DETECTION OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* IN MANGO PLANTS

by

GINA MERCIA SANDERS

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CONTENTS

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW: BACTERIAL BLACK SPOT OF MANGO IN SOUTH AFRICA

The mango (*Mangifera indica* L.) industry is the third largest subtropical industry in South Africa, with a gross value of R21 220 480, of which 24 464 tonnes were exported during the 1990 - 1991 season (Abstract of Agricultural Statistics, 1991). Since 1959, mango production has increased by a total of 20 329 tonnes per annum (Abstract of Agricultural Statistics, 1991). This increase may be ascribed to the fact that more fruit is being exported (Matthews, 1989), and there is a growing demand for fruit from the local market (Conradie & van Zyl, 1988). Several cultivars are popular among local consumers such as Zill, Tommy Atkins, Fascell and Sensation (Smith, 1982). More than 100 commercial mango cultivars are available worldwide (Smith, 1982). 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 (J.M. Kotzé, personal communication).

Bacterial black spot of mango (BBS) is an economically important disease in South Africa in that crop losses on certain farms with no spraying programmes have been recorded to be up to 50% (Viljoen & Kotzé, 1972). Major losses are due to unacceptably lesioned fruit and premature fruit drop. Total losses with respect to BBS have not been established, but due to well implemented spraying programmes, the disease has been kept in check (J. Colyn, personal communication).

BBS is a preharvest disease. The first signs of infection on leaves or fruit are tiny, water soaked spots. Symptoms are manifested on fruit as irregular, black, raised spots which later crack open to form characteristic star shaped lesions. New spots seldom appear on fruit after picking (Viljoen & Kotzé, 1972). Leaf infection is manifested by water soaked spots or dark lesions surrounded by yellow halos (Viljoen & Kotzé, 1972). On twigs and petioles, dark longitudinal lesions which later crack open, are also characteristic of black spot infection. Recently, signs of infection have also been observed on flowers of heavily infected trees (J. Lonsdale, personal communication).

No cultivars are entirely resistant to BBS (Tomer & Mullins, 1987). Differences in cultivar susceptibility with respect to BBS have been reported. Cultivars such as Haden, Kent, Irwin, Keitt and Smith are highly susceptible, whereas cultivars such as Fascell and Sensation are less susceptible (Smith, 1982).

BBS has been reported from the major mango producing areas of Letaba, Hoedspruit and districts of Barberton and Soutpansberg (Kotzé, et al., 1976). The global distribution of BBS includes the Indian subcontinent, Australia, South America, Taiwan, Reunion, Pakistan and Brazil (Bradbury, 1986).

Disease control is currently achieved with copper oxychloride applications (Frean, 1985), a mixture of copper oxychloride and zineb or cupric hydroxide (Vermeulen et al., 1992). Although the pathogen can be spread through the air by means of wind, from plant to plant, the disease is spread mainly through propagation of infected plant material (Viljoen & Kotzé, 1972). Propagation and maintenance of healthy material would be the ultimate solution to the problem.

The etiological agent of bacterial black spot was first described by Doidge in 1915 as a fast growing, Gram positive, peritrichously flagellated bacterium, and was subsequently named *Bacillus mangiferae* (Doidge, 1915). Khan and Kamal isolated another organism and classified it as *Erwinia mangiferae* Doidge (Breed et al., 1957). The actual causal organism was first isolated by Patel et al. (1948), who described it as a Gram negative organism with a single polar flagellum. Viljoen and Kotzé (1972) confirmed the similarity between an organism isolated by them and that of Patel et al. (1948) and tentatively suggested that the causal agent of bacterial black spot disease was *Pseudomonas syringae*.

However, Steyn et al. (1974) found sufficient differences between their isolate and *P. syringae* to warrant recognition as a separate species. Daniel et al. (1975) also identified the causal organism as *P. mangiferae*, but suggested that it's taxonomic position be redefined. The organism was concluded to be *Xanthomonas campestris* pv. *mangiferaeindicae*. In 1980 the designation was finally proposed as *Xanthomonas campestris* pv. *mangiferaeindicae* (Dye et al., 1980). This has subsequently been confirmed (Manicom and Wallis, 1984), based on physiological comparative studies, and electrophoretic protein profiles (van Zyl & Steyn, 1990).

In the course of the abovementioned studies, no reports regarding different virulence groups for *X. c.* pv. *mangiferaeindicae* were made. Due to findings presented in this study, a theory was postulated that *X. c.* pv. *mangiferaeindicae* exists as strains differing in virulence. Most research has been carried out on the role of exopolysaccharides (EPS) and lipopolysaccharides (LPS) in virulence of *Xanthomonas campestris* pv. *campestris, Erwinia amylovora* and several Pseudomonads (Chatterjee & Vidaver, 1986;

Leigh & Coplin, 1992). In this study, it was suggested that proteins play a role in virulence in *X. c.* pv. *mangiferaeindicae*. Proteinaceous virulence determinants have been reported predominantly for animal and human pathogens such as *Aeromonas salmonicida*, *Campylobacter fetus* subsp. *intestinalis* and *Staphylococcus aureus* (Hammond et al., 1984).

In order to increase yields, a plant improvement scheme is important. This involves the establishment of disease free orchards using certified healthy young trees obtained from disease free mother trees. Diagnosis of healthy mother trees is complicated by the fact that *Xanthomonas campestris* pv. *mangiferaeindicae* is a year round phylloplane resident (Manicom, 1986). An understanding of the epiphytic phase is of prime importance for disease control strategies (Leben, 1981). When the pathogen is in the epiphytic or survival phase, the metabolism is close to a standstill, and thus more resistant to inhibitors and harmful conditions (Leben, 1981). This has been found to be especially true for xanthomonads (Timmer et al., 1987) and pseudomonads (Schuster & Coyne, 1974; Hirano & Upper, 1983). These organisms can survive as epiphytic populations during adverse conditions (Leben, 1965; Hayward, 1974), using protective mechanisms such as gum production to survive environmental threats such as drying and solar radiation (Manicom, 1983). These epiphytic populations provide inoculum for dispersal although there is no manifestation of infection. Warm and humid conditions with intermittent spells of rain were found to be conducive to disease development (Viljoen & Kotzé, 1972).

Symptomless infected mother trees should be identified so that only healthy material is propagated. Propagative material obtained from infected trees used for the establishment of new orchards is one of the major methods of dispersal of the disease. Standard isolation techniques using nonselective medium, followed by identification using physiological and biochemical tests and final proof of pathogenicity using 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.

Bacterial infections on seeds (Claflin & Ramundo, 1987), and other plant tissues (Comstock & Irey, 1992; Nomé et al., 1980) are currently being screened using rapid, accurate, inexpensive techniques eg. selective media (Mulrean & Schroth, 1981; Schaad & Forster, 1985) or serological techniques such as dot - immunobinding assay (DIA) (Comstock & Irey, 1992), immunofluorescence (IF) (Duveiller & Bragard, 1992) and the enzyme - linked immunosorbent assay (ELISA) (Sherald & Lei, 1991). Serology has many applications in plant pathology, specifically in the elucidation of plant disease etiology, for

example, study of translocation and distribution of the pathogen (Alvarez & Lou, 1985; Lipp et al., 1992)

Selective media play an important role in the elucidation of disease epidemiology, and has been used for the detection of several pathogens such as *Clavibacter michiganensis* subsp. *sepedonicus* (de la Cruz et al., 1992), *X. c.* pv. *phaseoli* (Mabagala & Saettler, 1992) and *Erwinia* spp. (Pierce & McCain, 1992). Growth of non target bacteria can complicate the detection and identification of target bacteria from diseased plant material (Sijam et al., 1991). The growth of these non target organisms is greatly reduced using selective media, facilitating the determination of the ecology of the pathogen. It is of prime importance that the ecology of the pathogen is considered before successful control measures can be introduced (McGuire et al., 1986). 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 greatest possible application of serology is thus its implementation in commercial screening programmes, and has been implemented with a great deal of success by many researchers in plant virology and phytobacteriology (Bar - Joseph et al., 1979; Civerolo & Fan, 1982 and Lommel et al., 1982). These techniques vary in accuracy, simplicity, degree of variability and cost. The ELISA has been used most successfully on a commercial scale (Clark, 1981; Dosba et al., 1986; de Boer et al., 1988).

Initially, polyclonal antisera, also known as heterogeneous serum antibodies were used for detection of plant pathogens. These antibodies are raised by conventional immunisation (Hood et al., 1984). The antisera produced contain antibodies secreted by many antibody producing B - cell clones (Liddell & Cryer, 1991), and thus consist of more than one molecular species. Although polyclonal antisera are relatively quick and inexpensive to use, they have several drawbacks, the most important of which are variability of serum obtained from different animals and lower specificity (Pollock et al., 1984). These disadvantages showed the need for a more specific, reproducible tool for detection.

Monoclonal antibodies are the result of a somatic cell hybridisation first demonstrated by Köhler & Milstein in 1975 (Yelton & Sharff, 1981). These are known as homogeneous serum antibodies (Hood et al., 1984). Monoclonal antibodies are highly specific for a single epitope (Halk & de Boer, 1985). They have several advantages over polyclonal antisera, of which the unlimited quantities of highly specific antisera are the

most important. Monoclonal antisera, however, are time consuming and expensive to produce and in certain cases may be too specific (Halk & de Boer, 1985). The type of antiserum used in an assay will thus depend upon the purpose for which the assay is intended.

The purpose of this investigation was to raise monoclonal antibodies against *X. c.* pv. *mangiferaeindicae* and to develop a medium for selective isolation of this pathogen from field mango plant material for establishment of disease free orchards. These two techniques were then used concurrently to detect *X. c.* pv. *mangiferaeindicae* in mango mother material. Monoclonal antibodies were also used to investigate differences in virulence in *X. c.* pv. *mangiferaeindicae*.

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CHAPTER TWO

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST DIFFERENT ISOLATES OF *XANTHOMONAS CAMPESTRIS* PV *MANGIFERAEINDICAE*

ABSTRACT

Four differential virulent *Xanthomonas campestris* pv. *mangiferaeindicae* isolates recovered from mango black spot lesions, were used to raise monoclonal antibodies (mAb). Two immunisation approaches were followed: In the first, four groups of mice were immunised with one bacterial isolate respectively. The second approach involved immunisation of mice with the four pooled bacterial isolates. The spleens from each group except the pooled isolates were homogenised together for cell fusion. The resultant mAbs were characterised with regard to the antigen binding specificity and antibody class. All antibodies were of the IgG class and reacted with a proteinaceous epitope. The highest percentage of cross reactions with other organisms was observed with clone 2A/10B/2, while the lowest was found with clone 2C/3G/1.

2.1 INTRODUCTION

Bacterial black spot (BBS) of mango (*Mangifera indica* L.) is caused by *Xanthomonas campestris* pv. *mangiferaeindicae*. Cultivars differ in susceptibility to BBS, i.e. Keitt is more susceptible than Sensation (Smith, 1982). The first signs of infection on fruit and leaves are small water soaked spots which seldom appear after picking (Viljoen & Kotzé, 1972). Symptoms on fruit appear as irregular, black raised spots which later crack open to form star shaped cracks. On leaves, the dark lesions are surrounded by yellow halos (Viljoen & Kotzé, 1972). The disease is spread readily by means of infected grafting material. The use of certified grafting material combined with sanitation practices and chemical control can limit the spread of the disease as well as losses to the producer.

An accurate reproducible technique is required to detect latent infections to limit disease spraed and subsequent harvest losses. Serological methods have been successfully implemented for the detection of phytopathogenic bacteria in plant material (Halk & de Boer, 1985). Initially, polyclonal antibodies were used for the field detection of pathogens such as *X. c.* pv. *holcicola* (Leach et al., 1987). Problems associated with cross reactivity and variability of polyclonal antisera have created the demand for

monoclonal antibodies for diagnostic and taxonomic purposes (Halk & de Boer, 1985).

Monoclonal antibodies are superior to polyclonal antibodies as specific probes in terms of specificity and reproducibility, making them useful for the detection and identification of various plant pathogenic prokaryotes (Civerolo & Fan, 1982; Claflin & Ramundo, 1987; Leach et al., 1987; Yuen et al., 1987).

MAbs have also been used for pathovar differentiation (Benedict et al., 1987) and serodiagnostic studies (Lazarovits et al., 1987). For these purposes, mAbs have been raised against several pathovars of *X. campestris*, namely *X. campestris* pv. *citri* (Civerolo & Fan, 1982), *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *translucens* (Claflin & Ramundo, 1987; Lazarovits et al., 1987) and *X. campestris* pv. *oryzae* and *X. campestris* pv. *oryzicola* (Benedict et al., 1989).

Several techniques implementing antisera for field detection have been described such as the enzyme - linked immunosorbent assay (ELISA) (Sherald & Lei, 1991), dot immunobinding assay (DIA) (Comstock & Irey, 1992) and immunofluorescence (IF) (Duvellier & Bragard, 1992). Thusfar, only polyclonal antibodies have been implemented for elucidation of black spot disease etiology (Cronjé, 1988).

The purpose of this study was to raise and characterise monoclonal antibodies against different isolates of *Xanthomonas campestris* pv. *mangiferaeindicae* for the diagnosis of latent and existing infections of mother trees for the plant improvement programme.

2.2 MATERIALS AND METHODS

Bacterial isolates and antigen preparation

Four fruit isolates of *X. c*. pv *mangiferaeindicae* differing in virulence (Chapter Three) were used in the immunisation programme (Table 1). Isolates were cultured on Boost agar plates (Manicom, 1980) at 28°C for 72 hours. Cells were harvested in ¼ strength Ringer's solution (Oxoid), centrifuged at 23 000 g for ten minutes in a Sorvall Superspeed refrigerated centrifuge with a SS - 34 rotor and washed three times using sterile phosphate buffered saline, pH 7.0 (PBS).

Cells were resuspended in sterile PBS to a final concentration of 1.5×10^7 cells/ml determined by means of a Petroff - Hausser counting chamber using a Zeiss phase contrast microscope. Aliquots were prepared and frozen at -20°C until required for immunising mice or screening for antibody production.

Table 1 Difference in symptom expression of *Xanthomonas campestris* pv. *mangiferaeindicae* isolates from four virulence groups in Sensation mango fruit

Isolate	Percentage infection[a]	Lesion severity[b]
1F2	88.7	Severe
1F7	60.4	Less severe
1F5	42.5	Moderate
1F4	12.0	Mild

[a]   Percentage infection was determined from the formula no. lesions/no. symptomatic x 100

[b]   Lesion severity rating graded as follows: Severe = star shaped cracks surrounded by a yellow halo; Less severe = dark spots surrounded by a yellow halo; Moderate = water soaked spots with yellow halo; Mild = slight water soaking.

Immunisation of mice

Balb/C mice (six week old, males) obtained from the H.A. Grové Research Centre, Pretoria, South Africa were immunised intraperitoneally with 1.5×10^7 cells/ml in ½ ml PBS on days 0, 14, and 59. Five groups consisting of three mice were immunised. For preparation of mAbs four groups were respectively treated with one bacterial isolate. The remaining group was immunised with the pooled bacterial isolates. Polyclonal immune sera was prepared from the five groups of mice on day 24. Spleens of the mice were harvested on the 62nd day.

Production of hybridomas

Cell fusion between immune spleen cells and the hypoxanthine - guanine phosphoribosyl - transferase - deficient, nonsecreting mouse myeloma cells, Sp2/0 (Shulman et al., 1978) was performed according to the method of Galfré and Milstein (1981). Two cell fusions were performed differing in their source of splenocytes. In the first, spleens from each of the four groups of mice immunised with the individual isolates were homogenised together, while in the second, a spleen was removed from the group of mice immunised with the pooled quartet of bacterial virulence groups.

Cells thus obtained were fused 5 : 1 to myeloma cells using 40% (w\v) polyethylene glycol (Whittaker, M.A. Bioproducts, Walkersville, Maryland). The fused cells were suspended in hypoxanthine - aminopterin - thymidine (HAT) medium and distributed into 96 well tissue culture plates (Nunc, Roskilde, Denmark). HAT medium consisted of Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Ayrshire, Scotland) supplemented with $NaHCO_3$ (3.7g /litre), sodium pyruvate (1mM), amphotericin B (1 μg/ml), penicillin - streptomycin mixture (10 IU/ml and 10 μg/ml, respectively), hypoxanthine (100 μM), aminopterin (0.4 μM), thymidine (16 μM), and 10% donor horse serum, all of which were purchased from Highveld Biological (Pty.) Ltd., Kelvin, South Africa. Cultures were fed with fresh medium every third day.

After one week, aminopterin was excluded from the medium, and from the second week, hypoxanthine and thymidine were omitted as well. 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 upon reaching confluency and after determination of the type specificity of the secreted antibody against all bacterial isolates, subsequently cloned on soft agar as described by Liddell and Cryer (1991). Cloned hybridoma cultures were subcloned using the limiting dilution technique as described by Liddell and Cryer (1991) before being frozen away in 10% dimethyl sulphoxide, 20% horse serum, and 70% Dulbecco's modified Eagle's medium as described by Harlow and Lane (1988). 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 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 isolates of *X. c.* pv. *mangiferaeindicae* were mixed and used for coating 96 - well microtiter plates (Cooke Microtiter system M29A, Sterilin products, Middlesex, England) at 100 μl per well. The plates were incubated at 4°C overnight and were fixed with 70% methanol for 30 minutes. For specificity determination, four duplicate long rows of a microtiter plate were each covered with one of the four different isolates of *X. c.* pv. *mangiferaeindicae* at 10^7 cells/ml and treated as described above. Plates were blocked with 300 μl/well 0.5% casein (Merck, Darmstadt, Germany) in PBS, pH 7.4 for one hour at room temperature. After washing the plate with 0.1% Tween 20 in PBS, the wells received 50 μl undiluted hybridoma supernatant for screening and specificity determination.

After washing, the plates received 50 μl per well of goat - anti - mouse immunoglobulin G (H + L) - peroxidase conjugate (Dakopatts, Denmark) at a dilution of 1 : 5000 in 0.5% casein as described above. After final washing, 50 μl of substrate solution consisting of o - phenylenediamine (Sigma, St Louis, Missouri) (10 mg) and urea - hydrogen peroxide (BDH, Poole, England) (8 mg) in 10 ml citrate buffer (0,1M, pH 4,5) was added and allowed to develop in the dark at room temperature for 45 - 60 minutes.

Colour development was measured at 450 nm on an Anthos 2001 ELISA plate reader. Monoclonal antibodies produced from positive cultures were evaluated for cross reactivity with the four isolates, 1F2, 1F4, 1F7 and 1F5. Ringer's solution substituted for bacteria was used as a negative control. Signal to background ratios were determined for each antibody - antigen combination. Means of signal to background ratios were compared using the least significant difference test at the 95% confidence level as described by de la Cruz et al. (1992)

Determination of cross - reactivity

Polyclonal antisera (1/100) or culture supernatants from clones which exhibited a high degree of reactivity with *X. c*. pv. *mangiferaeindicae* were evaluated for cross - reactivity with related and non - related organisms as well as mango phylloplane inhabitants (Chapter Four). Cross - reactivity was determined by means of ELISA as described using the test organisms at 10^7 cells/ml Ringer's solution as solid phase antigen. The antigens were dispensed in triplicate rows per organism using Ringer's solution as negative control for antigen coating, while serum supplemented DMEM or unrelated supernatant substituted respectively for hybridoma supernatant or polyclonal antiserum as negative control. Absorbance values obtained were graded according to ELISA signal strength, with the following increments: 0 - 0.035 (-), 0.036 - 0.55 (1 +), 0.056 - 0.158 (2 +), 0.159 - 0.287 (3 +), 0.290 - 0.305 (4 +), 0.306 - 0.420 (5 +) and 0.420 and higher (6 +).

Determination of immunoglobulin class by ELISA

Hybridoma culture supernatants were dispensed in the appropriate antigen coated wells at 50 μl per well. After washing, the plates were probed with 1 : 1000 dilutions of heavy chain specific rabbit - anti - mouse immunoglobulin sera (Miles Research Products Division, Elkhart, Indiana) at 50 μl per well and incubated at room temperature for 30 minutes. The plate was developed with peroxidase conjugated swine - anti - rabbit immunoglobulin G (H + L) (Dakopatts, Denmark) and substrate solution as described.

Characterisation of bacterial epitopes by ELISA

Pooled isolates of *X. c.* pv. *mangiferaeindicae* (10^7 cells/ml) were shaken gently for 16 hours in 0.2% (w/v) trypsin (Miles Scientific, Illinois)/ PBS (pH 7.2), and subsequently used as coating antigen for ELISA as described. Untreated cells were used as positive control. Periodate oxidation of bacteria was performed by incubation of 10^7 cells/ml pooled isolates in 50 mM sodium glutamate buffer, pH 4.5 with 20 mM periodic acid (Merck, Darmstadt, Germany) at 4°C for one hour as described by Woodward et al. (1985). The reaction was stopped by addition of glycerol to a final concentration of 20 mM. Prior to coating microtiter plates, the depletion of periodate was verified by the absence of colour development upon addition of a few drops of 0.1M potassium iodide, using some glycerol free sample as positive control. Subsequently, the ELISA was carried out as described, using unrelated antiserum to substitute hybridoma culture supernatant as negative control.

*Optimisation of coating antigen concentration*

An ELISA was carried out as described using concentrations of *X. c.* pv. *mangiferaeindicae* ranging from 10^7 to 10^2 cells/ml as solid phase antigen, using mAb 2A/10B/2 as primary antibody. Values were considered positive if they were twice that of the background with a corresponding coefficient of variation of less than 10%.

2.3 RESULTS

Specificity assay of polyclonal antisera

Polyclonal antisera drawn from the five groups of immunised mice were evaluated for their antigen specificity in ELISA. All the mice reacted positively to the antigen challenges, the strongest response being measured with the combined antigen group (Fig. 1). The strong antigenicity of the least virulent isolate, 1F4 was remarkable, especially with antisera raised by immunisation with the more virulent strains.

This indicates an inversely proportional relationship between immunogenicity (ability to elicit antibody response) and antigenicity (ability to react with antibodies) of the surface structures of the different isolates of bacteria. Due to the extensive cross reactivity between isolates, no clear distinction could be made between the antigen groups with the polyclonal antisera.
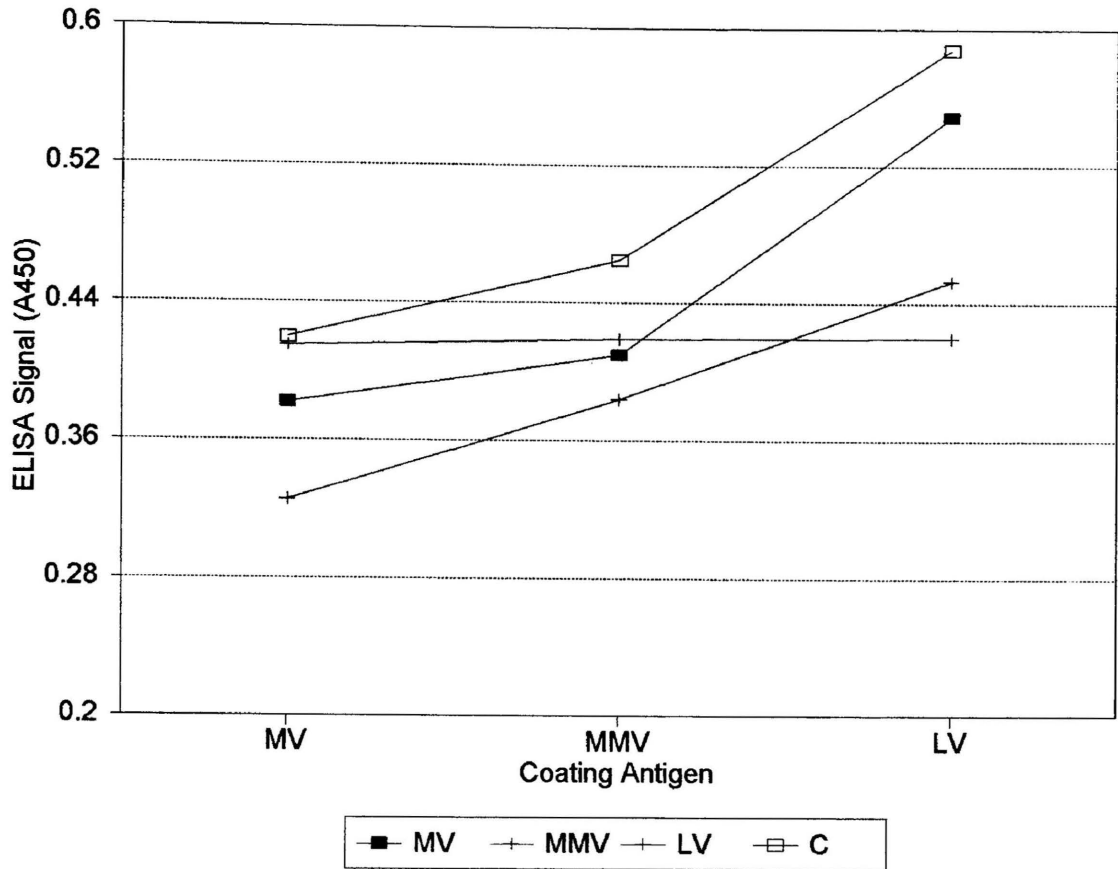
All codes designated in legend indicate immunogen utilised. Codes designated as follows: C = Combined isolates; MV = Most virulent; MMV = Moderately virulent; LV = Least virulent.

Fig. 1 Specificity of polyclonal antisera against immunised antigen groups of *Xanthomonas campestris* pv. *mangiferaeindicae* determined by means of ELISA on plates coated separately with each isolate.

Properties of monoclonal antibodies against the four antigen groups

A mixture of splenocytes derived from three mice, one from each virulence group of *X. c.* pv. *mangiferaeindicae* and splenocytes from one mouse immunised with each virulence group was fused with Sp2/0 myeloma cells. This fusion yielded a total of 1464 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. Cloning was carried out yielding three monoclonal hybridomas. Subcloning using limiting dilution yielded an average of 15 subclones per clone. Clones utilised for further evaluation were 2D/11B/A, 2C/3G/1, 2A/7B/2, 2D/12H/4C, 2D/11B/3A and 2A/10B/2. Specificity assays with the four isolates showed a consistent and strong reaction of all the monoclonal antibodies with the least virulent isolate, 1F4 and weakest

for the most virulent isolate, 1F2 (Table 2). Signal to background ratios of all the monoclonal antibodies were significantly higher with 1F4 than the other isolates (Table 2). No monoclonal antibody reacted with one isolate only, but all antibodies reacted positively with all isolates (Table 2), with apparently the same specificity. This mAb specificity was subsequently denoted XCM - 1.

Table 2 ELISA signal to background ratios showing cross reactivity of different monoclonal antibodies raised against four isolates of *Xanthomonas campestris* pv. *mangiferaeindicae*

Clone	Isolate			
	1F2	1F4	1F7	1F5
2D/12H/4C	3.75 a	4.89 b	4.41 ab	4.23 ab
2C/3G/1	4.70 ab	6.03 c	5.11 b	4.28 a
2D/1B/A	4.61 a	6.91 c	5.72 b	4.67 a
2A/7B/2	3.93 a	4.95 b	4.38 ab	3.82 a
2A/10B/2	3.75 a	4.89 b	4.44 ab	4.23 ab
2D/1B/A3	3.50 a	5.23 b	4.76 ab	5.97 b

Means in rows followed by the same letters do not differ significantly according to Fischer's protected least significant difference at the 95% confidence level

Determination of cross - reactivity

Clone 2A/10B/2 was the most cross - reactive, while 2C/3G/1 was the least cross - reactive. Most cross reactions were observed with *Pseudomonas syringae* pv. *syringae* isolates and other pathovars of *Xanthomonas campestris* (Table 3). No cross reactions were observed with phylloplane inhabitants, other species of *Xanthomonas* or non - related organisms, with the exception of a strong cross reaction of all three mAbs with an *Erwinia* sp. isolated from avocados (Table 3).

Determination of immunoglobulin class by ELISA

All clones were of the IgG class. Some were of the subclass 2a and the others were of the subclass 3. Due to their identical antigenic specificities determined by Western blot analysis (Chapter Three), these different mAb isotypes were designated XCM - 1(2a) and XCM - 1(3).

Table 3   Analysis of antibody binding specificity of three different monoclonal antibodies raised against *Xanthomonas campestris* pv. *mangiferaeindicae* by ELISA

Bacteria	Code[c]	Clone		
		2A/10/B 2	2C/3G /1	2D/12H/4 C
Bacillus licheniformis[a]	MAL	-	-	-
Erwinia sp[a]	E.s	4+	4+	4+
Micrococcus kristinae[a]	M.k	-	-	-
Ps syringae pv. *atrofaciens*[a]	3417	-	-	-
Ps. aeruginosa[a]	P.a	-	-	-
Ps. cepacia[a]	B18/G	-	-	-
Ps. fluorescens[a]	L908	-	-	-
Ps. maltophilia[a]	P.m	-	ND	ND
Ps. putida[a]	B11/V	-	ND	-
Ps. pv. *phaseolicola*[a]	3448	-	-	-
Ps. syringae[a]	W -1b	-	-	-
Ps. syringae[a]	P.s	-	ND	ND
Ps. syringae pv. *morsprunorum*[a]	CF8	-	-	-
Ps. syringae pv. *syringae*[a]	777	2+	-	1+
Ps. syringae pv. *syringae*[a]	3367	5+	-	-
Ps. syringae pv. *syringae*[a]	3346	3+	3+	2+
Ps. syringae pv. *syringae*[a]	3419	-	-	-
Ps. syringae pv. *syringae*[a]	3457	2+	-	-
Ps. syringae pv. *syringae*[a]	3367	6+	-	-
Ps. syringae pv. *syringae*[a]	3436	3+	-	-
Ps. syringae pv. *syringae*[a]	3344	2+	-	-
Ps. syringae pv. *syringae*[a]	3456	3+	1+	-
Ps. tabaci[a]	P.t	-	ND	ND
Ps. vesicularis[a]	B24/G	3+	-	-
Ps. viridiflava[a]	P.v	2+	-	-
Staphylococcus xylosus[a]	B38/V	-	-	-
Unidentified (phylloplane)[a]	MT8	1+	-	-
Unidentified (phylloplane)[a]	MT2	-	-	-
Unidentified (phylloplane)[a]	MT10	-	-	-
Unidentified (phylloplane)[a]	MT5	-	-	-
Unidentified (phylloplane)[a]	MT9	-	-	-
X. albilineans[b]	X.a	3+	-	1+
X. campestris pv. *campestris*[a]	X.c	6+	5+	6+
X. campestris pv. *mangiferaeindicae*[a]	X.m X.p	6+	5+	6+
X. campestris pv. *phaseoli*[a]	X.f	5+	3+	5+
X. fragariae[a]		-	-	-

[a]   Bacteria obtained from L. Korsten and E. van Zyl, Department of Microbiology and Plant Pathology, University of Pretoria

[b]   Organism obtained from D. Pillay, Department of Microbiology and Biochemistry, University of Durban - Westville

Results denoted ND indicates that the test was not done

[c]:   Sources of isolates as follows: 3456: *Zea mays*; 3419: PDCC 4375; L908, P.a., W -1b, B38/V, E.s., M.k., B24/G, B11/V: Avocado; 3346: *Chaetochloa lutescens*; 3367, 3436, 3367: Millet; 3457, 3417: Wheat; 3448: Pinto beans; 3344: Sudan grass; X.m., MAL, MT9, MT2, MT8, MT5, MT10: Mango; X.c.: Sugar cane; X.a.: Sugar cane; X.f.: Strawberry; X.p.: Bean leaves; P.m., P.t., P.s., P.v., 777, CF8: Unknown

Values designated for binding specificity as follows: - = negative reaction; + = positive reaction with greater value according to signal strength.

Characterisation of bacterial epitopes by ELISA

Antibody secreting clones 2A, 2C and 2D were evaluated. ELISA signals obtained using antigen treated with periodate were very similar to those of the positive control, ie. untreated, with the same concentration. However, following treatment with trypsin, the ELISA signal obtained was as low as the negative control (Fig. 2), indicating that the XCM - 1 epitope is proteinaceous.
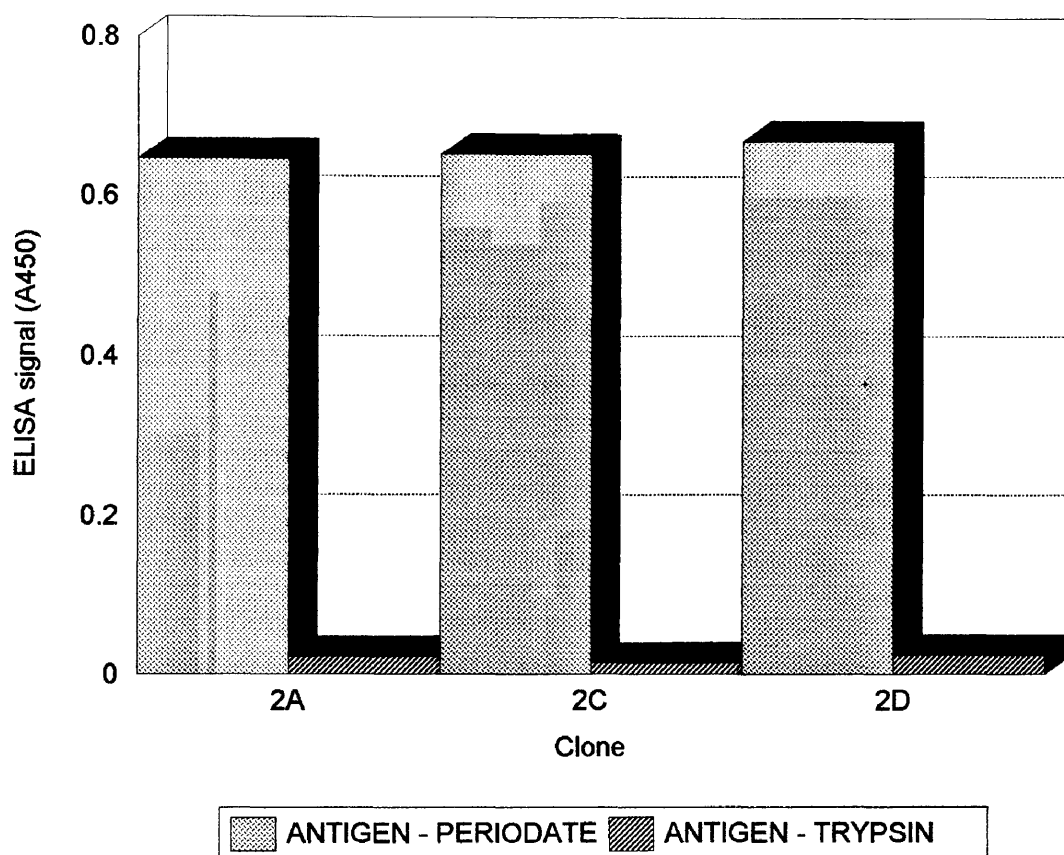
Fig.2   Determination of antigen binding specificity of monoclonal antibodies raised against isolates of *Xanthomonas campestris* pv. *mangiferaeindicae*   by means of ELISA using trypsin digestion and periodate oxidation of antigens.

Optimisation of coating antigen concentration

This was carried out using the strongest antibody producing clone, 2A/10B/2. Strong signals were obtained with 10^7 cells / ml to 10^5 cells/ml. The lowest cell count at which the signal to background ratio was greater than two was 10^6 cells/ml and the optimum cell concentration was 10^7 cells/ml (Fig. 3).

Fig. 3   ELISA signal of antibodies from clone 2A/10B/2 at different concentrations of pooled isolates of *Xanthomonas campestris* pv. *mangiferaeindicae* as solid phase antigen.

2.4 DISCUSSION

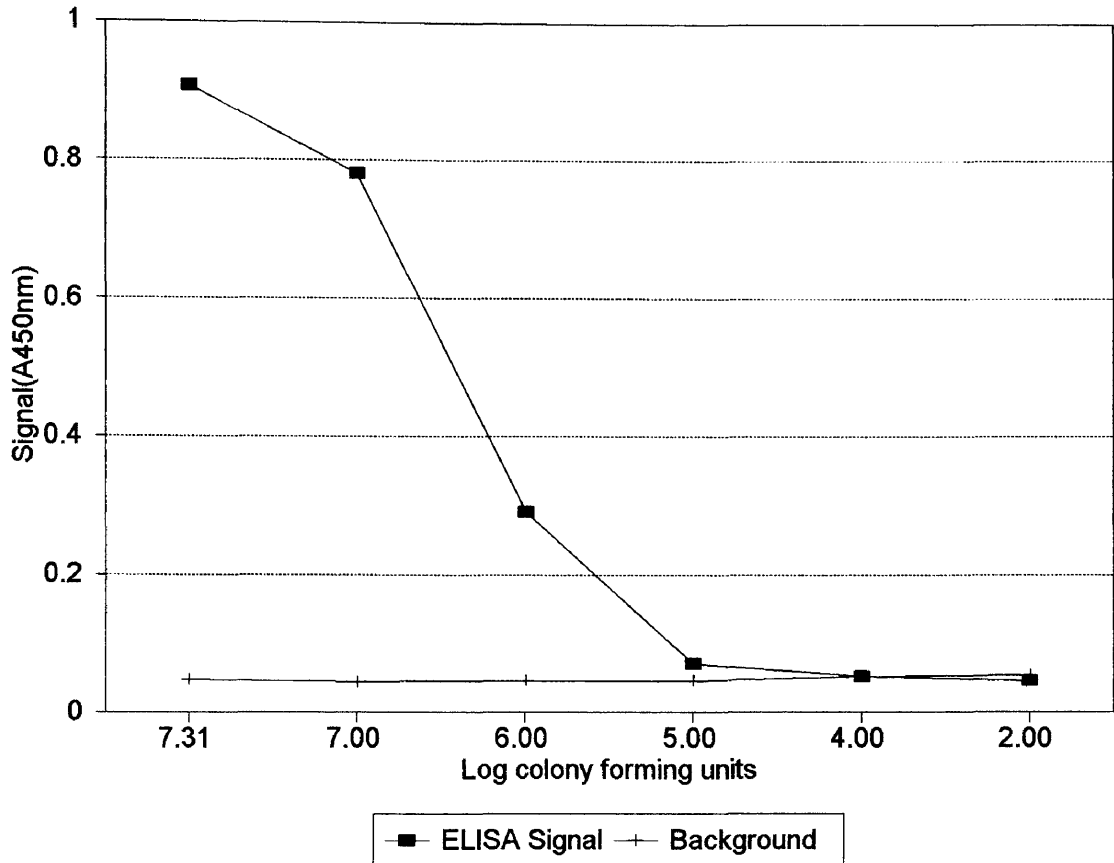Since all mice reacted positively to the antigen challenges, yielding high antibody titers, it may be concluded that all isolates of *X. c.* pv. *mangiferaeindicae* were immunogenic, similar to those described by de Boer et al., 1988. However, a remarkable reaction pattern was observed when testing the antisera from each isolate using isolates separately as solid phase antigens. The weakest reactions with all antisera was observed when the most virulent isolate (1F2) was used as solid phase antigen, while the strongest reactions were observed when the least virulent isolate (1F4) was used as solid phase antigen. An interesting observation was that similar absorbance values were obtained for all isolates when antisera raised against the least virulent isolate was tested.

No clear distinction could be made between the antigen groups with the polyclonal antisera. All mAbs obtained in the subsequent cell fusion were of the IgG2a and IgG3 subclass. However, similar results were obtained using mAbs, where significantly stronger reactions were observed for all mAbs with the least virulent isolate, 1F4. All mAbs reacted positively with all isolates and appeared to have similar specificities. Western blot analysis (Chapter Three) seemed to confirm this, since band patterns of all isolates probed with each mAb appeared to be similar. MAbs with at least four specificities have been raised against phytopathogenic bacteria (Benedict et al., 1989; McLaughlin et al., 1989; Vernon - Shirley & Burns, 1992).

These findings may imply that mAbs generated would not be suitable for detection of *X. c.* pv. *mangiferaeindicae* on mango plants. Different reaction patterns were observed for all isolates, but all reactions were strong. However, these mAbs would not be suitable for pathovar or serovar differentiation as described by Benedict et al. (1989) and Vernon - Shirley and Burns (1992), since the epitope with which the mAbs react does not appear to be unique to *X. c.* pv. *mangiferaeindicae.*

Although no specific attempts were made to achieve this, all mAbs reacted with proteinaceous epitopes. Western blot analysis (Chapter Three) revealed a concurrent band for all isolates at 38,9 kD. This molecular weight corresponds approximately to that of porins and OmpA which migrate closely together and both of which are highly immunogenic (Brown & Hormaeche, 1989; Franken et al., 1992). However, these surface proteins are often closely associated with, and difficult to separate from immunodominant lipopolysaccharide (LPS) (Poxton et al., 1985). MAbs reacting with protein epitopes were found to be most common when using outer membrane protein fractions (Kerr et al., 1992) or selected outer membrane proteins such as protein F or OmpA (Labadie & Desnier, 1992) for immunisation. Few reports have been made where mAbs react with protein epitopes following immunisation with whole cells (McLaughlin et al., 1989), since this type of antigen generally results in mAbs reacting with LPS epitopes (McLaughlin et al., 1989; Franken et al., 1992). It has also been reported that mAbs reacting with protein epitopes were more specific than those reacting with LPS epitopes (Franken et al., 1992).

Cross - reactions of antisera with certain *Ps. syringae* pv. *syringae* isolates and *X. campestris* pathovars will not adversely affect the effectivity of the monoclonal antibodies. The *Pseudomonas* isolates were from wheat or millet and chances of encountering these specific isolates upon the mango leaf or fruit surface are small.

Cross - reactions between other pathovars of *X. campestris* have been reported but are of minimal importance since pathovars are host specific (Alvarez & Lou, 1985). Similar results of cross reactive mAbs have been reported, but cross reactivity was limited to closely related organisms (Kerr et al., 1992) and related pathovars (Franken et al., 1992). The differences in cross reactivity patterns challenge the theory that all three mAbs have the same specificity, since mAb 2A/10B/2 was the most cross - reactive while 2C/3G/1 and 2D/12H/4C predominantly cross reacted with *Xanthomonas campestris* pathovars. The cross reaction of all monoclonal antibodies with the *Erwinia* sp. was unexpected, since it is completely unrelated to *X. c.* pv. *mangiferaeindicae.* This cross reaction may be ascribed to a common protein epitope.

The minimum antigen concentration required for a signal to background ratio greater than two was found to be 10^6 cells/ml. This was higher than 10^4 cells/ml reported for detection of *Erwinia carotovora* var. *atroseptica* (Cother & Vruggink, 1980). Similar detection levels were described for *X. albilineans* from sugar cane (Comstock & Irey, 1992). In order to improve detection levels, optimisation of the ELISA is required, since levels of 10^3 cells/ml have been detected when optimised systems or enhancement systems such as the streptavidin - biotin system have been incorporated into the ELISA (Yolken et al., 1983).

An ELISA assay using mAb XCM - 1 allows enhanced specificity of detection as compared to polyclonal antisera. Although no mAbs were raised which reacted with one isolate only, the potential for using these mAbs for field detection of *X. c.* pv. *mangiferaeindicae* is still great. Since all monoclonal antibodies react with all isolates, there is no danger that certain isolates will not be detected. The detection method best suited to field detection of phytopathogenic bacteria is the ELISA and has been implemented successfully in several crops (Clark, 1981). The application of these mAbs by ELISA could also prove to be useful for epidemiological studies and subsequent control of BBS in mango.

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CHAPTER THREE

AN INVESTIGATION INTO DIFFERENCES IN VIRULENCE
IN DIFFERENT ISOLATES OF *XANTHOMONAS CAMPESTRIS* PV.
MANGIFERAEINDICAE

ABSTRACT

Differences in virulence among isolates of *Xanthomonas campestris* pv. *mangiferaeindicae*, the causal agent of bacterial black spot of mango were investigated. Isolates of *X. c.* pv. *mangiferaeindicae* were grouped into four virulence groups based upon symptom expression. These isolates were tested for differences in virulence using inoculation assays. Similar results were obtained throughout. A relationship between mAb binding specificity and virulence of bacteria was shown by Western blot analysis. Clear differences in band patterns were observed between the most virulent isolate, 1F2 and the least virulent isolate, 1F4.

3.1 INTRODUCTION

In a previous paper (Chapter Two), monoclonal antibodies (mAbs) were prepared against four isolates of *Xanthomonas campestris* pv. *mangiferaeindicae*. These mAbs raised against different isolates of *X. c.* pv. *mangiferaeindicae* reacted with protein epitopes (Chapter Two) and showed different reaction patterns with the different isolates, suggesting that virulence factors might be proteinaceous.

Differences in virulence in bacteria have been well documented. Some isolates of *Pseudomonas solanacearum* from different geographical regions have been found to differ in virulence from other isolates (Sequeira & Graham, 1977; Boucher et al., 1986) and mutants of *Erwinia amylovora* deficient in exopolysaccharides differ in virulence from nondefective mutants (Ayers et al., 1979).

The cell envelope of Gram negative bacteria is a complex multilayered structure composed of a variety of structural molecules including proteins, phospholipids and a lipopolysaccharide (LPS) (Minsavage & Schaad, 1983). There are many proteins on the outer membrane although it is poor in enzyme activity. LPS and almost all proteins have sites exposed which can act as receptors for phages, colicins and donor cells in conjugation and can react with antibodies, enzymes and chemicals (Lugtenberg & van

Alphen, 1983).

Most research has been carried out on the role of exopolysaccharides (EPS) and lipopolysaccharides (LPS) in virulence of *Xanthomonas campestris* pv. *campestris*, *Erwinia amylovora* and several Pseudomonads (Chatterjee & Vidaver, 1986; Leigh & Coplin, 1992). EPS's have been positively correlated with virulence in *E. amylovora* (Ayers et al., 1979), *Ps. solanacearum* (Husain & Kelman, 1958; Ofuya & Wood, 1981) and *Agrobacterium tumefasciens* (Puvanesarajah et al., 1985).

To date, very little has been documented regarding the role of outer membrane proteins as a virulence determinant. Virulent strains of a fish pathogen, *Aeromonas salmonicida* have been shown to possess an additional protein layer known as the A layer or A protein and a similar protein layer has been found to play a role in virulence in the animal and human pathogens *Campylobacter fetus* subsp. *intestinalis* and *Staphylococcus aureus* (Hammond et al., 1984).

Western blot analysis using monoclonal antisera is a widely used technique in phytobacteriology (Franken et al., 1992) and has been used extensively for antibody characterisation (Wright et al., 1986; Segers et al., 1990; Labadie & Desnier, 1992) and diagnostic purposes (Kerr et al., 1992). Close examination of patterns obtained also makes it possible to identify different antibodies, making it a useful screening procedure during antibody production (Thomas et al., 1986).

The purpose of this study was to investigate differences in virulence in different isolates of *X. c.* pv. *mangiferaeindicae* by means of inoculation studies and Western blot analysis using specific monoclonal antibodies, and develop these antibodies as tools to study differences in virulence in different isolates.

3.2 MATERIALS AND METHODS

Isolation of organisms

Fruit and leaves from Sabre and Sensation trees with typical BBS symptoms were collected from Zebediela Estates, South Africa in December 1990. These were placed into insulated, cooled containers and transported to the laboratory where isolations were made immediately.

Plant material was surface disinfested by dipping into a 3% solution of sodium hypochlorite 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 periphery of the lesions, teased apart in sterile ¼ strength Ringer's solution (Oxoid) and streaked onto Standard I nutrient agar (Biolab) (STD I) and Boost agar (BA) plates (Manicom, 1980) and incubated at 28°C for 72 hours.

Virulence tests

Isolates were cultured on BA and STD I plates and incubated under conditions as described. Identity of the organisms were confirmed by means of standard physiological and morphological characteristics (Starr, 1981). Cells were harvested and suspended in ¼ strength Ringer's solution to a final concentration of 10^7 cells/ml by means of a Petroff - Hausser counting chamber with a Zeiss phase contrast microscope. Five mature Sensation fruit were inoculated by making a number of pricks on the fruit and dropping 10 μl prepared inoculum onto each wound. Two controls were used, namely, prick wounds only, and pricking with 10 μl sterile Ringer's solution on wounds. All wounds were covered with sterile cotton wool moistened with SDW, covered with plastic bags and incubated at an average temperature of 28°C for 12 days. Symptom development was monitored daily, and cotton wool replaced after each inspection.

Isolates were grouped according to percentage infection. In order to verify results, isolations were made from inoculated fruit, and reinoculated into mature Sensation fruit as described. Based upon symptom expression and percentage infection, determined by the ratio of total number of wounds to total number of wounds showing symptoms and expressed as a percentage, groups of isolates were designated. Symptom expression was also tested by inoculating three soft, mature Kent leaves using the detached leaf technique as described by Graham & Gottwald (1990). Fruit and leaves were evaluated daily, and final evaluation was carried out after 12 days. Leaves were evaluated according to a severity rating scale and percentage infection.

The severity rating scale was described as follows: 0; slight necrosis around edges of the wound, 1; necrosis around the wound edge with occasional water soaking, 2; same as rating 1 with a higher percentage infection, with some wounds cracked open, 3; severe necrosis around wound sites with a high percentage infection appearing water soaked. Micrographs of leaf lesions were taken at a 25 and 50 times magnification using a Zeiss stereo microscope. Representative organisms from each group were used for further

studies. All isolates were frozen away in glycerol as described by Sleesman & Leben (1978).

Sample preparation for SDS - Polyacrylamide gel electrophoresis (PAGE)

Suspensions of 10^7 cells/ml of all four virulence groups of *X. c.* pv. *mangiferaeindicae*, namely, 1F2, 1F4, 1F5 and 1F7 were centrifuged for 10 minutes at 4°C in a Hemle fixed speed microcentrifuge. Each pellet was suspended in 100μl sample buffer (2% sodium dodecyl sulphate, 10% glycerol, 5% v/v 2 - ß - mercaptoethanol and 0.001% bromophenol blue in 0.06M Tris - HCl, pH 6.8). These preparations were boiled for 5 minutes at 100°C and 20μl was loaded per lane.

SDS - PAGE

The Laemmli SDS - gel electrophoresis system using a 12% w/v acrylamide resolving gel and 4% stacking gel was performed in an OWL electrophoresis unit (OWL Scientific Plastics, Inc. Cambridge, MA 02139) (Laemmli, 1970). Two millimetre thick slab gels were used with a Tris - HCl buffer, pH 8.3. Molecular weight markers were used as follows: phosphorylase B (94 000 D), albumin (67 000 D), ovalbumin (43 000 D), carbonic anhydrase (30 000 D), trypsin inhibitor (20 100 D) and α - lactalbumin (14 400 D) (Boehringer Mannheim). Pre - electrophoresis was carried out at 60V for one hour and electrophoresis at 100V for 3 hours at room temperature using a Pharmacia ECPS 3000/200 power pack (Pharmacia, LKB Biotechnology, Sweden). Staining of proteins was done with a 0.2% Coomassie Blue solution in 45% methanol and 10% acetic acid for one hour. The gel was subsequently washed in a destaining solution consisting of methanol, acetic acid and water in a ratio of 5 : 1 : 5. The gel was cut into identical sections, each section destined for a different culture supernatant or antiserum.

Western blotting

Immunoblotting was carried out with a semi dry transblot apparatus (BioRad Laboratories, Richmond, CA). The proteins were blotted onto polyvinylidine - diflouride membrane (PVDF) (Milllipore corporation, Bedford) with 10mM 3 - cyclohexylamino - 1 - propane sulphonic acid (CAPS) buffer (Sigma), pH 9.0 at 10mA for 35 minutes at room temperature. After blotting, the membranes were incubated in incubation buffer (1% milk powder and 0.05% Tween 20 in 20mM Tris buffered saline pH 7.4). Membranes were transferred to undiluted culture supernatants from clones 2C/3G/1, 2A/10B/2 and 2D/11B/A or 1/100 dilution in incubation buffer of the polyclonal antisera raised against the least virulent isolate, 1F4 and the most virulent isolate, 1F2, and incubated for 18 hours at 10°C. After three washes with 0.1% milk powder in Tris buffered saline, pH 7.4,

membranes were incubated for one hour with peroxidase conjugated Goat - a - Mouse IgG (H + L) (Cappel, Worthington) diluted 1:500 in incubation buffer.

After the final wash, the membranes were developed in 0.06g 4 - chloro - 1 - naphtol (Sigma) in 20ml cold methanol added to 60μl 30% H_2O_2 in 100ml Tris buffered saline. The reaction was stopped by replacing the substrate buffer with distilled water after five minutes for the monoclonal supernatant and after 15 minutes for the polyclonal antisera.

3.3 RESULTS

Isolation of organisms

From a total of 35 disease samples, 27 isolates induced symptoms resembling BBS when inoculated into fruit. After reisolation, pure cultures of *X. c.* pv. *mangiferaeindicae* were recovered. The standard physiological and morphological tests performed, confirmed their identity. The two media did not adversely effect the virulence of the isolates. The nutritional requirements of *X. c.* pv. *mangiferaeindicae* are such that growth as well as slime production will be more prolific in a richer medium such as Boost agar.

Virulence tests

Upon reinoculation into fruit, a variety of symptoms were observed ranging from water soaked spots to typical star shaped lesions. Based upon symptom expression and percentage infection, isolates were assigned to four groups (Table 1).

Similar differences in symptom expression were observed for the various isolates with the detached leaf technique (Table 2). Very clear differences could be observed between symptoms produced by the isolates when examined microscopically (Fig 1). Isolate 1F2 was found to produce the highest percentage infection (Table 2) as well as the most severe symptoms, while isolate 1F4 produced the lowest percentage infection and the least severe symptoms, which is in accordance with their field properties.

Table 1 Isolates from four virulence groups of *Xanthomonas campestris* pv. *mangiferaeindicae* showing difference in symptom expression in Sensation mango fruit.

Isolate	Percentage infection [a]	Lesion severity [b]
1F2	88.7	Severe
1F7	60.4	Less severe
1F5	42.5	Moderate
1F4	12	Mild

[a] Percentage infection was determined from the formula: no. lesions/ no. symptomatic x 100

[b] Lesion severity rating on fruit graded as follows: Severe = star shaped cracks surrounded by a yellow halo; Less severe = dark spots surrounded by a yellow halo; Moderate = water soaked spots with a yellow halo; Mild = slight water soaking.

Table 2 Difference in symptom expression of different isolates of *Xanthomonas campestris* pv. *mangiferaeindicae* as indicated by percentage infection of detached Kent mango leaves

Isolate	Percentage infection	Severity rating[a]
1F2	44.2	3
1F7	12.9	1
1F4	22.4	0
1F5	28.2	2

[a] Severity rating: 0 = slight necrosis around the edges of the wound; 1 = necrosis around the wound edge with occasional water soaking; 2 = same as rating 1 with a higher percentage infection, some wounds cracked open; 3 = severe necrosis around wound sites with a high percentage infection, appearing water soaked with wounds on main veins cracked open and oozing exudate.

A

B

C

D

Fig. 1   Lesion development of different isolates of *Xanthomonas campestris* pv. *mangiferaeindicae* prick inoculated onto mature Kent mango leaves. Lesion development evaluated after 12 days 1F2 (A), 1F7 (B), 1F4 (C), 1F5 (D).

SDS - PAGE electrophoresis

A wide variety of bands were observed for all isolates. A concurrent band was observed for all isolates at 39kD (Fig. 2). Differences between isolates were most marked with isolate 1F7 were an extra band was observed at 23 kD, which appeared in close proximity to a concurrent band as a doublet (Fig. 2). This observation cannot be related to virulence since no such band was observed with isolate 1F2 (most virulent) and 1F4 (least virulent).
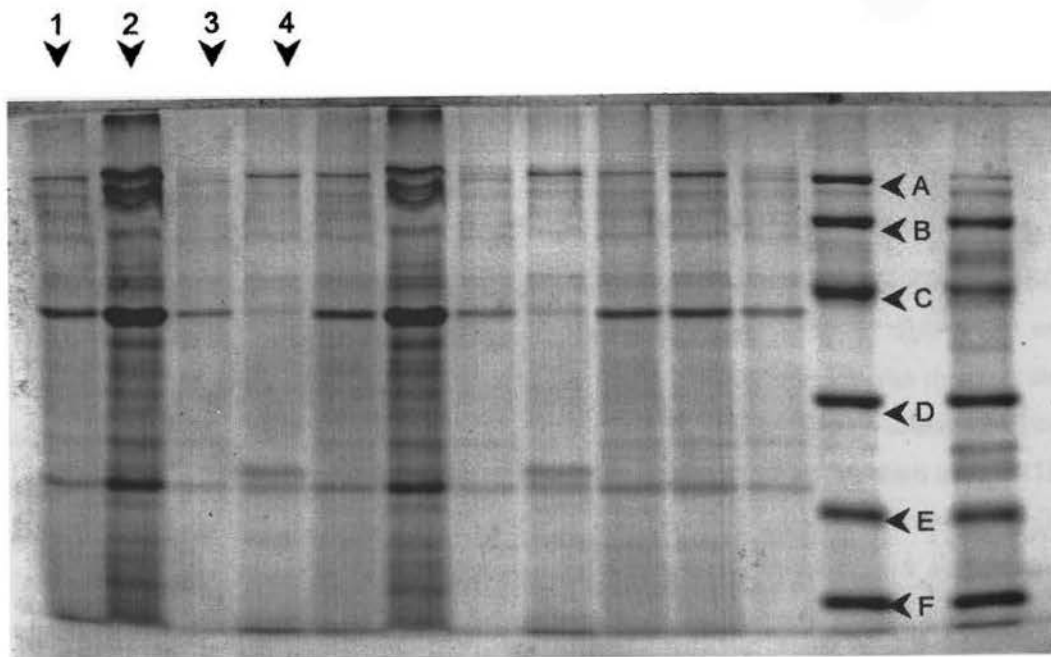
Fig. 2   SDS - PAGE electropherogram of different *Xanthomonas campestris* pv. *mangiferaeindicae* isolates. Electrophoresis was performed using a 12% (w/v) acrylamide resolving gel and a 4% stacking gel. Samples are: 1F2 (1), 1F4 (2), 1F5 (3), 1F7 (4). Standard molecular mass markers were: phosphorylase B (94 000 D) (A), albumin (67 000 D) (B), ovalbumin (43 000 D) (C), carbonic anhydrase (30 000 D) (D), trypsin inhibitor (20 100 D) (E), alpha - lactalbumin (14 400 D) (F).

Western blot analysis

The differences in reaction patterns generated by the antisera against the least and against most virulent isolates (Figure One, Chapter Two) may suggest that the two types of antisera recognise different antigens. To investigate this possibility, the SDS - PAGE electropherogram mentioned above was electroblotted onto a PVDF membrane and probed with antiserum against the most or least virulent isolate. Many bands were observed with both antisera (Fig. 3). No major differences were observed to substantiate the suggestion of different sets of different antigens recognised by two types of antisera. However, quantitative differences were observed which may explain the ELISA results depicted in Figure One, Chapter Two.

Another SDS - PAGE electropherogram was electroblotted and probed with mAbs XCM - 1(G3) and XCM - 1(G2a). The results in Figure 4 confirmed the identical antigenic specificity of the three mAbs tested. Thus lanes 1, 5 and 9 shows identical patterns obtained from isolate 1F2 with XCM - 1(G2a), another clone of XCM - 1(G2a) and XCM - 1(G3) respectively. Lane 10 is a duplicate of lane 9. The reactivities of isolate 1F4 with mAb XCM - 1(G2a) (lanes 2 and 6) reveal a qualitative difference with the most virulent isolate (1F2). Whereas isolate 1F2 had a characteristic band at 32kD, isolate 1F4 could be characterised by two bands at 31 and 33 kD. With the moderately virulent isolate (1F5) (lanes 3,7 and 11), a faint band of 32 kD could be observed, while no bands in the 30 - 33kD range were observed with moderately virulent isolate, 1F7 (lanes 4 and 8). In all isolates tested, the strongest band appeared at 39kD. The intensity of this band seemed to correlate with detectability of bands in the 30 - 33kD range, but not with bacterial counts in the loaded samples, which were identical for all lanes.
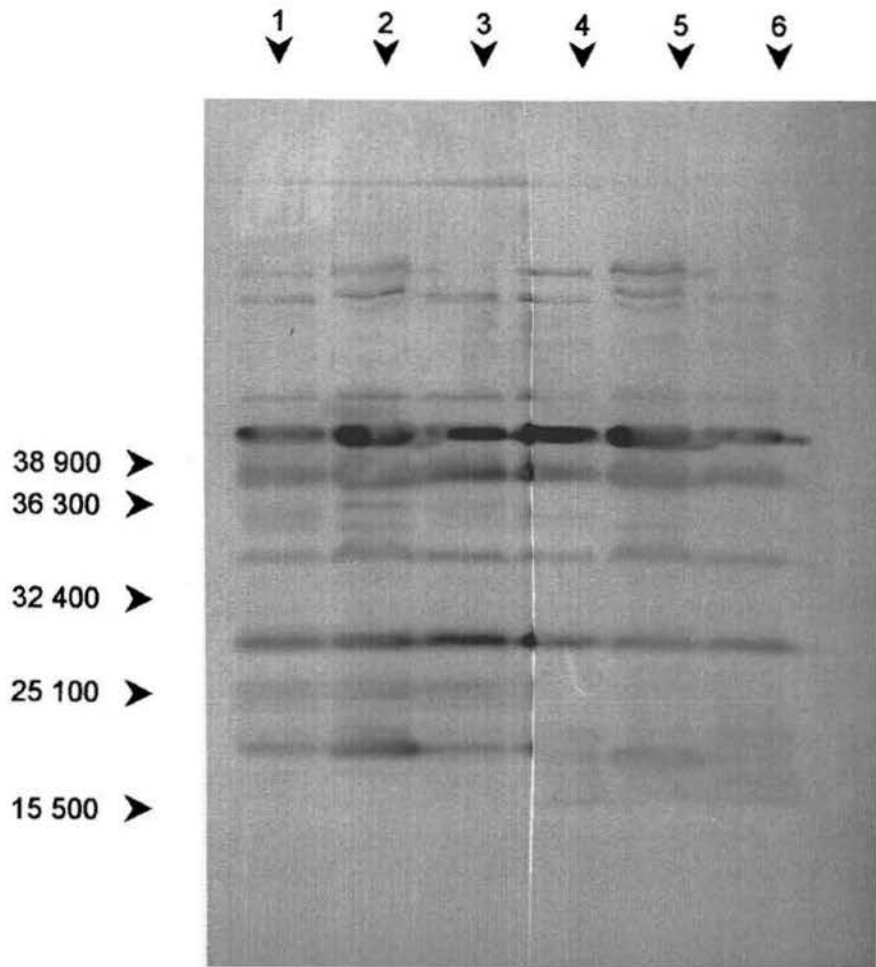
A

Fig. 3   Western blot of different *Xanthomonas campestris* pv. *mangiferaeindicae* isolates separated electrophoretically and probed with polyclonal antisera raised against isolate 1F2 (most virulent) (A) and antisera against isolate 1F4 (least virulent) (B). Samples are as follows: 1F2 (1), 1F4 (2), 1F5 (3), 1F2 (4), 1F4 (5), 1F5 (6). Molecular weights of relevant bands expressed in Daltons.
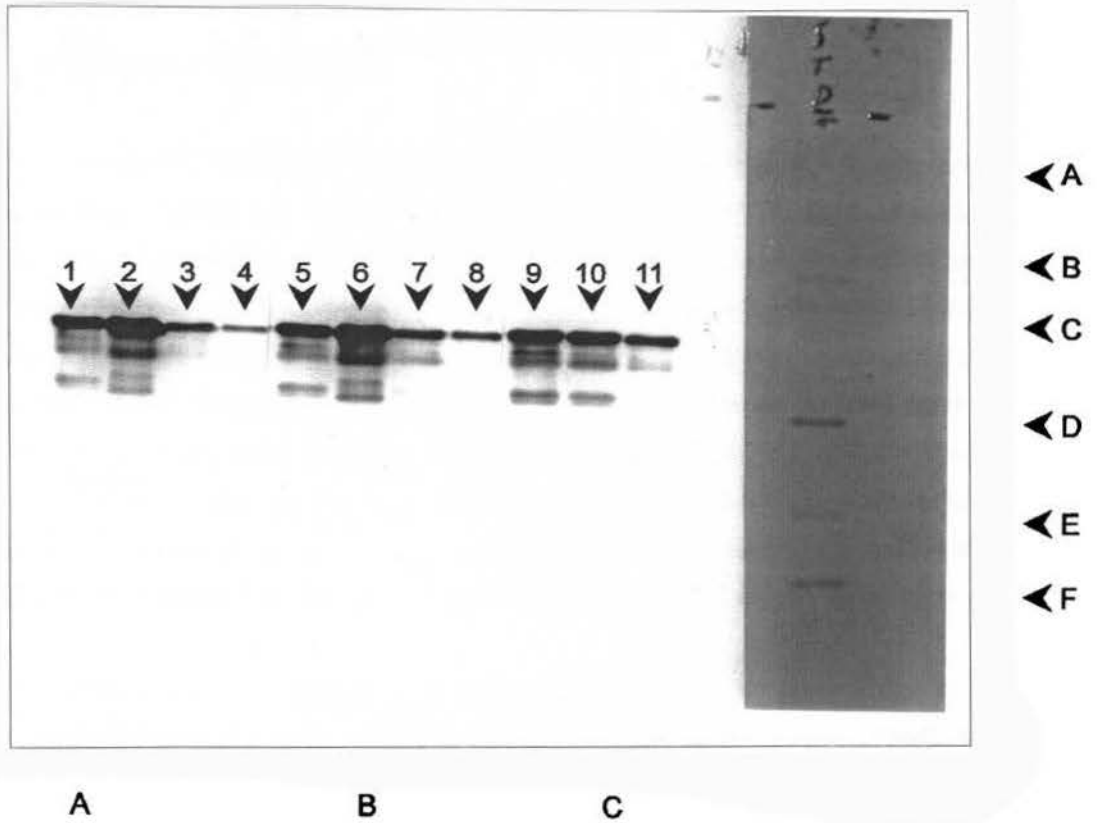
Fig. 4   Western blot of different *Xanthomonas campestris* pv. *mangiferaeindicae* isolates separated electrophoretically and probed with monoclonal antisera 2A/10B/2 (A), 2C/3G/1 (B), 2D/11B/A (C). Samples are as follows: 1F2 (1), 1F4 (2), 1F5 (3), 1F7(4), 1F2 (5), 1F4 (6), 1F5 (7), 1F7 (8), 1F2 (9), 1F2 (10), 1F4 (11). Molecular weight markers include: Phosphorylase B (94 000 D) (A); Albumin (67 000 D) (B); Ovalbumin (43 000 D) (C); Carbonic anhydrase (30 000 D) (D); Trypsin inhibitor (20 100 D) (E); Alpha - lactalbumin (14 400 D) (F).

3.4 DISCUSSION

The isolates collected for this study was grouped into virulence classes according to percentage infection as well as symptom severity with 1F2 being most virulent and isolate 1F4, least virulent. These results were found to be reproducible, and the detached leaf assay has been successfully used to evaluate virulence in *Pseudomnas syringae* pv. *syringae* on pears (Yessad et al., 1992) and *X. c.* pv. *citrumelo* on citrus (Graham & Gottwald, 1990). These findings were similar irrespective of whether tests were carried out on individual leaves per isolate or all isolates together on the same leaf (unpublished data). Isolates that were grouped as being moderately virulent showed variable results between the assays. This was found to be the the case with leaves and fruit in different stages of development as well as leaves of different cultivars (unpublished data).

Differences in virulence in plant pathogenic bacteria other than *X. c.* pv. *mangiferaeindicae* have been well documented (Chatterjee & Vidaver, 1986; Leigh & Coplin, 1992; Yessad et al., 1992). In contrast to the report by (Manicom & Wallis, 1984), my results show that *X. c.* pv. *mangiferaeindicae* exists as strains differing in virulence. All virulence tests clearly indicated that isolate 1F2 was most virulent and isolate 1F4 was least virulent. This provides the first concrete evidence for the existence of different virulence strains for *X. c.* pv. *mangiferaeindicae*, which were confirmed with Western blots probed with mAb XCM -1, the identity of which is described in Chapter Two

A LPS is a common virulence determinant and has been extensively implicated with virulence in animal pathogens such as *Salmonella spp.*, *Klebsiella pneumoniae* and *Haemophilus influenzae* (Hammond et al., 1984) and plant pathogens such as *E. amylovora*, and several *X. campestris* pathovars (Chatterjee & Vidaver, 1986). In this case, it was found that differences in amount of LPS did not play a role in virulence, since the reactions with proteins of all virulence groups was equally strong. No differences were observed in LPS profiles (unpublished data). Thus, it is clear that LPS does not play a role in virulence of *X. c.* pv. *mangiferaeindicae*.

Western blot analysis was extensively used to investigate differences in virulence, and are also widely used for characterisation of antigens (Benjamini & Leskowitz, 1991). Initially, it appeared as though the antisera against the least and most virulent isolates recognised different antigens (Fig 1, Chapter Two). The least virulent isolate (1F4) consistently showed the strongest reactions with both oligo - and monoclonal antibodies,

while weaker reactions were obtained with the more virulent isolate (1F2). Thus, an inversely proportional relationship exsists between ELISA signal strength and virulence.

Western blots of different virulence isolates probed with polyclonal antisera raised against either the least (1F4) or the most virulent isolate (1F2) did not reveal significant qualitative differences between the most and least virulent isolates. Similarly, different strains of *Rhizobium trifolii* could not be separated by polyclonal antisera (Wright et al., 1986). Although polyclonal antisera have been successfully implemented for field detection of single plant pathogens (Anderson & Nameth, 1990), cross reactivity is one of the most important problems with polyclonal antisera as a diagnostic tool. Polyclonal antibodies react with many antigenic determinants resulting in cross reactions with organisms sharing a common epitope (de Boer et al., 1988) leading to false positive results.

Qualitative differences, however, were observed when probing Western blots with mAbs. All clones of mAb XCM - 1 (Chapter Two), although extensively cross reactive with all the isolates, succeeded in detecting a qualitative difference between the least and most virulent isolates. The four isolates shared a concurrent band at 39 kD. Between 30 - 33kD differences in band pattern were observed, and in the case of 1F7 bands in this region were absent (Fig. 4). A correlation was observed between the intensity of this band and the detectability of the bands in the 30 - 33 kD region, despite equal cell counts in all samples. It has been shown that production and secretion of antigens may vary with growth conditions, and their concentration is not necessarily correlated with cell numbers (de Boer et al., 1988).

Most strain specific mAbs react only with one strain or virulence group and not with any other (Alvarez et al., 1985; Wright et al., 1986; Bouzar et al., 1988; Segers et al., 1990). In order to achieve such strain specific mAbs, specially bred mice must be used (Segers et al., 1990) or the virulence determinants such as LPS (Bouzar et al., 1988) must be isolated and used for immunisation. However, the aim of this sudy was to raise mAbs for the detection of *X. c.* pv. *mangiferaeindicae* in the field (Chapter Two) and no such steps were taken, since over specificity of monoclonal antisera may be as great a problem as low specificity of polyclonal antisera (Harlow & Lane, 1988).

From results obtained, I suggest that certain protein(s) may play a role in determining virulence of *X. c.* pv. *mangiferaeindicae*. It is also clear that mechanisms inducing virulence are poorly understood, despite extensive reasearch on suspected

determinants such as toxins, enzymes and polysaccharides. While these factors may induce certain visible symptoms, the total disease phenomenon has many properties (Preece, 1982). These results may be of importance to breeders when developing new cultivars for resistance to BBS, since evaluation using all isolates would give a better picture of the plant's resistance to this disease.

MAbs raised could thus be used as a probe in order to further elucidate the differences in virulence between isolates and determine the mechanisms of virulence as well as factors inducing virulence of *X. c.* pv. *mangiferaeindicae.*

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CHAPTER FOUR

A RAPID METHOD FOR DIFFERENTIATION OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* FROM OTHER XANTHOMONADS AND MANGO PHYLLOPLANE INHABITANTS

ABSTRACT

Several selective media were evaluated for differentiation and selective enrichment of *Xanthomonas campestris* pv. *mangiferaeindicae*. Boost broth supplemented with cycloheximide, methyl violet and methyl green (BVGA) selectively enriched low concentrations of the epiphytic stage of *X. c.* pv. *mangiferaeindicae* amongst other phylloplane inhabitants. The identity of the isolated organism was confirmed using *X. c.* pv. *mangiferaeindicae* - specific monoclonal antibodies in an enzyme - linked immunosorbent assay (ELISA).

4.1 INTRODUCTION

Bacterial black spot disease (BBS) of mango (*Mangifera indica* L.) is a pre - harvest disease caused by *Xanthomonas campestris* pv. *mangiferaeindicae*, which manifests as swollen, water soaked spots which crack open to expose the vascular tissue on leaves and twigs (Viljoen & Kotzé, 1972). Fruit symptoms range from small water soaked spots to characteristic star shaped lesions (Viljoen & Kotzé, 1972).

The main mechanism of spread of this disease is by the distribution of infected plants in the form of young trees grafted with infected material, which are often asymptomatic (Manicom, 1983). Diagnosis of pathogen free material is an arduous task since the causal organism is a year round phylloplane resident (Manicom, 1986), without any visible symptoms. Detection of this epiphytic phase of the pathogen is of primary importance in order to establish control measures and clean orchards (McGuire et al., 1986).

Selective or semi - selective media are an invaluable aid for the isolation of phytopathogenic bacteria from various sources, including plant tissue (Stead, 1992). These media can also be used for selective enrichment of target organisms prior to the use of other detection methods such as the enzyme - linked immunosorbent assay (ELISA).

Since techniques such as the ELISA require a minimum cell concentration of 10^4 cells/ml for successful detection (Alvarez & Lou, 1985; Civerolo & Fan, 1982; McLaughlin et al., 1989), a selective enrichment step is required, so that low cell concentrations upon or within the plant may be detected.

An ideal selective medium should be selective to at least the species level (Claflin et al., 1987). Several enrichment media have been described for pathovars of *Xanthomonas campestris*, namely *X. campestris* pv. *campestris* (Chang et al., 1991), *X. campestris* pv. *vesicatoria* (Sijam et al., 1991), *X. campestris* pv. *phaseoli* (Mabagala & Saettler, 1992), *X. campestris* pv. *dieffenbachiae* (Norman & Alvarez, 1989), *X. campestris* pv. *juglandis* (Mulrean & Schroth, 1981) and *X. campestris* pv. *translucens* (Schaad & Forster, 1985). No selective medium has been described for *X. c.* pv. *mangiferaeindicae*.

The purpose of this study was to evaluate available selective media and develop a semi - selective medium suitable for the differentiation of the epiphytic phase of *X. c.* pv. *mangiferaeindicae* from other phylloplane inhabitants in order to screen mango mother material for the presence of the pathogen by means of ELISA.

4.2 MATERIALS AND METHODS

Bacterial isolates

Test organisms used in this study were isolated from the mango phylloplane or obtained from L. Korsten and E. van Zyl (Department of Microbiology and Plant Pathology, University of Pretoria. Several Pseudomonads obtained from L. Korsten were supplied by Dr I. Roos, Infruitec, Stellenbosch). Isolation of phylloplane inhabitants was carried out by inoculating 9ml volumes of Standard I (Std I) nutrient broth (Biolab), with one gram of Keitt leaf discs punched out using a number 10 cork borer. Plant material was obtained from the University of Pretoria Experimental farm. After 48 hours incubation at 28°C, a 10 - fold dilution series was made as described by Harrigan & McCance (1966), and plated onto Std I agar. Plates were incubated as described.

Representative organisms were randomly isolated for use in this study and were grouped according to key physiological tests namely oxidation/fermentation, oxidase, catalase, Gram reaction, and colony morphology and colour. These isolates as well as test organisms were grouped into representative categories (Table 1).

All cultures were maintained on Std I agar except *Xanthomonas* and *Pseudomonas* spp. which were maintained on Boost medium (Manicom, 1986). Cultures were cryopreserved in 15% glycerol as described by Sleesman & Leben (1978).

Table 1   Bacterial isolates used to evaluate selectivity of media for the enrichment of *Xanthomonas campestris* pv. *mangiferaeindicae* from mango trees

Bacteria utilised	Source[a]	Designation	Representative group[b]
Acinetobacter lwoffii	B	Org B	4
Agrobacterium radiobacter	B	A.r	4
Bacillus licheniformis	B	B.l	3
Bacillus megaterium	B	B.m	3
Erwinia sp.	A	E.s	5
Klebsiella pneumoniae spp. *pneumoniae*	B	K.p.spp.p	5
MB1	A	MB1	6
MB4	A	MB4	6
MT10	A	MT10	6
MT2	A	MT2	6
MT5	A	MT5	6
MT6	A	MT6	6
MT7	A	MT7	6
MT9	A	MT9	6
Micrococcus kristinae	B	M.k	3
Pseudomonas syringae	B	W - 1b	2
Ps. marginales pv. *alfalfae*	B	P.m.pv.a	2
Ps. viridiflava	C	P.v	2
X. axonopodis	C	X.a	1
X. campestris pv. *campestris*	C	X.c.pv.c	1
X. campestris pv. *mangiferaeindicae*	A	X.c.pv.m	1
X. campestris pv. *phaseoli*	C	X.c.pv.p	1
X. campestris pv. *viticola*	C	X.c.pv.v	1
X. fragariae	C	X.f	1

[a]   A = This study; B = L. Korsten, Department of Microbiology and Plant Pathology, University of Pretoria; C = E.van Zyl, Department of Microbiology and Plant Pathology, University of Pretoria

[b]   1 = Xanthomonads; 2 = Pseudomonads; 3 = Bacilli and other Gram positive organisms; 4 = Non related organisms; 5 = Enterobacteria; 6 = Phylloplane organisms

Media

Media tested are listed in Table 2. In order to enhance the selectivity of less selective media (LCM and YS), 12 compounds viz. novobiocin (5 µg), chloramphenicol (10

μg), neomycin (10 μg), rifampicin (2 μg), oxytetracycline (30 μg), kanamycin (5 μg), gentamycin (10 μg), erythromycin (5 μg), tetracycline (10 μg), ampicillin (10 μg), streptomycin (10 μg) and penicillin G (10 μg) were evaluated against *Klebsiella pneumoniae* spp. *pneumoniae*, an *Erwinia* sp., MT10, a phylloplane isolate and *X. c.* pv. *mangiferaeindicae.* Ten millilitre volumes of molten STD I nutrient agar were inoculated with 200 μl of a cell suspension of *X. c.* pv. *mangiferaeindicae* with an optical density (O.D.) of 0,150 at 600nm, measured on an LKB Ultrospec spectrophotometer and poured into sterile petri dishes. Pre - impregnated antibiotic assay discs (Sigma) were aseptically placed onto the cooled medium. Three replicates per antibiotic were used. Sterile, untreated assay discs were used as negative controls. Plates were incubated as described, and after 48 hours, zones of inhibition were measured and expressed in millimetres.

Table 2   Selective media evaluated for detection of epiphytic *Xanthomonas campestris* pv. *mangiferaeindicae* from mango trees

MEDIUM	REFERENCE
Brilliant cresyl blue starch (BS)	Mulrean & Schroth, 1981
Boost violet green agar (BVGA)	Sanders et al., 1989
Cellobiose starch (CS)	Norman & Alvarez, 1989
Esculin trehalose (ET)	Norman & Alvarez, 1989
Glucose yeast extract chalk agar (GYCA)	Cruz et al., 1984
Lederberg's complete medium (LCM)	Tuinier & Stevens, 1989
MXP -	Claflin et al., 1987
Starch methionine (SM)	Chun & Alvarez, 1983
Standard 1 violet green agar (SVGA)	Sanders et al., 1989
SX -	Schaad & White, 1974
XMSM -	Juhnke & Des Jardin, 1989
XTS -	Schaad & Forster, 1985
Yeast salts (YS)	Dye, 1962

Evaluation of selective media. Three replicates were used in all the evaluations unless stated otherwise.

Efficiency of recovery on solid media. Cell suspensions of all organisms with an O.D. of 0,150 at 600nm, corresponding to an approximate count of 10^7 cells/ml were prepared. A 10 - fold dilution series was prepared as described. Five replicates of each test medium were inoculated with 0,1ml of either 10^3 or 10^2 cells/ml bacterial suspension. STD I agar was used as reference medium and inoculated in the same way. All plates were incubated at 27°C until confluent growth was achieved. Media were evaluated   by determining plating efficiencies as defined by Kado & Heskett (1970). Those media which yielded the highest ratio of growth relative to STD I were used for the next step of evaluation.

Efficiency of recovery from liquid media. A cell suspension of *X. c.* pv. *mangiferaeindicae* with an O.D. of 0,150 was prepared as described. Nine millilitre volumes of SVGA, BVGA, MXP and XTS were inoculated with 0,1ml bacterial suspension. Boost broth was used as a reference medium and inoculated and incubated in the same way. After 24, 48 and 72 hours, cell counts were made from each replicate. Media which yielded the highest counts of *X. c.* pv. *mangiferaeindicae* were used for further evaluation. Mean counts were compared using Fischer's protected least significant difference (LSD) test at the 95% confidence level as described by de la Cruz et al. (1992).

Optimisation of isolating mango phylloplane inhabitants

In order to determine the competing ability of *X. c.* pv. *mangiferaeindicae* with other phylloplane inhabitants in a limited volume, various methods were evaluated for optimum recovery of phylloplane inhabitants. Leaves were randomly picked from five different Keitt mango trees and pooled together. A total of one gram of leaf material was cut from the leaves using a number 10 cork borer. Several washing buffers were tested viz. ¼ strength Ringer's solution (Oxoid), 0,1% and 1% Tween 20, 0,05M and 0,025M ethylene diamine tetra - acetic acid (EDTA), 0,01% Triton X100, 0,01% and 0,05% sodium dodecyl sulphate (SDS) and 0,1% glycerol. Water and peptone phosphate buffer (McGuire et al., 1986) were also evaluated. All buffers were prepared in 9ml volumes in McCartney bottles with glass beads and sterilised. Three replicates per buffer were made. Leaf discs were shaken thoroughly using a vortex for one minute and then sonicated in an ultrasonic bath for one minute. A 10 - fold dilution series was prepared. Three replicates of of 0,1ml per dilution were plated out on STD I nutrient agar. Plates were incubated at 27°C for 48 hours. A second group of buffers were evaluated namely, Tween 80, yeast extract, glucose, L - glutamic acid, ammonium sulphate and 0,1% peptone as described by (Dickinson et al., 1975). These were shaken on a platform shaker for one hour.

In another experiment, maceration in phosphate buffer as described by Leben et al (1968) was carried out as well. Dilution series were prepared and incubation was carried out as described. Total counts per gram of leaf material were made and species diversity was determined by making counts of different fungi, bacteria and yeasts present. All data was statistically analysed as described.

Evaluation of medium selectivity

Mature Keitt mango leaves from the University of Pretoria Experimental farm were used for these evaluations. The optimum isolation technique was utilised namely, thorough shaking on a vortex for one minute in 0.025M EDTA, followed by ultrasonification for one

minute. A 10 - fold dilution series was prepared, and plated out on five replicates of each medium, with STD I agar as reference medium. Selectivity of each medium was expressed as the drop in total count for each medium relative to STD I. Selectivity was also evaluated using liquid medium. Five 9ml volumes of each medium were inoculated with one gram Keitt mango leaf discs and incubated at 27°C and total counts made every 24 hours up to 72 hours. Species diversity was determined for each medium as described. Leaf impressions were made by placing a 250g weight on either the ad - or abaxial surface pressing on the test medium for one minute. Counts per plate were made and selectivity was compared with that of Std I agar. Data was statistically analysed as described.

Competition with phylloplane inhabitants in optimal selective media

Five 9ml volumes of BVGA were inoculated with one gram of Keitt mango leaf discs and 200 μl of a 10^6 cells/ml *X. c.* pv. *mangiferaeindicae* added to each replicate. This was repeated with dilutions ranging from 10^5 to 10^2 cells/ml *X. c.* pv. *mangiferaeindicae*. Media were incubated at 27°C. Results were recorded after 72 hours by layering 100μl from each thoroughly shaken replicate onto 96 - well microtiter plates (Cooke Microtiter system M29A, Sterilin products, Middlesex, England). A total of 45 replicates were tested per dilution with uninoculated broth as negative control. The ELISA was carried out as described in Chapter Two.

4.3. RESULTS

Bacterial isolates

Eight isolates were chosen from the mango phylloplane population. Only one of the eight were Gram positive, all were catalase positive, three were oxidase negative and all had an oxidative metabolism (Table 3).

Table 3 Physiological and morphological characteristics of mango phylloplane isolates

ISOLATE	Gram reaction	Oxi/Ferm	Oxi[a]	Cat[a]	Morphology	Colony colour
MT10	-	Oxidative	+	+ +	Rods	Off white
MT9	-	Oxidative	+	+	Large rods	White
MT6	-	Oxidative	+	+	Rods	Off white
MT5	-	Oxidative	+	+ +	Short rods	Light orange
MB4	-	Oxidative	-	+	Crooked rods	Beige
MB1	-	Oxidative	-	+	Rods	Cream
MT2	-	Oxidative	-	+	Rods	Light orange
MT7	+	Oxidative	+ +	+	Large rods	Off white

Codes used as follows: Oxi/Ferm = Oxidation/Fermentation test; Oxi = Oxidase test; Cat = Catalase test

[a] (-) indicates a negative reaction; (+) indicates a positive reaction; (+ +) indicates a strong positive reaction

Media

Due to the growth of *Klebsiella pneumoniae* spp. *pneumoniae* and the *Erwinia* sp. on several selective media, antibiotic assays were deemed necessary. Of all the antibiotics tested, it was found that medium selectivity would be further enhanced by the addition of ampicillin (Table 4). None of the other antibiotics evaluated could be incorporated into a selective medium since most were inhibitory to *X. c.* pv. *mangiferaeindicae*.

Table 4   Antibiotic compounds screened for selective enhancement of bacterial growth
media

ANTIBIOTIC EVALUATED[a]	INHIBITION ZONES OF SELECTED ORGANISMS[b]			
	K.p.spp.p	E.s	MT5	X.c.pv.m
Novobiocin	0	0	32.3	17.3
Chloramphenicol	24	20.3	34.6	22.6
Neomycin	12	10.6	30.3	23
Rifampicin	0	0	36.6	26.6
Oxytetracycline	26.6	25.3	38	32.6
Kanamycin	106	11.3	28	22.6
Gentamycin	13.6	12.6	35	32.3
Erythromycin	0	0	31	28
Tetracycline	20.6	18.6	36.6	31
Ampicillin	0	18	36.3	0
Streptomycin	12	8.6	31.3	29.6
Penicillin G.	0	0	35.3	13

[a]   Pre - impregnated antibiotic assay discs were used.

[b]   Organisms coded as follows: K.p.spp.p = *Klebsiella pneumoniae* spp. *pneumoniae*; E.s = *Erwinia* sp.; MT5 = unidentified mango phylloplane inhabitant; X.c.pv.m = *Xanthomonas campestris* pv. *mangiferaeindicae*.

Data represents a mean total inhibition zone in millimetres including diameter of the antibiotic assay disc (6mm). Sterile, untreated discs were used as negative controls.

Efficiency of recovery on solid media. Three media, namely, BVGA, MXP and XTS were found most effective for *X. c.* pv. *mangiferaeindicae* growth with plating efficiencies of 110.5%, 52.3% and 50.9% respectively. Plating efficiencies of unrelated organisms and isolated phylloplane inhabitants on these media were low, being 1.3% and 7.8% for *Ps. viridiflava* and *Erwinia* sp. respectively (Table 5). High plating efficiencies in this medium were observed with *K. p.* spp. *pneumoniae* and MT5. Several other media, namely, YS, GYCA and LCM were found to promote growth of *X. c.* pv. *mangiferaeindicae* (Table 5), but selectivity was low. Most media evaluated inhibited the growth of all phylloplane isolates, with the exception of YS, GYCA and LCM (Table 5). Plating efficiency of *X. c.* pv. *mangiferaeindicae* was found to be 0% on SVGA, CS, XMSM, SM and BS.

Table 5 Plating efficiency of test bacteria on selective media relative to Standard 1 nutrient agar

Selective media[b]

BACTERIA[a]	YS	SVGA	BVGA	GYCA	CS	ET	MXP	LCM	XMSM	SM	XTS	BS	SX
X.c.pv.m	121.3	0	110.5	97.5	0	33.9	52.3	139.5	0	0	50.9	0	25.7
X.c.pv.c	84.7	131.1	146.4	93.5	0	0	98.4	103.9	93.8	40.6	112.3	0	106
X.c.pv.v	129.2	80.5	95.7	48.7	0	0	47.3	111.3	97.3	0	78.4	0	72.3
X.c.pv.p	39	44.3	66	100	0	0	97.6	47.6	92.2	0	87	42.3	52
X.a	100	84.3	94	118.6	0	0	14.2	94.5	0	93.8	95.6	0	94
X.f	18.3	0	0	21.2	0	0	8.4	17.2	0	0	53.6	0	0
W -lb	116.7	333	0	16.6	0	0	22.8	116.7	0	0	0	66.6	50
P.v	28.7	0	1.3	95.6	0	0	0	124.1	0	0	51.7	86.2	8.6
B.l	12.5	0	0	18.7	0	0	0	107.1	0	0	0	0	0
B.m	115.4	0	0	53.8	0	0	9.2	92.3	8.7	0	11.6	0	0
M.k	21.7	0	0	13.3	57.8	132.7	39.1	21.7	42.1	84.4	0	82.9	0
A.l	100	0	0	71.43	0	5.4	54	171.4	0	75.5	58.3	23.5	42.8
A.r	190.3	0	0	105.6	66.3	79.7	21	126.4	1.03	38	0	70.4	0
K.p.spp.p	113.4	56.7	94	26.8	19.3	30	3	88	8.66	23	0	47.3	91.4
E.s	92.8	0.68	7.8	80.4	0	0	0	65.7	0	0	0	0	48.6
MT10	91.9	0	0	72.2	0	0	0	109.6	0	0	0	0	0
MT9	36.8	0	0	145	0	0	0	63.1	0	0	0	0	0
MT6	106.7	0	0	10.5	0	0	0	73.3	0	0	0	0	0
MT7	3.6	0	0	93.5	0	0	0	70.9	0	0	0	0	0
MT5	12.6	0	54.3	7.14	0	0	0	35.4	0	0	0	0	93.6
MB4	119.2	0	0	89.4	0	0	0	126.9	0	0	0	0	0
MB1	56.3	0	0	134.4	0	0	0	111	0	0	0	0	0
MT2	47.7	10.5	0	78	0	5.26	0	147.3	0	0	0	0	0

[a] Bacterial codes utilised as follows: X.c.pv.m = *Xanthomonas campestris* pv. *mangiferaeindicae*; X.c.pv.c = *X. c.* pv. *campestris*; X.c.pv.v = *X. c.* pv. *viticola*; X.c.pv.p = *X. c.* pv. *phaseoli*; X.a = *X. axonopodis*; X.f = *X. fragariae*; W -1b = *Pseudomonas syringae*; P.v = *Ps. viridiflava*; B.l = *Bacillus licheniformis*; B.m = *B. megaterium*; M.k = *Micrococcus kristinae*; A.l = *Acinetobacter lwoffii*; A.r = *Agrobacterium radiobacter*; K.p.spp.p = *Klebsiella pneumoniae* spp. *pneumoniae*; E.s = *Erwinia* sp.; MT10, MT9, MT6, MT7, MT5, MB4, MB1, MT2 = unidentified phylloplane inhabitants.

[b] Selective media designated as follows: YS = Yeast salts; SVGA = Standard 1 violet green agar; BVGA = Boost violet green agar; GYCA = Glucose yeast chalk agar; CS = Cellobiose starch; ET = Esculin trehalose; MXP, XMSM, XTS, SX = no designation; LCM = Lederberg's complete medium; SM = Starch methionine; BS = Brilliant cresyl blue starch.

Plating efficiencies expressed as percentages relative to growth on Std I medium as defined by Kado & Heskett (1970).

Efficiency of recovery from liquid media. Based upon results obtained, BVGA, MXP, XTS and SVGA were selected for further evaluation. SVGA was included due to results obtained in previous experiments, where high counts were obtained in liquid media (unpublished data). After 24 hours, differences in counts between the test media were small, with greater differences observed after 48 hours (Fig. 1). After 72 hours, differences between test media were marked. Growth was found to be most prolific in XTS with a total count of 1,4 x 10^9 cells/ml followed by BVGA with 5,3 x 10^8 cells/ml (Fig. 1). Compared to XTS, growth in BVGA was slow at first, with counts of 0 cells/ml compared to 3,3 x 10^8 cells/ml after 24 hours. The counts obtained using these two media were found to be significantly higher than the counts obtained using MXP and SVGA with a count of 4,6 x 10^6 cells/ml in MXP after 72 hours (Fig. 1).

Isolation of mango phylloplane inhabitants

It was found that total counts obtained throughout, were in the order of 10^3 cells/gram leaf material (Table 1). Significant differences were observed only with 0,025M EDTA and 0,05% SDS. Counts obtained using all other buffers and isolation methods did not differ significantly from each other. Buffers such as 0,05M EDTA and Ringer's solution with 0,5% Tween 20 yielded counts as low as 5 x 10^2 and 7,3 x 10^2 cells/gram leaf material respectively. Total species diversity was generally low, the lowest being 3 and the highest, 10 in Ringer's solution with 0,5% Tween and 0,1% L - glutamic acid repectively. Buffers such as 0,05% SDS and 0,025M EDTA were found to produce the highest counts of 7,95 x 10^6 cells and 8 x 10^5 cells respectively per gram leaf material (Table 1). Although the counts obtained using 0,025M EDTA were lower than those of 0,05% SDS, the species diversity was higher, albeit only in terms of bacteria, the yields in terms of fungi was equivalent (Table 6). Other buffers such as 0,1% L - glutamic acid yielded low cell counts, but the species diversity was higher than any other buffer evaluated.
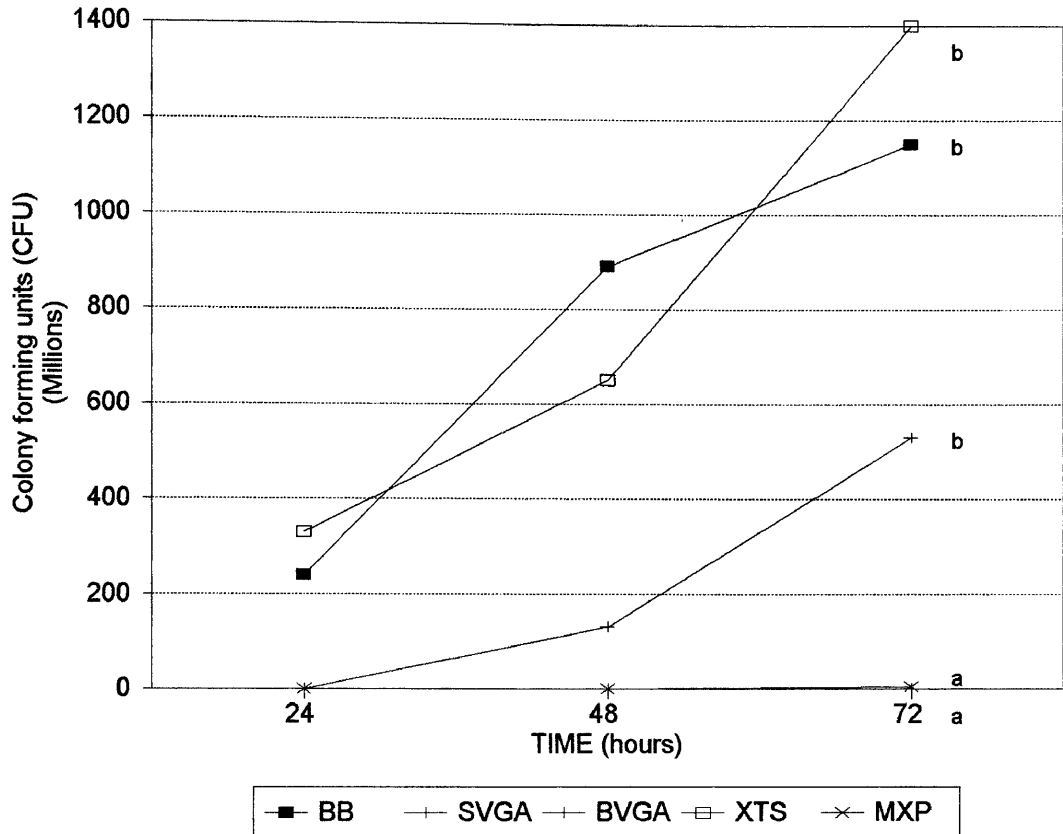
Fig. 1 A comparison of *Xanthomonas campestris* pv. *mangiferaeindicae* growth in selective media. Counts represent a mean value of five replicates plated onto Standard 1 nutrient agar, incubated at 28°C and evaluated after 72 hours. Media are coded as follows: SVGA = Std 1 violet green medium (Sanders et al., 1989); BVGA = Boost violet green medium (Sanders et al., 1989); BB = Boost medium (Manicom, 1983); XTS = XTS broth (Schaad & Forster, 1985); MXP = MXP medium (Claflin et al., 1987). Line graphs followed by the same letter do not differ significantly according to Fischer's Least significant difference test at the 95% confidence level.

Table 6 Evaluation of various buffers for the isolation of organisms from the mango phylloplane

Buffer utilised	Log Total count (cfu/ml)	Total species diversity
0.001% Tween 80	3.65 a	6
0.1% Yeast extract	3.56 a	5
0.1% Tween 80	3.90 a	9
0,1% Glucose	3.47 a	6
0.1% L - Glutamic acid	3.95 a	10
0.1% Ammonium sulphate	3.45 a	5
Phosphate maceration	3.98 a	9
0.05% SDS	6.90 c	4
0.01%SDS	3.06 a	3
0.025M EDTA	5.90 b	9
0.05M EDTA	2.69 a	6
0.01% Triton X100	4.21 a	6
0.01% Glycerol	3.33 a	9
Ringer's solution	3.13 a	9
Ringer's + 0.5% Tween 20	2.86 a	3
Ringer's + 1% Tween 20	3.23 a	2
Peptone phosphate buffer	2.25 a	9
Water	3.02 a	11

Means in columns followed by the same letter do not differ significantly according to Fischer's protected least significant difference at the 95% confidence level.

Evaluation of medium selectivity

The highest drop in total cell counts on solid media with respect to Std I were obtained on SVGA and BVGA with 100% decrease in total counts for each medium. XTS and MXP exhibited a drop of only 7% in total counts relative to Std I. Counts obtained in liquid media were high, especially after incubation for 72 hours. Counts were the lowest in XTS medium, with a total count of $1,06 \times 10^{10}$ cells/ml followed by SVGA and BVGA with counts of $3,6 \times 10^{10}$ cells/ml and $2,7 \times 10^{10}$ cells/ml respectively (Fig. 2). Counts were the highest in MXP, with a total count of $1,79 \times 10^{11}$ cells/ml (Fig 2). Total species diversity was low, with an average of two for MXP, XTS, SVGA and BVGA, and three for Std I. Similar results were obtained in the leaf impression studies which yielded average counts of 76, 1, 90, and 52 colonies per adaxial impression for MXP, BVGA, XTS and Std I respectively. Leaf impressions clearly showed differences in selectivity (Fig. 3).
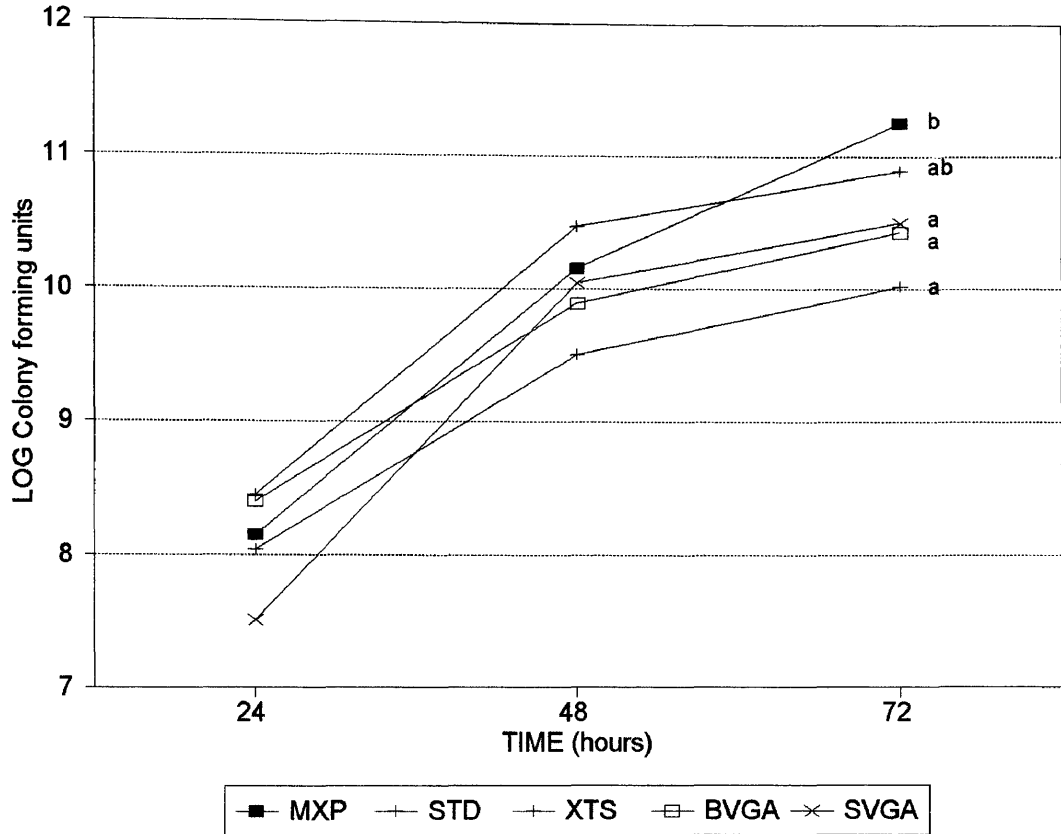
Fig. 2 A comparison of growth of mango phylloplane inhabitants in selective media. Counts are a mean of five replicates plated onto Standard 1 nutrient agar, incubated at 28°C and evaluated after 72 hours. Media are coded as follows: SVGA = Std 1 violet green medium (Sanders et al., 1989); BVGA = Boost violet green medium (Sanders et al., 1989); BB = Boost medium (Manicom, 1983); XTS = XTS broth (Schaad & Forster, 1985); MXP = MXP broth (Claflin et al., 1987). Line graphs followed by the same letter do not differ significantly according to Fischer's least significant difference test at the 95% confidence level.

MXP

STD 1

BVGA

XTS

Fig. 3   Comparison of selectivity of various media evaluated by means of the leaf
impression technique. Media coded as follows: XTS = XTS agar (Schaad &
Forster, 1989); BVGA = Boost violet green agar (Sanders et al., 1989); MXP =
MXP agar (Claflin et al., 1987); STD I = Standard I nutrient agar.

Determination of competing ability of Xanthomonas campestris pv. mangiferaeindicae with mango phylloplane inhabitants in BVGA by means of ELISA

Confluent growth was observed at all dilutions tested. The ELISA signal indicated a low count of *X. c.* pv. *mangiferaeindicae* at 10^6 cells/ml initial concentration with a gradual increase until 10^3 cells/ml, where a drop in signal was observed (Fig. 4).
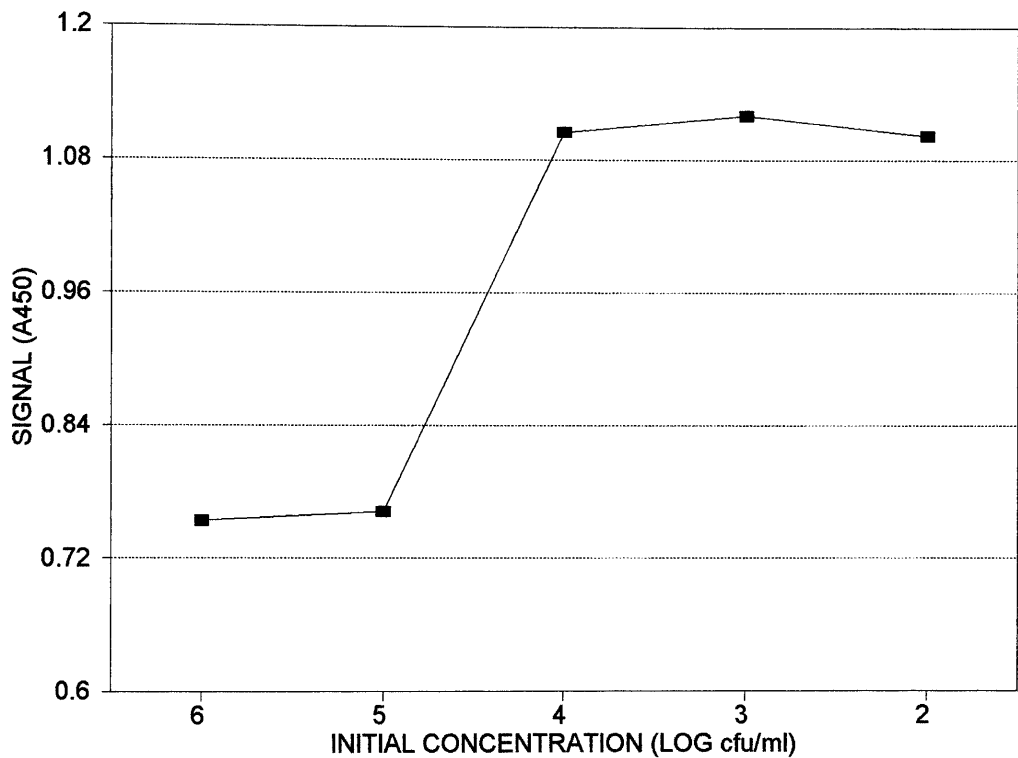

Fig 4: Competing ability of different *Xanthomonas campestris* pv. *mangiferaeindicae* concentrations with mango phylloplane inhabitants in BVGA medium (Sanders et al., 1989) determined by means of ELISA.

4.4 DISCUSSION

From the results obtained, it is clear that BVGA and XTS are the best semi - selective media for the enrichment of *X. c.* pv. *mangiferaeindicae* from the mango phylloplane. In all assays carried out which included solid and liquid media evaluation, plating assays and leaf impressions, it was found that both BVGA and XTS facilitated

growth of the target organisms, while inhibiting the growth of non - target organisms. High plating efficiencies were observed in both media, but only for *X. campestris* pathovars, thus conforming to the requirements for an ideal selective medium (Claflin *et al.*, 1987). However, the plating efficiency of BVGA was considerably higher than that of XTS, and may be ascribed to the fact that BVGA is a richer medium and contains less antibiotics than XTS.

In most media evaluated, with the exception of LCM, GYCA, and YS, growth of phylloplane and unrelated organisms was inhibited, exhibiting either low plating efficiencies or no growth at all. The low percentage growth of phylloplane organisms and most unrelated organisms may be ascribed to the fact that most media used in this evaluation were selective for pathovars of *Xanthomonas campestris* (Chun & Alvarez, 1983; Schaad & Forster, 1985; Claflin et al., 1987; Norman & Alvarez, 1989). High yields were also obtained using YS, GYCA and LCM. These media do not conform to the specifications for a good selective medium as defined by (Claflin et al., 1987), since they contain easily metabolisable compounds, and are thus able to sustain growth of a large variety of organisms.

Most selective media contain additives in order to inhibit certain groups of organisms, ie. antibiotics such as cycloheximide and cephalexin which were present in most media evaluated. The importance of additives for enhancement of selective media should not be underestimated. When the target organisms are slow growing, as is the case for *X. c.* pv. *mangiferaeindicae*, with doubling times often exceeding 100 minutes (Starr, 1981), it is of paramount importance to inhibit fast growing contaminants such as fungi and many Gram positive bacteria.

After inoculation, initial growth rates of *X. c.* pv. *mangiferaeindicae* were found to be low in BVGA as compared to XTS. With time, the growth rate increased, although the final counts obtained in BVGA were not as high as those in XTS. Slow initial growth may be ascribed to the fact that the organisms must adapt alternative metabolic pathways in order to utilise the medium successfully (Stanier et al., 1976).

Although initial growth in BVGA was slow compared to XTS, it was the most suitable for the selective isolation of *X. c.* pv. *mangiferaeindicae* from the mango phylloplane. Statistically, there was no difference between BVGA and XTS, but BVGA was selected as the best medium for selective isolation of *X. c.* pv. *mangiferaeindicae* because of the ease of preparation and low cost of this medium. In the initial stages of evaluation,

it was found that MXP and XTS also seemed promising. During later experiments however, it was found that MXP did not support the growth of *X. c.* pv. *mangiferaeindicae* as well as the other media evaluated. Total counts in MXP yielded 10^6 cells/ml whereas BVGA and XTS yielded 10^8 and 10^9 cells/ml respectively. Similarly, yields of phylloplane organisms in MXP was highest with total counts of 10^{11} cells/ml followed by XTS and BVGA, both yielding counts of 10^{10} cells/ml after 72 hours. All these assays confirmed that MXP was not suitable as a selective medium due to poor growth of *X. c.* pv. *mangiferaeindicae* and high counts of phylloplane organisms obtained. On the other hand, SVGA which seemed promising in earlier work (unpublished data) yielded the lowest counts of phylloplane inhabitants but no growth of *X. c.* pv. *mangiferaeindicae* after 72 hours and plating efficiency of SVGA was 0%.

During preliminary experiments of medium evaluation, it was found that the phylloplane inhabitants were highly successful in attaching to the leaf surface, since standard isolation techniques yielded very low counts and low species diversity, often limited to a single *Bacillus* sp. For epiphytic bacteria, it is a selective advantage to resist removal, and attachment of bacterial cells to foliage is an important step in certain plant diseases and plays an important role in epiphytic colonisation (Romantschuk, 1992).

One minute vortex shaking, followed by one minute in an ultrasonic bath in 0,025M EDTA was selected as the optimum isolation method of mango phylloplane inhabitants. Although the use of ultrasound has been reported to be successful for the removal of plant epiphytes without the use of surfactants (Haefele & Webb, 1982), poor results were obtained in this case. Dickinson et al. (1975) found that diluents such as quarter strength Ringer's solution, or distilled water supplemented with either peptone, yeast extract or L - glutamic acid were superior to distilled or tap water alone. No significant differences were found between these diluents in this study.

The use of surfactants such as SDS or compounds such as EDTA yielded significantly higher counts. Various bacterial surface molecules or structures have been implicated to function as adhesins, including proteins, polysaccharides and soluble molecules such as ß - 1,2 - glucan (Romantschuk, 1992).

Washing buffers containing compounds such as SDS and EDTA which are able to degrade these adhesins would result in the highest yields. Washing in 0,025M EDTA yielded the highest counts coupled with the highest species diversity. When determining the best isolation buffer and technique, a critical parameter, namely that of species

diversity must be considered in conjunction with total count. This factor is especially important when the isolation technique is used to determine medium selectivity. In order to obtain a true picture of the selectivity of the medium, it should be challenged with as wide a variety of organisms possible.

An important factor for differentiation of *X. c.* pv. *mangiferaeindicae* from mango phylloplane inhabitants is the accuracy with which they can be detected. Although selective media are considered to be accurate (Stead, 1992), increased accuracy can be afforded by combining selective media with a serological assay using monoclonal antibodies raised against *X. c.* pv. *mangiferaeindicae.* For these purposes, the ELISA is the most widely used and successful technique (Clark, 1981). This was found to be highly successful, since *X. c.* pv. *mangiferaeindicae* was capable of successfully competing for nutrients and growth factors. The absorbance values obtained are a reflection of the numbers of *X. c.* pv. *mangiferaeindicae*, and thus the competing ability. The initial low ELISA signal may be ascribed to the fact that above 10^7 cells/ml the ELISA signal is lower than at other cell concentrations. This was found using *Acinetobacter lwoffi* as solid phase antigen (Smith, 1988) and is due to cell clumping, and can be true for any whole cell antigen. A similar approach using a combination of monoclonal antibodies and selective media was successfully implemented by Norman & Alvarez (1989) for the detection of *X. c.* pv. *dieffenbachiae.* In comparison with other detection methods such as immunofluorescence colony staining and immunoisolation (Stead, 1992), this method is relatively quick.

The detection method of *X. c.* pv. *mangiferaeindicae* can play an important role in the control of BBS in South Africa, especially in the light of the mode of dispersal of this disease. Since the material is often asymptomatic, a reliable, sensitive method is required for detection. One of the most sensitive methods for detecting a pathogen in diseased or apparently healthy tissues is isolation onto a good selective medium (Stead, 1992). The use of the semi - selective medium BVGA, coupled with use of specific monoclonal antibodies can be used to detect the epiphytic phase on mother material and subsequently halt the spread of the disease. It can also be used to study the epidemiology of the disease and help to pinpoint optimal times for application of bactericides since when the numbers of the pathogen are at their lowest, it is the best time to apply control measures (Leben, 1981). If these factors are seriously considered and applied, the threat of this disease to the mango industry could be greatly reduced.

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CHAPTER FIVE

FIELD EVALUATION OF MONOCLONAL ANTIBODIES FOR THE DETECTION OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* ON MANGO PLANTS

ABSTRACT

Monoclonal antibodies raised against *Xanthomonas campestris* pv. *mangiferaeindicae* were tested in the field by Enzyme - linked immunosorbent assay (ELISA) in conjunction with selective media for detection of latent infections or epiphytic populations of *X. c.* pv. *mangiferaeindicae*. All positive controls produced a strong positive ELISA reaction. Positive tests were obtained from all areas tested and twigs were found to be better for detection than leaves, since positive reactions were mostly obtained with twigs. Only 4.5% of the 112 samples tested, tested positive on both twigs and leaves. This technique appears to be reliable, although further testing is required to confirm this.

5.1 INTRODUCTION

Bacterial black spot disease of mango (*Mangifera indica* L.) (BBS) caused by *Xanthomonas campestris* pv. *mangiferaeindicae* is a major threat to the mango industry in South Africa. Losses are mainly due to lesioned fruit, although premature fruit drop also increases losses (Viljoen & Kotzé, 1972). Besides the losses in revenue due to less acceptable fruit, this disease also limits distribution of more popular cultivars and limits the areas where mangoes can be cultivated successfully (Tomer & Mullins, 1987).

X. c. pv. *mangiferaeindicae* survives as epiphytic populations on the mango phylloplane and is found in lesions, cankers, buds as well as leaf litter (Manicom, 1983). Disease transmission is mainly via propagation of infected grafting material. Young trees may be asymptomatic in the nursery, but manifest disease when planted in the orchard (Viljoen & Kotzé, 1972). Strategies for control include sanitation practices and chemical control by means of regular sprays of copper oxychloride (Vermeulen et al., 1992). The most successful control measure would be indexing and certification of propagative material and subsequent establishment of disease free orchards.

A successful indexing programme requires a reproducible and accurate method for testing of material. In order to achieve this, a method is required that can detect the pathogen on young plants, as well as on mother material. Rapid and accurate techniques were previously not available for detection of latent infections or epiphytic populations of phytopathogenic bacteria. Standard methods requiring isolation and identification by means of physiological and biochemical analysis are time consuming and expensive, and not compatible with commercial practice. The use of serological assays are more rapid and reliable (Halk & de Boer, 1985). The use of mono - and polyclonal antibodies for screening of phytopathogens is widespread (Anderson & Nameth, 1990; Hsu et al., 1990; Kitagawa et al., 1992). Techniques such as the enzyme - linked immunosorbent assay (ELISA) (Alvarez & Lou, 1985; Sherald & Lei, 1991), immunofluorescence (IF) (Duvellier & Bragard, 1992) and dot - immunoassay (DIA) (Comstock & Irey, 1992) are widely used for this purpose. The ELISA has been used sucessfully for the detection of pathogens in seed (Claflin & Ramundo, 1987; Benedict et.al., 1989), plant tissue (Alvarez & Lou, 1985; Leach et al., 1987; Sherald & Lei, 1991) and potato tubers (Cother & Vruggink, 1980). The most significant characteristic of the ELISA is the ability to detect plant pathogens in much lower concentrations than is possible using classical immunoprecipitation methods (Clark, 1981). Further advantages of this technique include speed, versatility, quantitative precision and reproducibility as well as potential for automation (Clark et al., 1986). This detection method may be further enhanced by selective enrichment of the sample prior to testing by means of ELISA, and has been succcessfully implemented by Norman & Alvarez (1989) for the detection of *X. c.* pv. *dieffenbachiae* on aroids.

In previous chapters, the production of monoclonal antibodies against, and the development of a semi - selective medium for *X. c.* pv. *mangiferaeindicae* was described. The purpose of this study was to evaluate the efficiacy of the combined use of the enrichment media and monoclonal antibodies for field detection of *X. c.* pv. *mangiferaeindicae* in mango plants.

5.2 MATERIALS AND METHODS

Sampling

Mango leaf and twig samples were obtained from three different localities in the Transvaal Lowveld (Table 1). In addition, greenhouse material obtained from Nelspruit, served as a positive control, having tested positively for the presence of *X. c.* pv. *mangiferaeindicae* by ELISA prior to this evaluation. Asymptomatic twigs and leaves from trees in all areas were randomly collected from the entire tree, cut and rolled into newsprint and sealed in plastic bags.

All samples were placed into cool containers and transported to the laboratory where sample preparation was carried out immediately.

Sample preparation and indexing by ELISA

Twig sections and leaf discs were used for evaluation. Small twig sections, ca. $3mm^2$ were cut fom the entire sample, one gram of which was placed into three replicate 9ml volumes of BVGA (Sanders et al., 1989). Leaf discs were punched from a variety of leaves using a number 10 cork borer, and one gram of discs was placed into three 9ml volumes of BVGA. Tubes were incubated at 28°C for 72 hours. All samples were thoroughly shaken using a vortex and 100μl from each replicate and tissue type was layered onto each of three wells in a 96 - well microtiter plate (Cooke Microtiter system M29a, Sterilin Products, Middlesex, England). Uninoculated medium was used as a negative control. The ELISA was carried out as described in Chapter Two, using monoclonal antibody 2D/12H/4C as primary antibody. Samples were considered positive if the signal obtained was twice that of the background with a corresponding coefficient of variation of less than 15%.

5.3 RESULTS

A total of 112 samples were tested, of which 62 tested positive. Positive reactions were obtained from all the cultivars tested. The signal to background ratio of positive samples for all areas was low, but sufficient to be considered positive, with the exception of the positive control, which gave a high value (15.73). The highest percentage positive material was also obtained from the positive greenhouse material with 100% of the samples once again testing positive, followed by material from Tzaneen (74%) (Table 1).

The number of positive results obtained from twigs was considerably higher than the number of positive results obtained from leaves. The most marked differences in detection on twigs and leaves was observed in material from Hoedspruit where 95% of all the positive tests were on twigs and only 15% on leaves (Table 2). Similar distribution of positive reactions in leaves and twigs were observed in samples from Tzaneen. Of all the samples tested, for all areas, only five samples tested positive for leaves and twigs.

Table 1   Positive reactions from different localities and cultivars for the evaluation of monoclonal antibodies as detection method for *Xanthomonas campestris* pv. *mangiferaeindicae*

Locality	Cultivar	Total samples	Total percentage positive	Average positive signal
Hoedspruit	Keitt	15	39	3.43
	Heidi	10		
	Kent	15		
	Sensation	10		
Tzaneen	Kent	39	74	3.36
	Keitt	11		
Lisbon Estates	Keitt	8	12.5	2.53
Greenhouse	Tommy Atkins	1	100	15.73
	Sensation	1		
	Neldika	1		
	Heidi	1		

Table 2   A comparison of the incidence of positive tests for *Xanthomonas campestris* pv. *mangiferaeindicae* on mango twig and leaf samples using a combination of semi - selective media and specific monoclonal antibodies

Locality	No. samples tested	Total positive	Percentage twigs positive	Percentage leaves positive
Hoedspruit	50	20	95	15
Tzaneen	50	37	59.4	51.3
Lisbon	8	1	100	0
Greenhouse	4	4	75	50

5.3 DISCUSSION

Monoclonal antibodies previously prepared (Chapter Two) effectively detected *X. c.* pv. *mangiferaeindicae* in field samples. Field material may thus be quickly and accurately tested for the presence of the pathogen. Monoclonal antibodies have been used in this way for the detection of several other plant (Alvarez & Lou, 1985; de Boer et al., 1988; Bishop et al., 1989), insect (Olsen et al., 1990) and human pathogens (Kerr et al., 1992). A total of 55.4% of all samples tested positive for *X. c.* pv. *mangiferaeindicae* even

though all material was asymptomatic. All samples were taken from symptomless trees or trees which had manifested symptoms in previous seasons but were asymptomatic at present. These findings highlight the absolute necessity of screening material prior to use for grafting purposes. It was found that positive reactions were obtained from all cultivars and localities tested. This factor is important since the detection system must successfully detect the pathogen on all mango material irrespective of area or cultivar.

Since many positive reactions were obtained, the specificity of the antisera may be questionable, and it's subsequent use as a tool for detection in doubt. In Chapter Two, the cross reactivity of these monoclonal antibodies were evaluated. Cross reactions were only observed with certain *Ps. syringae* pv. *syringae* isolates, other pathovars of *X. campestris* and an *Erwinia* sp. Most of these organisms are of no concern since thay are host specific (Alvarez & Lou, 1985). However, the *Erwinia* sp., although isolated from avocado, could also occur on the mango phylloplane.

BVGA does not support significant growth of the *Erwinia* sp., thus eliminating a great deal of possible false positive results. Growth of *Erwinia* sp. could be completely eliminated by the addition of ampicillin to the medium (Chapter Two). This approach has been successfully implemented for identification of *X. c.* pv. *dieffenbachiae* from aroids (Norman & Alvarez, 1989). This highlights the importance of the complementarity of these two techiques for indexing purposes.

It is important that all parameters of this indexing system be optimised and standardised, in order to be reproducible and reliable. Hybridoma technology has the potential for producing an unlimited amount of highly specific monoclonal antibodies, which are ideal reagents for diagnostic purposes (Halk & de Boer, 1985). These hybridomas constantly secrete antibodies which are constantly specific in terms of specificity and isotype (Pollock et al., 1984). The components of the selective media are also all known factors, and medium of exactly the same quality and nutritional content can be repeatedly produced.

The sampling method is the most difficult method to standardise and optimise due to the uncertainty as to the exact location of the organism on, or within the plant. It is also not known whether, following infection, translocation of the organism within the plant is systemic. From the results obtained, it would seem that the best material for indexing purposes are twigs, since the highest incidence of positive tests were obtained from twigs.

The most difficult section of the indexing programme to control is the sampling method. It is not possible to test every twig, and for this reason the pathogen may not be detected and results cannot be regarded as completely accurate. Trees testing positive, can be regarded as positive, since no false negative reactions were obtained during the preliminary evaluation of this system. Such trees should be removed from the mother block. Trees which test negative, should be re - tested at a later stage and monitored for symptom development, especially when conditions are favourable for disease development. In so doing, trees can be certified as pathogen free, preventing indiscriminate spread of the disease.

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5.1 INTRODUCTION

Bacterial black spot disease of mango (*Mangifera indica* L.) (BBS) caused by *Xanthomonas campestris* pv. *mangiferaeindicae* is a major threat to the mango industry in South Africa. Losses are mainly due to lesioned fruit, although premature fruit drop also increases losses (Viljoen & Kotzé, 1972). Besides the losses in revenue due to less acceptable fruit, this disease also limits distribution of more popular cultivars and limits the areas where mangoes can be cultivated successfully (Tomer & Mullins, 1987).

X. c. pv. *mangiferaeindicae* survives as epiphytic populations on the mango phylloplane and is found in lesions, cankers, buds as well as leaf litter (Manicom, 1983). Disease transmission is mainly via propagation of infected grafting material. Young trees may be asymptomatic in the nursery, but manifest disease when planted in the orchard (Viljoen & Kotzé, 1972). Strategies for control include sanitation practices and chemical control by means of regular sprays of copper oxychloride (Vermeulen et al., 1992). The most successful control measure would be indexing and certification of propagative material and subsequent establishment of disease free orchards.

A successful indexing programme requires a reproducible and accurate method for testing of material. In order to achieve this, a method is required that can detect the pathogen on young plants, as well as on mother material. Rapid and accurate techniques were previously not available for detection of latent infections or epiphytic populations of phytopathogenic bacteria. Standard methods requiring isolation and identification by means of physiological and biochemical analysis are time consuming and expensive, and not compatible with commercial practice. The use of serological assays are more rapid and reliable (Halk & de Boer, 1985). The use of mono - and polyclonal antibodies for screening of phytopathogens is widespread (Anderson & Nameth, 1990; Hsu et al., 1990; Kitagawa et al., 1992). Techniques such as the enzyme - linked immunosorbent assay (ELISA) (Alvarez & Lou, 1985; Sherald & Lei, 1991), immunofluorescence (IF) (Duvellier & Bragard, 1992) and dot - immunoassay (DIA) (Comstock & Irey, 1992) are widely used for this purpose. The ELISA has been used sucessfully for the detection of pathogens in seed (Claflin & Ramundo, 1987; Benedict et.al., 1989), plant tissue (Alvarez & Lou, 1985; Leach et al., 1987; Sherald & Lei, 1991) and potato tubers (Cother & Vruggink, 1980). The most significant characteristic of the ELISA is the ability to detect plant pathogens in much lower concentrations than is possible using classical immunoprecipitation methods (Clark, 1981). Further advantages of this technique include speed, versatility, quantitative precision and reproducibility as well as potential for automation (Clark et al., 1986). This detection method may be further enhanced by selective enrichment of the sample prior to testing by means of ELISA, and has been succcessfully implemented by Norman & Alvarez (1989) for the detection of *X. c.* pv. *dieffenbachiae* on aroids.

In previous chapters, the production of monoclonal antibodies against, and the development of a semi - selective medium for *X. c.* pv. *mangiferaeindicae* was described. The purpose of this study was to evaluate the efficiacy of the combined use of the enrichment media and monoclonal antibodies for field detection of *X. c.* pv. *mangiferaeindicae* in mango plants.

5.2 MATERIALS AND METHODS

Sampling

Mango leaf and twig samples were obtained from three different localities in the Transvaal Lowveld (Table 1). In addition, greenhouse material obtained from Nelspruit, served as a positive control, having tested positively for the presence of *X. c.* pv. *mangiferaeindicae* by ELISA prior to this evaluation. Asymptomatic twigs and leaves from trees in all areas were randomly collected from the entire tree, cut and rolled into newsprint and sealed in plastic bags.

All samples were placed into cool containers and transported to the laboratory where sample preparation was carried out immediately.

Sample preparation and indexing by ELISA

Twig sections and leaf discs were used for evaluation. Small twig sections, ca. $3mm^2$ were cut fom the entire sample, one gram of which was placed into three replicate 9ml volumes of BVGA (Sanders et al., 1989). Leaf discs were punched from a variety of leaves using a number 10 cork borer, and one gram of discs was placed into three 9ml volumes of BVGA. Tubes were incubated at 28°C for 72 hours. All samples were thoroughly shaken using a vortex and $100\mu l$ from each replicate and tissue type was layered onto each of three wells in a 96 - well microtiter plate (Cooke Microtiter system M29a, Sterilin Products, Middlesex, England). Uninoculated medium was used as a negative control. The ELISA was carried out as described in Chapter Two, using monoclonal antibody 2D/12H/4C as primary antibody. Samples were considered positive if the signal obtained was twice that of the background with a corresponding coefficient of variation of less than 15%.

5.3 RESULTS

A total of 112 samples were tested, of which 62 tested positive. Positive reactions were obtained from all the cultivars tested. The signal to background ratio of positive samples for all areas was low, but sufficient to be considered positive, with the exception of the positive control, which gave a high value (15.73). The highest percentage positive material was also obtained from the positive greenhouse material with 100% of the samples once again testing positive, followed by material from Tzaneen (74%) (Table 1).

The number of positive results obtained from twigs was considerably higher than the number of positive results obtained from leaves. The most marked differences in detection on twigs and leaves was observed in material from Hoedspruit where 95% of all the positive tests were on twigs and only 15% on leaves (Table 2). Similar distribution of positive reactions in leaves and twigs were observed in samples from Tzaneen. Of all the samples tested, for all areas, only five samples tested positive for leaves and twigs.

Table 1 Positive reactions from different localities and cultivars for the evaluation of monoclonal antibodies as detection method for *Xanthomonas campestris* pv. *mangiferaeindicae*

Locality	Cultivar	Total samples	Total percentage positive	Average positive signal
Hoedspruit	Keitt	15	39	3.43
	Heidi	10		
	Kent	15		
	Sensation	10		
Tzaneen	Kent	39	74	3.36
	Keitt	11		
Lisbon Estates	Keitt	8	12.5	2.53
Greenhouse	Tommy Atkins	1	100	15.73
	Sensation	1		
	Neldika	1		
	Heidi	1		

Table 2 A comparison of the incidence of positive tests for *Xanthomonas campestris* pv. *mangiferaeindicae* on mango twig and leaf samples using a combination of semi - selective media and specific monoclonal antibodies

Locality	No. samples tested	Total positive	Percentage twigs positive	Percentage leaves positive
Hoedspruit	50	20	95	15
Tzaneen	50	37	59.4	51.3
Lisbon	8	1	100	0
Greenhouse	4	4	75	50

5.3 DISCUSSION

Monoclonal antibodies previously prepared (Chapter Two) effectively detected *X. c.* pv. *mangiferaeindicae* in field samples. Field material may thus be quickly and accurately tested for the presence of the pathogen. Monoclonal antibodies have been used in this way for the detection of several other plant (Alvarez & Lou, 1985; de Boer et al., 1988; Bishop et al., 1989), insect (Olsen et al., 1990) and human pathogens (Kerr et al., 1992). A total of 55.4% of all samples tested positive for *X. c.* pv. *mangiferaeindicae* even

though all material was asymptomatic. All samples were taken from symptomless trees or trees which had manifested symptoms in previous seasons but were asymptomatic at present. These findings highlight the absolute necessity of screening material prior to use for grafting purposes. It was found that positive reactions were obtained from all cultivars and localities tested. This factor is important since the detection system must successfully detect the pathogen on all mango material irrespective of area or cultivar.

Since many positive reactions were obtained, the specificity of the antisera may be questionable, and it's subsequent use as a tool for detection in doubt. In Chapter Two, the cross reactivity of these monoclonal antibodies were evaluated. Cross reactions were only observed with certain *Ps. syringae* pv. *syringae* isolates, other pathovars of *X. campestris* and an *Erwinia* sp. Most of these organisms are of no concern since thay are host specific (Alvarez & Lou, 1985). However, the *Erwinia* sp., although isolated from avocado, could also occur on the mango phylloplane.

BVGA does not support significant growth of the *Erwinia* sp., thus eliminating a great deal of possible false positive results. Growth of *Erwinia* sp. could be completely eliminated by the addition of ampicillin to the medium (Chapter Two). This approach has been successfully implemented for identification of *X. c.* pv. *dieffenbachiae* from aroids (Norman & Alvarez, 1989). This highlights the importance of the complementarity of these two techiques for indexing purposes.

It is important that all parameters of this indexing system be optimised and standardised, in order to be reproducible and reliable. Hybridoma technology has the potential for producing an unlimited amount of highly specific monoclonal antibodies, which are ideal reagents for diagnostic purposes (Halk & de Boer, 1985). These hybridomas constantly secrete antibodies which are constantly specific in terms of specificity and isotype (Pollock et al., 1984). The components of the selective media are also all known factors, and medium of exactly the same quality and nutritional content can be repeatedly produced.

The sampling method is the most difficult method to standardise and optimise due to the uncertainty as to the exact location of the organism on, or within the plant. It is also not known whether, following infection, translocation of the organism within the plant is systemic. From the results obtained, it would seem that the best material for indexing purposes are twigs, since the highest incidence of positive tests were obtained from twigs.

The most difficult section of the indexing programme to control is the sampling method. It is not possible to test every twig, and for this reason the pathogen may not be detected and results cannot be regarded as completely accurate. Trees testing positive, can be regarded as positive, since no false negative reactions were obtained during the preliminary evaluation of this system. Such trees should be removed from the mother block. Trees which test negative, should be re - tested at a later stage and monitored for symptom development, especially when conditions are favourable for disease development. In so doing, trees can be certified as pathogen free, preventing indiscriminate spread of the disease.

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CHAPTER SIX

DISCUSSION

The mango industry is one of the most important subtropical industries in South Africa (Abstract of Agricultural Statistics, 1991). Bacterial blackspot disease (BBS) is an economically important disease in South Africa which results in unmarketable fruit and subsequent loss of revenue. Well implemented copper oxychloride spraying programmes has kept the disease under control (J. Colyn, personal communication). Since the main mechanism of disease distribution is via propagation of infected plant material (Viljoen & Kotzé, 1972), and no cultivars are entirely resistant to this disease (Tomer & Mullins, 1987), maintainance of healthy nursery material is important. Asymptomatic trees can easily become a source of inoculum due to the epiphytic phase and latency of *Xanthomonas campestris* pv. *mangiferaeindicae*.

In order to establish a means by which diseased material, albeit asymptomatic, can be identified, a quick, accurate and relatively inexpensive technique is required. Although selective media are considered to be accurate (Stead, 1992), and are widely used for diagnostic purposes (Bashan & Assouline, 1983; de la Cruz et al., 1992), increased accuracy can be afforded by combining the implementation of selective media with a serological assay using monoclonal antibodies. Use of semi - selective medium, BVGA, coupled with monoclonal antibodies raised against *X. c.* pv. *mangiferaeindicae*, was found to be a successful method for the detection of the epiphytic phase as well as latent infections of *X. c.* pv. *mangiferaeindicae* on mother material and could help prevent the spread of the disease.

The first logical step in the investigation was to isolate the pathogen from field infections, in order to obtain virulent isolates for monoclonal antibody production. Differences in virulence were consistently detected and were categorised according to percentage infection as well as symptom severity. Virulence classes included most virulent (1F2) and least virulent (1F4). Differences in virulence in plant pathogenic bacteria other than *X. c.* pv. *mangiferaeindicae* have been well documented (Chatterjee & Vidaver, 1986; Leigh & Coplin, 1992; Yessad et al., 1992), and a theory was postulated that *X. c.* pv. *mangiferaeindicae* also existed as strains differing in virulence.

These different isolates were subsequently used individually and pooled to raise monoclonal antibodies (mAbs) for detection of a wide spectrum of isolates. No clear distinction could be made between the different virulence groups by polyclonal antisera. All mAbs raised were IgG2a and IgG3 subclasses. All mAbs reacted positively with all four virulence isolates and appeared to have the same specificity (XCM - 1). Western blot analysis confirmed this, since band patterns of all isolates probed with each mAb appeared to be similar. MAbs with at least four specificities have been raised against phytopathogenic bacteria (Benedict et al., 1989; McLaughlin et al., 1989; Vernon - Shirley & Burns, 1992). The differences in cross reactivity patterns with related and non - related organisms challenge the theory that all three mAbs have the same specificity, since mAb 2A/10B/2 was the most cross - reactive and 2C/3G/1 and 2D/12H/4C predominantly cross reacted with *X. campestris* pathovars.

All mAbs reacted with proteinaceous epitopes and could be used as a probe to further elucidate the differences in virulence between isolates. Initially, differences in virulence were postulated to be due to differences in lipopolysaccharide (LPS) structure or composition. LPS has been positively correlated with virulence in plant pathogens such as *E. amylovora*, and several *X. campestris* pathovars (Chatterjee & Vidaver, 1986). In this case, it was found that differences in amount of LPS did not play a role in virulence, since the reactions with proteins of all virulence groups was equally strong. No differences were observed in LPS profiles (unpublished data). Since no differences were observed in the amount of LPS or LPS profiles, it may be concluded that LPS does not play a role in virulence of *X. c.* pv. *mangiferaeindicae*.

From the results obtained, it was deduced that certain protein(s) may play a role in determining virulence of *X. c.* pv. *mangiferaeindicae*, since Western blot analysis revealed qualitative differences between the isolates and a concurrent band for all the isolates at 38,9 kD. This molecular weight corresponds approximately to that of the porins and OmpA which migrate closely together (Brown & Hormaeche, 1989; Franken et al., 1992). The intensity of this band appeared to be correlated with the intensity of the bands where differences between the isolates were observed. Although no reports have been made regarding differences in virulence (Manicom & Wallis, 1984), these differences provide the first concrete evidence for the existence of different virulence strains for *X. c.* pv. *mangiferaeindicae* the identities of which were confirmed with Western blots probed with mAb XCM -1.

An enzyme - limked immunosorbent assay (ELISA) using mAb XCM - 1 allowed enhanced specificity of detection as compared to polyclonal antisera. Since all monoclonal antibodies reacted with all isolates, false negative reactions may be minimised. Cross - reactions of antisera with certain *Pseudomonas syringae* pv. *syringae* isolates and *X. campestris* pathovars will not affect the effectivity of the monoclonal antibodies as an indexing tool. *Pseudomonas* isolates were from wheat or millet and would not be encountered upon the mango leaf or fruit surface. Cross - reactions between other pathovars of *X. campestris* have been reported but are of minimal importance since pathovars are host specific (Alvarez & Lou, 1985). The cross reaction of all monoclonal antibodies with the *Erwinia* sp. was unexpected, since it is completely unrelated to *X. c.* pv. *mangiferaeindicae.* Similar results of cross reactive mAbs have been reported, but cross reactivity was limited to closely related organisms (Kerr et al., 1992) and related pathovars (Franken et al., 1992). These mAbs would not be suitable for pathovar or serovar differentiation as described by Benedict et al. (1989) and Vernon - Shirley and Burns (1992), since the epitope with which the mAbs react does not appear to be unique to a specific virulence strain.

BVGA and XTS were found to be the best semi - selective media for the enrichment of *X. c.* pv. *mangiferaeindicae* from the mango phylloplane. Solid and liquid media evaluation, plating assays and leaf impressions revealed that both media facilitated growth of the target organisms while inhibiting the growth of non - target organisms. High plating efficiencies were observed in both media for only *X. campestris* pathovars, thus conforming to the requirements for an ideal selective medium (Claflin & Ramundo, 1987).

During preliminary experiments of medium evaluation, it was found that phylloplane inhabitants were highly successful in attaching to the leaf surfaces, since standard isolation techniques yielded very low counts and low species diversity, often limited to a single *Bacillus* sp. It is a selective advantage for epiphytic bacteria to resist removal from foliage and attachment of bacterial cells is an important step in certain plant disease and epiphytic colonisation (Romantschuk, 1992). One minute vortex shaking, followed by one minute in an ultrasonic bath in 0,025M EDTA was selected as the optimum isolation method of mango phylloplane inhabitants. Although the use of ultrasound has been reported to be successful for the removal of plant epiphytes without the use of surfactants (Haefele & Webb, 1982), poor results were obtained in this study. No significant difference was found between the diluents described by Dickinson et al. (1975). The use of surfactants such as sodium dodecyl sulphate (SDS) or compounds such as ethylenediamine tetraacetic acid (EDTA) yielded significantly higher total counts

coupled with high species diversity.

Various bacterial surface molecules or structures function as adhesins, which include proteins, polysaccharides and soluble molecules such as ß - 1,2 - glucan (Romantschuk, 1992). Washing buffers containing compounds such as SDS and EDTA which are able to degrade these adhesins would result in the highest yields.

Field evaluation of the combined use of monoclonal antibodies and selective media was found to be an accurate, reproducible way of detecting *X. c.* pv. *mangiferaeindicae* in mango plants. A total of 55.4% of all samples were positive indicating the presence of *X. c.* pv. *mangiferaeindicae*. Positive trees included Keitt, Kent and to a lesser extent, Sensation. Although these trees were asymptomatic, the pathogen was present and could cause disease under favourable conditions.

The monoclonal antibody evaluated in these assays indicated a high number of positive samples. These findings give rise to speculation as to the specificity of the antisera, and it's subsequent usefulness as a tool for detection of *X. c.* pv. *mangiferaeindicae*. The only cross reactions which may limit the efficiacy of the monoclonal antibodies are those with the *Erwinia* sp. However, BVGA does not support significant growth of the *Erwinia* sp., thus eliminating most false positive results. Growth of the *Erwinia* sp. could be completely eliminated by the addition of ampicillin to the medium. This thus highlights the importance of the complementarity of these two techiques for indexing purposes.

The parameters of this indexing system must be standardised and optimised. It is also not known whether, following infection, translocation of the organism within the plant is systemic, comlicating the sampling procedure. It was found twigs were the best material for indexing purposes since the highest incidence of positive tests were obtained from this material.

The detection system described in this study has proved to be successful for the detection of *X. c.* pv. *mangiferaeindicae* in mango plants. This may thus be used to screen mother material prior to use. An indexing programme has been initiated for this purpose and was highly successful until a total loss of signal was encountered. This resulted in the production of a second set of monclonal antibodies for the commercial programme. The benefit of these antibodies is twofold, in that they are of such a nature that they may also be used to study disease etiology. The mango industry may benefit widely using these

monoclonal antibodies as well as the detection system as a whole.

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SUMMARY

DETECTION OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* IN MANGO PLANTS

The yearly losses incurred by bacterial blackspot disease are high. Often trees are asymptomatic, with the pathogen either in the resident phase or latent stage of infection. Detection of the pathogen in these asymptomatic trees is one of the most important means of controlling the disease. Isolates which consistently differed in virulence were isolated from symptomatic mango plants. These isolates could be categorised into four groups based upon differences in virulence. Monoclonal antibodies (mAbs) were successfully raised using separate and pooled isolates for immunisation. MAbsraised were of the IgG class and reacted with a proteinaceous epitope. These monoclonal antibodies could distinguish between different virulence groups of *Xanthomonas campestris* pv. *mangiferaeindicae* by means of Western Blot analysis. These antibodies were used along with a selective medium, BVGA for detection of epiphytic populations as well as latent infections in mango. An enrichment step prior to the enzyme - linked immunosorbent assay (ELISA) is important, since bacterial counts on trees with latent infections are too low to result in a positive signal. These techniques in combination are thus useful for detection and monitoring of the pathogen, which may play an important role in controlling the spread of the disease.

OPSOMMING

OPSPORING VAN *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* OP MANGO PLANTE

Bakteriese swartvlek is 'n ekonomies belangrike siekte vir die Suid Afrikaanse mango bedryf. Swartvlek besmette bome kan simptoomloos wees, maar met die patogeen in 'n latente of epifitiese fase. Opsporing van simptoomlose bome is een van die belangrikste beheermaatreels. Isolate wat verskille in virulensie getoon het was geisoleer vanaf besmette mangoplante. Die isolate was gekarakteriseer volgens hul virulensieverskille. Monoklonale teenliggame (mKt) was suksesvol berei met die afsonderlike en gesamentlike isolate as antigeen in die immuniseringsprogram. MKt berei resorteer in die IgG klas en reageer met 'n proteienagtige epitoop. Hierdie mKts kon onderskei tussen die verskillende virulensiegroepe van *Xanthomonas campestris* pv. *mangiferaeindicae* d.m.v. "Western Blot" analise. Die mKts was gebruik tesame met die selektiewe medium BVGA vir opsporing van die epifitiese populasies en latente infeksies in mango's. Die verrykingsstap voor die ELISA (enzyme - linked immunosorbent assay) is belangrik aangesien bakterietellings op bome met latente infeksies te laag is om 'n positiewe ELISA sein te kry. Die gekombineerde tegnieke is dus bruiksaam vir opsporing en monitering van die patogeen en kan sodoende 'n belangrike rol in siektebeheer speel.