RAPID METHOD FOR THE DETECTION AND QUANTIFICATION OF *BOTRYTIS CINEREA* IN PLANT TISSUES

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ABSTRACT

Monoclonal antibody to *B. cinerea* (BC-58) was used to develop a plate-trapped antigen enzyme linked immunosorbent assay (PTA-ELISA) to detect and quantify *Botrytis* antigens in boysenberry flowers. The ability of antibody BC-58 to detect *B. cinerea* in extracts from artificially infected boysenberry flowers was assessed. Results showed that the antigen could be detected in latent infections. Antibody BC-58 sensitivity to heat treatment of the antigen, incubation conditions, and the detection limit were also investigated. Autoclaving at 121°C reduced the sensitivity of the antibody. Additionally, the incubation of the antigen at 4°C overnight produced higher absorbance values at 405 nm than incubation at 37°C for 2 h. The detection and quantification of *B. cinerea* antigen was reliable within 0-16 µg dried mycelium per ml of PBS buffer and at least 8 x 10^3 spores.

Keywords: *Botrytis cinerea,* PTA-ELISA, boysenberry flowers, monoclonal antibody.

INTRODUCTION

Botrytis cinerea, the pathogen responsible for grey mould, causes substantial losses of soft fruits, vegetables and cut flowers. The most commonly infected fruit include grape, apple, strawberry, boysenberry, raspberry, pear and tomato. Flower infections often remain dormant until the fruit ripens (Dashwood & Fox 1988). The difficulties inherent in the detection of the fungus at the latent stage necessitate the development of rapid and easy-to-use detection methods such as immunoassays. The ability to detect latent infections in plant tissues should prove useful not only for early disease management but also for identifying infected berries post-harvest.

Recently, a number of antibodies raised to *Botrytis cinerea* have been developed (Dewey et al. 2000). These can be used to detect and quantify the fungus in extracts from plant tissues in plate-trapped antigen-enzyme-linked immunosorbent assays (PTA-ELISAs). However, the efficacy of these antibodies varies considerably (Dewey et al. 2000). Bossi & Dewey (1992) raised a monoclonal antibody (BC-KH4) to *B. cinerea* which has proven useful in immuno-localisation of *B. cinerea* and *B. fabae* on plant surfaces and within plant tissues. The antibody functions only at specific dilutions of the plant extract. Thus, it is difficult to use this antibody in the quantification of *B. cinerea* by ELISA in green plant extracts with high accuracy. The objectives of this work were to optimise a rapid PTA-ELISA method to detect and quantify *B. cinerea* in infected plant tissues, and to assess the efficacy of two monoclonal antibodies (BC-56 and BC-58) to *B. cinerea* in detecting the pathogen in artificially infected boysenberry flowers.

MATERIALS AND METHODS

Optimisation of PTA-ELISA method for detecting *Botrytis cinerea*

Monoclonal antibodies BC-56, BC-58 and positive standard were obtained from Dr R. Cernusko (Georg-August University, Goettingen, Germany). *Botrytis cinerea* spores

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were extracted from PDA plate cultures using 10 ml of sterile water. The spore concentration was adjusted to 8.35 x 10^5/ml with Phosphate Buffered Saline (PBS; 0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.025% KH_2PO_4, pH 7.2). Four serial dilutions of 8.4 x 10^4, 8.4 x 10^3, 8.4 x 10^2 and 8.4 x 10^1 spores/ml were used. In this experiment, the efficacy of two monoclonal antibodies to *B. cinerea* (BC56 and BC58) to detect BC spores was assessed, as was incubation temperature and duration. For each of the dilutions 100 µl was put in six wells of a 96 wells microtiter plate (plate A). Plate B was prepared in the same manner. For the positive standards 100 µl of 0, 2, 4, 6, 8, 16 AgU was run in parallel with the samples. Plate A was incubated at 4°C overnight and Plate B was incubated at 37°C for 2 hours. Following incubation, the wells were decanted and rinsed three times with wash buffer (PBS + Tween 80, PBST). The plates were dried with a paper towel and 200 µl of blocking buffer (3% non-fat dried milk) was added to each test well, and incubated at 37°C for 2 hours. The wells were washed three times with PBST. Subsequently, triplicate wells were incubated with antibodies BC-56 and BC-58 in each plate for 1 h at 37°C. Again, the wells were washed as described earlier. This was followed by the incubation with the secondary antibody, streptavidin alkaline phosphatase conjugate diluted 1:50 in PBST, for 1 h at 37°C, and subsequently with the alkaline phosphatase substrate, 4-Nitrophenyl disodium orthophosphate in diethanolamine buffer. The enzyme reaction was finally stopped by the addition of 100 µl of 3M NaOH to each well, and the colour intensity was measured at 405 nm using Fluostar (type 0403) microplate reader.

Detection of *B. cinerea* in artificially infected boysenberry flower

The ability of the Mab (BC-58) to detect *B. cinerea* in flowers was assessed by ELISA using extract from some artificially infected boysenberry flowers. Spores were obtained from a 2-week old *B. cinerea* cultured on oatmeal agar at room temperature. The spores were extracted with 10 ml of distilled sterile water, and the spore concentration was adjusted to 10^5spores/ml. Boysenberry laterals at mid-bloom were collected and open flowers were labelled with tags. Two to three laterals were placed in each of six flasks containing tap water. At least four flowers/flask were inoculated with 50 µl of the spore suspension (5 x 10^3 spores/flower). Flowers in one of the flasks (control) were inoculated with 50 µl of distilled water. The flowers were incubated at room temperature for 10, 9, 8, 7 and 6 days.

After incubation, 2 flowers from each flask were crushed separately in a mortar with PBS in a 1:4 (w/v) ratio. The extract was centrifuged at 1500 g for 5 min to remove cell debris. The supernatant was further diluted to 1:9 (v/v) with PBS. The diluted supernatant (100 µl) was used to coat the wells of a microtiter plate. The assay was run in triplicate as described previously. The plates were incubated at 4°C overnight and MAb BC-58 was used in the assay. This antibody was chosen because in experiment one, antibody BC-56 failed to detect the antigen. The sensitivity of the antibody to heat treatment was also assessed in this experiment. All the diluted samples above were autoclaved at 121°C for 20 min. These samples were also run alongside non-autoclaved samples and standards.

RESULTS AND DISCUSSION

All absorbance values are an average of replicate readings. The variation in absorbance values between samples on the same plate was ± 0.015 or less. Figure 1 shows the reference standard calibration curve obtained for *B. cinerea* mycelium (300 µg/ml) with serial dilutions from 16 µg/ml to 2 µg/ml and expressed as antigen units, which are equivalent to µg dried mycelium per ml of PBS buffer. A linear relationship between the absorbance and AgU was obtained within this range with a positive correlation coefficient of 0.9954. This suggests that the predictability of the levels of the antigen will be reliable within the range. Dewey et al. (2000) obtained a similar result although they used a different antibody (BC.12CA4). They reported a linear relationship between absorbance and antigen concentration between 0 and 20-antigen units/ml.

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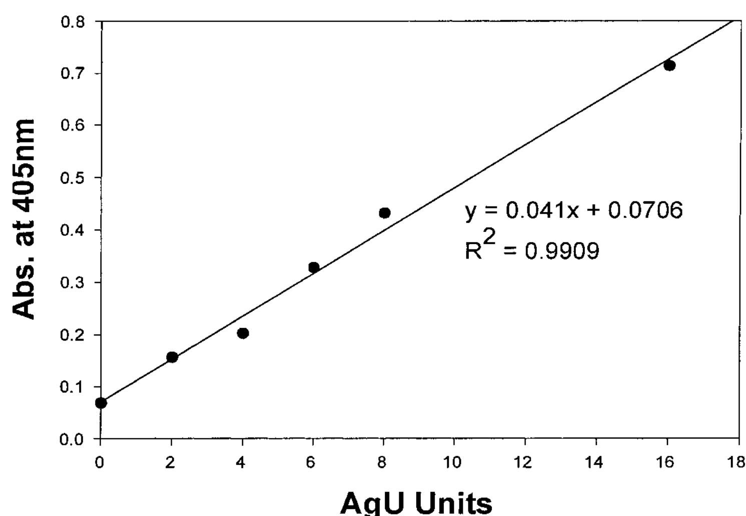

FIGURE 1: Absorbance values from PTA-ELISA of BC-58 with positive standard of *B. cinerea* mycelium.

Antibody BC-56 was unable to detect the antigen (Table 1), however antibody BC-58 detected the antigen at a concentration of at least 8000 spores for both incubation periods. The antibody showed higher absorbance values if the samples were incubated at 4°C overnight than when the samples were incubated at 37°C for 2 hours. This difference may be due to fewer antigens binding to the surface of the plate in samples incubated for a shorter inoculation period.

TABLE 1: The mean absorbance at 405 nm for a range of *B. cinerea* spore concentrations and two different antibodies for short or long incubation periods.

	Number of spores/well				
	8400	840	84	8.4	0
Incubated at 4°C overnight					
BC-56	0.089	0.080	0.098	0.077	0.078
BC-58	0.815	0.113	0.090	0.065	0.069
Incubated at 37°C					
BC-56	0.078	0.072	0.085	0.091	0.077
BC-58	0.627	0.101	0.086	0.079	0.075

The antibody BC-58 was able to detect the antigen in artificially inoculated boysenberry flowers (Table 2). There was a marked increase in the levels of the detectable antigen as the time between inoculation and sampling increased. A possible explanation is that during incubation, more mycelia were formed with the increasing incubation time and hence, increased detectable antigen. Also, increased spore germination has been found to increase the absorbance values of this assay (Bossi & Dewey 1992).

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TABLE 2: Mean absorbance at 405 nm for artificially infected boysenberry flowers that had been incubated for various times. The experiment was carried out with autoclaved and non-autoclaved samples and standards.

	Incubation Period (days)					
Treatments	Control	10	9	8	7	6
Non-autoclaved	0.089	1.27	0.965	0.456	0.237	0.192
Autoclaved	0.096	0.136	0.129	0.131	0.101	0.112

The antibody used in this experiment was sensitive to heat treatment as indicated by the very low absorbance values of those samples that were autoclaved compared to the non-autoclaved samples. This indicates that the autoclaving of *B. cinerea* reduced the ability of this antibody to bind to the antigen, suggesting that BC 58 essentially recognises heat-labile molecules that are expressed on the surface of the spores or conidia. However, most genus-specific and near-isolate specific *B. cinerea* monoclonal antibodies can recognise the antigen even with heat treatment of up to 120°C (Meyer & Dewey 2000).

CONCLUSIONS

The results presented in this paper demonstrated that the incubation of test samples at 4°C overnight improved the overall sensitivity of the PTA-ELISA procedure. This method can be successfully used to detect and quantify *B. cinerea* in boysenberry flowers after infection. This immunoassay will be a useful research tool in the management of grey mould. Further investigation is required to evaluate the specificity of this antibody, because it may recognise all species of *Botrytis* as well as some unrelated fungi.

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