Production of monoclonal antibodies against different strains of *Xanthomonas campestris* pv. *mangiferaeindicae* and detection methods.

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ABSTRACT

Xanthomonas campestris *pv* mangiferaeindicae *is the cause of bacterial blackspot of mango. Fruit isolates of* X. c. *pv.* mangiferaeindicae *which differed in terms of virulence were used to produce monoclonal antibodies. Differences in virulence were confirmed using plant inoculation studies. These antibodies cross react with all the virulence groups, and it is most useful for field detection of latent and existing infections. A selective medium was also developed which might be used by nurserymen together with antibodies to determine infection in the field. This work is of great importance to nurseries and the technique can be used by prospective buyers of plants to determine whether they are free of bacterial blackspot.*

INTRODUCTION

The mango industry is of economic importance in South Africa, the gross value of which amounted to R21 220 480, (Abstract of Agricultural Statistics, 1991). Eighteen cultivars are under cultivation in South Africa but only a few are suitable for export, namely Zill, Tommy Atkins, Kent, Keitt, Haden and Irwin (Kotzé, pers comm.).

This disease affects different cultivars with varying intensity. Cultivars such as Haden, Kent, Irwin, Keitt and Smith are susceptible, whereas cultivars such as Fascell and Sensation are less susceptible (Smith, 1982). All these cultivars are thus liable for a degree of loss in yield with a concurrent drop in export revenue.

Bacterial blackspot (BBS) is a preharvest disease with new spots seldom appearing on fruit after picking and symptom expression occurs on all aboveground plant parts in various ways (Viljoen & Kotzé, 1972). Major losses are due to unacceptably lesioned fruit and premature fruit drop.

Disease control is at present being affected by copper oxychloride applications (De Wet, 1981; Frean, 1985), a mixture of copper oxychloride and zineb and cupric hydroxide (Bot, Sweet, Krause & Hollings, 1988). The disease is spread mainly via the propagation of infected plant material (Viljoen & Kotzé, 1972). Although these treatments have limited sucess, propagation of healthy plants would be the ultimate solution to the problem. Diagnosis of healthy mother trees for grafting material is complicated by the fact that *X. c.* pv. *mangiferaeindicae* is a year round phylloplane resident (Manicom, 1986) without any visible symptoms. Infected mother trees should thus be identified so that only healthy material is propagated.

Standard isolation techniques using nonselective medium, followed by identification using physiological and biochemical tests and final proof of pathogenicity with Koch's postulates have been used up to now to identify the pathogen. However, these tests are time consuming and not practical for large scale commercial screening.

Infected plant material is screened using rapid, accurate, inexpensive techniques such as selective media (Mulrean & Schroth, 1981; Schaad & Forster, 1985) or serological techniques such as dot-immunobinding assay (DIA) (Calfin & Ramundo, 1987), immunofluorescence (IF) (Schaad, 1978) and the enzyme-linked immunosorbent assay (ELISA) (Civerolo & Fan, 1982). However, the ELISA has been used the most often on a commercial scale with the most success (Dosba, Lansac, Pecheur, Teyssier, Piquemal & Michel, 1986; De Boer, Wieczorek & Kummer, 1988). Selective media can also play an important role in the elucidation of disease epidemiology. The growth of non target organisms is greatly reduced using selective media, facilitating the determination of the ecology of the pathogen.

Detection of the pathogenic as well as the epiphytic phase of the pathogen could be greatly improved by a combination of selective media and the implementation of serological techniques. The purpose of this investigation was to raise monoclonal antibodies against and develop a medium for the selective cultivation of *Xanthomonas campestris* pv. *mangiferaeindicae* from field mango plant material.

MATERIALS AND METHODS

Isolation of organisms.

Fruit and leaves from Sabre and Sensation trees with typical BBS symptoms were collected from Zebediela Estates in December 1990.

These were transported to the laboratory where isolations were made immediately. Plant material was surface sterilized in a 3% solution of NaOCl for one minute, followed by three rinses in sterile distilled water (SDW). Isolations were made from young lesions in various stages of development, ranging from water soaked spots to larger lesions which had already cracked open. Small sections were cut from the edges of the lesions, teased apart in sterile Ringer's solution (Oxoid) and streaked onto Standard I nutrient agar (Biolab).

Plates were incubated at 28°C for 72 hours. Bacterial isolates for selective media evaluation were isolated or obtained from L. Korsten and E. van Zyl (Department of Microbiology and Plant Pathology, University of Pretoria). Isolation of phylloplane inhabitants was done by inoculating 10mℓ volumes of Std I nutrient broth (Biolab) with one gram of leaf discs. After incubation for 48 hours at 28°C, a 10 – fold dilution series was made as described by Harrigan & McCance (1966) and plated onto Std I nutrient agar. Plates were incubated as described. Dominant organisms were isolated for use in this study. Cultures were maintained on Std I nutrient agar exept for *Xanthomonas* and *Pseudomonas* spp. which were maintained on Boost medium (Manicom, 1986). Cultures were frozen away in glycerol as described by Sleesman & Leben (1978).

Pathogenicity tests.

Isolates were grown up on Boost agar plates (Manicom, 1980). The cells were harvested and suspended in Ringer's solution to a final concentration of 10^7 cells/mℓ. Sensation fruit was inoculated by prick inoculating 10$\mu\ell$ of inoculum on a minimum of 30 sites per fruit. Prick wounds only and prick inoculation of

10μ Ringers solution served as negative controls. All wounds were covered with sterile cotton wool moistened with SDW, covered with plastic bags and incubated at room temperature, average 28°C for 12 days. Symptom development was monitored daily. The isolates were grouped according to symptom development (Table 1). In order to verify these results, isolations were made from inoculated fruit, grown up as described and reinoculated into fruit as described. Based upon symptom expression, four groups of isolates were described (Table 2). Symptom expression was also tested by inoculating leaves using the detatched leaf technique as described by Graham & Gottwald (1990). Leaves were observed daily, and final evaluation was carried out after seven days. Representative organisms from each group were used for further studies. All isolates were frozen away as described.

Bacterial isolates and antigen preparation.

Representative isolates of *X. c.* pv *mangiferaeindicae* were used in the immunisation programme. These organisms were grown up and harvested under conditions as described. Cells were harvested in Ringer's solution, centrifuged at 5 000 r.p.m. for ten minutes and then washed three times using sterile phosphate buffered saline pH7,0 (PBS). Cells were resuspended in sterile PBS to a final concentration of 1,5 $\times$ 10^7 cells/mℓ. This was then used to immunise mice and screen for antibody production.

Immunisation of mice. Balb/C mice were obtained from the H.A. Grove Research Centre, Pretoria, South Africa. Isolates from each of the four groups were separately used to immunise four groups of three six week old mice. The four isolates were pooled and used to immunise one group of three mice. Immunisation was carried out as described by Verschoor, Coetzee & Visser (1989).

Production of hybridomas.

Murine myeloma cells (sp2/0) were obtained from the Onderstepoort Veterinary Research Institute. Cell fusion between immune spleen cells and the hypoxanthine – guanine phosphoribosyl – transferase – deficient, nonsecreting mouse myeloma cells (Sp2/0) was performed according to the method of Galfre and Milstein (1981). Two cell fusions were performed. The first fusion involved the pooling of spleen cells from one mouse from each virulence group, and the second, from one mouse immunised with pooled isolates. The culture supernatants were screened for the presence of specific antibody when the cultures approached confluency. Positive cultures were transferred to 24 – well multidish plates and cloned on soft agar upon reaching confluency and after determination of the type specificity of the secreted antibody against all bacterial isolates. Cloned hybridoma cultures were subcloned as described by Verschoor, Coetzee & Visser (1989) before being frozen away in 10% dimethyl sulfoxide, 20% horse serum, and 70% Dulbecco's modified Eagle's medium. Culture supernatants drawn for screening and antibody characterisation were from confluent cultures matured for at least three days.

Antibody screening and specificity assay

Screening of polyclonal antisera, hybridoma culture supernatants and the determination of the specificity of the monoclonal antibodies obtained were done by the enzyme – linked immunosorbent assay (ELISA) (Engvall, 1980) using whole bacteria as the solid phase antigen. For screening, equal volumes of 10^7 cells/mℓ Ringer's solution from each of the four strains of *X. c.* pv. *mangiferaeindiae* were mixed. For specificity determination, four duplicate long rows of a microtiter plate were covered individually with the different isolates of *X. c.* pv. *mangiferaeindicae* at 10^7 cells/mℓ. The ELISA was carried out as described by Verschoor, Coetzee & Visser (1989). Colour development was measured at 450 nm on an Anthos 2001 multiplate scanner.

Determination of immunoglobulin class by ELISA

Hybridoma culture supernatants were dispensed in duplicate short rows of the appropriate antigen coated wells at 50 $\mu\ell$ per well. After washing, the plates were probed with a 1:1 000 dilution of heavy chain specific rabbit-anti-mouse immunoglobulin serum (Miles Research Products Division, Elkhart, Indiana) dispensed in duplicate long rows on the plate and incubated at room temperature for 30 minutes. The plates were developed with peroxidase conjugated swine-anti-rabbit immunoglobulin G (H + L) (Dakopatts, Denmark) and treated further as described.

Selective media

Media used in this evaluation included Std I nutrient agar and broth. Selective media evaluated were XTS (Schaad & Forster, 1985), BVGA, SVGA (Sanders, Korsten & Kotzé, 1989) and MXP (Claflin, Vidaver & Sasser, 1987).

Efficiency of recovery from liquid media

A cell suspension of *X. c.* pv. *mangiferaeindicae* with an O.D. of 0,200 was prepared. Three replicates of 10mℓ volumes of each medium was inoculated with 0,1mℓ cell suspension. After 24 hours, a 10-fold dilution was prepared as described by Harrigan & McCance (1966) for each replicate and plated onto STD I nutrient agar. This was repeated at 24 hour intervals with the last evaluation taking place after 72 hours. All plates were incubated at 27°C for 72 hours. Media which yielded the highest counts of *X. c.* pv. *mangiferaeindicae* were used for further evaluation.

Evaluation of medium selectivity

Five 10 mℓ volumes of each medium was inoculated with one gram leaf discs and incubated for 72 hours at 27°C. Samples were taken from each replicate every 24 hours. A 10-fold dilution series was prepared as described and total counts made.

RESULTS

Isolation of organisms

A total of 35 isolations were made, and of these, eight did not produce symptoms when re-inoculated into fruit. The isolates, in general did not grow well on Standard I nutrient agar, but grew better on Boost medium. However, this resulted in excessive slime formation. Media used did not have an effect on the differences in the isolates. A total of eight dominant phylloplane isolates were chosen from the total population observed. Only one of the eight were Gram positive, all were catalase positive, three were oxidase negative and all have an oxidative metabolism.

Pathogenicity tests

Isolates were reinoculated into fruit and a variety of symptoms were observed. The isolates were then grouped according to percentage infection (Table 3). A variety of symptoms were observed ranging from small water soaked spots to typical star shaped lesions. The isolates were then assigned into four groups based upon symptom expression. Differences in symptom expression were observed for the various isolates using the detached leaf technique. Differences were clearly illustrated with the detached leaf technique. In accordance with what was found during the original pathogenicity study, isolate 1F2 was found to produce the highest percentage of infection as well as the most severe symptoms. In general, the symptoms obtained were in accordance with the original findings. Upon re-isolation, pure cultures of *X. c.* pv. *mangiferaein-*

TABLE 1: Grouping of isolates according to symptom development on Sensation fruit

Isolate	No Pricks	No symptomatic	No asymptomatic	Percentage infection
1F6	50	34	16	68
1F5	47	20	27	42.5
1F1	55	23	33	41.8
1F4	50	6	44	12
2F7	45	8	37	17.7
1F2	53	47	6	88.7
1F7	48	29	19	60.4

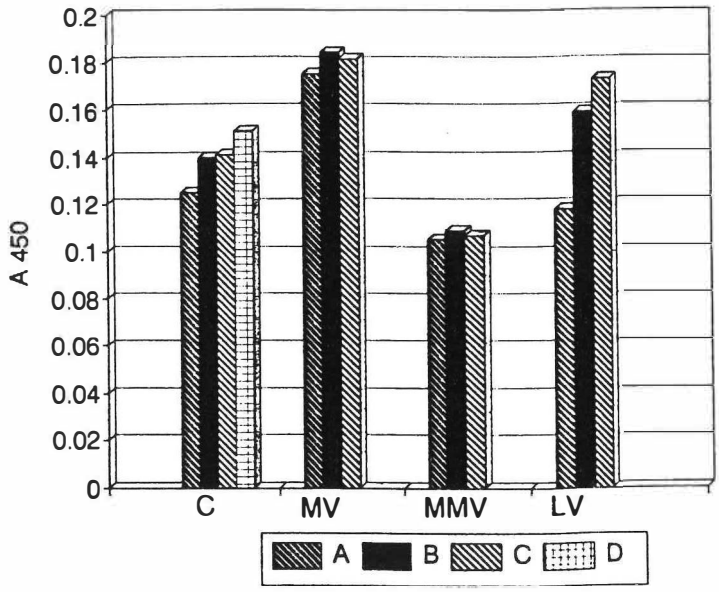
TABLE 2: Disease severity rating for the reaction of Sensation mango fruit after inoculation with strains of *Xanthomonas campestris* pv *mangiferaeindicae*

	V1: Highly virulent	V2: Virulent	V3: Mildly virulent	V4: Avirulent/ low virulence
	Fast symptom development, dark spots, beginning to crack open.	Black spot with water soaked edges.	Water soaked spot, surrounded by a yellow halo.	No symptoms.
Isolate	1F2	1F6/1F7	1F1/1F5	2F7/1F4

All fruit inoculated at the same time and evaluated at the same time (after 12 days.)

TABLE 3: Differences in isolates from symptomatic Sensation fruit as indicated by percentage infection of detached mango leaves.

Isolate	Severity rating	Percentage infection
1F2	3	44.19
1F7	1	12.9
1F4	0	22.4
1F5	2	28.2



Letters represent individual mice per antigen group: C = combined antigen group; MV = most virulent isolate; MMV = moderately virulent isolate; LV = least virulent isolate.

Fig 1 Specificity of polyclonal antisera against immunised antigen groups of *Xanthomonas campestris* pv. *mangiferaeindicae*

TABLE 4: ELISA signal to background values of polyclonal antisera against all virulence groups of *Xanthomonas campestris* pv. *mangiferaeindicae*.

Combination	A450	Signal background	%CV[A]
MV:MV	0,382	8,68	9,42
MV:MMV	0,325	7,38	15,38
MV:C	0,415	9,43	3,85
MV:LV	0,420	9,54	5,00
MMV:MV	0,41	9,31	5,61
MMV:MMV	0,384	8,72	16,67
MMV:LV	0,419	9,52	16,47
MMV:C	0,465	10,56	10,75
LV:MV	0,549	12,47	15,12
LV.MMV	0,453	10,29	9,93
LV:LV	0,42	9,54	5,47
LV:C	0,588	13,25	3,91

A: Standard coefficient of variation (Standard deviation/mean × 100). Antigen groups designated as follows: MV = most virulent; MMV = moderately virulent; LV = least virulent; C = combined antigen group.

TABLE 5: A comparison of oligoclonal culture supernatants against the four antigen groups of subcultured and fresh isolates of *X. c.* pv. *mangiferaeindicae*

Subcultured Isolates	
Antigen group[A]	Specificity[BC] frequency distribution
MV; MMV; LV; C	5,35
MV	1,78
MMV	0,59
LV	1,19

Fresh isolates	
Antigen group[A]	Specificity[BC] frequency distribution
MV; MMV; LV; C	19,4
MV	5,5
MMV	1,38
LV	15,27

A: Antigen groups named as follows: MV = most virulent; MMV = moderately virulent; LV = least virulent; C = combined antigen group.
B: The frequency is calculated as the number of oligoclonals expressing that specificity out of a total 168 screened.
C: Frequency calculated as described out of 142 screened.

dicae were obtained from all lesons thus verifying that the lesions obtained were indeed due to this organism. Very clear differences could be observed between the isolates.

Specificity assay of polyclonal antisera

Polyclonal antisera was obtained from all mice in each antigen group. The antisera was assayed individually to test their reactivity against the bacteria. A background signal was obtained by using normal mouse serum on the antigen coated wells. All the mice reacted positively to the antigen challenges (Fig 1). Polyclonal immune sera was tested for cross reactivity with the different antigen groups. All antisera raised reacted the most strongly with the combined antigen group. The weakest cross reactions, with the exeption of the most virulent group, were with their corresponding antigens (Table 4). No clear distinction could thus be made between the antigen groups with the polyclonal antisera.

Properties of monoclonal antibodies against the four antigen groups

A mixture of spleen cells derived from three mice, one from each virulence group of *X. c.* pv. *mangiferaeindicae* and spleen cells from one mouse immunised with each virulence group was fused with Sp2/0 myeloma cells. This fusion yielded a total of 1 464 viable oligoclonal hybridoma cultures, of which 329 tested positive for antibody production against the antigens. The oligoclonal culture supernatants were then tested against each of the antigen groups. It was found that the highest specificity frequency distribution was a combination of the combined virulence group and all the other antigen groups, while the lowest was the moderately virulent antigen group (Table 5). Cloning was carried out yielding eight monoclonal antibodies. Subcloning yielded an average of 15 subclones per clone. Two monoclonal subclones from each clone with the highest signal to background ratio were chosen for further evaluation.

Efficiency of recovery from liquid media

Based upon results obtained, BVGA, MXP, XTS and SVGA were evaluated further. SVGA was incorporated due results obtained in previous experiments (unpublished data). Upon evaluation of SVGA, BVGA, MXP and XTS in broths, growth was found to be most prolific in XTS with a total count of 1,4 $\times$ 10^9 cells/mℓ after 72 hours with BVGA producing 5,3 $\times$ 10^8 cells/mℓ, both of which are sufficient for a positive ELISA signal (Fig 2). Due to the poor growth of *X. c.* pv. *mangiferaeindicae* in SVGA, this medium was not evaluated further.

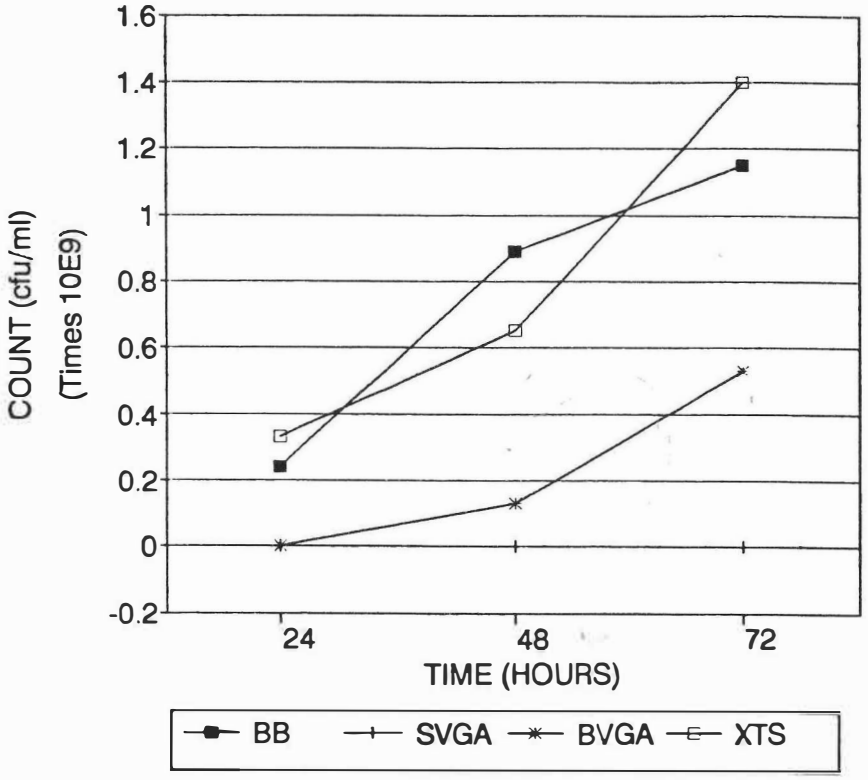


Fig 2: A comparison of growth of *X. c.* pv. *mangiferaeindicae* in selective media. Counts are a mean of 5 replicates plated onto Standard I nutrient agar, incubated at 28°C and evaluated after 72 hours. Media are coded as follows: SVGA = Std I-violet-green agar; BVGA = Boost-violet-green agar; BB = Boost broth; XTS = XTS broth.

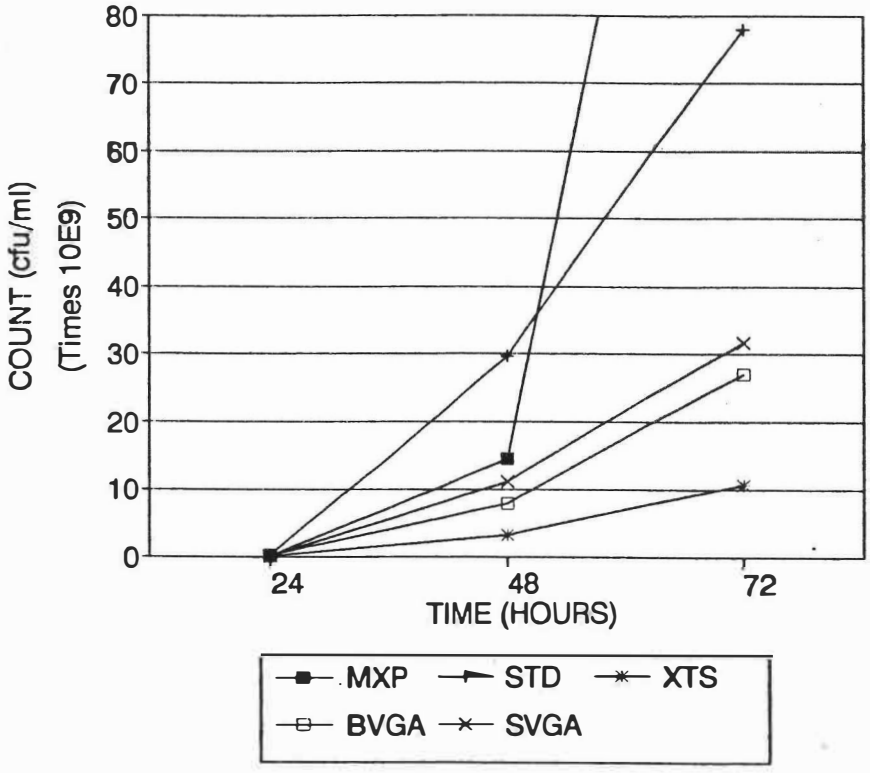


Fig 3: A comparison of growth of phylloplane inhabitants in selective media. Counts are a mean of 5 replicates plated onto Standard I nutrieng agar, incubated at 28°C and evaluated after 72 hours. Media are coded as follows: SVGA = Std I-violet-green agar; BVGA = Boost-violet-green agar; BB = Boost broth; XTS = XTS broth.

Evaluation of medium selectivity

Counts obtained in general were high, especially after incubation for 72 hours. Counts were the lowest in XTS medium, followed by BVGA and SVGA. Counts were found to be the highest in MXP (Fig 3).

DISCUSSION

Differences in virulence in bacteria have been well documented. Certain strains of *Xanthomonas campestris* pv. *vesicatoria* have been found to be pathogenic on pepper plants, but not on tomato (Canteros, Minsavage, Bonas, Pring & Stall, 1991). The differences in virulence between the isolates may be ascribed to several factors, such as toxins, enzymes, growth factors and polysaccharides (Preece, 1982). Studies were carried out in order to determine whether differences in enzyme activity could explain the differences in virulence. However, no differences were observed in the enzymes tested (G. Sanders, unpublished). Virulence factors should thus be considered as a possible interaction of factors (Preece, 1982). Considerable evidence suggests that these mechanisms are either requisites for basic pathogenicity or virulence factors that increase disease severity (Keen & Staskawicz, 1988).

Mice were kept on an immunisation programme for eight weeks prior to fusion, which eventually yielded monoclonal antibodies of the IgG 2b class. Serum obtained too soon after immunisation may be unsuitable for ELISA due to the predominace of the IgM antibodies, part of the primary immune response (Clark, 1981). Monoclonal antibodies were raised against isolates differing in virulence. Some of the monoclonal antibodies obtained were able to distinguish between the various isolates, although their frequency was not as high as those which cross reacted with all the isolates. Monoclonal antibodies were raised against different isolates of *Haemophilus paragallinarum,* where similar results were obtained, and immunodominance of isolates was implicated (Verschoor, Coetzee & Visser, 1989). It was suggested in this case that differences in the lipopolysaccharide O-chain lengths resulted in the differences in antibody activity. However, it was determined that the *X. c.* pv. *mangiferaeindicae* specific monoclonal antibodies reacted with a protein epitope (G. Sanders, unpublished), thus differences in O-chain length could not elucidate differences of antibody activity in this case.

BVGA and XTS medium were found to be the most selective. Ideally, selective media should be selective to at least the species level (Claflin, Vidaver & Sasser, 1987). Although these media do not conform to these requirements, selectivity is sufficient to enrich *X. c.* pv. *mangiferaeindicae* to levels required for detection. Detection of the epiphytic phase of this organism is of paramount importance if it's ecology is to be studied and control established (McGuire, Jones & Sasser, 1986). Furthermore, selective media can be used in conjunction with monoclonal antibodies in order to detect latent infections and so stop the spread of the disease in this manner.

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