Evaluation of the Global Access Diagnostics Botrytis rapid test method

FINAL REPORT TO WINE AUSTRALIA

Project Number: **CSU 2301**

Principal Investigator: **Christopher C Steel**

Research Organisation: **Charles Sturt University**

Date: **1st March 2024**

Project CSU 2301 Evaluation of the Global Access Diagnostics botrytis rapid test method

Chief Investigator and author:
Prof Christopher C Steel

Institution:
Gulbali Institute
Charles Sturt University
Locked Bag 588
Wagga Wagga
New South Wales 2678

Citing this report

This report may be cited as 'Steel, C C (2024), Evaluation of the Global Access Diagnostics Botrytis rapid test method, CSU 2301, Charles Sturt University, Wagga Wagga, NSW, 39 pp'.

Disclaimer:

1. Abstract

Grapes are typically assessed for grey mould by visual inspection, which is subjective and prone to error. This project investigated Botrytis Alert, a commercially available rapid antigen detection kit as an alternative to current industry practices for grey mould (*Botrytis cinerea*) detection. Six wine industry participants analysed 376 grape samples and a further 346 samples were analysed by Charles Sturt University. The genus specific nature of the Botrytis Alert kit provided a definitive and rapid quantitative determination of the presence of grey mould in grape samples, superior to visual assessments and other assessment measures, currently used in the wine industry.

2. Executive summary

Botrytis cinerea responsible for grey mould is a fungus that frequently attacks grape bunches, particularly if climatic conditions are conducive to infection. Aside from a loss of yield, wine made from grey mould affected grapes suffers from various faults, such as a loss of red wine colour, browning in white wine, formation of mouldy and earthy characters and diminished desirable aromas and flavours. As with any plant disease, early detection and ideally quantification of grey mould of grapes is essential for management decisions, including timing of harvest and grape processing procedures. Unfortunately, in the case of grey mould, the fungus responsible for the disease is often hidden from view within the interior of the bunch. Furthermore, the disease is harder to identify on red-skinned grape varieties and can sometimes be confused with other physiological or pathological disorders of the vine. Current wine industry practises largely rely on visual assessment of vineyards and grapes at harvest however this subjective method is fraught with difficulty given the fact that the fungus is often hidden within the interior of the bunch. A number of alternative methods for grey mould assessment are carried out by some grape growers and wine makers. These include measurement of gluconic acid, activity of the fungal enzyme laccase (which is responsible for wine oxidation), and in some cases measurement of grape and / or wine colour. Other methods of assessment, such as molecular methods (e.g. qPCR) and chemical analysis for fungal metabolites (e.g. ergosterol) are only applicable for the research laboratory and are not readily applied to a field situation where a rapid determination of the amount and severity of grey mould is required, (e.g. immediately prior to harvest). A further technique involving immunodetection where monoclonal antibodies are employed to detect fungal antigens does have potential to be packaged into a rapid field-based kit and a number of such devices have already been commercialised.

This project investigated the use of a commercially available Botrytis antigen detection kit, Botrytis Alert, that employs a hand-held reader. This kit is specific for Botrytis and will not detect other fungi, thereby removing the uncertainty that is often associated with subjective methods of detection.

The Botrytis Alert kit was evaluated alongside visual assessments of grey mould contamination and measurements of ergosterol, a sterol unique to fungal cells that can be used to quantify the amount of fungal biomass present in a grape sample. In laboratory studies, using hand harvested grapes from a number of vineyard sites and grape varieties, the Botrytis Alert antigen detection kit was found to be more reliable than visual assessments of grey mould. The linear relationship between the amount of fungus present in a grape sample and the signal intensity of the Botrytis Alert antigen detection kit was confirmed by adding known amounts of freeze-dried *B. cinerea* mycelium to clean grape sample homogenates.

Aside from the studies conducted in the research laboratory, the Botrytis Alert antigen detection kit was also evaluated by six wine industry participants. The industry participants used the Botrytis Alert antigen detection kit alongside whatever method they routinely used for grey mould evaluation. Analysis of the data gathered by the wine industry participants further confirmed that visual assessment of grey mould contamination is prone to error.

Some samples originally assessed (by visual inspection) as contaminated with grey mould by the wine industry participants were found to be uninfected when assessed with the Botrytis Alert antigen detection kit. Conversely some samples that were visually assessed as disease free were subsequently found to contain *B. cinerea*, and in some cases this was at relatively high levels of contamination. For other winery operators who were maybe more skilled in visually assessing grapes for grey mould, there was stronger correlation between their assessment of the amount of grey mould present and the signal intensity of the Botrytis Alert antigen detection kit.

The genus specificity of the Botrytis Alert antigen detection kit makes it a significantly more reliable and accurate method for Botrytis grey mould detection and quantification, than visual assessments and other methods currently used by the wine industry for Botrytis monitoring. Furthermore, the kit is suitable for field use, requires minimal training, and a result is obtained in a matter of minutes.

3. Background

Grey mould of grapes caused by the filamentous fungus *Botrytis cinerea* is one of several fungal diseases that affects the grapevine. Aside from affecting grape yield, the negative impact this fungus has on grape and wine quality is a serious issue for the wine industry (Steel, et al. 2013). Management of the disease during the growing season is hampered by a lack of suitable control options, furthermore the fungus frequently colonises the interior of the grape bunch and is hidden from view. Despite this, monitoring the vineyard by the naked eye is still the predominant method used to not only detect grey mould of wine grapes but also to estimate the amount of disease present in a vineyard or batch of grapes at harvest.

A number of alternative methods to visual inspection of vineyards to ascertain grey mould occurrence and severity have been explored and these have been described previously (Iland, et al. 2024, Steel 2021). Some of these alternative methods lack the specificity for *B. cinerea* and most are not suitable for the rapid analysis of grape samples during vintage. Of the previously reviewed methods, detection and potentially quantification of *B. cinerea* by measuring the levels of Botrytis antigens was highlighted in a previously funded Wine Australia research project as the most suitable for a wine industry setting (Steel 2021). The methodology involved in antigen detection is suitable for rapid field-based kits and has been applied to numerous situations where rapid diagnosis or detection is required, such as detection of human and animal infections (e.g. Coronavirus detection during the Covid 19 pandemic). The technology is not new, in fact a number of monoclonal antibodies were raised to *B. cinerea* more than thirty years ago, with the goal to develop an immunoassay for grey mould spoilage in horticultural crops (Dewey, et al. 1993, Meyer and Dewey 2000).

In the last decade or more, a number of companies have commercialised this technology and developed rapid detection kits that are genus-specific for Botrytis (Dewey, et al. 2013). The kits use the BC-12.CA4 monoclonal antibody, that is specific for the genus Botrytis and does not detect other fungi associated with the rotting of grapes, such as Aspergillus and Penicillium (Steel – unpublished observations). Optimization of the methodology to improve user friendliness and ease of use in a field situation has resulted in the recent development of a rapid Botrytis antigen kit named Botrytis Alert. The Botrytis Alert kit employs a cube reader that fits in the palm of the hand (https://www.botrytisalert.co.uk/) (Mologic 2024). A countdown timer is incorporated into the cube reader such that readings are recorded and stored after 10 minutes, thus eliminating operator errors when readings are not recorded at exactly 10 minutes after initiating the assay. As is the case with all such devices that employ a lateral flow device, there is a non-linear response in terms of the signal intensity with respect to the amount of Botrytis antigen present at higher readings greater than approximately 120 (Steel 2021), due to the antibody binding sites becoming saturated.

4. Project Aims

The aims of this project were to evaluate the suitability of Botrytis Alert, a commercially available lateral flow device (LFD) rapid antigen detection kit developed by Global Access Diagnostics (GADx), and trading as Mologic, (https://www.botrytisalert.co.uk/) (Mologic 2024) for the measurement of botrytis bunch rot in grape samples collected in the vineyard, or must samples taken at the weighbridge. To achieve this goal six wine industry participants were identified to trial the kit in an industry setting during the 2023 vintage. The industry participants were asked to compare Botrytis Alert for Botrytis detection and quantification with whatever method they typical used to assess Botrytis during vintage. Separate to this, the research team at Charles Sturt University (CSU) at Wagga Wagga collected grape bunches affected with grey mould from a number of vineyards in NSW and VIC. These bunches were visually assessed for the severity of grey mould infection and analysed using the Botrytis Alert antigen detection kit. The grape samples were also analysed for ergosterol, a fungal sterol indicative of fungal contamination which can be used to estimate the amount of fungal biomass present in a plant tissues (Porep, et al. 2014).

5. Methods

5.1 Validation of the Botrytis Alert antigen detection kit

Validation of the Botrytis Alert antigen detection kit by the research team at CSU, involved the collection of grape bunches from field sites with varying levels of grey mould infection as assessed by visual inspection. Aside from measuring Botrytis antigens in bunch homogenates, the fungal sterol ergosterol was quantified to determine the amount of fungal biomass present in a sample.

Isolates of *B. cinerea* were also freeze dried and known weights of the freeze-dried mycelium powder added to grape homogenates in order to obtain a calibration curve for fungal biomass versus signal intensity (SI) of the Botrytis Alert antigen detection kit. To achieve this, four *B. cinerea* isolates (ET4, TN080, TN162, and VRU0019) from the Botrytis culture collection of the Gulbali Institute at CSU were grown in Potato Dextrose Broth (PDB) for 7 days at 25 °C, after which time the cultures were filtered (Whatman number 1 paper) and the mycelium washed three times with sterile distilled water (SDW). The mycelium was harvested using a sterile spatula by gently scrapping the surface of the filter paper. The harvested mycelium was then freeze-dried and subsequently ground to a fine powder using liquid nitrogen and a pestle and mortar. Known weights of the freeze-dried fungal powder were then added to grape bunch homogenates, previously determined to be free of grey mould contamination based on analysis of Botrytis antigens. To ensure a uniform suspension of the freeze-dried mycelium powder in the grape juice, mixtures were shaken for a minute three times over an hour at room temperature. Control samples consisted of grape homogenates without the addition of any freeze-dried mycelium. The grape juice homogenates containing different amounts of freeze-dried mycelium by weight, were then assayed using the Botrytis Alert antigen detection kit as described above. The re-constituted fungal material in grape homogenates was then analysed for ergosterol and Botrytis antigens using the methods as described above.

5.2 Grape Sampling

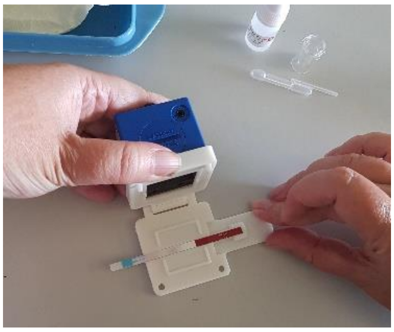
Grape bunches (346 individual grape bunches encompassing 10 different wine grape varieties) were sampled from 7 wine regions in SE Australia between February and April 2023 at grape maturity. The 8 regions included vineyards in Canberra, Hilltops, Hunter Valley, Mildura, Orange, Riverina, Rutherglen and Tumbarumba. Each bunch was visually assessed (by the naked eye) for grey mould severity, and the results recorded. Individually bunches were then homogenised, and aliquots of homogenates stored at – 20 °C for subsequent analysis.

5.3 Botrytis Antigen Analysis

Frozen grape bunch homogenates were thawed to room temperature, centrifuged (3000 *g*, 15 mins) and the supernatants collected for analysis using the Botrytis Alert antigen detection kit according to the manufacturer's instructions (Mologic 2024). In brief, this involved taking a 40 µL sample of the homogenate using a calibrated bulb pipette provided in the kit and adding this to 4 mL of sample buffer i.e. equivalent to a 1/100 dilution. Once mixed, a Botrytis test strip was dipped in the diluted sample for 15 seconds. The test strip was then immediately placed in the cube reader and the timer started (Figure 1). The test strip contains a control (C) and a test (T) line, and after ten minutes the signal intensity (SI) of the control and test line are recorded and stored until the operator initiates another test. The cube reader returns signal intensity (SI) readings of 0 to approx. 250. SI test line readings of < 15 are indicative that Botrytis is absent from the sample, while readings of > 15 indicate that Botrytis is present. The greater the amount of Botrytis (i.e. Botrytis antigens) that is present, the higher the SI reading for the test line. Thus, the kit provides a quantitative estimate of the amount of botrytis (and hence grey mould severity) in a grape sample.

(a) 

(a) A test strip is dipped into a grape sample diluted (1/100) in buffer for 15 seconds. (Sample dilution is achieved by taking a 40 µL sample using the dispensing bulb pipette provided with the kit and adding this to 4 mL of the sample buffer).

(b) 

(b) After 15 seconds the test is placed in the holder for the cube reader.

(c) 

(c) The cube reader is activated with a card and the 10-minute countdown timer commences.

(d) 

(d) The image indicates that there are 9 minutes and 55 seconds to go until this test is complete.

(e) 

(e) After 10 minutes have elapsed, the test reading is displayed on the screen. In this image, the signal intensity (SI) is 48, indicative that this sample is positive for B. cinerea *contamination. The reading remains on the screen until the operator initiates another test.*

Figure 1: The Botrytis Alert rapid antigen detection cube reader.

5.4 Ergosterol Analysis

After thawing to room temperate, grape bunch homogenates were analysed for the fungal sterol, ergosterol using a modified method as previously described (Steel, et al. 2020). In brief, homogenised grape must (20 g) was weighed into a 250 mL round bottom flask. Samples were saponified via a base hydrolysis reaction in a solution containing 50 mL Milli-Q water, 75 mL methanol, 50 mL ethanol (absolute, 100 % v/v) and 10 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 (25 mL). The filtrates were then transferred to a 250 mL separatory funnel. The flask was rinsed with 25 mL methanol and added to the separatory funnel. Ergosterol was extracted from the aqueous phase with 50 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 5 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 previously described (Steel, et al. 2020). 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).

5.5 Wine industry evaluation of the Botrytis Alert antigen detection kit

Six wine industry participants (identified as W1 to W6 in this report), from a range of operators and locations in SE Australia were invited to participate in the evaluation of the Botrytis Alert antigen detection kit developed by GADx. The participants were selected to ensure that a range of different grape growing regions and wine industry enterprises were included in the study. Prior to commencing the study, training on the use of the kit was provided either on site or via an instructional video created by the CSU research team involved with the project. Industry participants were asked to ideally analyse 50 to 100 samples using the Botrytis Alert kit and to also assess these samples for Botrytis by whatever technique they routinely used in their industry setting. Each industry participant was provided with an excel sheet to record details of their samples and their results. At the conclusion of vintage, the industry participants sent their data sheet containing details of their analyses and sample results to project team at CSU for data analysis.

5.3 Data Analysis

For data analysis the results from all experiments were imported into Matlab version 9.12.0.2009381 (R2022a -The Mathworks, Natick) for scatter plots with polynomial fittings.

6. Results/Discussion

6.1 Botrytis antigen and ergosterol analysis of grape bunches with visible symptoms of grey mould, collected from seven wine grape growing regions.

Grape bunches with different levels of perceived grey mould contamination, (based on visual assessment), were analysed for ergosterol and Botrytis antigens using the Botrytis Alert antigen detection kit. Samples that were found to have a signal intensity reading of 150 or greater with the Botrytis Alert antigen detection device were excluded from some of the analyses. The rationale behind this decision was twofold, first there is a non-linear response when measuring SI readings with any lateral flow device that relies on the intensity of a colour reaction at readings of greater than 100 to 150 and secondly, a grape grower should clearly be able to detect grey mould with the naked eye if the bunch is so severely affected with grey mould that it has a signal intensity reading of greater than 100.

Positive correlations were observed for all combinations of visual assessment, SI of the Botrytis Alert antigen detection kit (GADx SI) and ergosterol however the strongest correlation was observed in all instances where the Botrytis Alert antigen detection kit was compared with ergosterol rather than the other two parameters. Results are presented below, firstly for all whites and red varieties combined, and then subsequently for a selection of single varieties.

White grape (mixed varieties and wine regions) samples (n = 175) were analysed and data for the samples that had SI readings of < 150 are presented in Figure 2. For these samples the strongest correlation was found when the SI of the Botrytis Alert antigen detection kit was compared with measurements of ergosterol (r^2 = 0.5002). This was a stronger correlation than that for visual assessment and either the Botrytis Alert antigen detection kit (r^2 = 0.07644), or visual assessment and ergosterol (r^2 = 0.04835) (figure 2).

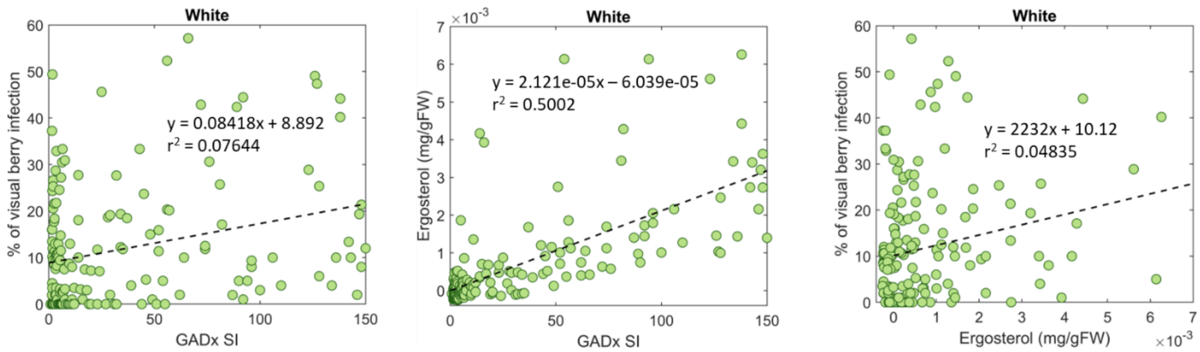


Figure 2: *Analysis of 175 grape bunches (white varieties) for grey mould infection based on visual assessment, Botrytis antigens and ergosterol content. Only bunches that had a SI reading of <150 are included in the analysis. GADx SI = Signal intensity of the Botrytis Alert antigen detection kit.*

Similar conclusions were made when the data for the red varieties was analysed (n = 93). The correlation between the SI of Botrytis Alert antigen detection kit and measurements of ergosterol (r^2 = 0.7759) was much stronger than when visual assessment of grey mould was compared with either the SI of Botrytis Alert device (r^2 = 0.1807) or ergosterol (r^2 = 0.2034) (Figure 3). As above, this indicates that visual assessment of grey mould was less accurate than either ergosterol or antigen detection.

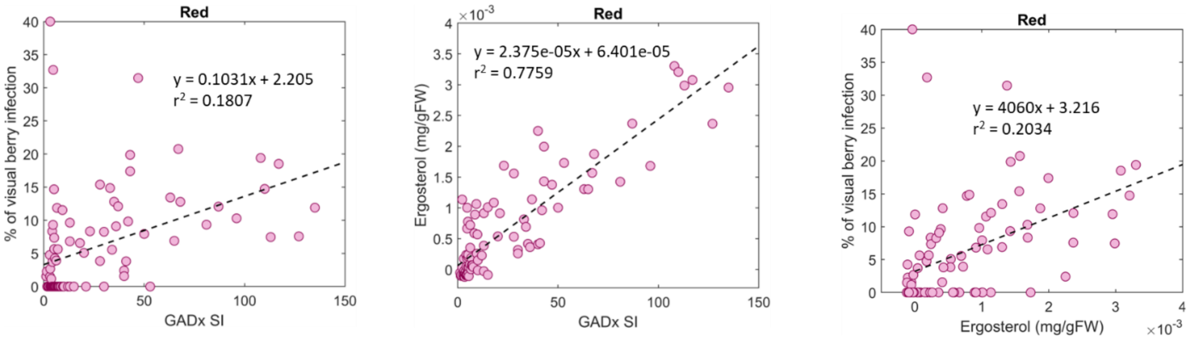


Figure 3: *Analysis of 93 grape bunches (red varieties) for grey mould infection based on visual assessment, Botrytis antigens and ergosterol content. Only bunches that had a SI reading of <150 are included in the analysis. GADx SI = Signal intensity of the Botrytis Alert antigen detection kit.*

Figure 4 below shows the combined results obtained for all red and white varieties examined for all bunches that were found to have a SI of < 150 when analysed using the Botrytis Alert antigen detection kit. The r^2 values for the three combinations were as follows, visual assessment vs. SI of the Botrytis Alert antigen detection kit, r^2 = 0.09955, ergosterol vs. SI of the Botrytis Alert antigen detection kit, r^2= 0.5496 and visual assessment vs. ergosterol r^2 = 0.06557.

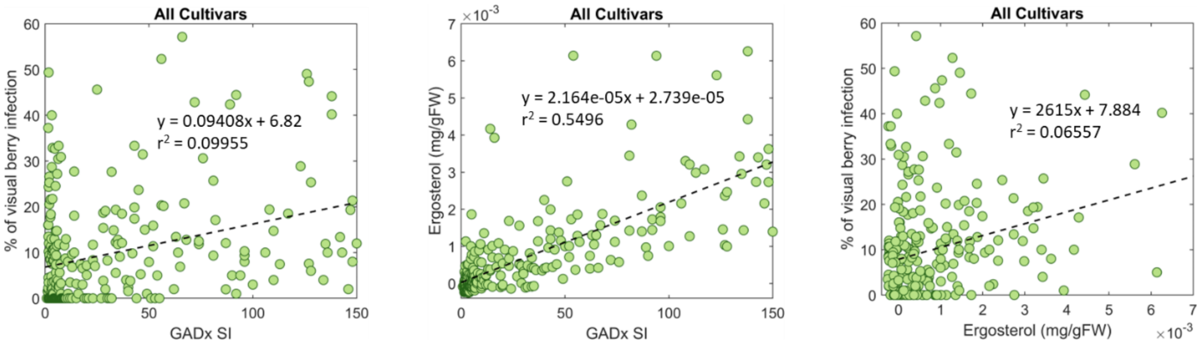


Figure 4: *Analysis of 268 grape bunches (both white and red varieties), for grey mould infection based on visual assessment, Botrytis antigens and ergosterol content. Only bunches that had a SI reading of <150 are included in the analysis. GADx SI = Signal intensity of the Botrytis Alert antigen detection kit.*

The greater the number of grape varieties included in an analysis, then the greater the scatter in the data, suggesting that grape variety, or possibly grape region was influencing the results. To illustrate this point, data from one grape growing region (i.e. Canberra) and one grape variety (i.e. Shiraz) is presented below in figure 5, where greater r^2 values were calculated than when data for all varieties was combined. The strongest correlation (r^2 = 0.8956) was still found for the SI of the Botrytis Alert antigen detection kit vs. ergosterol analysis.

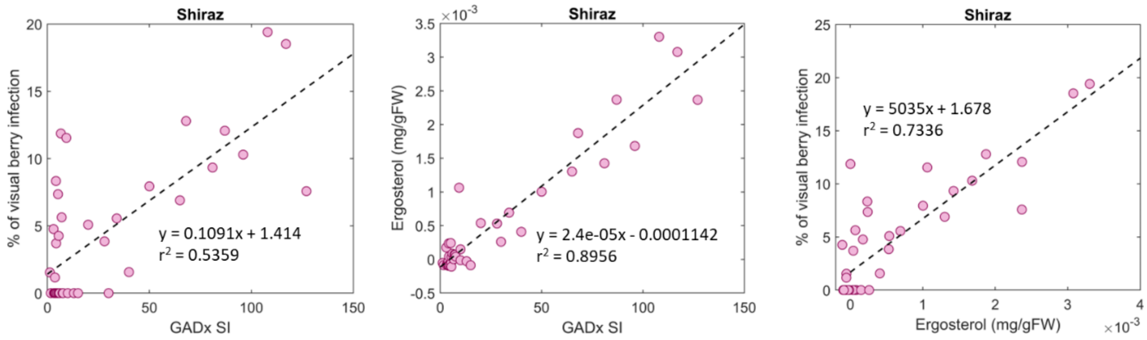


Figure 5: *Analysis of 42 Shiraz grape bunches for grey mould infection based on visual assessment, Botrytis antigens and ergosterol content. Only bunches that had a SI reading of <150 are included in the analysis. GADx SI = Signal intensity of the Botrytis Alert antigen detection kit.*

In the next example, results for one grape variety Chardonnay, collected from two cool climate grape growing regions, Orange and Tumbarumba were combined in the analysis. As above the strongest correlation in the analysis was when the data for the Botrytis Alert antigen detection kit were compared with the ergosterol content of the grape sample (r2 = 0.7767).

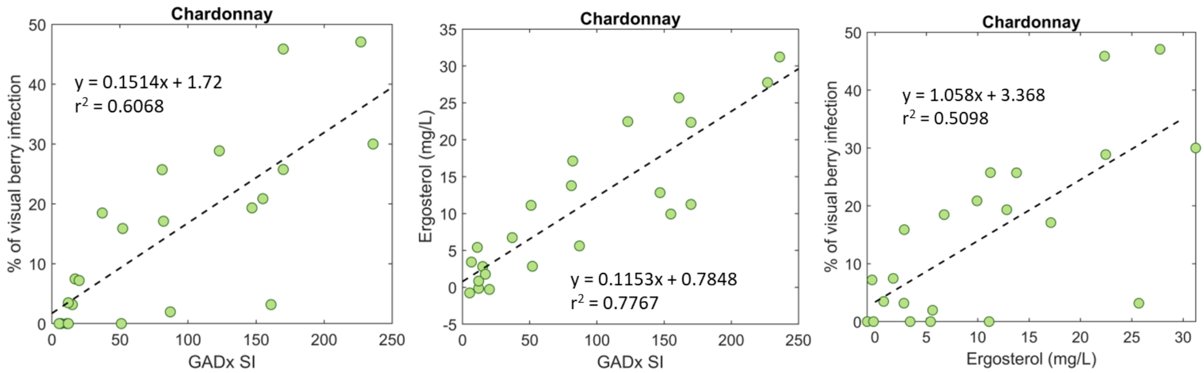


Figure 6: *Analysis of 22 Chardonnay grape bunches for grey mould infection based on visual assessment, Botrytis antigens and ergosterol content. In this analysis, all bunches were included, even those that had a SI with the Botrytis Alert antigen detection kit of greater than 150.*

Figure 7 below shows the analysis of 44 Sauvignon Blanc grape bunches collected from four grape growing regions (Griffith, Mildura, Orange and Wagga Wagga). As above for the other varieties examined, the strongest correlation was found between the Botrytis Alert antigen detection kit (GADx SI) and ergosterol analysis in the Sauvignon Blanc grape samples. It should be noted in this data set, that a number of these bunches were scored as having between 10 and 30% grey mould infection based on visual assessment, yet low amounts of ergosterol and botrytis antigens were recorded. This further confirms the inaccuracies and reliability issues with visual assessments of grey mould.

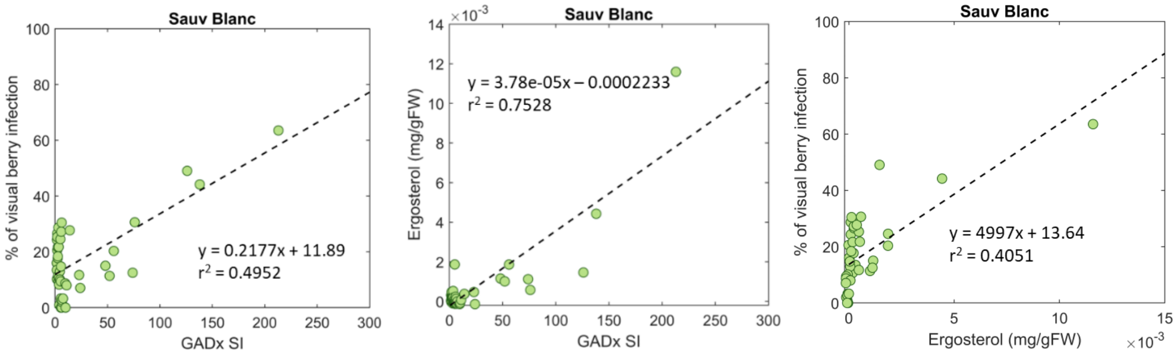


Figure 7: *Analysis of 44 Sauvignon Blanc grape bunches for grey mould infection based on visual assessment, Botrytis antigens and ergosterol content. In this analysis, all bunches were included, even those that had a SI with the Botrytis Alert antigen detection kit of greater than 150.*

Additional data for the other individual grape varieties, as well as data for samples, even those that had high levels of grey mould infection and returned SI readings of the Botrytis Alert antigen detection kit of greater than 150, can be found in Appendix 5.

6.2 Botrytis antigen analysis of grape bunches with atypical symptoms of grey mould and /or other pathological / physiological disorders

The specificity of the Botrytis Alert antigen detection kit for Botrytis was evident when grape bunches were analysed that had a rotten or mouldy appearance or were damaged in some other way and had an appearance that was not typical of grey mould. Some examples are shown in the table below together with some potential conclusions.

Variety	Wine region	Observation	SI Test	SI Control	Conclusions
Furmint	Hill Tops	Sunburn	5.6	270	Botrytis is absent
Chardonnay	Orange	Other rots?	6.6	247	Botrytis is absent
Chardonnay	Orange	Other rots?	15	248	Botrytis is absent or at very low levels
Pinot Noir	Orange	Brown colour	5.1	275	Botrytis is absent
Pinot Noir	Orange	Mycelium, uneven ripening	35	254	Botrytis present at relatively low levels. Other fungi are also present
Pinot Noir	Orange	Sun burn	2.2	273	Botrytis is absent
Pinot Noir	Orange	Sour rot, vinegar smell	3.2	258	Botrytis is absent
Riesling	Tumbarumba	Unidentified mycelium	25	230	Botrytis present at relatively low level. Other fungi are also present.
Riesling	Tumbarumba	Sooty Mould	3.6	267	Botrytis is absent

***Table 1:** Botrytis Alert analysis of grape bunches with rots and other disorders that did not appear to be grey mould. SI Test and SI Control relate to the signal intensity of the Botrytis Alert antigen detection kit.*

Some bunches were assessed as having atypical grey mould symptoms, probably as a result of other fungi growing concurrently on the same bunch. Antigen analysis confirmed that *B. cinerea* was also present in these bunch samples. Some examples of these bunch samples are in Table 2 below. From these results it can be concluded that Botrytis was present in all five of these grape bunches, even though typical symptoms of grey mould were not immediately apparent by visual assessment.

Variety	Wine region	Observation	SI Test	SI Control
Riesling	Tumbarumba	Unidentified mycelium	66	262
Riesling	Tumbarumba	Unidentified mycelium plus caterpillar	127	245
Riesling	Tumbarumba	Other fungi present	56	239
Chardonnay	Orange	Other fungi present	82	247
Chardonnay	Orange	Sour rot	134	241

***Table 2:** Botrytis Alert analysis of grape bunches with rots that appear to be mixed infections. SI Test and SI Control relate to the signal intensity of the Botrytis Alert antigen detection kit.*

6.3 Wine industry evaluation of the Botrytis Alert antigen detection kit

The aim of this part of the research was for wine industry personnel to evaluate the applicability of the Botrytis Alert kit for their winery operations during vintage. To this end, the wine industry participants were asked to evaluate the Botrytis Alert antigen detection kit alongside whatever method that they used routinely for the evaluation of grey mould in an industry setting at vintage.

Botrytis Alert antigen detection kits were distributed to the six wine industry participants (identified as W1 to W6), involved in this project in February / March 2023. On site training was provided for three of the participants while online training via an instructional video was carried out for the other three participants. A seventh wine industry participant also agreed to be involved with the trial but was unable to do so as vintage was nearing completion when the project commenced.

The level of engagement varied between the different participants. Aside from visual estimations of Botrytis contamination, carried out by all of the wine industry participants, gluconic acid, laccase, and red colour estimations were conducted by W1, W2 and W3 respectively, while W1 also looked at antigen detection in ferments.

The wine industry participants collectively analysed 376 samples for the presence of Botrytis antigens. Results for each of the six participants are presented below. The data for the six wine industry participants is presented separately, as each operator would have carried out their routine evaluation of grey mould differently (e.g. differences in the way visual assessment was carried out, and differences in interpretation of percentage infection).

6.3.1 Comparisons between visual estimations, gluconic acid and antigen detection.

W1 analysed a total of 86 grape samples, encompassing 13 different grape varieties. Samples were collected directly from the vineyard, at the weighbridge as sampling was taking place for the assessment of fungicide MRL requirements and colour assessment, as well as must samples taken at the Maselli in line analyser. Some later samples were also taken in the tank before or during fermentation. Aside from visual assessment, gluconic acid concentrations were also determined enzymatically. Grape juice and ferment samples were analysed with the Botrytis Alert antigen detection kit.

W1 is a winery that also produces dessert late-harvest Botrytised wine, so in the case of this wine industry participant, there was interest in confirming the presence of *B. cinerea* with respect to noble rot as opposed to grey mould. Aside from grape samples from the vineyard, ferments were also analysed. For this wine industry participant only, the amount of *B. cinerea* present in these samples was also calculated with reference to the calibration curve for signal intensity of the Botrytis Alert antigen detection kit vs. fungal dry weight (as calculated in section 6.4). Because of the high amount of *B. cinerea* present in grape samples destined for Botrytised wine, all grape and ferment samples were diluted prior to analysis using the Botrytis Alert antigen detection kit. The level of dilution varied from 1 in 20 to 1 in 25 depending upon the sample.

W1 sampled grapes from four blocks identified as block 1 to 4 and discussed below separately.

Results for Block 1

Block 1 was divided in to two sub-blocks identified as Block 1A and Block 1B and grapes samples were analysed three times from these two sections of the one vineyard. This was carried out to evaluate the progression of noble rot in April and May 2023. (Table 3).

		Grape sampling date		
Block		23/04/23	9/05/23	14/05/23
1A	Botrytis Alert SI test[1]	15	20	31
	Sample dilution factor[2]	20	25	25
	Botrytis Alert SI Control	236	220	223
	Gluconic acid (g / L)	0.68	1.07	2.46
	Botrytis concentration[3] (mg dry wt. fungus / g fresh wt. of ferment)	0.95	1.73	2.91
1B	Botrytis Alert SI test[1]	9.7	29	28
	Sample dilution factor[2]	20	25	25
	Botrytis Alert SI Control	230	226	227
	Gluconic acid (g / L)	1.21	1.61	1.65
	Botrytis concentration[3] (mg dry wt. fungus / g fresh wt. of ferment)	0.49	2.69	2.59

Table 3: *Analysis of Semillon grapes from Block 1 destined for late-harvest Botrytised wine at three time points in the vineyard. [1]Botrytis Alert SI test reading relates to the diluted sample. [2]Sample dilution factor relates to the dilution carried out for the Botrytis Alert antigen detection. [3] Botrytis concentration was calculated with reference to a standard curve obtained from the addition of known amounts of freeze-dried mycelium to a Shiraz grape homogenate known to be free of* B. cinerea *contamination and calculated using the following formula, Botrytis content (g DW/kg FW) = 0.0043 x SI - 0.017, with SI being the SI test result from the cube reader.*

The grapes from these two sub-sections of Block 1 were then combined and the ferments of these grapes were analysed in June 2023 at three successive time points during the fermentation process (Table 4).

	Ferment sampling date		
	6/06/23	9/06/23	26/06/23
Botrytis Alert SI test[1]	88	58	66
Sample dilution factor[2]	20	25	25
Botrytis Alert SI Control	230	205	198
Gluconic acid (g / L)	3.05	3.02	3.01
Botrytis concentration[3] (mg dry wt. fungus / g fresh wt. of ferment)	7.23	5.81	6.67

Table 4: *Analysis of late-harvest Botrytised wines made from Semillon grapes from a blend of grapes from blocks 1A and 1B at three time points during the fermentation process. [1]Botrytis Alert SI test reading relates to the diluted sample. [2]Sample dilution factor relates to the dilution carried out for the Botrytis Alert antigen detection. [3] Botrytis concentration was calculated with reference to a standard curve obtained from the addition of known amounts of freeze-dried mycelium to a Shiraz grape homogenate known to be free of B. cinerea contamination and calculated using the following formula, Botrytis content (g DW/kg FW) = 0.0043 x SI - 0.017, with SI being the SI test result from the cube reader.*

Results for Blocks 2 and 3.

Similar to block 1 above, grape samples were analysed from two vineyards, (identified as blocks 2 and 3), before harvest to evaluate the amount of noble rot in April 2023. For these blocks, the grapes were crushed separately keeping the grapes from the two different sections of the vineyard apart. Ferments were analysed in June 2023 at three successive time points during the fermentation process. Again, all samples were diluted prior to analysis using the Botrytis Alert antigen detection kit, the dilution varying from 1 in 20 to 1 in 25 depending upon the sample. Data for both grape and ferment samples for Botrytised dessert wine production are presented in Table 5.

Block		Grape	Ferments		
		23/04/2023	6/06/2023	9/06/2023	26/06/2023
2A	Botrytis Alert SI test	61	132	129	132
	Sample dilution factor[1]	20	20	25	25
	Botrytis Alert SI Control	187	202	198	205
	Gluconic acid (g / L)	1.76	1.63	1.60	1.56
	Botrytis concentration[2] (mg dry wt. fungus / g fresh wt of ferment)	4.91	11.01	13.44	13.77
2B	Botrytis Alert SI test	67	130	123	94
	Sample dilution factor[1]	20	20	25	25
	Botrytis Alert SI Control	217	225	225	205
	Gluconic acid (g / L)	1.88	1.82	1.80	1.81
	Botrytis concentration[2] (mg dry wt. fungus / g fresh wt of ferment)	5.42	10.84	12.80	9.68
3A	Botrytis Alert SI test	84	97	106	101
	Sample dilution factor[1]	20	20	25	25
	Botrytis Alert SI Control	208	198	218	220
	Gluconic acid (g / L)	1.27	3.79	3.74	3.79
	Botrytis concentration[2] (mg dry wt. fungus / g fresh wt of ferment)	6.88	8.00	10.97	10.43
3B	Botrytis Alert SI test	98	108	97	112
	Sample dilution factor[1]	20	20	25	25
	Botrytis Alert SI Control	245	224	204	203
	Gluconic acid (g / L)	0.66	2.18	2.12	2.19
	Botrytis concentration[2] (mg dry wt. fungus / g fresh wt of ferment)	8.09	8.95	10.00	11.62
3C	Botrytis Alert SI test	99	124	115	122
	Sample dilution factor[1]	20	20	25	25
	Botrytis Alert SI Control	219	241	209	223
	Gluconic acid (g / L)	1.06	2.28	2.24	2.28
	Botrytis concentration[2] (mg dry wt. fungus / g fresh wt of ferment)	8.17	10.32	11.94	12.69

Table 5: *Analysis of Semillon grapes from Blocks 2 and 3 and the subsequent late-harvest Botrytised wines made from these grapes at three time points during the fermentation process. Botrytis Alert SI test readings relate to the diluted sample. [1] Sample dilution factor relates to the dilution carried out for the Botrytis Alert antigen detection. [2] Botrytis concentration was calculated with reference to a standard curve obtained from the addition of known amounts of freeze-dried mycelium to a Shiraz grape homogenate known to be free of B. cinerea contamination and calculated using the following formula, Botrytis content (g DW/kg FW) = 0.0043 x SI - 0.017, with SI being the SI test result from the cube reader.*

Results for Block 4

In the case of Block 4, only the grapes rather than the ferment were sampled, and at one time point, but at six different locations in the one vineyard before harvest to evaluate the amount of noble rot in April 2023. As for blocks 1 to 3, the samples were diluted prior to analysis using the Botrytis Alert antigen detection kit, the dilution varying from 1 in 10 to 1 in 50 depending upon the sample. The results are presented in Table 6. The SI results for the samples from locations 1, 2 and 4 indicate that the dilution factor for these three samples was too great, since this resulted in SI readings of < 15.

	Sample location[3]					
	1	2	3	4	5	6
Botrytis Alert SI test	14	12	51	15	22	52
Sample dilution factor[1]	10	20	25	25	50	50
Botrytis Alert SI Control	229	241	231	192	210	231
Gluconic acid (g / L)	0.07	0.1	0.73	0.3	0.91	7.27
Botrytis concentration[2] (mg dry wt. fungus / g fresh wt of ferment)	0.43	0.69	5.06	1.19	3.88	10.33

Table 6: *Analysis of W1 grapes from Block 4 of a Semillon vineyard destined for late-harvest Botrytized wine. Grapes were sampled on 24th April 2023. Grapes were sampled from six separate sites in the one block. Botrytis Alert SI test readings relate to the diluted sample.*

[1] *Sample dilution factor relates to the dilution carried out for the Botrytis Alert antigen detection.*

[2] *Botrytis concentration was calculated with reference to a standard curve obtained from the addition of known amounts of freeze-dried mycelium to a Shiraz grape homogenate known to be free of* B. cinerea *contamination and calculated using the following formula, Botrytis content (g DW/kg FW) = 0.0043 x SI - 0.017, with SI being the SI test result from the cube reader.*

[3] *Sample location relates to different places in the one vineyard. The purpose was to examine the distribution of Nobel rot* (B. cinerea*) across a single vineyard block).*

The results for blocks 1 to 4 discussed above confirm the presence of *B. cinerea* in these grape and ferment samples. The SI values of the Botrytis Alert antigen detection kit are higher for the ferments than they are for the equivalent grape samples. This concentrating effect of the antigen is to be expected during grape processing. The results also confirm the stability of the antigen during the fermentation process.

Gluconic acid as a measure of grey mould contamination.

W1 routinely measures gluconic acid as an indicator of grey mould ('bad Botrytis') as opposed to noble rot ('good Botrytis'). W1 analysed 43 grapes samples consisting of the following grape varieties, Cabernet Sauvignon, Chardonnay, Durif, Merlot, Pinot Noir, Red Frontignac, Ruby Cabernet, Sauvignon Blanc, Semillon, Shiraz and Traminer.

Gluconic acid (up to 0.4 g / L) was detected in a number of grape samples that were scored as being free of grey mould based on visual assessment (Figure 8a). This could be due to misidentification of grey mould. Gluconic acid formation is not unique to *B. cinerea*. *Aspergillus*, another species of fungus, also infects grapes under warm dry conditions and can produce significantly more gluconic acid than *B. cinerea* (Lorenzini, et al. 2013,Shindia, et al. 2006).

Similar results were also obtained with the Botrytis antigen detection kit. A number of samples were scored as free of grey mould contamination, yet the presence of Botrytis antigens indicated otherwise (Figure 8b).

Gluconic acid was detected in some samples that tested both positive and negative for the presence of Botrytis antigens.

For three of the grape samples analysed by W1, the SI of the Botrytis Alert antigen detection kit was > 180, of these, two had gluconic acid levels of > 1 g /L while the level of gluconic acid in the third sample was only 0.2 g /L. (Figure 8c).

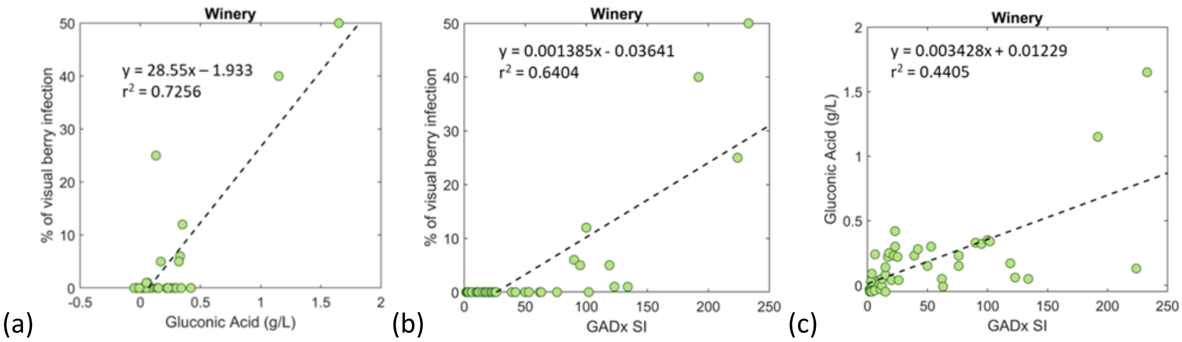


Figure 8: *Analysis of 43 grape bunches by W1 for grey mould infection based on visual assessment, Botrytis antigens and gluconic acid content. All bunches analysed by W1 that were assessed for grey mould, irrespective of the SI with the Botrytis Alert antigen detection kit were include in the analysis. GADx SI = Signal intensity of the Botrytis Alert antigen detection kit.*

6.3.2 Comparison between visual estimations, laccase activity and antigen detection.

W2 analysed 29 samples, of these only two were recorded as having grey mould contamination based on visual observations. In addition to assessing the samples using the Botrytis Alert kit, W2 also analysed all samples for laccase using the syringaldazine assay.

W2 analysed 27 samples that were recorded as free of any grey mould contamination based on visual assessment. Of the 27 apparently clean samples, two of the Shiraz samples had SI readings of 23 and 53 indicating the presence of Botrytis. These two Shiraz grape samples were negative for laccase. All the other samples assessed as being free of grey mould contamination returned SI values of < 12, confirming the absence of Botrytis (Table 7).

W2 also analysed two grape samples (one Chardonnay and one Shiraz) that they identified as having symptoms of grey mould, although the level of grey mould severity as assessed by visual inspection, was not recorded. Both of these samples returned signal intensity readings with the Botrytis Alert kit of 179 and 197 respectively, indicative of a relatively high level of grey mould contamination. These two samples also tested positive for the presence of laccase.

These grey mould affected samples were then mixed with additional grapes of the same variety that were assessed as free of grey mould contamination (exact proportions not recorded).

These mixed samples were analysed as described above. Both tested negative for laccase but returned SI values with the Botrytis Alert kit of 26 and 49 respectively, indicative that *B. cinerea* could still be detected in these samples that were diluted with healthy uninfected grapes (Table 8). This suggests that the Botrytis Alert kit is a more sensitive method for determining grey mould contamination than is determination of laccase enzyme activity.

Variety	SI Test	SI Control	Laccase activity[1]
Chardonnay	2.5	259	0
Chardonnay	2.7	271	0
Chardonnay	2.9	281	0
Chardonnay	3.9	275	0
Chardonnay	3.9	276	0
Chardonnay	10	255	0
Chardonnay	11	270	0
Chardonnay	12	280	0
Durif	1.8	256	0
Durif	2.6	260	0
Durif	2.7	257	0
Durif	4.8	258	0
Muscat Gordo Bianco	1.7	260	0
Semillon	2.2	268	0
Semillon	2.6	257	0
Semillon	2.9	279	0
Semillon	3.7	255	0
Semillon	3.7	282	0
Shiraz	0	242	0
Shiraz	4.1	248	0
Shiraz	6.6	266	0
Shiraz	8	248	0
Shiraz	25	267	0
Shiraz	53	260	0
White Frontignac	3	286	0
White Frontignac	5.7	275	0
White Frontignac	9.3	276	0

Table 7: *Levels of Botrytis antigens and laccase activity in 27 grape samples assessed as free of grey mould based on visual assessment. Botrytis antigens were detected in two of these 27 grape samples.*

[1]Laccase activity expressed as enzyme Units / mL based on oxidation of syringaldazine determined using a Kone lab.

Sample number	Variety and sample details	SI Test	SI Control	Laccase activity[1]
1	Chardonnay grapes with visual symptoms of grey mould	179	223	0.02
1 a	Chardonnay grapes with visual symptoms of grey mould mixed with uninfected grapes.	26	267	0
2	Shiraz grapes with visual symptoms of grey mould	197	247	0.09
2 a	Shiraz grapes with visual symptoms of grey mould mixed with uninfected grapes.	49	277	0

Table 8: *Levels of Botrytis antigens and laccase activity in 2 grape samples (1 and 2) that had symptoms of grey mould contamination as detected by the naked eye. These same two samples were then mixed with uncontaminated grapes assessed as free of grey mould based on visual assessment and re-analysed. [1]Laccase activity expressed as enzyme Units / mL based on oxidation of syringaldazine determined using a Kone lab.*

6.3.3 Comparison between visual estimations, red colour and antigen detection.

W3 concentrated on only red grape varieties and had a focus on colour measurements as an indicator of grape quality. W3 analysed 186 grape samples comprising eight different red varieties sampled from a number of different grape-growing regions between April and May 2023. Visual assessment was scored either as negative (i.e. absent) or positive (i.e. present) for grey mould but no indication of the extent (i.e. severity) of grey mould infection was recorded.

The visual assessment for grey mould contamination conducted by W3 did not correlate with the results obtained with the Botrytis Alert antigen detection kit. As an example, out of 35 samples analysed from one of the eight vineyards examined, 26 of these samples were scored as free of grey mould. Despite this, the SI readings for the antigen detection device for these 26 samples were all > 50.

Furthermore 17 of these 26 samples had SI readings that were >100 (Table 9). Typically, grey mould is immediately apparent to the naked eye in a sample that has a SI reading of greater than 100 with the Botrytis Alert antigen detection device.

Combining the data for all 186 samples analysed by W3 revealed that there was no correlation between the level of Botrytis antigens as determined by the SI readings of the Botrytis Alert antigen detection kit and any of the parameters used for colour estimation i.e. Abs at 420nm (r^2 = 0.01191), Abs at 520nm (r^2 = 0.0001971), colour intensity (r^2 = 0.0894) and hue (r^2 = 0.0111) (Figure 9).

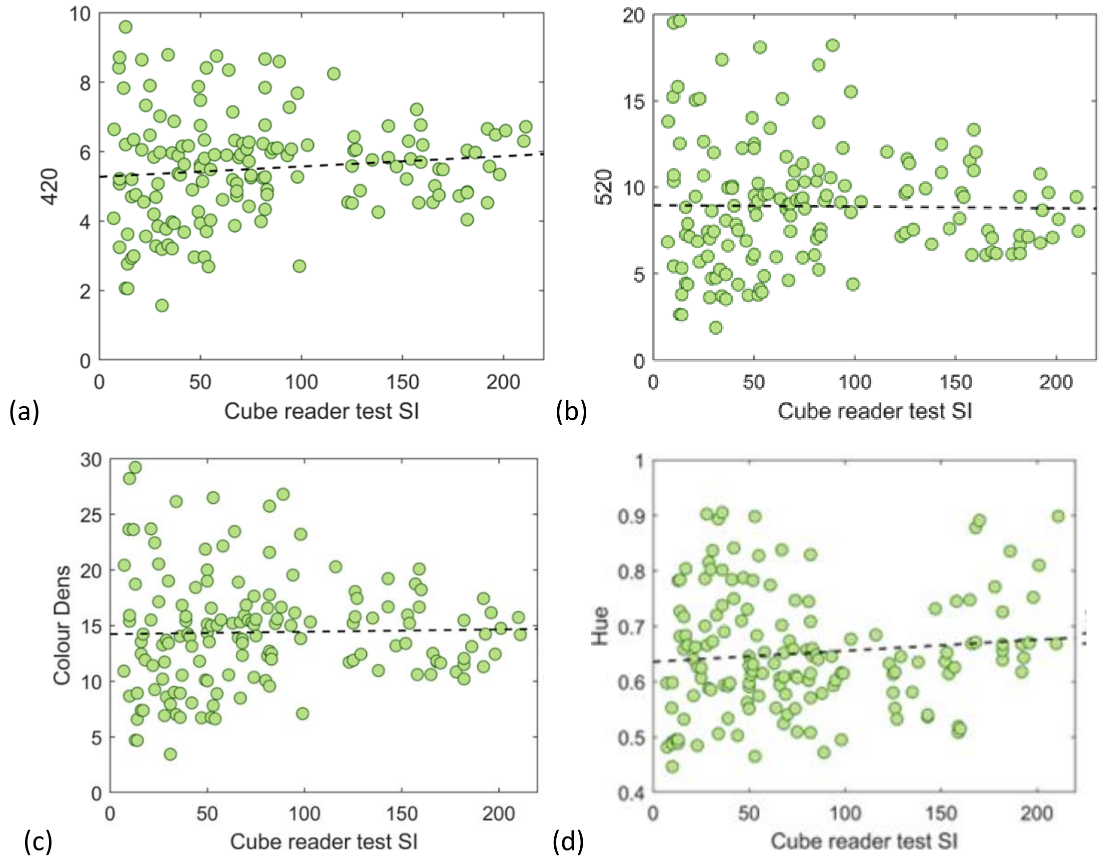


Figure 9: Colour quality parameters for 186 grape samples analysed with the Botrytis Alert antigen detection kit (Cube reader test SI), (a) Abs_{420nm} (r^2 = 0.01191), (b) Abs_{520nm} (r^2 = 0.0001971), (c) colour density (r^2 = 0.0005737) and (d) Hue (r^2 = 0.0111).

Variety[1]	SI Test	SI Control	Abs $_{420nm}$	Abs $_{520\ nm}$	Hue	Colour Density	Hue F	Visual assessment
SHZ	23	271	3.560	5.684	0.626	9.245	0.000	NEG
SHZ	37	280	3.931	6.624	0.594	10.555	0.003	NEG
CAS	57	268	5.909	9.613	0.615	15.522	0.000	NEG
CAS	64	255	8.348	15.114	0.552	23.462	0.038	POS?
CAS	66	284	5.188	8.735	0.594	13.923	0.006	NEG
CAS	68	266	5.084	8.356	0.608	13.441	0.038	NEG
CAS	74	273	6.273	11.379	0.551	17.653	0.003	NEG
CAS	75	274	5.317	8.761	0.607	14.079	0.003	NEG
CAS	80	250	3.990	6.070	0.657	10.060	0.001	NEG
CAS	82	265	6.764	11.001	0.615	17.765	0.006	NEG
CAS	82	260	8.665	17.059	0.508	25.724	0.043	NEG
CAS	98	271	5.272	8.556	0.616	13.828	0.004	NEG
SHZ	99	282	2.703	4.397	0.615	7.099	0.004	NEG
CAS	123	259	4.542	7.179	0.633	11.720	0.001	NEG
CAS	126	268	6.031	9.767	0.617	15.799	0.006	NEG
SHZ	127	262	6.060	11.385	0.532	17.445	0.034	NEG
CAS	135	267	5.760	9.915	0.581	15.675	0.007	NEG
CAS	138	265	4.259	6.704	0.635	10.963	0.000	NEG
SHZ	143	268	5.823	10.861	0.536	16.684	0.037	NEG
CAS	147	249	5.570	7.614	0.732	13.184	0.051	POS
SHZ	152	240	5.212	8.190	0.636	13.402	0.040	NEG
CAS	158	259	4.528	6.078	0.745	10.606	0.038	NEG
SHZ	159	291	6.767	13.315	0.508	20.081	0.031	NEG
SHZ	160	282	6.192	12.032	0.515	18.224	0.020	NEG
CAS	165	254	4.539	6.071	0.748	10.609	0.041	NEG
CAS	168	272	4.748	7.074	0.671	11.822	0.002	NEG
CAS	168	256	5.498	6.263	0.878	11.761	0.049	POS
CAS	170	259	5.486	6.158	0.891	11.643	0.053	POS
CAS	178	267	4.722	6.123	0.771	10.845	0.044	NEG
CAS	182	258	4.043	6.162	0.656	10.205	0.025	NEG
CAS	186	252	5.972	7.147	0.836	13.119	0.133	POS
CAS	192	258	4.527	6.783	0.667	11.310	0.011	NEG
SHZ	196	285	6.484	9.678	0.670	16.162	0.072	NEG
CAS	198	253	5.341	7.101	0.752	12.442	0.025	POS
CAS	201	256	6.604	8.151	0.810	14.755	0.162	POS

Table 9: *Levels of Botrytis antigens, colour quality parameters and visual assessment for the presence (POS) or absence (NEG) of grey mould contamination in 35 grape samples collected from one grape growing region in South Australia. Samples are ranked from lowest to highest test signal intensity with the Botrytis Alert antigen detection kit. [1]CHD = Chardonnay, SHZ = Shiraz.*

6.3.4 Wine industry participants that compared the antigen detection device only with visual estimations of grey mould contamination.

Three of the wine industry participants (W4, W5 and W6), only evaluated the Botrytis Alert antigen detection kit with respect to visual assessment. This is understandable, as for a large proportion of the grape and wine industry, grey mould incidence (as is the case with other vine pests and diseases), is assessed only by visual inspection with the naked eye.

W4 operates a number of vineyards in SE Australia. This collaborator focused on grape samples collected from the vineyard and analysed 99 grape samples that were collected at various time points from 1st to the 24th March 2023. The percentage of infected berries in the bunch and the percentage of the bunch infected with grey mould were recorded along with visual observations of grey mould occurrence (Table 10). Based on visual assessment, 18 of the 99 grape samples analysed by W4 were recorded as having symptoms associated with grey mould. However, these observations did not correlate with the results obtained with the Botrytis Alert antigen detection kit. The SI readings of the Botrytis Alert kit for six of the samples that were identified as having grey mould was < 15. Similarly 16 samples that the grower identified as being free of Botrytis had SI readings of the Botrytis Alert kit > 40. Given the specificity of the antigen detection kit, these findings suggest that grey mould was mis-identified by W4, and that symptoms were confused with either a different fungal pathogen or a physiological problem which might have included sun damage, berry shrivel or some other undefined disorder.

Variety [1]	SI Test	SI Control	% of bunch infected	% infected berries in bunch
PIG	5.8	273		
CHA	7.6	247		
MER	8	272		
SAB	8.5	246	8	12
CHA	9	246		
COL	9.6	263		
CAS	11	265		
CHA	11	269		
CHA	11	245		
PIG	11	263		
CHA	12	237		
CHA	12	264		
CHA	13	268	1	5
PIG	13	247		
SAB	13	254	8	12
CHA	14	262	1	5
CHA	14	252	1	5
SHZ	14	233		
CHA	15	268	1	5
CHA	15	266		
SHZ	15	274		
SHZ	15	257		
CHA	16	263	1	5
PIG	16	243		
PIG	16	271		
CHA	17	260	1	5
CHA	17	270		
CHA	17	239		
GOR	17	258		
MOS	17	249		
PIG	17	259		
WHF	17	261		
SAB	18	249	5	5
CAS	19	262		
GOR	19	260		
PIG	19	257		
CHA	20	260	1	5
CHA	20	272	1	5
CHA	20	273		
CHA	20	237		
PIG	20	228		
PIG	20	261		
SHZ	20	279		
CHA	21	265		
PIG	21	262		
SHZ	21	273		

SHZ	21	262		
CHA	22	259		
PIG	22	246		
PIG	22	263		
CHA	23	247		
PIN	23	264		
FIA	24	264		
SAB	24	252	5	5
SHZ	25	261		
PIG	26	229		
SAB	26	257	8	12
SAB	26	283		
SAB	26	252		
CAS	27	204		
CHA	27	252		
SAB	27	275	5	5
SHZ	27	261		
CHA	28	268	1	5
CAS	29	268		
SAB	30	263	8	12
CAS	31	266		
SHZ	31	265		
CHA	32	281		
FIA	32	245		
PIG	33	269		
FIA	34	252		
PIG	34	241		
PIG	34	275		
PIG	34	250		
PIG	34	262		
SHZ	34	261	5	
SHZ	34	273		
SEM	35	237		
CHA	36	254	1	5
FIA	36	248		
PIG	36	254		
FIA	43	267		
PIG	44	254		
PIG	44	247		
PIG	45	240		
SHZ	46	240		
SAB	48	255		
PIG	49	263		
SHZ	49	258		
RHR	50	248		
PIG	55	259		
CHA	56	262		
CAS	59	254		
SAB	61	273	5	5
PIG	63	270		
SHZ	68	257		
SHZ	69	278		
PIG	85	242		

***Table 10**: Assessment of 99 grape samples for visual symptoms of grey mould and presence of Botrytis antigens using the Botrytis Alert antigen detection kit conducted by W4. Samples are ranked from lowest to highest test signal intensity with the Botrytis Alert antigen detection kit.*

[1] CAS = Cabernet Sauvignon, CHA = Chardonnay, GOR = Muscat Gordo, FIA = Fiano, MER = Merlot, MOS = Moscato Giallo, PIG = Pinot Gris, RHR = Rhine Riesling, SAB = Sauvignon Blanc, SHZ = Shiraz and WHF = White Frontignac.

W5 and W6 examined 7 and 3 grape samples respectively (Table 11). Despite the low number of samples analysed by W5 and W6, and the rather vague observations on the amount grey mould estimated to be present, the results from the antigen detection device are consistent with the visual observations of grey mould contamination.

Industry Participant	Grape variety	SI Test	SI Control	Grower observation[1].
W5	Petit Verdot	59	249	25%
W5	Petit Verdot	66	252	25%
W5	Petit Verdot	5.7	268	0%
W5	Colombard	90	246	25%
W5	Colombard	92	251	25%
W5	Colombard	4.3	244	0%
W5	Montepulciano	10	249	Not recorded
W6	Shiraz	21	287	Not recorded
W6	Chardonnay	39	286	Bit of Botrytis - visual 30%
W6	Merlot	9.6	306	Not recorded

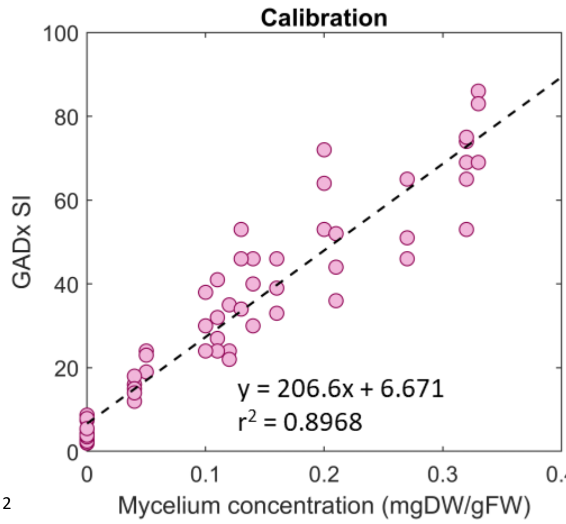
Table 11. Assessment of 10 grape samples for visual symptoms of grey mould and presence of Botrytis antigens using the Botrytis Alert antigen detection kit conducted by W5 and W6. [1]Grower observations are reproduced verbatim as submitted by the industry participants.

6.4 The relationship between Botrytis Alert antigen detection kit LFD SI and *B. cinerea* biomass

In order to clarify the quantification of Botrytis biomass present in a grape sample that was assessed with the Botrytis Alert antigen detection kit, four isolates of *B. cinerea* from the culture collection of the Gulbali Institute were grown in potato dextrose broth (PDB), the mycelium harvested, freeze-dried and known amounts of dry weight (mg) of the combined isolates added to a known weight (g) of Shiraz grape juice homogenate that was confirmed to be free of *B. cinerea* based on prior analysis using the Botrytis Alert antigen detection kit.

The Shiraz grape juice homogenates containing the suspension of the freeze-dried *B. cinerea* mycelium were then analysed using the Botrytis Alert antigen detection kit as described above and SI recorded.

There was a linear relationship between the amount dry weight of mycelium added to the grape homogenate and the SI of the Botrytis Alert antigen detection kit (r^2 = 0.8968). All control samples that were not amended with fungal mycelium had SI readings of < 10 (Figure 10).



2

Figure 10: *Relationship between dry weight of a suspension of freeze-dried* Botrytis cinerea *mycelium in Shiraz grape juice homogenate and the signal intensity of the Botrytis Alert antigen detection kit (Gadx SI). Freeze-dried mycelium from four isolates of B. cinerea (ET4, TN080, TN162 and VRU0019) was combined for this experiment.*

From the above data, the amount of Botrytis biomass present in grape samples was then calculated using the following formula;

Botrytis content (g DW/kg FW) = 0.0043 x SI - 0.017, with SI being the SI test result from the cube reader.

7. Outcome/Conclusion

Several conclusions can be drawn from this research. Based on the assays performed by some of the wine industry participants, one could conclude from the data that antigen detection is an inaccurate method and provides a poor estimation of the presence and amount of grey mould in a grape sample. An alternative and more plausible conclusion, is that current methods and in particular visual observations, are flawed when used for the assessment of grey mould contamination of grapes.

The Botrytis Alert detection kit is specific for the genus Botrytis, and *B. cinerea* is the only species within this genus that infects grapes and causes grey mould. Thus, there is no doubt, that if Botrytis antigens are found in a grape sample, then *B. cinerea* must be present. This leads one to conclude that the second of the two explanations above is therefore the correct one.

It is not surprising that visual observations are somewhat inaccurate for the assessment of grey mould contamination of grapes. Grey mould is often hidden from view within the interior of the bunch and out of immediate sight to the naked eye. Furthermore, detecting grey mould on red skinned varieties is more difficult than on white skinned varieties. It may be that some assessors mistook other diseases and physiological disorders, (such as sour rot, berry shrivel and heat damage) with the symptoms of grey mould.

Assessing grey mould contamination on the basis of colour quality measurements is flawed. Grape colour is influenced by many factors in the vineyard. Abiotic factors such as the temperature of the growing season and water availability have significant effects on the concentration of the berry anthocyanins responsible for red colour in the skin of the grape (Iland, et al. 2024). While Botrytis infection can lead to diminished pigmentation in grapes, it is generally at the post-crushing stage that laccase and other oxidative enzymes that are of fungal origin (Steel, et al. 2013), are likely to play a role in the loss of red colour.

Measuring laccase and gluconic acid do not appear to be as sensitive as measuring Botrytis antigens for the detection of grey mould of grapes. The values of these assays are that they provide other information that cannot be obtained by measuring fungal antigens. Laccase provides an indication of the likelihood of oxidation while gluconic acid and the ratio to glycerol can be an indicator of grey mould as opposed to noble rot. However, neither laccase nor gluconic acid are specific to the growth of *Botrytis cinerea*, since both can be produced by other fungi that could potentially attack grapes at harvest (Barad, et al. 2014,Guetsky, et al. 2005,Lorenzini, et al. 2013,Meunier and Steel 2009) .

Assessment of grey mould contamination of grapes by quantifying the amount of ergosterol present in a grape sample has been investigated under laboratory (Porep, et al. 2014) and wine industry conditions (Porep, et al. 2015). Ergosterol is present in virtually all filamentous fungi and yeasts, so as is the case with other measures of fungal metabolites and enzymes, ergosterol is not specific for *B. cinerea*. Nevertheless, measuring the amount of ergosterol present in a batch of grapes does provide an indication of the amount of total fungal biomass present (Steel, et al. 2020). Measurements of ergosterol are not readily applicable to a field situation. Sophisticated analytical equipment is required, and a minimum analysis time is of the order of hours to days depending upon the sample number.

Measuring grey mould based on the presence of *B. cinerea* antigens is a much more specific method of detecting and quantification of grey mould. Furthermore, in kit form, the presence or absence of *B. cinerea* and hence grey mould can be confirmed in a matter of minutes.

Informal feedback received form one or more of the industry participants in this research project indicated that the Botrytis Alert antigen detection kit was simple to use and could be incorporated into their wine production processes that involve onsite grape processing. Other participants indicated that they thought the kit was easy to use and could see the value in obtaining a rapid assessment of grey mould during vintage, while another participant felt the Botrytis Alert kit was more suitable for assessment for grapes in the vineyard prior to arrival at the winery. Future research might involve evaluation of a kit such as Botrytis Alert for vineyard sampling during the growing season to assess the progression of Botrytis infections and inform harvest decisions around timing. Some participants were less enthusiastic about adopting antigen detection of Botrytis. This may be a sign that they believe (potentially in error) that their existing methods for grey mould detection and quantification are satisfactory.

With respect to the relationship between the Botrytis Alert antigen detection kit and the amount of *B. cinerea* biomass present in grape tissue based on the measurement of ergosterol in the grape bunches analysed by the CSU researchers, this relationship was stronger for individual grape varieties than it was for all grape varieties combined.

There are several possible reasons for this. Firstly, the amount of ergosterol and / or antigen produced by *B. cinerea* varies depending upon the grape variety the fungus is growing on. Alternatively, the amount of ergosterol and / or antigen produced by *B. cinerea* may be influenced by the climatic conditions of the grape growing region and even the growth phase of the fungus, (e.g. sporulating vs mycelial growth). All of these explanations are plausible and would require further investigation.

It is a well-known fact that eukaryotic cells regulate the amount of sterol, (ergosterol in the case of fungal cells), in response to the temperature of the environment. Higher amounts of sterols are found in the cell membrane when an organism is growing under warmer conditions and conversely low amounts when the same organisms is grown under cool conditions (Aaronson, et al. 1982).

Steel et al. (2020) demonstrated that the average amount of ergosterol in three *B. cinerea* isolates increased from 1.45 (+/- 0.21) mg/ g dry weight of mycelium when grown at 15 °C to 3.22 (+/- 0.85) mg/ g dry weight of mycelium when grown at 27.5° C. This variability with temperature may explain the less than perfect relationship between ergosterol and measurements of fungal antigens when examining infected grape bunches from different grape growing regions which will vary in climatic conditions.

The nature and chemical composition of the antigens produced by *B. cinerea* have not been characterised. Furthermore, it is not known if or how factors such as the composition of the grape tissue (i.e. variety), the growth phase of the fungus, and environmental factors (e.g. temperature, light) influence antigen expression. One can draw parallels here with laccase production by *B. cinerea*, which is only expressed when the fungus is invading the grape tissue and when a suitable substrate is present.

One or all of the above explanations may account for some of the discrepancies between antigen production and the amount of grey mould present in grapes when examining multiple grape varieties and regions together. More work is thus needed to characterise the nature of the Botrytis antigen and the factors that lead to its expression by the fungus.

Despite these caveats, detection of grey mould using Botrytis Alert appears to be a significantly more reliable and accurate method than some of the other methods for grey mould detection currently employed by the wine industry.

8. Recommendations

A more objective measure of grey mould contamination is required for the wine industry. Some of the advantages and disadvantages of current methods have been reviewed previously (Steel 2021). The wine industry needs to be aware that existing methods of grey mould assessment are in some cases inaccurate, potentially flawed, and misleading. Whatever method is used must be one that is simple that requires minimal training, is applicable to a winery situation, accurate, quantitative and rapid, particularly during vintage. Use of a lateral flow device to detect Botrytis antigens, such as the Botrytis Alert antigen detection kit is such a tool that meets these requirements.

It is recommended that measuring Botrytis antigens should be adopted by the wine industry as an alternative method to provide a more definitive estimation of the presence of *B. cinerea*, and hence grey mould contamination in wine grape processing.

9. Appendix 1: Communication

Wine Australia Innovation News. New rapid Botrytis test may be a gamechanger, Publ. 19th May 2023. https://www.wineaustralia.com/news/articles/new-rapid-botrytis-test-may-be-a-gamechanger

Findings from the work were presented at the 16th Wine Tasmania Field Day held in Launceston on 24th October 2023 where approx. 100 wine industry participants attended.

An ASVO produced podcast on the findings and conclusions from this project, will be made available by ASVO in early 2024.

Articles are in preparation for a wine industry journal, and a peer-reviewed scientific journal.

10. Appendix 2: Intellectual Property Identify the intellectual property and/or valuable information arising from the research.

Nil

11. Appendix 3: References

Aaronson, L.R., A.M. Johnston, and C.E. Martin (1982) The effects of temperature acclimation on membrane sterols and phospholipids of *Neurospora crassa*. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism 713, 456 - 462. doi: https://doi.org/10.1016/0005-2760(82)90265-X.

Barad, S., S.B. Horowitz, I. Kobiler, A. Sherman, and D. Prusky (2014) Accumulation of the Mycotoxin Patulin in the Presence of Gluconic Acid Contributes to Pathogenicity of Penicillium expansum. Molecular Plant-Microbe Interactions 27, 66-77.

Dewey, F.M., A.H. Banham, R.A. Priestley, B. Martin, C. Hawes, S.I. Phillips, and P.W. Wareing (1993) Monoclonal antibodies for the detection of spoilage fungi. International Biodeterioration & Biodegradation 32, 127-136. doi: 10.1016/0964-8305(93)90045-4.

Dewey, F.M., C.C. Steel, and S.J. Gurr (2013) Lateral Flow Devices to rapidly determine levels of stable Botrytis antigens in table and dessert wines. American Journal of Enology and Viticulture 64, 291-295.

Guetsky, R., I. Kobiler, X. Wang, N. Perlman, N. Gollop, G. Avila-Quezada, I. Hadar, and D. Prusky (2005) Metabolism of the flavonoid epicatechin by laccase of *Colletotrichum gloeosporioides* and its effect on pathogenicity on avocado fruits. Phytopathology 95, 1341-1348.

Iland, P., P. Dry, T. Proffitt, S. Tyerman, C. Collins, V. Pagay, and C.C. Steel (2024) The Grapevine: from the science to the practice of growing vines for wine (Patrick Iland Wine Promotions Pty Ltd: Adelaide, South Australia, Australia).

Lorenzini, M., M. Azzolini, E. Tosi, and G. Zapparoli (2013) Postharvest grape infection of *Botrytis cinerea* and its interactions with other moulds under withering conditions to produce noble-rotten grapes. Journal of Applied Microbiology 114, 762-770. doi: 10.1111/jam.12075.

Meunier, M. and C.C. Steel (2009) Effect of *Colletotrichum acutatum* ripe rot on the composition and sensory attributes of Cabernet Sauvignon grapes and wine. Australian Journal of Grape and Wine Research 15, 223-227.

Meyer, U.M. and F.M. Dewey (2000) Efficacy of different immunogens for raising monoclonal antibodies to *Botrytis cinerea*. Mycological Research 104, 979-987.

Mologic, 2024. "Botrytis Alert", (Global Access Diagnostics, Bedford Technology Park, Thurleigh, Bedfordshire, MK44 2YA, UK 2024), https://www.botrytisalert.co.uk/, Access date 1st March 2024

Porep, J.U., S. Mrugala, M.S.P. Nikfardjam, and R. Carle (2015) Online Determination of Ergosterol in Naturally Contaminated Grape Mashes Under Industrial Conditions at Wineries. Food and Bioprocess Technology 8, 1455-1464.

Porep, J.U., R. Walter, A. Kortekamp, and R. Carle (2014) Ergosterol as an objective indicator for grape rot and fungal biomass in grapes. Food Control 37, 77-84. doi: http://dx.doi.org/10.1016/j.foodcont.2013.09.012.

Shindia, A.A., G.A. El-Sherbeny, A.E. El-Esawy, and Y.M.M.M. Sheriff (2006) Production of gluconic acid by some local fungi. Mycobiology 34, 22 - 29. doi: 10.4489/myco.2006.34.1.022.

Steel, C.C., "Determining thresholds for bunch rot tolerance in wine and detection of unwanted fungal aromas", (Wine Australia, Adelaide, South Australia 2021), pp. 100.

Steel, C.C., J.W. Blackman, and L.M. Schmidtke (2013) Grapevine bunch rots: Impacts on wine composition, quality, and potential procedures for the removal of wine faults. Journal of Agricultural and Food Chemistry 61, 5189-5206. doi: 10.1021/jf400641r.

Steel, C.C., L.J. Schwarz, Y. Qiu, C. Schueuermann, J.W. Blackman, A.C. Clark, and L.M. Schmidtke (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.

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

Prof Christopher C Steel
Gulbali Institute
School of Agricultural, Environmental and Veterinary Sciences
Charles Sturt University
Wagga Wagga

Dr Lachlan J Schwarz
Gulbali Institute
School of Agricultural, Environmental and Veterinary Sciences
Charles Sturt University
Wagga Wagga

Dr Aude Gourieroux
Gulbali Institute
School of Agricultural, Environmental and Veterinary Sciences
Charles Sturt University
Wagga Wagga

Darby Taguiam (CSU) is thanked for assistance with subculturing fungal isolates, Dilhani Perera (CSU) is thanked for assistance in sample extraction and Liang Jiang (CSU) is thanked for assistance with data analysis.

13. Appendix 5 Additional graphical analysis of other grape varieties collected by the CSU research team and not included in the main body of this report.

In all of these graphs and analyses, all bunches were included, irrespective of the SI reading with the Botrytis Alert antigen detection kit. Caution is advised in interpreting SI values of greater than 150.

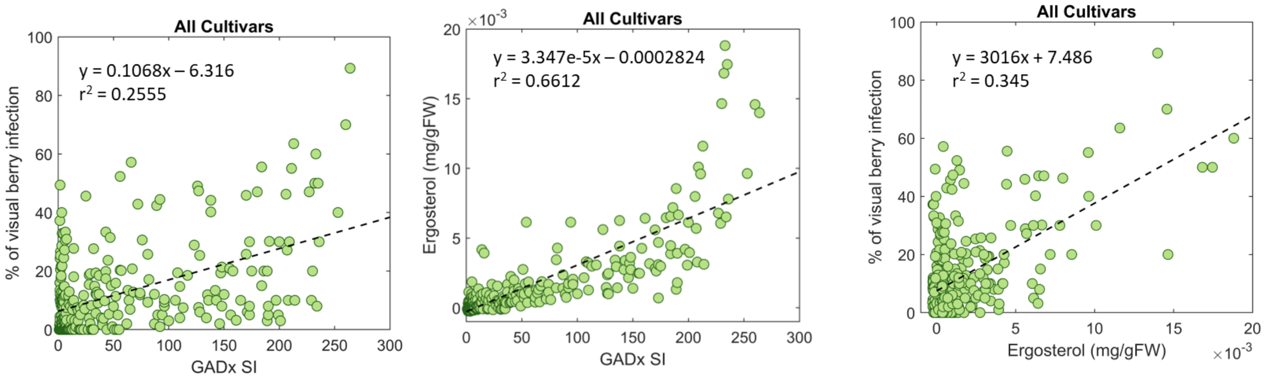
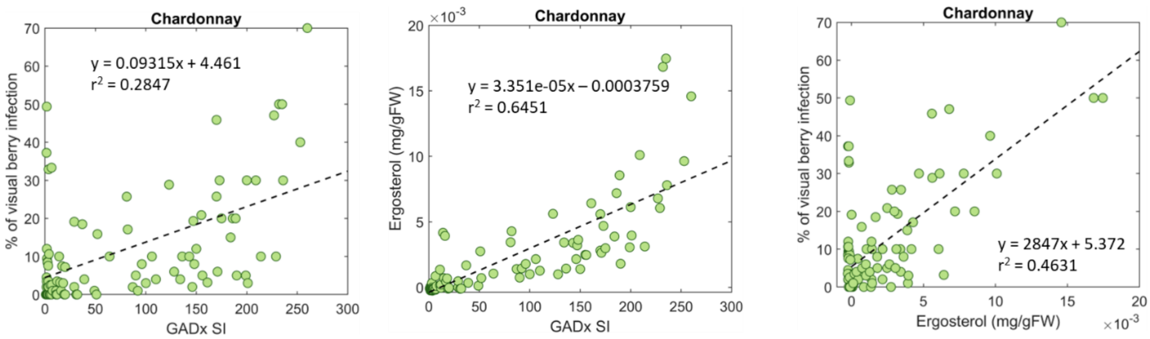
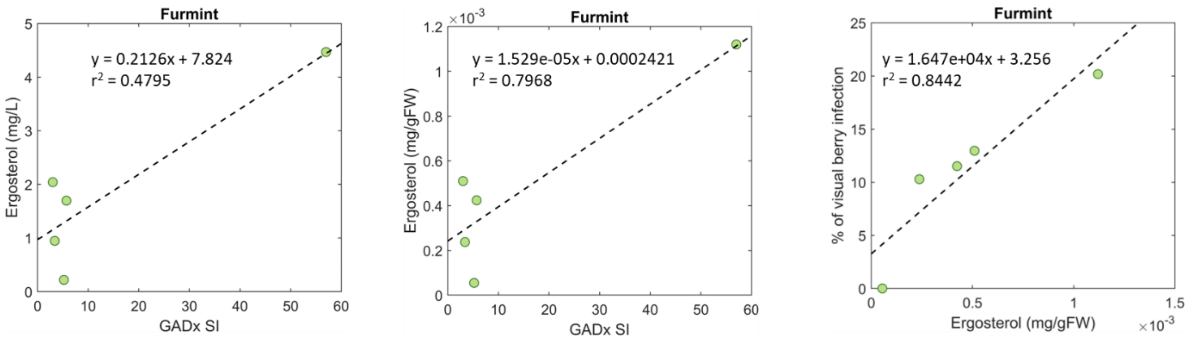


Figure 12: *Analysis of 312 grape bunches (all varieties, both white and red), for grey mould infection based on visual assessment, Botrytis antigens and ergosterol content. In this analysis, all bunches were included, irrespective of the SI reading with the Botrytis Alert antigen detection kit. GADx SI = Signal intensity of the Botrytis Alert antigen detection kit.*

Figure 13: *The figures below show the graphical representation of all varieties irrespective of the SI reading with the Botrytis Alert antigen detection kit.*



Chardonnay n = 108



Furmint, n = 5

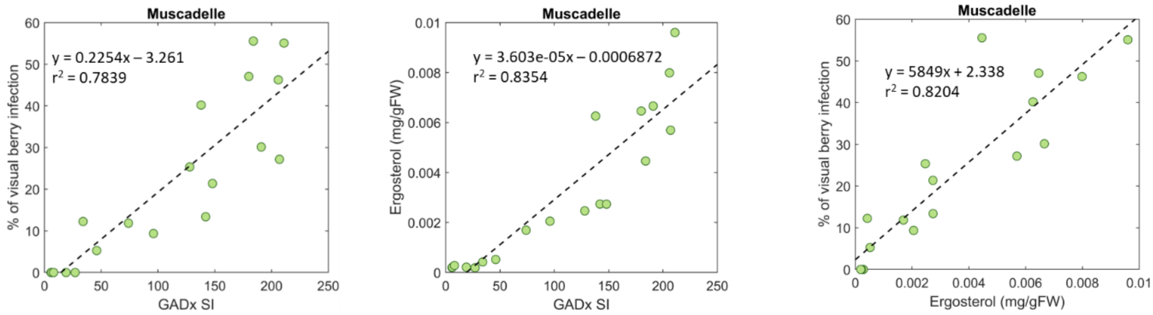
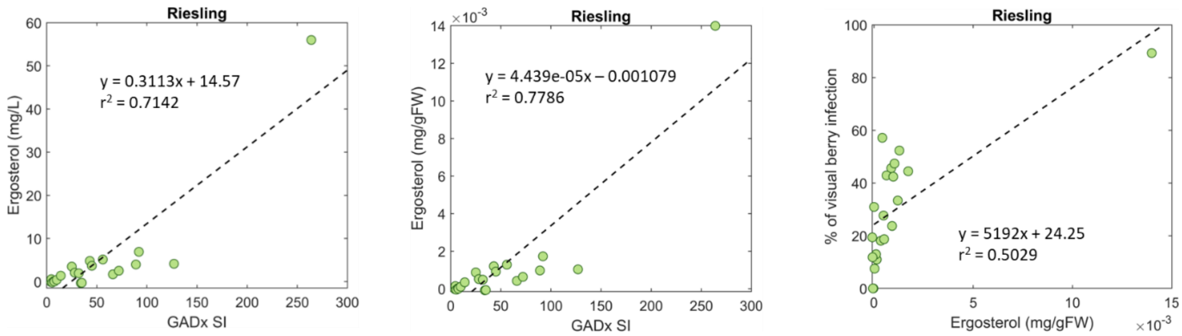
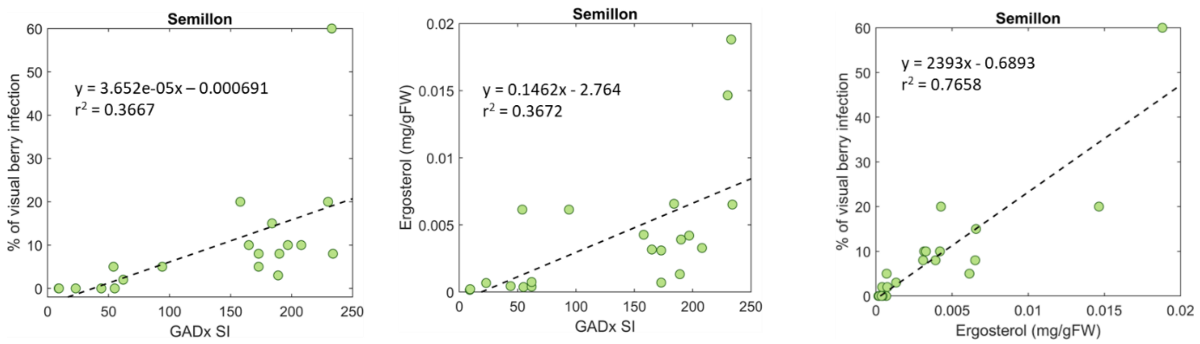


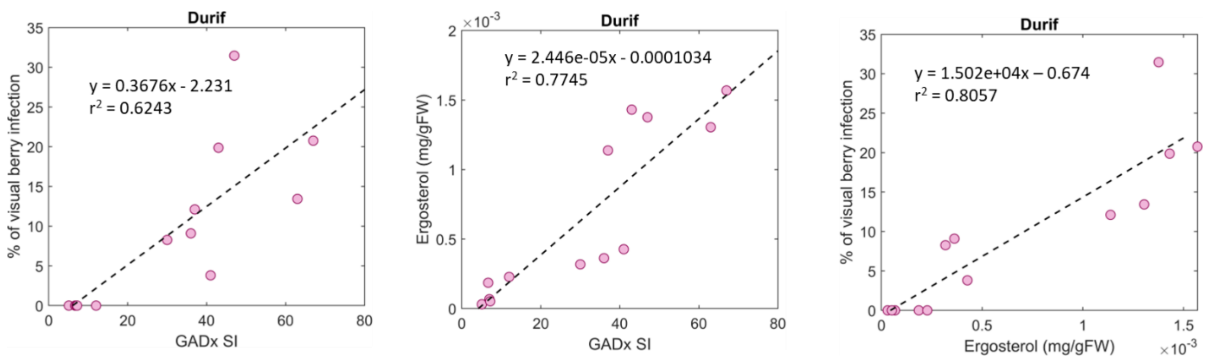
Figure Muscadelle n = 19



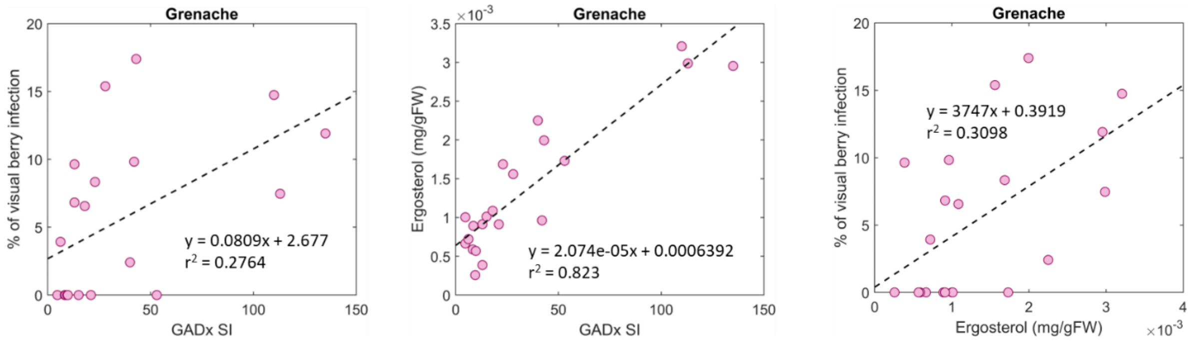
Riesling n = 21



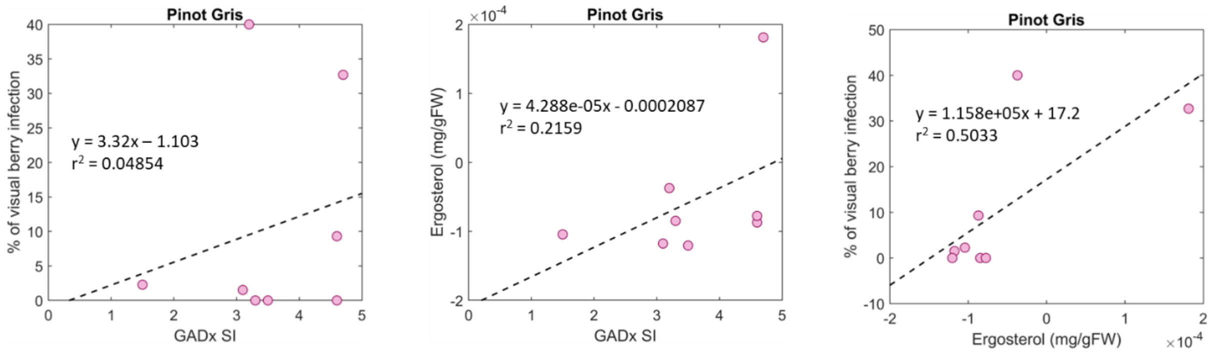
Semillon n = 21



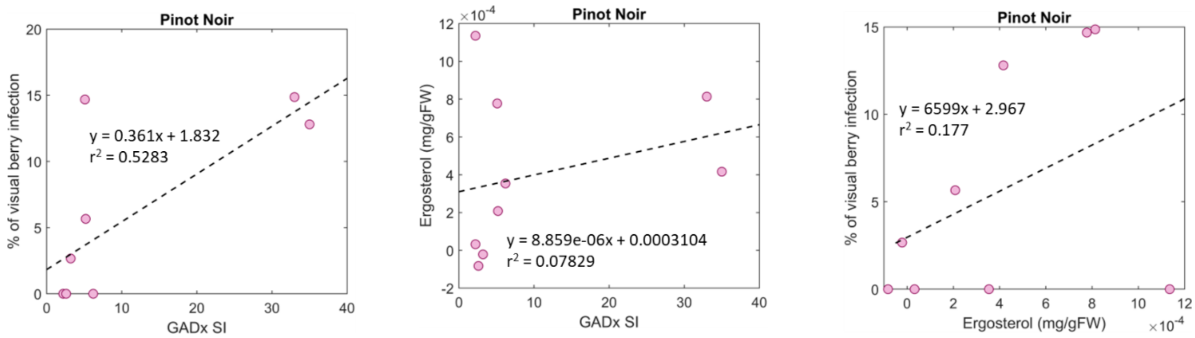
Durif n = 13



Grenache n = 21



Pinot Gris n = 8



Pinot Noir n = 9