OPTIMISATION OF PTA-ELISA DETECTION AND QUANTIFICATION OF *BOTRYTIS CINEREA* INFECTIONS IN GRAPES

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ABSTRACT

Botrytis cinerea can be detected in juice pre- and postharvest using the monoclonal antibody BC-12.CA4 in plate trapped antigen-enzyme linked immunosorbent assays (PTA-ELISA). The amount of antigen predicted by PTA-ELISA was found to increase as grape juice samples were progressively diluted. This effect was also observed in *B. cinerea*-contaminated boysenberry and blackcurrant juice samples. Detection of *B. cinerea* infections by PTA-ELISA was improved by incubation of grapes at 20°C for 48 h and was similar to visual assessments of infected grapes. PTA-ELISA is faster (1-3 days) than visual *B. cinerea* assessments (10-14 days). PTA-ELISA measurements can be standardised, removing human bias in determining *B. cinerea* infection levels.
Keywords: latent infection, monoclonal antibody, BC-12.CA4, immunoassay, juice.

INTRODUCTION

Botrytis cinerea Perk. Fr. (grey mould) is a ubiquitous pathogen of berries and other soft fruits and a major problem for wine producers. The fungus causes bunch rot in grapes, with a devastating impact on the quality and quantity of harvestable fruit.

Levels of *B. cinerea* fluctuate in response to variations in weather and management practices. Identification of latent infections is achieved by visual inspection of individual berries after 6-12 days incubation in the laboratory. However, this is time consuming, labour intensive and one must be able to positively identify sporulating *B. cinerea* amongst the other fungi present on/in grapes. The presence of bird damage, sour rot and the use of mechanical vine trimming can spread the fungus to otherwise healthy berries. Machine harvesting damages grapes resulting in postharvest fungal growth and the built up of fungal metabolites in juice. This can cause "tainting" of wine, altering properties such as flavour and colour and interfering with maturation (Bossi & Dewey 1992).

An effective assay to detect and quantify levels of latent infections pre-harvest would enable infected berries and lines of fruit to be quickly identified so that management can be modified to reduce the expression of these latent infections. Modifications might include, but are not limited to, harvesting at a lower Brix level, additional fungicide application or blending with other fruit with lower levels of *B. cinerea*.

Plate-trapped antigen enzyme-linked immunosorbent assays (PTA-ELISAs), employing monoclonal antibodies (MAbs) raised against specific fungal molecules, have the potential to provide a rapid method to detect *B. cinerea* levels throughout the season (Obanor et al. 2002; Meyer & Dewey 2000). This work investigated the ability of the genus-specific, monoclonal antibody BC-12.CA4 to detect latent *B. cinerea* infections in grapes at different stages of the growing season. BC-12.CA4 recognises a heat stable, carbohydrate epitope found predominantly on the surface of *B. cinerea* hyphae (Meyer

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& Dewey 2000). It is reported to provide accurate quantification from 0 to 20 Antigen units, equivalent to 0 to 20 μg freeze dried mycelia/ml and covering a range of 0-5% infection in harvested grapes (Dewey et al. 2000).

MATERIALS AND METHODS

Samples

Grapes were sampled to obtain a range of levels of *B. cinerea* from two varieties given different *B. cinerea* disease control regimes. Samples were obtained from two Hawke's Bay vineyards at three sampling times: pre bunch closure, early veraison and harvest. The grape varieties, Semillon and Chardonnay, were exposed to different *B. cinerea* disease control regimes. Vines were left untreated (both varieties), treated with fungicides (commercial spray programme, Chardonnay) or with a biological control agent (BOTRY-Zen™, Semillon). Samples, consisting of four or five bunches of grapes, were obtained from each treatment at both vineyards at each sampling time. Juice was extracted by crushing 20 randomly selected berries/bunch in sterile polyethylene bags and storing 2 ml aliquots (-20°C) in sterile Eppendorf tubes for later use in the immunoassays.

Grape juice samples of Semillon and Chardonnay were obtained from Hawke's Bay, and consisted of juice extracted from harvested grapes, henceforward referred to as postharvest juice sample. Similarly, pressed boysenberry and blackcurrant juice samples were obtained from Berryfruit Export Ltd., Nelson, and HortResearch, Lincoln, respectively.

PTA-ELISA assays

PTA-ELISAs were performed according to Dewey et al. (2000). The monoclonal antibody BC-12.CA4 and secondary antibody-enzyme conjugate (anti-mouse polyvalent immunoglobulins peroxidase conjugate) were obtained from Adgen and Sigma, respectively, and antigen standard (equiv. 4 BAgU/μl) was supplied by F.M. Dewey (Dept Vitic. and Enol., Uni. Calif., Davis, USA). Working volumes for all reagents were 100 μl per well, and all assays were performed in duplicate wells. Absorbance was read at 450 nm (A450 nm) and *B. cinerea* antigen units (BAgU) were calculated by inverse prediction from the linear section of the standard curve. Using inverse prediction, samples containing levels of *B. cinerea* contamination from 0-20 BAgU can be determined. This is equivalent to 0–5% infection in harvested berries (Dewey et al. 2000).

Samples and antigen standards were diluted in phosphate buffered saline (PBS, 0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.025% KH_2PO_4, pH 7.2) and antibodies were diluted 1:4 and 1:5000, in PBST (PBS containing 0.05% Tween 80). A set of antigen standards equivalent to 8, 4, 2, 1, 0.5, 0.25 and 0.125 BAgU/ml were included in each plate to allow quantification of sample antigen levels. Negative and positive controls consisted of PBS and antigen standard, respectively. The mean value for PBS was subtracted from each sample value prior to quantification, to minimise background absorbance.

Visual assessments

For each treatment and sampling time, from both vineyards, 20 frozen (24 h at -20°C) berries per bunch (selected at random) were placed on damp sterile paper towels in sterile plastic trays and incubated for approximately 10-12 days at room temperature. Trays were covered in sterile plastic bags to retain humidity and bags removed after 8-10 days to encourage sporulation. Individual berries were assessed under a stereomicroscope (x 10-20 magnification) for the presence of sporulating *B. cinerea*.

Experiments

Initial PTA-ELISAs were performed on undiluted juice samples, extracted from grapes taken at different stages of the growing season and exposed to different treatment regimes. PTA-ELISA measurements were compared with visual assessments of *B. cinerea* berry incidence. Grapes used for PTA-ELISA and visual assessments were obtained from the same bunches. The immunoassays performed on undiluted juice consistently failed to detect low levels of antigen in samples. Therefore the effect of sample incubation and juice dilution on detection sensitivity was further examined.

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For juice dilution experiments, 18 juice samples from each sampling time were selected to cover a range of absorbance levels based on the PTA-ELISA results from undiluted juice samples. These juice samples were either used undiluted or diluted in PBS to 5% juice concentration and assayed using PTA-ELISA as described above. The effect of juice dilution was also tested on four boysenberry and two blackcurrant postharvest juices. In total, over 200 juice samples were tested undiluted, and 72 samples were tested undiluted and at 5% juice concentration.

To determine the effect of incubation time and temperature on PTA-ELISA detection sensitivity, 18 grape juice samples were selected randomly from each sampling time (pre bunch closure, veraison, harvest and postharvest juice) in the juice dilution experiments. Samples were halved and incubated separately at 4 and 20ºC for a period of 72 hours. Sub-samples of juice (300 µl) were removed at 24 h intervals, stored at -20ºC and diluted in PBS to 5% juice prior to their use in immunoassays. To further demonstrate the effect of dilution, four postharvest juice samples were selected based on their positive absorbance readings after 48 h incubation at 20°C. The four juice samples were diluted in PBS to give final juice concentrations of undiluted (100), 50, 20, 10 and 5% juice and immunoassayed as described above.

To further explore the optimum concentration of juice required to allow accurate quantification of antigen levels, PTA-ELISAs were performed on juice obtained from visibly infected berries (selected from the visual assessments) diluted in PBS to give juice concentrations of 1, 0.5, 0.1 and 0.01%. The juice sample was prepared as described above.

Descriptive statistical analysis was used to compare visual assessments with the detection of *B. cinerea* by PTA-ELISA. Analysis of variance and regression analysis (Minitab Version 12.1) were used to determine the effect of juice dilution and incubation on absorbance levels.

RESULTS AND DISCUSSION

Grapes sourced during the season and postharvest from different vineyards and/or fungicide management programmes were expected to express different levels of *B. cinerea* disease. Although disease data is presented for the different treatments and varieties, it is beyond the scope of this study to discuss the effect of fungicide treatments on disease control. The aim of this study was to compare two methods (visual assessments versus PTA-ELISA) for determining *B. cinerea* infections of grapes.

Calibration

The linear section of the calibration curve used for inverse prediction of absorbance readings (A450 nm) to antigen units (BAgU) is given in Figure 1. Calculation of antigen levels, via inverse prediction, requires construction of a standard curve employing low concentrations of antigen. The omission of data points above 2 BAgU and inclusion of concentrations below 2 BAgU results in a linear plot from which low antigen levels can be predicted. Inclusion of data points above 2 BAgU would result in a line of best fit intercepting the Y-axis at approximately 0.4. Consequently, samples with absorbance values below 0.4 would fail to be detected by inverse prediction. Limiting data points to below 2 BAgU results in a linear plot intercepting the Y-axis at 0.1 and increases the sensitivity of the assay (Fig. 1). Therefore the threshold for quantifying *B. cinerea* in juice samples using PTA-ELISA is approximately 0.1 absorbance units at A450 nm, although positive detection can occur below the threshold value.

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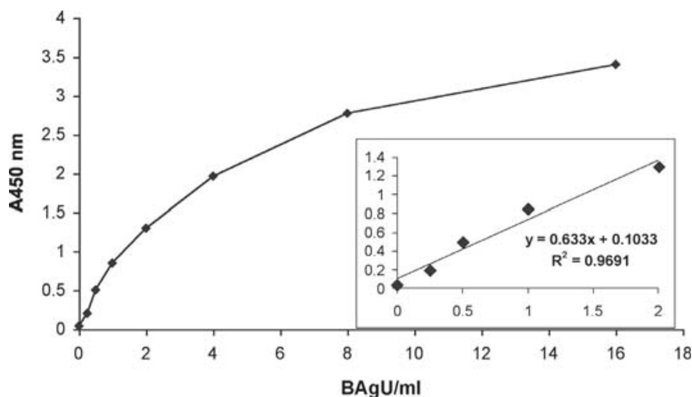

FIGURE 1: Standard calibration curve and the linear section (inset) of standard curve used for prediction of sample antigen levels.

PTA-ELISA and visual assessments

Results for PTA-ELISA and visual assessments of *B. cinerea* infection in grapes are given in Table 1. PTA-ELISA assays, performed on undiluted samples, were found to consistently underestimate the incidence and quantity of fungal infection indicated by visual assessment of incubated grapes (Table 1). However, the number of samples with positive absorbance values was found to generally increase as the season progressed (Table 1). Less than 1% of a total of 438 samples assayed had absorbance levels exceeding the lower threshold for detection by PTA-ELISA (A450 approx. 0.1). The assay failed to detect any antigen in pre bunch closure, veraison and three out of four harvest samples. Therefore, the effect of juice dilution and incubation of juice samples on *B. cinerea* detection using PTA-ELISA was further investigated.

Dilution of juice

Mean absorbance values increased ($P<0.05$) when samples were diluted to 5% (Table 2), raising the number of samples with detectable antigen levels from 0 to 33% and 12 to 44% in harvest and postharvest juice samples, respectively (Table 2). None of the veraison samples assayed undiluted produced absorbance values within the detectable range, but visual inspection of incubated grapes indicated a 20% level of infection. Assays performed at 5% juice revealed an increase in absorbance in one out of 18 veraison samples (6%). Pre bunch closure samples showed no significant increase in absorbance at 5% juice, although visual assessments showed that 15-40% of samples contained *B. cinerea*. The increase in absorbance values when grape juice samples were diluted to 5% juice was also observed in boysenberry and blackcurrant juice samples (Table 2). Subsequent serial dilution of grape juice samples showed that the increase in absorbance follows a first order kinetics (Fig. 2).

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TABLE 1: *Botrytis cinerea* berry infection for Chardonnay and Semillon grapes sourced from Hawke's Bay vineyards throughout the season as determined by visual assessment of frozen and incubated berries and PTA-ELISA using undiluted juice extracts.

Sampling time	Variety and fungicide treatment	Visual assessment (%)	Samples (%) above PTA-ELISA threshold[4]	Samples (%) with positive reading but below PTA-ELISA threshold[4]
PBC[1]	Chardonnay, BZ[2]	< 1	0	0
PBC	Chardonnay, untreated	< 1	0	0
PBC	Semillon, CS[3]	< 1	0	5
PBC	Semillon, untreated	< 1	0	0
Veraison	Chardonnay, BZ	< 1	0	52
Veraison	Chardonnay, untreated	< 1	0	4
Veraison	Semillon, CS	1	0	10
Veraison	Semillon, untreated	9	0	5
Harvest	Chardonnay, BZ	8	0	8
Harvest	Chardonnay, untreated	44	4	100
Harvest	Semillon,CS	5	0	100
Harvest	Semillon, untreated	35	0	100
Postharvest[5]	Chardonnay, BZ	–	0	100
Postharvest	Chardonnay, untreated	–	0	100
Postharvest	Semillon, CS	–	0	100
Postharvest	Semillon, untreated	–	25	100

[1]PBC = Pre bunch closure.
[2]BZ = Sprayed with BOTRY-Zen™.
[3]CS = Commercial spray programme.
[4]PTA-ELISA threshold at 0.1 absorbance units. Absorbance above this threshold can be used for quantification of BAgU using inverse prediction.
[5]Postharvest juice samples.

TABLE 2: Percentage of grape, boysenberry and blackcurrant juice samples containing detectable levels of antigen as well as corresponding mean PTA-ELISA A450 nm readings and the predicted mean BAgU/ml values in undiluted and 5% juice for non-incubated samples. Mean berry infection incidence (%) as assessed visually is also presented.

	PTA-ELISA						
	Undiluted juice			5% juice			
Sampling time	%	A450	BAgU	%	A450	BAgU	Visual[5]
PBC[1]	0	0	0	0	0	0	20
Veraison	0	0.005	0	6	0.015	0	20
Harvest	0	0.02	0	33	0.064	0	50
Postharvest[2] grape	12	0.063	0	44	0.11	0.3	-
Boysenberry[3]	75	0.296	0.4	75	0.534	14.9	-
Blackcurrant[4]	0	0.036	0	50	0.129	0.3	-

[1]PBC = Pre bunch closure.
[2]Postharvest juice samples.
[3]Postharvest boysenberry, sample size n=4.
[4]Postharvest blackcurrant, sample size n=2.
[5]Visual assessment data is based on sub-set of 72 original grape juice samples, selecting juices across the various absorbance-spectra when assessed undiluted.

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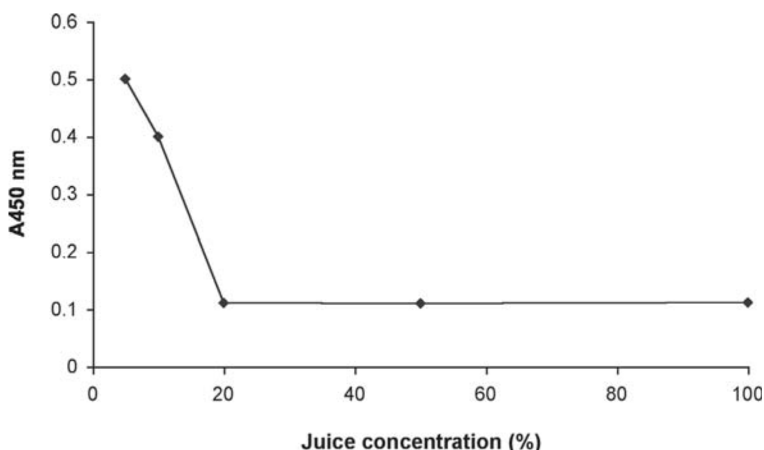

FIGURE 2: A450 nm absorbance readings at various levels of postharvest grape juice dilution in PBS. Juice was assayed after 48 h incubation at 20°C.

PTA-ELISA of juice obtained from visibly infected berries, progressively diluted from 1-0.01% in PBS, revealed that absorbance had ceased increasing at 0.33% (Table 3). The amount of antigen predicted in undiluted juice from PTA-ELISA of diluted samples increased as juice was diluted down to 0.01%. Antigen levels calculated at 0.1 and 0.01% dilutions were similar, with a predicted 2813 and 3002 BAgU/ml, respectively (Table 3). Results indicate that even at 5% juice, predicted antigen levels are likely to be underestimated and dilution to 0.1% may have revealed considerably more samples to contain detectable levels of antigen. To improve quantification, juice samples should be diluted to at least 0.1% in PBS prior to ELISA. However, the exact concentration of juice required to give accurate predictions has yet to be determined and may be found to vary among grape samples.

TABLE 3: Absorbance values and predicted antigen concentrations at low juice concentrations in juice from grapes with 100% incidence of *Botrytis cinerea*. Antigen levels in diluted juice were determined by inverse prediction. These values were used to calculate the corresponding levels of antigen in undiluted juice.

Dilution (% juice)	A450 nm in diluted juice	Predicted BAgU/ml in diluted juice	Calculated BAgU/ml for undiluted juice
1	2.19	3.64	364
0.5	2.23	3.71	740
0.33	2.36	3.95	1187
0.25	2.28	3.82	1527
0.14	1.83	3.01	2106
0.1	1.72	2.81	2813
0.01	0.98	1.49	3002

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Dewey et al. (2000) reported that PTA-ELISAs employing BC-12.A4 to detect *B. cinerea* in grapes, do not require careful dilution of juice samples prior to assay. However, results presented here indicate that 5% juice extract showed a considerable improvement in the ability to detect *B. cinerea* compared to undiluted juice. Further dilution to 0.1% was required to provide more accurate quantification of antigen levels. The rise and fall in absorbance in PTA-ELISA is a phenomenon known as the "Hook effect" and well recognised in immunology. High concentrations of solutes have been reported to increase the rate of antigen desorption from the plate surface (Virology Down Under 2003) and the settling of suspended particles may interfere with antigen-plate binding. However, at the low concentrations of juice and therefore low levels of suspended matter in juice samples, the "Hook effect" was still evident. This suggests that antigen-plate binding is more likely to be limited by competitive binding of solutes rather than the settling of particulates.

Incubation at 4 and 20ºC

Incubation temperature and period affected ($P<0.05$) absorbance readings, with best and most consistent absorbance readings after 48 h incubation at 20°C (Table 4). Visual assessments showed that 50% of the veraison and harvest samples contained *B. cinerea*. No visual inspection data was available for postharvest juice samples.

The largest increase in antigen levels was observed in postharvest samples incubated at 20ºC for 48 hours (Table 4). The number of postharvest samples in which antigen could be detected at 20°C increased by 89% (from 44 to 84%, Table 4); whereas at 4ºC it only increased by 5% (from 44 to 46%, Table 4). At 20ºC after 48 h, the number of harvest samples in which antigen could be detected (51%) was similar to levels of infection indicated by visual inspection (50%). However, at 4ºC detection level increased by 5% (from 33 to 35%, Table 4). However, pre bunch closure and veraison samples were well below detection levels obtained from visual assessments. This means visual assessments may overestimate, or PTA-ELISA may underestimate, levels of *B. cinerea* infections for pre bunch closure and veraison samples.

Dewey & Brasier (1988) reported that overnight coating resulted in higher signals. However, they studied prolonged incubation in the wells, whereas in our experiments the juice itself was incubated to encourage *B. cinerea* development in the juice sample rather than increasing the absorption period, which in our experiments always consisted of overnight incubation.

TABLE 4: Percent of samples containing detectable levels of antigen before (0 h) and after incubation at 4 and 20ºC for 24, 48 and 72 h in comparison with visual assessments.

		4ºC			20ºC			
Sampling time	0 h	24 h	48 h	72 h	24 h	48 h	72 h	Visual assessment[3]
PBC[1]	0	0	0	0	0	6	6	25
Veraison	6	6	6	6	6	7	8	50
Harvest	33	33	35	35	40	51	51	50
Postharvest[2]	44	44	46	46	69	84	84	-

[1]PBC = Pre bunch closure
[2]Postharvest grape juice samples
[3]Visual assessment data is based on sub-set of 21 original grape juice samples, selecting juices across the various absorbance-spectra when assessed at 5% juice.

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CONCLUSIONS

PTA-ELISAs, employing BC-12.CA4, have been successfully used to detect and quantify *B. cinerea* in harvested grapes. However, the levels of fungus present in grape juice were often below the threshold detectable by this assay. Incubation of crushed grape juice at 20ºC for 48 hours and/or dilution in PBS prior to assay may be used to amplify the detection of antigen to within the range detectable by PTA-ELISA.

Although, it was suggested that one of the benefits of using BC-12.CA4 is its ability to detect and quantify antigen levels in undiluted juice samples (Dewey et al. 2000), dilution of the juice samples increased sensitivity. This is in agreement with the research by Dewey et al (2000) who reported increased sensitivity when diluting with non-contaminated grape juice. Further work is required to determine the concentration of juice and incubation period at which antigen levels can be accurately determined.

ACKNOWLEDGEMENTS

We would like to thank the New Zealand Winegrowers and the New Zealand Foundation for Research, Science and Technology for funding this research. Thanks to Dr Molly Dewey for *Botrytis cinerea* antigen, advice and on-going discussions.

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