P L A N T B R E E D I N G A N D S E E D S C I E N C E
Volume 46 no. 2 2002

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EVALUATION OF POLYCLONAL AND MONOCLONAL ANTIBODIES FOR DETECTION AND DETERMINATION OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS*

ABSTRACT

Immunochemical diagnostics of *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckerman and Kotthoff) Davis et al., the causal agent of bacterial ring rot of potato, is based on the use of high quality polyclonal and monoclonal antibodies. The specificity and sensitivity of the polyclonal Po–Cs 6 and monoclonal Mn–Cs 1 antibodies for detection and identification of *C. michiganensis* subsp. *sepedonicus* in pure culture and extracts of potato tuber samples were compared by double antibody sandwich enzyme–linked immunosorbent assay (DAS ELISA). The polyclonal antibody Po–Cs 6 cross–reacted with strains of several *C. michiganensis* subspecies, *Pantoea agglomerans*, and weakly with *Pseudomonas fluorescens* and *Ralstonia solanacearum* (race 3) in all serological tests. After cross–absorption with these bacteria, the threshold level of homologous antigen NCPPB 3467 was 10^7 cfu/ml in DAS ELISA. The monoclonal antibody Mn–Cs 1 did not cross react with other *michiganensis* subspecies or with other plant pathogenic bacteria tested. The monoclonal antibody Mn–Cs 1 cross–reacted very weakly with the saprophytic bacteria *P. agglomerans* and *P. fluorescens.* The threshold level of homologous antigen NCPPB 3467 was 10^4 cfu/ml in DAS ELISA. The monoclonal antibody Mn–Cs 1 proved to be more sensitive and specific than the polyclonal antibody Po–Cs 6 in all serological tests.

Key words: bacterial ring rot of potato, cross reactions, DAS ELISA, sensitivity, specificity

INTRODUCTION

Bacterial ring rot of potato, caused by *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckerman and Kotthoff) Davis *et al.*, is a quar–antine disease for states of the EU and the region of EPPO (Smith *et al.*, 1997). In 1996, the disease was recorded for the first time in the Czech Republic in a sample of potato seed of the variety Sante, imported to the Czech Republic from West European countries (Kokošková & Pánková, 1998). Since that time, a systematic effort has been made to eliminate the ring rot pathogen from seed potato production in the Czech Repub–

Communicated by Piotr Masojć

…*Paper presented during International Seed Health Conference PTFit – IHAR 2000 "Seed Health as Quality Criterion" 9 – 11 Oct 2000*

lic. Bacteriological analyses of potato tuber samples are based on immunofluoresence antibody stain (IFAS) tests and tests of pathogenic–ity on eggplant according to the requirements of EU directive No. 93/85/ECC. As an alternative to serological assays, polymerase chain reaction (PCR) tests have been developed using published primers (Mills *et al.*, 1997), however also this technique does not always provide explicit results.

Investigation and preparation of new antibodies for determination and detection of *C. michiganensis* subsp. *sepedonicus* is important be–cause of the heterogeneity of the pathogen (De Boer, 1987). Changes of immunochemical properties can occur as a consequence of variability in climatic conditions and the selection of appropriate cultivars (Kokošková *et al.*, 2000). Polyclonal and monoclonal antisera can be used to detect pathogens by immunochemical techniques such as en–zyme–linked immunosorbent assay (ELISA) and IFAS in symptomless ring rot infections during the growing season (Janse and Van Vaerenbergh, 1987; Gudmestad *et al.*, 1991). Monoclonal antibodies generally are more sensitive and specific and are able to detect low con–centrations of the target bacterium with a high degree of accuracy (De Boer *et al.*, 1988; Westra *et al.*, 1994).

The goal of study was to compare the reliability of polyclonal and monoclonal antibodies for detection of *C. michiganensis* subsp. *sepedonicus* in potato plants. The both antibodies were evaluated for the purpose to be used in ELISA for rutine testing of potato samples in di–agnostic laboratories in future.

MATERIAL AND METHODS

Bacteria and plant samples

Two strains of *C. michiganensis* subsp. *sepedonicus,* no. 3467 and no. 3279, received from the National Collection of Plant Pathogenic Bacte–ria (NCPPB), Harpenden, Great Britain, were used as standards in all serological tests. Nine strains of plant pathogenic and two saprophytic bacteria (Table 2) were received from the Czech Collection of Microor–ganisms, Brno, the Czech Republic. Two strains each of *C. michiganensis* subsp. *insidiosus* and *Erwinia amylovora* and 20 strains of *C. michiganensis* subsp. *sepedonicus* were maintained in the Collection of Plant Pathogenic Bacteria at the Bacteriology Department of the Research Institute of Crop Production, Prague, the Czech Repub–lic. Strains of *C. michiganensis* subspecies and *Curtobacterium* sp. were cultured on C medium (Snieszko and Blonde, 1943) at 23 °C for 4 days. Strains of *Erwinia* sp., *Pantoea* sp. and *Xanthomonas* sp. were grown on nutrient beef–peptone medium at 26 °C for 1–2 days and strains of *Pseudomonas* sp. on King B' medium at 24 °C for 2 days (King *et al.*, 1954). All bacteria were maintained on beads in nutrient broth at –70 °C.

The extracts of potato samples were prepared according to EU direc–tive No. 93/85/ECC. In experiments, the tuber extracts evaluated as positive in IF test 6 months ago, were used. The tuber extracts were tested in dilution 1:1, 1:10, 1:100 and 1:1000

Antibodies

The polyclonal antiserum was prepared in rabbits against formalde–hyde– fixed cells of *C. michiganensis* subsp. *sepedonicus* NCPPB 3467 (Kokošková and Pánková, 1998). This crude antiserum was used for slide agglutination (SA) and Ouchterlony double diffusion (ODD) tests. The immunoglobulin (IgG) fractions were used for DAS ELISA.

The monoclonal antibody was separated from hybridoma culture fluid resulting from fusions using mice immunized with whole cells and extracellular soluble fraction of *C. michiganensis* subsp. *sepedonicus* NCPPB 3467 (Pánková and Kokošková, in press). The purified mixture of IgG fractions isolated from hybridoma liquid culture was used for double antibody sandwich enzyme–linked immunosorbent assay (DAS ELISA).

For polyclonal and monoclonal antibodies, the optimal concentration of tested range of IgG (0.1– 0.4 mg/ml), IgG–AP (1:50 – 1:400) and anti–gen (10^7 –10^3 cells/ml) were selected to DAS ELISA test.

Methods

The crude antiserum was used in SA tests (Claflin and, 1977) and in ODD tests (Ouchterlony, 1948). The separated IgG fraction was tested by DAS ELISA (Clark and Adams, 1977). The evaluation of ELISA sig–nals is shown in Table 1. The results of all samples evaluated in serological tests were summarized from 2 replications.

Table 1

Evaluation of DAS ELISA signals – the absorbance value at 405 nm

–	Negative reaction (below background)	< 0.2
+	Weakly positive reaction	0.21 – 0.29
++	Medially positive reaction	0.30 – 0.49
+++	Strongly positive reaction	> 0.50

Specificity of polyclonal and monoclonal antibodies was evaluated with the above–mentioned plant–pathogenic and saprophytic bacteria used at concentrations of 10^7 – 10^5 cfu/ml by DAS ELISA (Table 2).

Sensitivity of polyclonal and monoclonal antibodies also was evalu–ated with 37 pure cultures of *C. m. sepedonicus* used at concentrations of 10^7 –10^3 cfu/ml and with tuber extracts from 40 potato samples by DAS ELISA (Fig. 1).

Table 2

The specificity of the polyclonal Po–Cs 6 and the monoclonal Mn–Cs 1 antibodies in DAS ELISA

Isolate	Po–Cs 6 [cfu/ml]			Evaluation	Mn–Cs1 [cfu/ml]			Evaluation
	107	106	105		107	106	105	
Clavibacter michiganensis subsp. *sepedonicus* NCPPB1 3467	0.50	0.30	0.15	++	0.79	0.60	0.85	++
Clavibacter michiganensis subsp. *sepedonicus* NCPPB 3279	0.50	0.19	0.10	++	1.21	0.76	0.74	+++
Agrobacterium tumefaciens CCM2 2835	0.00	0.10	0.15	–	0.12	0.17	0.18	–
Clavibacter michiganensis subsp. *insidiosus* RICP3 12/5	0.20	0.19	0.11	(+)	0.19	0.20	0.20	–
Clavibacter michiganensis subsp. *michiganensis* CCM 1635	0.22	0.20	0.10	+	0.11	0.17	0.18	–
Clavibacter michiganensis subsp. *nebraskensis* CCM 2749	0.20	0.10	0.10	–	0.20	0.20	0.14	–
Curtobacterium flaccumfaciens CCM 2403	0.20	0.10	0.10	–	0.20	0.20	0.15	–
Erwinia amylovora RICP 8/95	0.00	0.00	0.00	–	0.20	0.20	0.10	–
Erwinia carotovora subsp. *carotovora* CCM 1008	0.11	0.09	0.18	–	0.20	0.18	0.14	–
Erwinia chrysanthemi CCM 989	0.03	0.00	0.09	–	0.12	0.15	0.20	–
Pantoea agglomerans CCM 2406	0.30	0.25	0.20	++	0.15	0.22	0.20	(+)
Pseudomonas fluorescens CCM 2115	0.23	0.22	0.20	+	0.21	0.20	0.19	(+)
Pseudomonas syringae pv. *syringae* CCM 4073	0.12	0.05	0.02	–	0.20	0.20	0.16	–
Ralstonia solanacearum CCM 2505	0.23	0.20	0.10	+	0.20	0.18	0.14	–
Xanthomonas vesicatoria CCM 2102	0.00	0.00	0.00	–	0.18	0.15	0.12	–

[1] NCPPB – National Collection of Plant Pathogenic Bacteria, Harpenden, Great Britain
[2] CCM – Czech Collection of Microorganisms, Brno, Czech Republic
[3] RICP – Collection of Plant Pathogenic Bacteria of Dept. of Bacteriology, Research Instituite of Crop Production, Prague, Czech Republic
– negative reaction, (+), +, ++, +++ intensity of positive reaction

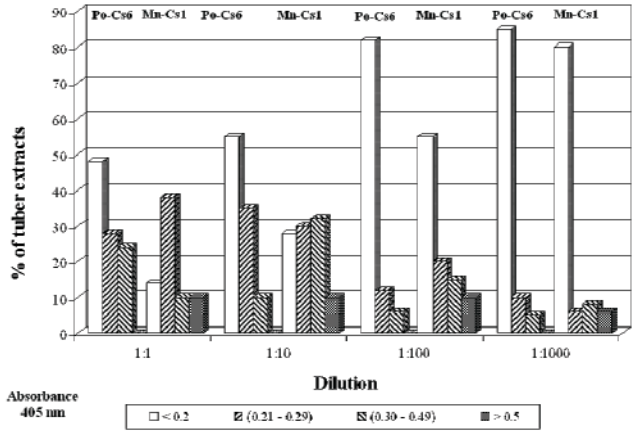

Fig. 1 Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato extracts with polyclonal Po–Cs 6 and monoclonal Mn–Cs 1 antibodies by DAS ELISA

RESULTS

The monoclonal Mn–Cs 1 and polyclonal Po–Cs 6 antibodies produced against the ring rot pathogen were compared using several serological methods.

The polyclonal antibody Po–Cs 6 was mainly used in SA and ODD tests. In these tests, the cultured *C. m.* subsp. *sepedonicus* isolates were used only in concentrations of 10^9 cfu/ml. The separated IgG fractions reacted positively in DAS ELISA. The optimal dilutions for identifica–tion of *C. m.* subsp. *sepedonicus* isolates by DAS ELISA were 0.2 mg/ml IgG, 1:100 IgG–AP, and the antigen in a concentration of 10^7 cfu/ml. *C. michiganensis* subsp. *sepedonicus* was identified to concentration of 10^4 cfu/ml, however only in 7 strains of 37 tested (Table 3). The optimal di–lutions of the potato extracts were 1:1 and 1:10 (Fig. 1) .

The polyclonal antibody Po–Cs 6 cross–reacted with strains *C. michiganensis* subsp. *insidiosus* RICP 12/5, subsp. *michiganensis* CCM 1635, *P. agglomerans* CCM 2406, and weakly with *P. fluorescens* CCM 2115 and *R. solanacearum* NCPPB 2505 (race 3) in the serological tests SA, ODD and DAS ELISA (Table 2).

The mixture of monoclonal antibodies Mn–Cs 1 did not react in SA and ODD tests. The separated IgG fraction reacted positively in DAS ELISA. The optimal dilutions for identification of *C. michiganensis* subsp. *sepedonicus* isolates by DAS ELISA were 0.1 mg/ml IgG, 1:250 IgG–AP, and the antigen in a concentration of 10^4 cfu/ml. Of 37 isolates of *C. michiganensis* subsp. *sepedonicus* tested still 22 isolates were identified in concentration 10^3 cfu/ml (Table 3). The optimal dilutions of the potato extracts were 1:10 and 1:100 (Fig. 1).

Table 3

Determination of *Clavibacter michiganensis* subsp. *sepedonicus* with polyclonal Po–Cs 6 and monoclonal Mn–Cs 1 antibodies in DAS ELISA

Absorbance value (405 nm)	Number of isolates									
	Po–Cs6 [cfu/ml]					Mn–Cs1 [cfu/ml]				
	10^7	10^6	10^5	10^4	10^3	10^7	10^6	10^5	10^4	10^3
< 0.2	8	10	16	30	/	1	1	1	1	15
(0.21 – 0.29)	21	26	21	7	/	12	13	10	11	20
(0.30 – 0.49)	8	1	0	0	/	18	17	18	15	2
> 0.5	0	0	0	0	/	6	6	8	10	0
Evaluation	++	+	(+)	–	/	++	++	+++	+++	–

– ⟶ negative reaction; (+), +, ++, +++ ⟶ intensity of positive reaction;
cfu/ml ⟶ concentration of isolates tested

The monoclonal antibody Mn–Cs 1 did not cross–react with related *C. michiganensis* suspecies or with other plant pathogenic bacteria tested. The monoclonal antibody Mn–Cs 1 very weakly cross–reacted with the saprophytic strains *P. agglomerans* CCM 2406 and *P. fluorescens* CCM 2115 (Table 2).

DISCUSSION

ELISA is considered to be a good compromise between sensitivity and specificity of detection, and ease and expense of application. This accounts for the present day popularity of ELISA for routine detection of plant pathogens (De Boer *et al.*, 1988, Gudmestad *et al.*, 1991).

The monoclonal antibody Mn Cs–1 was more sensitive than polyclonal antibody Po–Cs 6, when cells of *C. michiganensis* subsp. *sepedonicus* from pure cultures in concentrations 10^7 – 10^3 cfu/ml were tested and when *C. michiganensis* subsp. *sepedonicus* was detected in potato extracts of tuber samples in all dilutions (1:1, 1:10, 1:100 and 1:1000) tested, too.

The polyclonal antibody Po–Cs 6, mainly used in SA and ODD tests, determined the ring rot pathogen only at concentrations of 10^9 cfu/ml. The optimal dilutions for identification of *C. michiganensis* subsp. *sepedonicus* isolates by DAS ELISA were 0.2 mg/ml IgG, 1:100 IgG–AP, and the optimal concentration of 10^7 cfu/ml for the antigen. Determination was still possible at 10^4 cfu/ml. These results support those of Underberg & Sander (1991). The optimal dilutions of the potato extracts were 1:1 and 1:10 (Fig. 1). In our experience, the dilution 1:10 was more reliable. There was less difference between replications at the 1:1 dilution and less difficulty with background reactions with starch grains.

The mixture of monoclonal antibodies Mn–Cs 1 did not react in SA and ODD tests. The optimal dilutions for identification of *C. m.* subsp. *sepedonicus* isolates by DAS ELISA were 0.1 mg/ml IgG, 1:250 IgG–AP, and at antigen concentrations of 10^4 cfu/ml, but till to concentration 10^3 cfu/ml were isolates identified. These results support those of De Boer *et al.* (1988). The optimal dilutions of the potato extracts were 1:10 and 1:100 (Fig. 1). The 1:100 dilution seemed to be more reliable than the 1:10 for detection of *C. michiganensis* subsp. *sepedonicus* because more reduced background reactions variability.

Monoclonal antibody Mn Cs–1 was also more specific than polyclonal antibody Po–Cs 6 when tested against various plant pathogenic and saprophytic bacteria.

The polyclonal antibody Po–Cs 6 cross–reacted with strains of *C. m.* subsp. *insidiosus, C. michiganensis* subsp. *michiganensis*, *P. agglomerans,* and weakly with *P. fluorescens* and *R. solanacearum* (race 3) in the SA, ODD and DAS ELISA tests (Table 2). The occurrence of false–positive reactions due to the presence of cross–reacting saprophytic bacteria in the extracts is considered as a serious drawback to the use of polyclonal antibodies (De Boer, 1982; de Boer 1988; Griep *et al.*, 1998). The preparation of polyclonal antisera sufficiently specific against *C. m.* subsp. *sepedonicus* is very difficult because of the strong serological relationship between *C. michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *michiganensis,* and *C. michiganensis* subsp. *insidiosus* (De Boer, 1982; De Boer 1988, Kokošková *et al.*, 2000). In our studies, cross–reactions with the saprophytic bacteria *P. agglomerans* and *P. fluorescens* are the most troublesome. According to Miller (1984), *P. fluorescens* is the most frequent cross–reacting bacterium in serological tests. However, the potential presence of *P. agglomerans*, *P. fluorescens,* and *R. solanacearum* in extracts of potato tuber samples must be considered, because these bacteria may be present in the rhizosphere or within tissues of potato plants. The potential for signifi–cant cross–reactions remain in polyclonal antisera against *michiganensis* subspecies even after absorption with cross–reacting bacteria. Therefore, specificity of polyclonal antibody Po–Cs 6 probably would not be acceptable even after cross–absorption.

Cross–reactivity is a problem with all serodiagnostic tests. However, monoclonal antibodies react with a single antigenic determinant, mak–ing specificity potentially much better. The advantage of monoclonal antibodies over polyclonal antisera are their high levels of specificity and uniformity (De Boer, 1987). The monoclonal antibody Mn–Cs 1 cross–reacted with neither *C. michiganensis* subspecies nor with other plant pathogenic bacteria tested. The monoclonal antibody Mn–Cs 1 only very weakly cross–reacted with *P. agglomerans* and *P. fluorescens*. Using ELISA, the monoclonal antibody Mn–Cs 1 proved to be more sen–sitive and specific than polyclonal antibody Po–Cs 6. In future, monoclonal Mn–Cs 1 or both polyclonal Po–Cs 6 and monoclonal Mn–Cs

1 antibodies combined are considered to be used in ELISA for detection of *C. michiganensis* subsp. *sepedonicus* from potato tuber extracts.

ACKNOWLEDGMENTS

We wish to thank the Ministry of Agriculture of the Czech Republic for a support project No. 320/4308 that included this work.

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