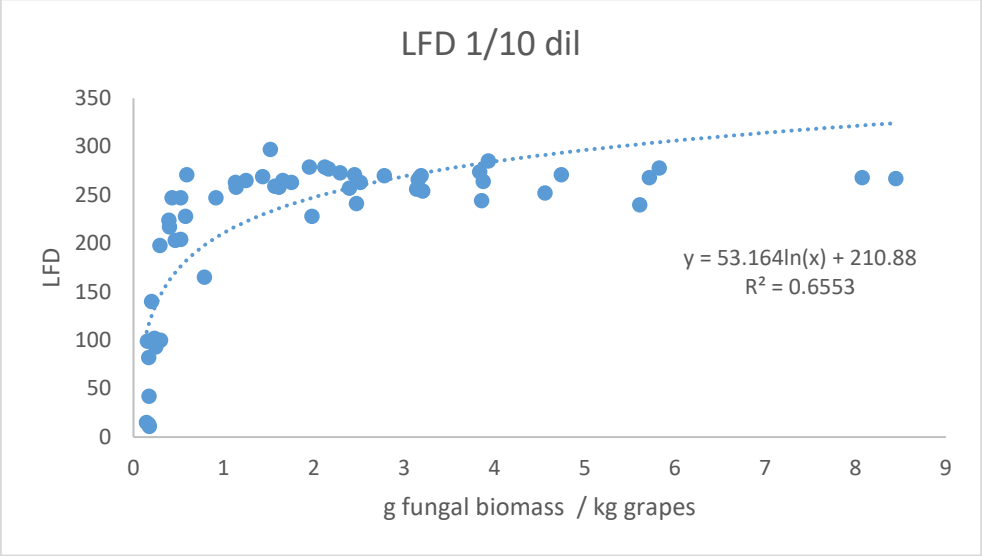


Figure 22. Measurement of Botrytis antigens using a 1/10 dilution vs the fungal biomass present in the grapes as determined by ergosterol equivalents.

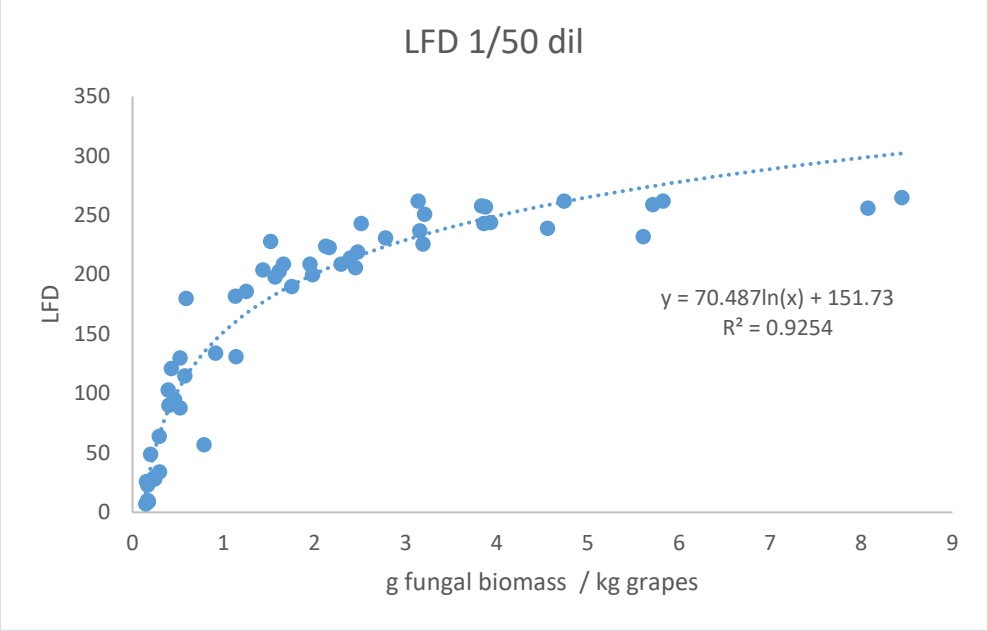


Figure 23. Measurement of Botrytis antigens using a 1/50 dilution vs the fungal biomass present in the grapes as determined by ergosterol equivalents.

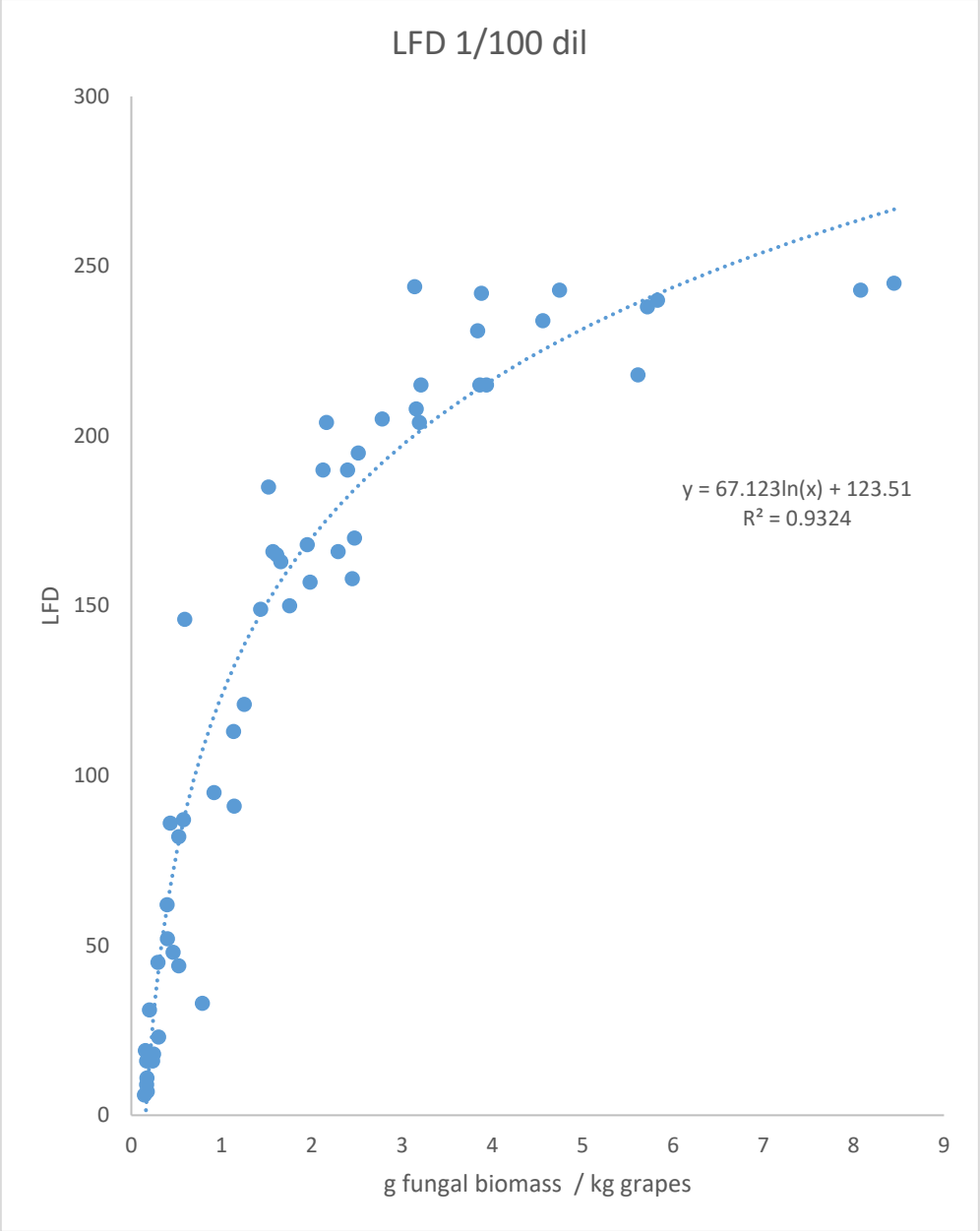


Figure 24. Measurement of Botrytis antigens using a 1/100 dilution vs the fungal biomass present in the grapes as determined by ergosterol equivalents over the 0 to 9 g of fungal biomass per kg fresh weight of grapes range.

While still a curved relationship, examination of the data for homogenates diluted 1/100 and examined over the 0.5 to 3 g fungal biomass / kg fresh weight of grapes reveals a more linear relationship (Figure 25).

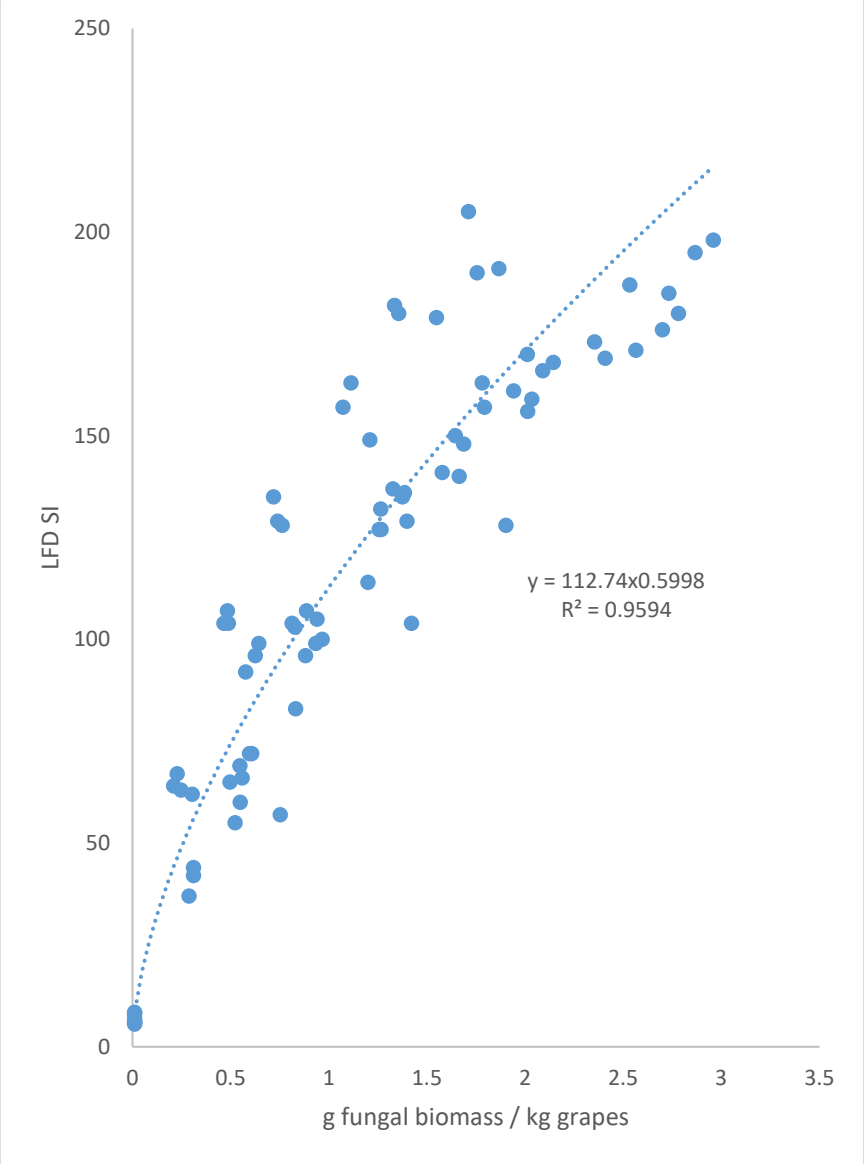


Figure 25. Measurement of Botrytis antigens using a 1/100 dilution vs the fungal biomass present in the grapes as determined by ergosterol equivalents over the range of 0 to 3.5 g of fungal biomass per kg fresh weight of grapes.

7. Outcome/Conclusion

Despite the fact that the outputs for the project were revised part way through the research, the objectives of the original application were met. Thresholds for grey mould contamination of Chardonnay grapes from one vineyard and one vintage were established and the methodology for the use of ergosterol as a quantitative indicator of fungal biomass and grey mould contamination was refined. Data for possible thresholds for grey mould contamination of Cabernet Sauvignon grapes were also obtained although it is acknowledged that further investigation is required. Measuring the fungal sterol ergosterol as an indicator of fungal contamination and further validation of the methodology using a range of *Botrytis cinerea* isolates from various regions and vintages was achieved, as was the impact of culture conditions particularly growth temperature. Our observation that the ergosterol content of Botrytis mycelium increases with an increase in temperature is consistent with studies carried out on other fungi (Aaronson, et al. 1982).

A wide range of techniques encompassing imaging, molecular, enzymatic assays and chemical analysis for detecting and potentially enumerating *B. cinerea* in grey mould infected grapes were investigated and compared. In many cases these observations were compared with data for the ergosterol analysis and hence the amount fungal biomass present in a grape sample.

All of the techniques investigated in this research project have advantages and disadvantages for evaluating grey mould contamination of wine grapes. These are summarised in Table 9. One option for the wine industry is to consider a combination of these techniques. Factors to be taken into consideration include the cost of operation, required skill set of the operator, access to specialised equipment, the time for analysis, specificity for *Botrytis cinerea* and applicability for a field or winery receival area situation.

Imaging techniques including assessment with the naked eye are prone to error particularly with regards to assessing any grey mould that might be hidden from view within the interior of a bunch. Detecting grey mould using visual inspection with the naked eye is more difficult on dark skinned varieties, and in this respect, hyperspectral imaging techniques offer an advantage over the naked eye. Quantitative PCR is prone to errors because of the multinucleate nature of *B. cinerea* (Büttner, et al. 1994,Shirane, et al. 1988) although the time frame to process samples is relatively quick (i.e. hours). While the LAMP assay is a sensitive method for Botrytis detection, the technique does not provide a quantitative estimate of Botrytis contamination.

Several commercially available enzymatic kits are available for the estimation of gluconic acid in grape samples. Some sample preparation is required but these kits provide a rapid analysis time and

indication of fungal contamination. A bench top spectrophotometer is required and compared to other techniques that require access to sophisticated equipment used in analytical chemistry measurement of gluconic acid by enzymatic analysis is a relatively simple and fast technique. Gluconic acid is not specific for Botrytis. Some of the other fungi associated with the rotting of grapes, such as Aspergillus species, produce greater amounts of gluconic acid than Botrytis (Steel 2014), as do *Penicillium* species (Lorenzini, et al. 2013). Even a low level of either of these other two bunch rot pathogens present in a grey mould affected bunch could potentially skew the data.

As is the case with gluconic acid, the fungal sterol, ergosterol is not unique to Botrytis. All fungi including yeasts contain ergosterol(Bloch 1983,Mercer 1984), consequently the yeast microflora present on the berry surface contributes to the amount of ergosterol detected in grape homogenates. These background levels of ergosterol will vary from vineyard to vineyard and season to season. Despite these limitations, in a vineyard affected with grey mould, analysis of ergosterol provides an accurate estimate of the amount of Botrytis present. The amount of ergosterol produced per g dry mass of fungal mycelium is comparable for all of the bunch rotting fungi. In mixed bunch rot situations ergosterol provides an indication of the total amount of fungal contamination on a fungal dry mass per kg fresh weight of grapes basis. There was a strong linear relationship (R^2 = 0.9669) between the amount of ergosterol detected and the number of grape berries infected with grey mould in the Chardonnay grape berry mixture experiment. Measuring ergosterol allows the amount of fungal biomass (i.e. g dry weight of fungus) in a batch of grapes to be calculated. The principal disadvantage of this technique is the time required for analysis which is currently two days. With some automation of the work, the time frame could potentially be reduced but the complexity of conducting an organic solvent extraction, followed by evaporation and HPLC data analysis means that the technique would require a minimum of a full day. A 24 to 48 hour turn around to obtain information on the amount of Botrytis present in a batch of grapes is unlikely to be acceptable for most wineries during vintage. The technique might however be useful in monitoring the fungal load of the vineyard in the weeks or days leading up to harvest.

Measuring Botrytis antigens using a lateral flow device (LFD) represents one of the quickest methods for measuring Botrytis in grapes. Homogenisation and subsequent centrifugation of a sample may take up to 20 minutes while the actual analysis time for the antigens takes ten minutes per sample with one minute intervals in between samples. The methodology is suited to low levels of Botrytis contamination and is specific for Botrytis. Other fungi such as Aspergillus, Penicillium, and yeast species on the berry surface are not detected (Dewey, et al. 2000) Steel, unpublished observations). This project evaluated one commercially available kit for Botrytis antigen detection produced by Mologic Ltd (UK). While the technology is not new (Dewey, et al. 2013), and there are other kits that

have been available previously, the advantage of this kit is the field applicability. The hand-held cube reader that is used to measure the SI in the LFD test strip measures approximately 38 x 38 x 34 mm and can easily be held in the palm of the hand. The sensitivity of the kit necessitates that sample dilution is required. Work conducted in this project routinely used a 1/100 dilution for most samples in order to obtain a quantitative estimate of the amount of Botrytis present. Using this dilution, a LFD SI reading of 50 was found to be equivalent to approximately 0.1 g dry mass of Botrytis per kg fresh weight of grapes, based on the measurement of ergosterol. Due to the non-linear response of the device, samples that return a LFD SI of >120 should be diluted. If quantification isn't required and a grower simply wants to determine if grey mould is present or not, then a 1/10 dilution is normally sufficient. If a reading of < 15 is obtained then it can be assumed that the sample is free of Botrytis contamination. As the LFD is specific for Botrytis, it cannot be assumed that samples that return a LFD SI reading of < 15 are free from other fungi associated with the rotting of grapes. The Mologic kit is currently not available for sale in Australia. If the Australian wine industry were to adopt this technology, then a local distributor would need to be found.

The list of techniques to quantify grey mould contamination of grapes in this study is not exhaustive. There are other ways that grey mould could be either detected or quantified in grape samples which haven't been investigated in the current project. Some of these have been evaluated previously by the authors and a brief summary is provided here. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR) in conjunction with chemometric modelling and machine learning algorithms has been investigated to discriminate berries infected with either Botrytis, Aspergillus or Penicillium with some success (Schmidtke, et al. 2019). Less success was achieved with whole bunches limiting the usefulness of this method for bunch rot estimation for the wine industry. Fungal contamination of grapes and other food products leads to the formation of volatile organic compounds (VOCs) that have mouldy earthy characters (La Guerche, et al. 2006,Steel, et al. 2013). These can be used to detect fungal contamination and could potentially be used as a quantifiable marker of grey mould contamination of grapes (Schueuermann, et al. 2019,Steel, et al. 2020). The procedure to assay grapes samples for the presence of these undesirable aroma compounds requires access to sophisticated equipment such as GC/MS and the time required for analysis is of the order of days. As such, assay of grey mould contamination based on VOCs measurements is unlikely to be adopted by the wine industry unless more rapid methods can be developed. A further method using quantification of fungal chitin has been investigated for the detection of powdery mildew in grapevine leaves (Ayliffe, et al. 2013). In unpublished work we found that the phenolic profile of grape berry tissue infected with *B. cinerea* precluded measurements of chitin using fluorometric measurements

(Steel 2014). For the above reasons, these three alternative methods for quantifying grey mould contamination of wine grapes were excluded from the current study.

The project has led to an improved understanding of the advantages and the disadvantages of the different methods for Botrytis detection and quantification which will allow grape growers and winemakers to decide which of these methods are the most suitable for Botrytis estimation. Quantification of grey mould contamination of grapes based on the ergosterol content and measurement of Botrytis antigens using a field-based kit have been evaluated and found to be reliable measures of grey mould. Findings from the project will lead to improved methods of Botrytis detection and quantification of grey mould contamination. This has the potential to reduce the economic losses this major disease of grapes has on the wine industry.

Table 9. Comparison of the different methods of Botrytis detection and quantification evaluated in this study.

Technique	Process time for 20 samples	Specificity for Botrytis	Detection of hidden Botrytis *	Vineyard / weighbridge applicability	Operator skills required	Equipment requirements
Inspection with the naked eye (e.g. walk through vineyard)	Minutes	Potential errors depending upon operator skills	No - unless the bunch is dissected	Yes	Basic training	No specialised equipment
Hyperspectral imaging	Hours	Yes	No - unless the bunch is dissected	Possible with future adaptation	Ability to interpret data	Hyperspectral camera, appropriate software
Gluconic acid by enzymatic analysis	Hours	No	Yes – berry homogenates	Winery lab rather than vineyard	Low level of training, skills in basic lab analysis	UV/ VIS spectrophotometer
qPCR	Hours	Yes	Yes – berry homogenates	Possible with portable field qPCR machine	High level of skills in molecular biology	Thermocycler
LAMP	Hours	Yes, but not quantitative	Yes – berry homogenates	No	High level of skills in molecular biology	Nanodrop spectrophotometer
Ergosterol analysis	Days	No	Yes – berry homogenates	No	High level of skills in analytical chemistry	HPLC, equipment associated with organic solvent extraction
Antigen detection	Minutes	Yes	Yes – berry homogenates	Yes	Low level of training, skills in basic lab analysis	Hand-held lateral flow device cube reader

* Hidden Botrytis relates to grey mould that might be hidden from view, for example within the interior of a tightly packed cluster.

8. Recommendations

Quantifying the amount of grey mould present in a grape sample using a lateral flow device (LFD) for the detection of Botrytis antigens is a relatively rapid and simple process. It is recommended that this technology should be adopted for the assessment of grey mould contamination of wine grapes. The device manufactured by Mologic Ltd is currently not available for purchase in Australia, so a suitable distributor needs to be found. Homogenates routinely need to be diluted 1/100 in order to obtain a semi-quantitative result. For grape samples severely affected with grey mould contamination, further dilution beyond 1/100 is required, although with such samples the presence of Botrytis would be obvious by visual inspection with the naked eye. Assaying a 1/10 dilution in advance will determine the presence or absence of Botrytis. Presence of Botrytis using a homogenate diluted 1/10 can be assumed if the LFD SI reading is greater than 15.

If a grape grower seeks to know how much total fungal load is present in a batch of grapes prior to harvest and a result isn't required in less than two days, then measuring the ergosterol content of the grape sample is recommended. This would require access to HPLC instrumentation and skills in organic solvent extraction which may preclude the technique from in-house assessment in some wineries.

Using ergosterol as a determinant of grey mould contamination, our previous work proposed that thresholds for Botrytis contamination of Chardonnay grapes is between 0.28 and 0.87 g dry mass of fungus / kg fresh mass of grapes (Steel, et al. 2020). Based on the data obtained from Shiraz field samples collected in 2021 and using a 1/100 dilution of a berry homogenate, this equates to a Botrytis LFD SI of between 55 and 110.

Based on the experiment involving the individual detached Chardonnay berries and the experiment that investigated whole bunches of Chardonnay, Cabernet Sauvignon, Semillon and Shiraz, homogenates diluted 1/100 that return a LFD SI reading of 100 or 50 equate to 0.8 or 0.2 mg of ergosterol per kg fresh weight of grape sample. It is recommended that berry homogenates that return a LFD SI reading of >100 should be diluted further.

Irrespective of the method used to detect and quantify Botrytis, the vineyard or winery receival area sampling regime needs to be taken into account. If a certain part of the vineyard is known to have a history of bunch rot problems, then growers should initially evaluate these areas. With this in mind growers need to be cognisant of the sampling regime used and sample collection to ensure a true representative sample of the vineyard block is taken.

Additional studies on the impact grey mould contamination of grapes has on wine made from infected grapes would allow measurements of ergosterol and Botrytis antigens to be correlated with the likely level of wine quality that can be expected when the grapes are vinified.

In the absence of a Botrytis antigen detection kit, enzyme assays for gluconic acid provide an indication of overall fungal contamination and these are recommended if a mixture of bunch rotting organisms such as Aspergillus and / or Penicillium are suspected. Investigating the performance of additional gluconic acid enzyme assay kits for bunches infected with a mixture of Botrytis and other bunch rotting fungi is a possible avenue for future research.

As Botrytis attacks a wide range of horticultural crops other than grapevines, some of the techniques investigated in this research project are applicable to other horticulture industries. Ergosterol is not unique to Botrytis but is found in all fungal species. So, measuring fungal sterols may be an option to detect and quantify fungal contamination in other host plant–pathogen combinations. Furthermore, the Botrytis antigen detection kit used in this study could be equally applied to other horticultural industries where grey mould contamination needs to be monitored.

9. Appendix 1: Communication

Publications

Steel C C, Schwarz, L J, Qiu, Y, Schueuermann C, Blackman, J, Clark, A C and Schmidtke, L M 2020. Thresholds for Botrytis bunch rot contamination of Chardonnay grapes based on the measurement of the fungal sterol, ergosterol. Australian Journal of Grape and Wine Research **26**, 79–89.

Steel, C, Qiu, Y and Schwarz, L 2020. Possible biomarker for botrytis: could the measurement of the fungal sterol ergosterol be the answer? Wine and Viticulture Journal, Spring 2020 Volume 35, pp42, 44, 46, 48 & 50.

Qiu, Y and Steel, C C 2020. Comparison of Botrytis infection quantification methods using laboratory infected Cabernet sauvignon grape bunches. Grapevine Management Guide, 2020 -21. Editor; Fahey, D. Pp. 11- 13. Publ: NSW DPI, Orange, NSW.

Industry presentations and extension activities

Presentations

Qiu, Y., Steel, C., Blackman, J., Clark, A., & Schmidtke, L. (2019). Comparison of quantification methods for *Botrytis cinerea* biomass in grape bunches. Poster session presented at 17th Australian Wine Industry Technical Conference (AWITC 2019), Adelaide, Australia.

Steel, C. C. (2021). Bunch rots of grapes – detection and impacts on wine quality. Australasian Plant Pathology Society - NSW Branch Meeting, Orange, NSW. 9th April 2021.

Webinars

Steel, C C. Techniques for determining the levels of grey mould contamination of grapes. NWGIC Webinar, 28th October 2020.

Steel, C C. Quantitative methods for Botrytis grey mould detection and estimation in grapes. AWRI Webinar, 28th January 2021.

Television and radio news broadcasts.

Chris Steel - CSU Research: Measuring bunch rot impact on wine quality. March 2019.

Prime 7 Riverina News

WIN News Wagga Wagga

Regional Radio Victoria

ABC Radio Riverland

Appendix 2: Intellectual property

No intellectual property has arisen from the research.

Appendix 3: References

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Appendix 4: Staff that have been engaged on the Project.

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Appendix 5 - gBlocks gene fragment sequence

GCTATGAGTGACCCAAGGGCACGTCG AGATCCTGATCCTCGATGGGGACCCTA
ACCCTAACCCGGGGGGCCAGATACGC TTTACCTTGGTTCGACTGTAGTCCCTAG
GAACGCCCTCGGAGTGTCCTAGGAAT GCCCCCGGTGAGCCCTTGGTCTAAAG
CCGTATAGGTGACTAGTTAACCCCATAT AGTTTGTGCGAGTACACTACTACCGGT
GAGCAGGCTGTAATTTCAATGTGCAGA ATCTGTCCCCGGTGAGCCCAGGTCACC
TTGCAATGAGTGGACAGCATGTTTGAA ATGCGATTAATTGTTGCTCCCGGTGAG
CCCACTAAATAATTCTGAGAGTTGGCC ATCTCATATTTCATCCCCGGTGAGCCCA
AGATATACTTACCTAGAGGTGGTAAGT ATGGAGAGCAATTTCTCGGAAATTAAT
TTTATTTAGTGGAATCAATTGTGGGAG CAGAGCCTATACAAAAGGAGTC