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Application of polyclonal and monoclonal antibodies for the detection of Xanthomonas campestris pv. campestris in crucifer seeds using immunofluorescence microscopy

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Abstract

Polyclonal and monoclonal antibodies (PCAs and MCAs) were tested for the detection of *Xanthomonas campestris* pv. *campestris* (Xcc) in cabbage seeds using immunofluorescence microscopy (IF). It was concluded that PCA 94, MCAs 20H6, 2F4, 18G12 and a mixture of MCAs 20H6, 18G12, 2F4 and 16B5 could be used to detect Xcc in seed extracts when 5 min and 2.5 h shaking of seeds are used as extraction methods. The reliability of confirming suspect colonies with MCAs and PCA 94 in IF depended in part on the seed lot tested and the antibody used. Some virulent Xcc strains derived from seed lots, did not react with MCAs 10C5, 2F4, 18G12, 17C12 and 16B5. On the other hand, saprophytic isolates obtained from one seed lot cross-reacted with MCA 17C12 and to a lesser extent with MCAs 2F4, 18G12 and PCA 94. No relationship was found between IF-reactions of Xcc strains using MCAs and reactions of Xcc strains in pathogenicity testing. Xcc and *X. c.* pv. *amoraciae* (Xca) could in general not be distinguished on the basis of reactions with MCAs and PCAs. Also in pathogenicity tests Xcc and Xca were hard to distinguish.

Additional keywords: pathogenicity testing, virulence, dilution-plating, YDC- agar, NSCA, NSCAA, nutrient starch medium, antibiotics.

Introduction

Xanthomonas campestris pv. *campestris* (Xcc), the causal agent of black rot in crucifers, is a seed-transmitted plant-pathogenic bacterium (Richardson, 1990). The use of disease-free seed is an important way to prevent establishment and spread of this pathogen. For detection of Xcc in seeds, techniques should be both specific and sensitive. Serological assays potentially meet these requirements. Schaad (1978) and Schaad and Donaldson (1980) used immunofluorescence microscopy (IF) with polyclonal antibodies (PCAs) to identify colonies and to detect Xcc in cabbage seed extracts, respectively. However, cell counts in IF were not reported, although they could give information on the specificity and sensitivity of IF. Alvarez and Lou (1985) used ELISA with PCAs to detect Xcc in leaf disk samples from field-grown cabbage. Yuen et al. (1987) used ELISA with monoclonal antibodies (MCAs) to detect and to differentiate strains of Xcc isolated from cabbage black rot lesions collected from the field. Franken et al. (1992) recently evaluated MCAs for identification of Xcc strains in IF, an enzyme

immunoassay and a dot-blot immunoassay.

The aim of the present study was to investigate the application of MCAs, produced against Xcc (Franken et al., 1992) as compared to a specific polyclonal antiserum (PCA 94), for detection of Xcc in crucifer seeds with IF. IF was chosen because this test was considered to be the most sensitive method for the detection of seed-borne bacteria (Franken and Van Vuurde, 1990). For direct detection of Xcc in seed extracts, cell counts in IF were used for comparison of MCAs with PCA 94.

Materials and methods

Monoclonal and polyclonal antibodies. All antibodies used in this study were described by Franken et al. (1992). MCAs were purified using ammonium sulphate precipitation or cation-exchange chromatography. PCA 94, MCA 20H6 (5 mg ml $^{-1}$), MCA 16B5 (5 mg ml $^{-1}$), MCA 2F4 (5 mg ml $^{-1}$), MCA 18G12 (5 mg ml $^{-1}$), 10C5 (2.5 mg ml $^{-1}$) and 17C12 (5 mg ml $^{-1}$) were used in dilutions of 1:100, 1:100, 1:100, 1:30, 1:30, 1:10 and 1:100, respectively. The mixture of MCA (Mix-MCA) was prepared by mixing equal volume aliquots of MCA 20H6 (5 mg ml $^{-1}$), MCA 16B5 (5 mg ml $^{-1}$), MCA 2F4 (5 mg ml $^{-1}$) and MCA 18G12 (5 mg ml $^{-1}$) and used at a 1:100 dilution.

Bacterial strains. All cultures used in this study are listed in Table 1.

Immunofluorescence microscopy. Naturally contaminated seed lots from various origins were tested with IF (10 000 seeds per seed lot). To each sample of 10 000 seeds 100 ml of saline (0.85% NaCl) was added. All samples were shaken for 5 min and 2.5

Table 1. Bacterial strains used in immunofluorescence microscopy and pathogenicity testing for *Xanthomonas campestris* pv. *campestris*.

Bacteria	Strain designation	Reveived from[1]
Bacillus subtilis	Bsub 6633	5
Erwinia herbicola	Eher 732, Eher 1062, Eher 1063,	4
	Eher 83	1
E. carotovora subsp. *carotovora*	Ecar 139	1
Pseudomonas marginalis	Pmar 86/1118	4
P. viridiflava	Pvir 540	1
P. cichorii	Pcich 478	4
Xanthomonas campestris pv. *amoraciae*	Xca 373, Xca 374	1
X. c. pv. *campestris*	Xcc 102, Xcc 364, Xcc 367, Xcc 671,	1
	Xcc 8-6, Xcc 9-48, Xcc 9-4, Xcc 9-30N,	2
	Xcc 8-19-1	2

[1] 1 = Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands; 2 = Author; 3 = D.L. Pinnow, Georgia, USA; 4 = Plant Protection Service, Wageningen, the Netherlands; 5 = American Type Culture Collection, Maryland, USA.

h at room temperature. Twenty μl per subsample (undiluted and 10× diluted) was fixed onto multitest slides (8 or 5 mm diameter). Indirect IF was done as described by Van Vuurde et al. (1983). At least 25 microscope fields were counted (field coefficient = 18, objective magnification ×63, internal magnification ×1.25 or ×1.00, ocular magnification ×10). Cell counts were converted to the number of fluorescent cells per ml. For analysis of variance, all cell counts were transformed to logarithms of fluorescent cells per ml (log cells per ml). To include zero readings one cell per ml was added to each cell count.

Plating. Plating was done on NSCA (nutrient starch cycloheximide agar; Schaad and Donaldson, 1980) and NSCAA (NSCA with the addition of nitrofurantoin and vancomycin; Randhawa and Schaad, 1984) as described by Franken et al. (1991). Suspect colonies from NSCA and NSCAA were confirmed on YDC (yeast extract-dextrose-calcium carbonate agar; Schaad, 1988), in IF using PCA 94 and MCAs, and pathogenicity testing. A seed lot was considered to be infested with Xcc, when YDC-positive (yellow mucoid) colonies produced typical black discolouration of the veins in the pathogenicity test, described by Schaad (1982).

Pathogenicity tests. Cultures were grown for 24-48 h on a growth factor medium or on YDC agar. For all pathogenicity tests, cultivars 'Septa' (*Brassica oleracea* var. *capitata*) and 'Tardis' (*Brassica oleracea* var. *gemmifera*) were used.

The pathogenicity test, published by Schaad (1982), was used as a reference (pathogenicity test 1). For this pathogenicity test, light milky cell suspensions ($10^7 - 10^8$ cells ml^{-1}) were made in distilled water and inoculations took place immediately after preparing the suspensions. At least two plants of each cultivar were inoculated per isolate. Plants were incubated at 22-25 °C.

Pathogenicity test 2 was a modification of a pathogenicity test suggested by B. Schrijver, Bejo Zaden B.V. (personal communication). In this test, seeds free of Xcc and fungal pathogens and with germination above 90% were treated with AAtiram (50% a.i. thiram), Rovral (50% a.i. iprodione) and Benlate (50% a.i. benomyl), according to standard procedures prescribed by the manufacturers, to avoid possible interference by fungi after inoculation. Twenty-five seeds per filter paper disk were germinated according to the Rules of the International Seed Testing Association (ISTA, 1985). After 3-4 days, seed coats were removed from the seedlings and cotyledons were dipped for 5 min into cell suspensions of pure cultures or YDC-positive colonies. Contact of the cell suspensions with the filter paper was avoided. After inoculation, filter papers were put onto humid silversand in a small tray. Incubation took place at 22-25 °C, and a minimum of 12 hours light per day was given. Symptoms appeared within 4-10 days. Typical Xcc-symptoms were initially papery brown to black localised lesions on margins of infected cotyledons and at the end of the middle vein of cotyledons (often V- shaped). With proceeding infection, cotyledons and hypocotyls often became water-soaked. Cotyledons finally collapsed. Controls were bacterial strains of *Erwinia herbicola, E. carotovora* subsp. *carotovora, Pseudomonas marginalis, P. viridiflava, P. cichorii, Bacillus subtilis.*

Table 2. Cell counts in immunofluorescence microscopy using monoclonal and polyclonal antibodies, produced against *Xanthomonas campestris* pv. *campestris*.

Seed lot	Antibodies	Extraction method (log cells per ml)[1]	
		5 min shaking	2.5 h shaking
B188	PCA 94	2.00	2.44
	MCA 20H6	0.00	0.00
	MCA 2F4	0.00	0.00
	MCA 18G12	0.00	0.00
	Mix-MCA	0.00	1.00
	MCA 10C5	0.00	0.00
	MCA 17C12	0.00	1.50
	MCA 16B5	1.71	0.00
B189	PCA 94	0.00	4.28
	MCA 20H6	3.88	4.47
	MCA 2F4	3.84	4.87
	MCA 18G12	3.97	4.05
	Mix-MCA	3.91	4.81
	MCA 10C5	3.62	4.27
	MCA 17C12	4.24	1.09
	MCA 16B5	0.00	1.00
B190	PCA 94	2.70	1.45
	MCA 20H6	1.20	2.65
	MCA 2F4	2.59	1.20
	MCA 18G12	2.59	1.00
	Mix-MCA	0.85	1.27
	MCA 10C5	1.59	2.20
	MCA 17C12	0.00	0.00
	MCA 16B5	0.00	0.00
B196	PCA 94	2.65	1.24
	MCA 20H6	2.00	1.15
	MCA 2F4	0.00	1.45
	MCA 18G12	0.00	1.39
	Mix-MCA	0.00	1.27
	MCA 10C5	0.00	0.00
	MCA 17C12	2.18	1.30
	MCA 16B5	0.00	0.00
B197	PCA 94	4.54	6.17
	MCA 20H6	4.87	5.70
	MCA 2F4	4.46	6.05
	MCA 18G12	4.26	5.94
	Mix-MCA	4.39	5.65
	MCA 10C5	3.90	6.03
	MCA 17C12	0.00	2.79
	MCA 16B5	1.09	0.00

Table 2. (Continued).

Seed lot	Antibodies	Extraction method (log cells per ml)[1]	
		5 min shaking	2.5 h shaking
B209	PCA 94	4.84	5.71
	MCA 20H6	4.53	5.89
	MCA 2F4	4.23	6.00
	MCA 18G12	4.36	5.84
	Mix-MCA	4.28	5.69
	MCA 10C5	3.54	5.38
	MCA 17C12	0.00	3.93
	MCA 16B5	0.00	0.00
B213	PCA 94	4.69	4.84
	MCA 20H6	4.09	4.83
	MCA 2F4	4.39	4.70
	MCA 18G12	4.51	5.10
	Mix-MCA	4.55	5.39
	MCA 10C5	1.09	4.52
	MCA 17C12	3.07	3.50
	MCA 16B5	0.00	0.00
B214	PCA 94	1.15	3.02
	MCA 20H6	1.00	0.00
	MCA 2F4	4.95	3.54
	MCA 18G12	4.69	2.98
	Mix-MCA	3.10	1.75
	MCA 10C5	0.00	4.21
	MCA 17C12	0.00	0.00
	MCA 16B5	0.00	0.00
B215	PCA 94	1.50	0.00
	MCA 20H6	1.09	2.55
	MCA 2F4	0.00	1.20
	MCA 18G12	0.00	1.20
	Mix-MCA	0.85	1.27
	MCA 10C5	0.00	0.00
	MCA 17C12	1.15	1.15
	MCA 16B5	0.00	0.00
B216	PCA 94	4.48	4.21
	MCA 20H6	4.40	4.25
	MCA 2F4	2.83	4.18
	MCA 18G12	3.66	4.23
	Mix-MCA	3.55	4.21
	MCA 10C5	3.49	3.83
	MCA 17C12	1.33	1.39
	MCA 16B5	0.00	0.00

[1] Means of 2 replications; standard error of differences of means = 0.905.

Table 3. Confirmation of colonies, derived from crucifer seed lots, with YDC-medium, IF and pathogenicity testing.

Seed lot	Number of[1] colonies tested	YDC result[2] (number of positive colonies)	IF result[3] (number of positive colonies)							Pathogenicity test[4] (number of positive colonies)
			PCA 94	MCA						
				20H6	2F4	18G12	Mix	10C5	17C12	16B5
B188	13	0	0	0	0	0	0	0	0	0	0
B189	12	6	6	6	6	6	6	6	1	0	6
B190	9	0	0	0	0	0	0	0	0	0	0
B196	3	0	0	0	0	0	0	0	0	0	0
B197	15	10	10	10	9	9	10	8	4	0	10
B209	15	7	6	7	5	4	6	3	2	0	7
B213	20	16	16	16	9	8	12	14	3	1	16
B214	20	0	2	0	2	1	1	0	4	0	0
B215	16	0	0	0	0	0	0	0	0	0	0
B216	5	0	0	0	0	0	0	0	0	0	0

[1] The number of colonies tested is the number of colonies transferred from NSCA or NSCAA to YDC.
[2] The number of positive colonies is the number of colonies which showed typical colony morphology and pigment production on YDC.
[3] The number of positive colonies is the number of colonies from YDC which showed good staining of the whole cell wall in IF.
[4] The number of positive colonies is the number of colonies which showed symptoms 'typical' for *Xanthomonas campestris* pv. *campestris*.

Results

Screening seed lots with IF using polyclonal and monoclonal antibodies. The results of screening seed lots with IF using MCAs and PCA 94 are summarized in Table 2. The cell counts in IF (expressed as log cells per ml) varied significantly with (i) the seed lot tested, (ii) the extraction method and (iii) the antibodies used ($P < 0.001$). Although in most cases, more fluorescent cells were found after 2.5 h than after 5 min shaking, exceptions were found for some combinations of seed lots and antibodies. For example, seed lot B189 tested with MCA 17C12, seed lot B190 tested with PCA 94 and seed lot B214 tested with MCA 18G12 yielded higher cell counts after 5 min than after 2.5 h shaking. PCA 94, MCAs 20H6, 2F4, 18G12 and the Mix-MCA generally gave high cell counts, whereas cell counts obtained with MCAs 17C12, 10C5 and 16B5 were generally lower or nearly zero. However, exceptions were found, e.g. when using PCA 94 and 5 min shaking a negative result was obtained for seed lot B189 whereas high cell counts were obtained with MCA 17C12. The same was valid when comparing cell counts obtained with resp. MCA 20H6 and MCA 16B5 for seed lot B188 using 5 min shaking.

For seed lots B197, B209 and B213 usually high cell counts were obtained (cell counts expressed as log cells per ml varied generally between 4 and 6). This, however, also depended on the antibodies used.

In general, the fluorescence intensity of cells in IF-slides stained with MCA 10C5 was inferior to the fluorescence intensity of cells stained with other MCAs and PCA 94. On the other hand, the fluorescence intensity of cells stained by MCA 20H6 was superior to that of cells stained by other MCAs and PCA 94 (not shown).

In the plating assays corresponding to the IF tests from Table 2, seed lots B189, B197, B213 using 5 min and 2.5 h shaking and seed lot B209 using 2.5 h shaking were found positive (infested).

The use of polyclonal and monoclonal antibodies for identification of suspect colonies. Suspect colonies were transferred from NSCA and NSCAA to YDC. PCA 94 and MCAs were used to identify colonies from YDC. MCAs 2F4, 18G12, 10C5 and 17C12 reacted with lower numbers of colonies in IF than PCA 94 and MCA 20H6 (Table 3). MCA 16B5 generally gave negative IF-results for all colonies tested. For most seed lots using PCA 94, MCA 20H6 and the Mix-MCA a good correlation was found between YDC results and confirmations by IF, e.g. for PCA 94 with seed lots B189, B197, B209 and B213, MCA 20H6 with seed lots B189, B197, B209 and B213. The Mix-MCA reacted generally in a similar way as PCA 94 and MCA 20H6, except for seed lot B213, for which some 'false-negative' colonies (i.e. Xcc colonies not reacting with the antibodies) were found. For seed lot B214 some YDC-negative colonies (saprophytic isolates) were found to cross-react with PCA 94, MCAs 2F4, 18G12, 17C12 and the Mix-MCA in IF. The results of the pathogenicity tests correlated well with the YDC-results and IF-confirmations using MCA 20H6 and PCA 94; nearly one hundred per cent of the YDC-positive and IF-positive (using PCA 94 and MCA 20H6) results were positive in the pathogenicity tests.

Comparison of IF-reactions to reactions in pathogenicity tests. To investigate a possible correlation between reactions in IF and the degree of virulence, several known

Table 4. Relationship between reactions in IF using several antibodies and the degree of virulence on cultivars 'Septa' and 'Tardis'.

Strains	Reaction of several strains in pathogenicity tests[1]				Reactions with monoclonal antibodies in IF[1]					
	pathogenicity test 1		pathogenicity test 2		20H6	16B5	17C12	2F4	18G12	10C5
	Septa	Tardis	Septa	Tardis						
Xcc 102	+	+	+	+	−	++	+	±	+	±
Xcc 8-19-1	++	++	++	++	++	−	±	±	±	+
Xcc 9-30 N	±/−	±/−	±	±	−	+	+	−	−	−
Xcc 8-6	±/−	±/−	±	±	−	+	+	−	+	−
Xcc 9-4	+	+	++	±	+	+	+	−	−	+
Xcc 671	++	+	+	+	−	+	+	−	+	−
Xcc 367	++	++	++	+	++	+	+	+	+	+
Xcc 364	+	+	+	+	−	+	+	+	+	+
Negative controls	−	−	−	−	−	−	−	−	−	−

[1] ++ = very strong reaction; + = strong reaction; ± = weak reaction; − = no reaction.

reference Xcc strains were tested in IF and pathogenicity test 1 and 2. Table 4 shows the reactions of some reference Xcc strains with MCAs in IF and their reaction on plants. Low virulent strains 9-30 N and 8-6 and virulent strains 102 and 671 did not react with MCA 20H6. MCA 16B5 and 17C12 generally reacted equally well with low virulent strains 9-30 N and 8-6 and highly virulent strains such as Xcc 367 and Xcc 364. MCA 2F4 did not react with low virulent strains 9-30 N and 8-6 and virulent strain 671. MCA 18G12 reacted with strains 8-6 (low virulence) and 367 (high virulence) but not with 9-30 N (low virulence) and 9-4 (moderate virulence). MCA 10C5 did not react with strains 9-30 N and 8-6 (low virulence) but did also not react with strain 671 (moderate virulence). Neither of the negative control strains produced symptoms in these pathogenicity tests, nor did they react with any of the antibodies tested.

In a separate experiment differences between Xcc strains 364 and 367, and Xca strains 373 and 374 were evaluated. These strains were tested in IF and inoculated on plants of cultivars 'Septa' and 'Tardis' using pathogenicity test 1 and 2. In IF, Xca 373 reacted with all the MCAs tested. Xca 374 did not react with MCA 10C5, but reacted weakly with the other MCAs. Xcc 364 failed to react with MCA 20H6, but reacted with all other MCAs. Xcc 367 and Xca 373 reacted identically with the MCAs in IF (results not shown).

In pathogenicity test 1, inoculation with Xca strains 373 and 374 occasionally resulted in appearance of localized brown to black discolouration of one vein and chlorotic leaf spots on cultivars 'Tardis' and 'Septa' (no systemic symptoms). Xcc strains 364 and 367 produced on both cultivars systemic black discolouration of the veins, followed by appearance of leaf lesions and desiccation of the lesion tissue. In pathogenicity test 2 on cultivar 'Septa', 96% ± 3.7% (25 seedlings per isolate, two

replicates per isolates) of the cotyledons were speckled (necrotic spots) after infection by Xca strains 373 and 374. For Xcc strains 364 and 367, 14% ± 18.2% of the cotyledons was speckled. Also on cultivar 'Tardis' speckled cotyledons were found for Xca strains 373 and 374 (41% ± 24.8%), whereas no speckled cotyledons were found for Xcc 364 and 367. However, all plants of both cultivars also showed 'typical Xcc-symptoms' in pathogenicity test 2 after inoculation with Xcc and Xca strains, viz. initially papery brown to black localised lesions on margins of infected cotyledons and at the end of the middle vein of cotyledons (often V-shaped). With proceeding infections, cotyledons and hypocotyls often became water-soaked. Cotyledons finally collapsed.

In both pathogenicity tests the reactions on cultivar 'Tardis' were somewhat weaker than on cultivar 'Septa'. Also the incubation time was longer on cultivar 'Tardis' than on cultivar 'Septa'.

Discussion

Screening seed lots with IF using polyclonal and monoclonal antibodies. Since IF is considered to be the most sensitive serological assay (Franken and Van Vuurde, 1990), this test was used to screen seed lots. The results presented in this study (Table 2) generally agreed well with results obtained earlier using antibodies from hybridoma supernatants (Franken, 1990). Cell counts depended on the antiserum, extraction method, and the seed lot used. The reasons for these interactions are not completely clear. Some explanations may be given. First, the expression of epitopes may differ between seed lots. This would explain why e.g. for MCA 16B5, that reacted with more Xcc strains (pure cultures) in IF than any other MCA used in this study (Franken, 1990; Franken et al., 1992), low cell counts were obtained. Second, seed lots may contain serologically different strains, of which some are predominant in a specific seed lot and do not react with e.g. MCA 16B5. In addition, phase variation ('a phenomenon which involves a spontaneous switch from the synthesis of one given cell-surface component or structure to another, antigenically distinct form of that component or structure' (Singleton and Sainsbury, 1987)) may be present within a Xcc strain. Third, saprophytes and seed components may affect the growth and serological reactions of Xcc. Strong evidence for one of these explanations has, however, not been found, yet.

As shown in Table 2, the Mix-MCA gave almost equal cell counts as PCA 94, MCA 20H6, MCA 2F4 and MCA 18G12, although this varied per seed lot and extraction method. No additional effect was noted by using a mixture of antibodies compared to using one MCA. However, the result may depend on the specific dilutions and antibodies used.

The dilution-plating results obtained in the present study did not completely correspond with results from earlier studies (Franken, 1990; Franken et al., 1991). In the latter studies seed lots B188, B190, B196, B214, B215 and B216 were found positive in dilution-plating whereas they were found negative in this study. This may be explained by the decrease in viability of Xcc cells during storage of the seed lots as is known for *Pseudomonas syringae* pv. *phaseolicola* (Taylor et al., 1979). However, the presence of dead or 'dormant' cells does not need to have special implications for the characteristics of IF as such, since dead or 'dormant' cells can still be detected with IF.

On the basis of indexing a limited number of seed lots, it is concluded that PCA

94, MCA 20H6, MCA 2F4, MCA 18G12 and the Mix-MCA may be used to detect Xcc in seed extracts with IF. A prerequisite is that seed lots should be screened by using two extraction methods, viz. 5 min and 2.5 h shaking to minimize the risk that seed lots are incorrectly identified as being 'healthy'. To assess the risk of false-negative and false-positive reactions in more detail, and the occurrence of interactions between seed lots and extraction methods, IF is now used in combination with dilution-plating for routine indexing of crucifer seed lots using 5 min and 2.5 h shaking. When using both dilution-plating and IF, additional information on the health status of a seed lot will be obtained. Moreover, information on the sensitivity of IF as compared to dilution-plating will become available, which will help to decide whether IF-positive seed lots which are negative in dilution-plating should be considered infested or not. Studies to investigate correlations between IF and dilution-plating are now being conducted with more seed lots.

The use of polyclonal and monoclonal antibodies for identification of suspect colonies by IF. When using PCA 94, MCA 20H6 and the Mix-MCA in IF generally good correlations were obtained between IF and YDC confirmation tests. However, when confirming 315 colonies derived from dilution-plating assays done earlier with seed lots B188, B189, B189, B213 and B216 (Franken, 1990), YDC-positive and IF- positive results corresponded for 98.2 and 80% when using PCA 94 and MCA 20H6, respectively. Especially for seed lots B213 and B216 not all YDC-positive results could be confirmed by MCA 20H6 whereas positive reactions with PCA 94 almost completely corresponded with YDC-positive colonies (A.A.J.M. Franken, unpublished). Other antibodies were not tested in this particular case. The IF- tests on colonies with MCAs confirmed to a large extent the results with respect to the direct detection of Xcc in seed extracts. However, by using MCAs 2F4, 18G12, 16B5, 10C5 and 17C12 for confirmation of suspect colonies the risk of 'false-negative' reactions is larger than when using PCA 94 and MCA 20H6.

It should be noted that growth rate differences between strains, derived from the same seed lots, were frequently observed on NSCA, NSCAA, YDC and the growth factor medium. These observations suggest that colonies vary in extracellular polysaccharide production and may contain alterations in the cell envelope (Cadmus et al., 1976; Kamoun and Kado, 1990; Kidby et al., 1977), that may seriously influence the reliability of the serological reactions. This emphasizes the fact that more information is needed to establish a possible relationship between physiological conditions and serological reactions of Xcc strains.

The question may also arise whether strains change in their reactions with MCAs in IF or in pathogenicity tests after prolonged storage on slants or after repeated passage through plants. This, however, has not been noted during this or other studies (Yuen et al., 1987).

Reactions in IF compared to reactions in pathogenicity testing. No correlation was found between reactions of Xcc strains on plants of two susceptible cultivars and reactions with MCAs in IF. For an exact analysis of the correlation between reactions with MCAs in IF and the degree of virulence, however, pathogenicity testing on different and more diverse cultivars of several *Brassica* species is necessary.

Differentiation of Xca and Xcc strains was not possible on the basis of reaction with

the PCA 94 and the MCAs in IF. The cross-reaction of Xca strains with antibodies raised against Xcc strains was reported earlier and mentioned as a potential problem in detection and identification of Xcc strains (Alvarez and Lou, 1985; Franken, 1990; Franken et al., 1992). Options to be considered for distinguishing Xca from Xcc strains are e.g., sodium dodecyl sulphate-polyacrylamide gel electrophoresis and fatty-acid profiling, isozyme analysis and DNA hybridization methods. For seed testing and phytosanitary certification, however, pathogenicity testing will often remain necessary for 100% reliability. As suggested by Machmud and Black (1985) Xca causes leaf spot, which is not caused by Xcc. They also found that disease severity as a result of Xca infection varied among cabbage cultivars and that optimum temperatures for Xcc and Xca may differ. In the present study, Xca gave considerably more necrotic spots on cotyledons in pathogenicity test 2 than Xcc. In pathogenicity test 1 symptoms caused by Xcc were more severe and tended to be more systemic than symptoms caused by Xca. The differences noted are in part quantitative differences, and depend in part on the cell concentration applied and cultivar used. For confirmation of colonies by pathogenicity testing, it is therefore difficult to distinguish Xca from Xcc, especially when weakly virulent Xcc strains are isolated from seed lots. Few reports with information about seed infection by Xca are available, whereas numerous reports are available on seed infection with Xcc (Machmud and Black, 1985; Richardson, 1990). The role and significance of Xca in causing a major disease of crucifers is therefore not clear. Therefore, it is advisable to consider seed lots infested when colonies are found positive on YDC and in pathogenicity testing, regardless of whether individual colonies should be regarded as Xca or Xcc.

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