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ELISA OPTIMISATION FOR MONITORING *BACILLUS SUBTILIS* IN PREHARVEST BIOCONTROL PROGRAMMES

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Abstract

Enzyme linked immunosorbent assay (ELISA) was optimised for more effective monitoring of *Bacillus subtilis* in avocado field spray programmes using natural antagonists. Parameters of the ELISA that were optimised included, incubation temperature and -time, fixatives, blocking buffers, washing buffers, antibody incubation time, conjugate concentration, conjugate incubation time and enzyme- substrate reaction time. Incubation conditions of 4ºC for 24 h gave the highest signal/background value, the baking method was found more applicable due to its 3 months storage potential without loss of ELISA signals. Bovine serum albumin gave the highest signals when used as a blocking solution. When comparing the optimised ELISA protocol with the one used previously, a higher signal/ background value were obtained with the former ELISA.

1 Introduction

Disadvantages associated with the use of chemicals and their detrimental effects on human health and the environment have resulted in an increased interest in alternative control strategies such as biological control. Biological control has been evaluated successfully at a pre- and postharvest level for control of avocado fruit diseases using *Bacillus subtilis* sprays (Korsten *et al.,* 1993). When bacteria are applied as biocontrol agents in the field, their populations should be monitored at several intervals starting from the time of application (Spurr and Knudsen, 1985). This information is necessary to improve biocontrol efficacy through better formulation, minimising wasteful applications and optimising spray scheduling (Knudsen and Spurr, 1987).

Different indirect methods such as leaf imprints and -washings and direct scanning electron microscopy (SEM) have been evaluated to detect antagonist populations after field applications in avocado orchards (unpublished data). The indirect techniques gave lower viable counts compared to total higher counts with the SEM. These techniques were found to be either inaccurate or not suited to large scale monitoring of field sprays. Bacterial populations have been successfully monitored using antibodies (Schaad *et al.,* 1990). Subsequently, monoclonal antibodies were produced against the antagonist (Unpublished data).

The purpose of this study was to optimise the ELISA technique for efficient and rapid monitoring of antagonists in field biocontrol studies.

2. Materials and methods

2.1 Monoclonal antibodies

Isolate B246 originally isolated form the avocado phylloplane and obtained from L. Korsten (Department of Microbiology and Plant Pathology, University of Pretoria) was used for the optimisation study. Monoclonal antibodies secreted by clone 6B7E5 (IgG2b) which gave the highest optical density reading when tested against B246, was selected for the optimisation studies.

2.2 ELISA optimisation

The ELISA technique described by Verschoor *et al.* (1989) was used throughout the optimisation study, changing only the various steps under investigation. A total of 11 replicates were used per assay and a negative control (Dulbecco's modified Eagle's medium (DMEM)) was included in each batch. B246 suspensions (100 *µl)* were incubated in 96-well microtiter plates (Cooke Microtiter system M29A, Sterilin products, Middlesex, England) for either 1, 2 or 4 h or overnight at temperatures of either 4, 24 and 37ºC or were air dried for 1 h under a heating lamp and laboratory fan. Various fixatives were compared viz. 70, 90 and 100 % methanol; 70, 80 and 100 % ethanol; 60 and 70 % - 1- butanol; 0.25, 0.5 and 0.75 % formaldehyde; 0.25, 0.5 and 0.75 % glutaraldehyde (After I h of fixation with glutaraldehyde plates were washed 3 times with 100 mM glycine in phosphate buffered saline (PBS) pH 7.2); 0. 1 % sodium dodecyl sulphate (SDS) in carbonate buffer; 60, 70 and 80 % isopropanol; 6 M urea in carbonate buffer; 50, 60 or 70 % acetic acid. Fixatives were incubated at room temperature for 1 h. The most effective blocking buffer were selected comparing 0.5, 1, 1.5, % skim milk in PBS; 0.5, 1, 1.5 % gelatine in PBS and 1, 3 and 5 % bovine serum albumin (BSA). The blocking solutions were incubated for 1, 2 h or overnight. Different washing buffers were compared using 0.5 % Tween 20 in PBS, 1 % BSA in PBS, 0.5 % casein in PBS and PBS. Optimum primary antibody incubation time (30, 45, and 60 min), optimum conjugate concentration (Peroxidase conjugated goat-anti-mouse IgG; heavy and light chain specific, Dakopatts, Denmark) (1: 100, 1: 1000, 1:5000, and 1: 10000), conjugate incubation time (30, 45, and 60 min) and enzyme-substrate reaction time (10, 20, 30, 40, 50, and 60 min) using urea peroxide and orthophenylene diamine in citrate buffer were determined. Superiority of the optimised ELISA was determined by comparing it with the ELISA technique previously used according to Verschoor *et al.* (1989).

Values were considered positive if they were twice that of the background with a corresponding coefficient of variation of less than 10 %.

3. Result

3.1 ELISA optimisation

A comparison of various incubation temperatures showed that the highest signal/background ratio was achieved at VC for 24 h and that the lowest standard coefficient of variation was observed when micrititer plate was baked under a heating lamp and fan (Table 1). Of the various fixative concentrations evaluated, the 0.5% glutaraldehyde, 6M urea and 0.75% formaldehyde gave the highest signal/background ratio (Table 2). The coefficient of variation for the three fixatives were less than 10%. 60% 1 - butanol, 80% isopropanol, 70% acetic acid and 0.25% glutaraldehyde gave a zero coefficient of variation (Table 2). Differences were also found between the various blocking solutions used (Table 3). Both 1% and 5% BSA concentrations

gave the highest signal/background ratio and the coefficient of variation was also within acceptable limits. A 2 h incubation time for 1 % BSA as blocking solution gave the highest signal/background ratio (Table 3). Tween 20/PBS gave the highest signal/background ratio coupled with the lowest coefficient of variation when used at a concentration of 0.5 %, thus making it more effective. Although the incubation time of the primary antibody gave the highest value after 45 min, the coefficient of variation was more than 10%. An incubation time of 60 min was more acceptable. A conjugate concentration of 1 : 5000 gave the highest signal/background ratio and 45 min incubation time of conjugate dilution 1 : 5000 also gave the highest signal/background ratio. All conjugate concentrations gave an acceptable coefficient of variation. The highest signal/background ratio for different for enzyme - substrate reaction times were obtained after 30 min, which also gave the highest coefficient of variation. The coefficient of variation after 50 and 60 min was more acceptable.

Discussion

When comparing the optimised ELISA protocol with the one used previously (Verschoor *et al.,* 1989) a higher signal/background ratio was obtained with the former ELISA protocol and it is therefore more suitable to detect different concentrations of *B. subtilis* in field studies.

Optimisation of ELISA for commercial screening is of utmost importance, in order to rule out variation between batches. It is therefore important, when establishing a new immunoassay, to set acceptable levels of precision, and to achieve an inter-assay variation of less than 10 % (McClaren *et al.,* 1981).

Since a variety of ELISA protocols have been described for various applications and antigen - antibody systems (Korsten et al.,1990; Temeyer *et al.,* 1986; Verschoor *et al.,* 1989), it was deemed necessary to optimise this particular system to suit it's purpose, namely detection of low concentrations of antigen in the field.

Throughout this study the optimum dilution, time and concentration chosen had a coefficient of variation of less than 10 %. The highest signal/ background ratio was obtained with incubation temperatures of 4ºC for 24 h. This adsorption procedure seemed to give a more uniform coating. The same conditions were obtained by Korsten *et al.* (1990) when optimising the ELISA to detect *Pseudomonas syringae* on avocado. In contrast, Verschoor *et al* (1989) pointed out the advantage of baking with a heating lamp and laboratory fan, which include storing the plates at 4ºC for at least 3 months with no significant loss of ELISA - signal. BSA (1 %) gave the highest signal/background value when used as a blocking solution and Tween 20/PBS as a washing buffer. This is in accordance with a report by (McClaren *et al.,* 1981) who found that nonspecific binding of the enzyme conjugate to the coated solid phase can be reduced by including Tween 20 and BSA in the medium. When compared to the ELISA protocol used previously (Verschoor *et al.,* 1989) a higher signal/background ratio was obtained with the optimised ELISA and is therefore more suited to detection of different concentrations of *B. subtilis* in field studies.

The sensitivity, speed and large amount of samples that can be processed (Clark, 1981) are attributes of ELISA. This method is therefore more efficient when compared to leaf imprint, washing and SEM techniques. The ELISA method can be used to monitor *B. subtilis* throughout the season, which will give information to improve biocontrol effectiveness through better formulation, adjustment of dosage and spray scheduling.

References

Clark, M.F., 1981. Immunosorbent assays in plant pathology. Annual Review of Phytopathology 19 : 83 - 106.

Korsten, L., De Villiers, E.E., Weimer, F.C. and Kotz6, J.M., 1993. A review of biological control of postharvest diseases of subtropical fruits. In: Champ, B.R., Highley, E. and Johnson, G.I. (Eds). Postharvest handling of tropical fruits. Proceedings of an International conference held at Chiang Mai, Thailand. Watson, Ferguson and Company : 172 - 185.

Korsten, L., Sanders, G.M. and Kotz6, J.M., 1990. Practical application of monoclonal antibodies in the detection of bacterial diseases of subtropical crops. Acta Horticulturae 275 : 745 - 751.

Knudsen, G.R. and Spurr, H.W., 1987. Field persistence and efficacy of five bacterial preparations for control of peanut leaf spot. Plant Disease 71 442-445.

McClaren, M.L., Lillywhite, J.E. and Andrews, C.S., 1981. Indirect enzyme linked immunosorbent assay (ELISA): practical aspects of standardization and quality control. Medical Laboratory Sciences 38 : 245 - 251.

Schaad, N.W., SOle, S., Van vuurde, J.W.L., Vruggink, H., Alvarez, A.M., Benedict, A.A., De Wael, L. and Van Laere, 0., 1990. In: Klement, Z., Rudolph, K. and Sands, D.C. (Eds). Methods in Phytobacteriology. Akadémiai Kiadò, Budapest : 153 - 190.

Spurr, H.W. and Knudsen, G.R., 1985. Biological control of leaf diseases with bacteria. In: Windels, C.E. and Lindow, S.E. (Eds). Biological control on the phylloplane. The American Phytopathological Society St. Paul. Minnesota : 45 - 62.

Temeyer, K.B., Haufler, M. and Pruett, J.H., 1986. Development of an improved ELISA for antibody detection and use in production of a hybridoma secreting a monoclonal antibody specific for crystal protein of *Bacillus thutingiensis ssp. israelensis.* Journal of Economic Entomology 79 : 1287 - 1293.

Verschoor, J.A., Coetzee, L. and Visser, L., 1989. Monoclonal antibody characterization of two field strains of *Haemophilus paragallinanan* isolated from vaccinated layer hens. Avian Diseases 33 : 219 - 225.

Table 1 Effect of incubation time and -temperature on ELISA signal using *Bacillus subtilis* as solid phase antigen and supernatant from clone 6B7E5 as primary antibody

INCUBATION TIME	INCUBATION TEMPERATURE	SIGNAL/BACKGROUND RATIO	%CV[a]
24 h	4 C	11.35	4.85
24 h	25 C	7.23	6.48
24 h	37 C	4.23	12.77
	BAKE	8.44	4.63
4 h	4 C	6.53	5.12
2 h	4 C	9.46	9.11
1 h	4 C	7.04	5.33
4 h	25 C	4.84	5.63
2 h	37 C	8.10	10.24
4 h	37 C	4.83	12.81
2 h	37 C	5.36	7.43
1 h	37 C	6.25	13.69

[a] Coefficient of variation: standard deviation x 100/mean

Table 2 Effect of fixation method on ELISA signal using *Bacillus subtilis* as solid phase antigen and supernatant from clone 6B7E5 as primary antibody

FIXATIVE	SIGNAL/BACKGROUND RATIO	%CV[a]
70% methanol	4.33	6.17
90% methanol	4.33	11.00
100% methanol	5.50	5.45
70% ethanol	7.00	15.70
80% ethanol	3.67	6.88
100% ethanol	3.03	11.00
60% 1-butanol	4.00	0
70% 1-butanol	2.58	7.64
0,25% formaldehyde	4.14	7.70
0,5% formaldehyde	2.07	5.09
0,75% formaldehyde	14.00	7.14
Sodium dodecyl sulphate	3.00	25.10
60% isopropanol	3.50	10.90
70% isopropanol	12.00	6.00
80% isopropanol	4.00	0
6M urea	14.00	6.29
50% acetic acid	2.50	11.40
60% acetic acid	2.50	24.40
70% acetic acid	0.60	0
0,25% glutaraldehyde	4.33	0
0,5% glutaraldehyde	16.00	6.25
0,75% glutaraldehyde	7.00	29.60

[a] Coefficient of variation: standard deviation x 100/mean

Table 3 Effect of different blocking solutions on effectiveness of the ELISA using *Bacillus subtilis* as solid phase antigen and supernatant from clone 6B7E5 as primary antibody

BLOCKING SOLUTION	SIGNAL/BACKGROUND VALUE	%CV[a]
1% Bovine serum albumin	15.45	5.02
3% BSA	9.00	8.44
5% BSA	12.86	10.80
0,25% Casein	7.06	12.80
0,5% Casein	7.86	12.90
0,75% Casein	5.24	6.86
0,5% Skim milk	8.57	9.58
1% Skim milk	9.29	11.60
1,5% Skim milk	9.17	5.67
0,5% Gelatine	7.65	10.60
1% Gelatine	7.86	13.73
1,5% Gelatine	4.76	11.60

[a] Coefficient of variation: standard deviation x 100/mean