COMPARISON OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* FROM MANGO PRODUCTION AREAS IN SOUTH AFRICA

by

NOMPHELO GANTSHO

Submitted in partial fulfillment of the requirements for the degree of M.Sc. (Plant Pathology)

In the Faculty of Natural and Agricultural Sciences
Department of Microbiology and Plant Pathology
University of Pretoria

September, 2001

DEDICATION

To the memory of

my grandmother

Nofezile Maud Gantsho

Born 01 January 1912

and

Died 13 April 1995

MAY HER SOUL REST IN PEACE

"I am the resurrection and the life. He who believes in Me, though he may die, he shall live."

John 11 : 25

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to the following:

Professor L. Korsten for her guidance and constructive criticism during the preparation of this thesis;

The National Research Foundation for my bursary and partially funding of the project;

Marius Boshoff and Gina Swart for their efficient help through-out this work;

The University of Pretoria for the opportunity to do the M.Sc.;

I am deeply grateful to my family, Nompinda, Mlindi, Thenjiwe, Thembinkosi and Sixolile for their unfailing support and encouragement;

To Zuki, for all the help with the typing of this thesis as well as your support through-out;

The people of the 9th floor and the people in ground floor for the help in analysing the results;

To my friends for always being there for me;

Finally, but most important, MY HEAVENLY FATHER.

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

The mango, *Mangifera indica* L., originated from the India/Burmese border region (Kwee and Chang, 1985). Today, it is grown throughout the subtropics and tropics and is one of the world's most important crops (Schroeder, 1991a). In terms of world production, South Africa is a relatively small producer and exporter (Van Zyl, 1998). The mango tree is prone to attack by a number of pathogens resulting in major economic losses in the industry (Korsten *et al.*, 1992). The most important diseases of mango in South Africa are powdery mildew, blossom blight, bacterial black spot (BBS), anthracnose and soft brown rot (Korsten *et al.*, 1992). Of these, BBS is the most important, particularly in newly established orchards. Bacterial black spot is a preharvest disease which causes reductions in yield and market value of fruit. This disease is also of significant importance in other mango producing countries such as the islands of the Indian Ocean, south east Asia and Australia (Pruvost and Luisetti, 1991). In these tropical and subtropical countries, BBS is a serious necrotic threat and can cause losses of up to 50% in untreated orchards (Manicom and Wallis, 1984; Pruvost *et al.*, 1995).

Bacterial black spot is caused by *Xanthomonas campestris* pv. *mangiferaeindicae* which is a Gram negative motile rod with a single flagellum which forms apigmented colonies on nutrient agar (Pruvost *et al.*, 1998). Leaf symptoms consist of small, dark brown, angular spots. Similar spots will develop on small fruit (Manicom, 1986). However, as fruit develop, lesions enlarge which crack open releasing a gummy exudate. Stem lesions often occur as elongated black cankers which crack and release gum (Pruvost and Manicom, 1993). The pathogen can occur as an epiphytic colonist of leaves (Manicom, 1986; Pruvost *et al.*, 1990), buds (Pruvost *et al.*, 1993), and fruit of mango (Pruvost and Luisetti, 1991). During its epiphytic stage, it can multiply and infect the plant during favourable rainy weather conditions (Pruvost *et al.*, 1990). Disease severity increases during windy rains which promote wounding of leaf surfaces (Boshoff *et al.*, 1999). The pathogen, *X. c.* pv. *mangiferaeindicae* is not only pathogenic on mango, but also on other plant species belonging to the family *Anacardiaceae* e.g. cashew and pepper tree (Pruvost and Manicom, 1993).

In the 1970s, classification of xanthomonads was according to the species or botanical family of the host from which they were isolated (Dye *et al.*, 1980). To differentiate between pathovars of *X. campestris,* a plant suspected of being the host for that specific pathogen was inoculated (Lazo *et al.,* 1987). This practice proved to be tedious, time-consuming, subjective, and subject to a surprising number of artifactual influences. New methods have subsequently been

developed to rapidly identify and classify closely related pathogenic bacteria based on genomic fingerprinting approaches. Gagnevin *et al.* (1997) compared genetic diversity of strains of *X. c.* pv. *mangiferaeindicae* by restriction fragment length polymorphism (RFLP) analysis and obtained groups consistent with those described by Some and Samson (1996). However, comparative studies of this nature have not been conducted in South Africa.

Until recently, the relatedness of bacterial isolates has been determined by testing for one or several phenotypic markers, using methods such as sero-, phage-, and biotyping as well as antibiotic susceptibility testing (Farber, 1996; Pooler *et al.*, 1996). However, these techniques are often too expensive, or too insensitive for use in routine diagnosis (Louws *et al.*, 1994). Manicom and Wallis (1984) gave a comprehensive report comparing apigmented strains isolated from mango mostly from South Africa. Some and Samson (1996), have evaluated the relationships among strains of *X. c.* pv. *mangiferaeindicae* by isoenzyme analysis of esterase, phosphoglucomutase and superoxide dismutase, and they have shown that the strains could be classified into eight groups. In South Africa, the method of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has shown considerable promise in classifying and identifying bacterial strains at a molecular level (Van Zyl, 1990). Van Zyl (1990), differentiated phytopathogenic pseudomonads and xanthomonads up to a sub-species level based on these protein profiles. Sanders (1993), differentiated between isolates using monoclonal antibodies according to their differences in virulence.

Detailed characterisation of the genetic variability among strains of *X. c.* pv. *mangiferaeindicae* has increased, along with the prevalence and increasing economic importance of the bacterium (Gagnevin *et al.*, 1997). Most characterization involves the use of the molecular techniques (Gagnevin*et al.*, 1997). Molecular typing methods which involve DNA analysis can offer many advantages over traditional techniques (Farber, 1996). One advantage is that since DNA can always be extracted from bacteria, all bacteria should be typeable. Another is that the discriminatory power of DNA-based methods is greater than that of phenotypic procedures (Farber, 1996).

Mango cultivars vary in susceptibility to BBS and farmers are advised that planting of susceptible cultivars e.g. Haden, Kent, Keitt, Irwin, Lippens, Brooks, Zill, Ruby and Eldon, be avoided (Schroeder, 1991b). Limited control of the disease with copper compounds in regions where the disease is endemic, has been achieved (Gagnevin *et al.*, 1997), and integrated disease management is therefore recommended (Pruvost and Manicom, 1993). Planting of wind-breaks around orchards (Visser, 1995), applying biocontrol agents (Korsten *et al.*, 1992) and timely copper sprays, can decrease disease incidence (Manicom and Wood, 1981). However, the

number of copper sprays is dependent on rainfall, susceptibility of the cultivar used, and quantity of inoculum available in the orchard (Manicom and Wood, 1981). One or two additional winter sprays, together with pruning of infected twigs, can reduce inoculum in the rainy season and therefore improve control significantly (Manicom and Wood, 1981). However, Boshoff *et al.* (1999), recently observed that current BBS control measures are inadequate and that extensive spraying with copper is becoming uneconomical. Furthermore resistance to copper based compounds has been reported for the bacterial plant pathogens *Pseudomonas syringae* pv. *tomato* (Bender and Cooksey, 1986), *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *syringae* (Stall *et al.,* 1986).

In view of this, a study was undertaken to compare different isolates of *X. c.* pv. *mangiferaeindicae* obtained from different mango cultivars viz. Keitt and Kent and from different production areas viz. Bavaria and Constantia Estates. The investigation entailed comparison of isolates based on:

- Biochemical and physiological characteristics
- Virulence and copper resistance
- Protein profiles
- Polymerase chain reaction

REFERENCES

Bender, C. L. and Cooksey, D. A. 1986. Indigenous plasmids in pathogens *Pseudomonas syringae* pv. *tomato*: Conjugative transfer and role in copper resistance. *Journal of Bacteriology* 165: 534-541.

Boshoff, M., Kotzé, J. M. and Korsten, L. 1999. Control of bacterial black spot of mango. *South African Mango Growers' Association Yearbook* 18: 35-38.

Dye, D. W., Bradbury, J. F., Goto, M. S., Hayward, A. C., Lelliot, R. A. and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Review of Plant Pathology* 59: 153-168.

Farber, J. M. 1996. An introduction to the hows and whys of molecular typing. *Journal of Food Protection* 59: 109-1101.

Gagnevin, L., Leach, J. E. and Pruvost, O. 1997. Genomic variability of *Xanthomonas* pathovar *mangiferaeindicae*, agent of mango bacterial black spot. *Applied and Environmental Microbiology* 63: 246-253.

Kwee, L. T. and Chang, K. K. 1985. Diseases and disorders of mango in Malaysia. Tropical Press, Kuala Lumpur.

Korsten, L., Lonsdale, J. H., de Villiers, E. E. and de Jager, E. S. 1992. Preharvest biological control of mango diseases. *South African Mango Growers' Association Yearbook* 12: 72-78.

Lazo, G. R., Roffey, R. and Gabriel, D. W. 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment-length polymorphism. *International Journal of Systematic Bacteriology* 37: 214-221.

Louws, F. J., Fulbright, D. W., Stephens, C. T. and de Bruin, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* 60: 2286-2295.

Manicom, B. Q. 1986. Factors affecting bacterial black spot of mangoes caused by *Xanthomonas campestris* pv. *mangiferaeindicae. Annals of Applied Biology* 109: 129-135.

Manicom, B. Q. and Wallis, F. M. 1984. Further characterization of *Xanthomonas campestris* pv. *mangiferaeindicae. International Journal of Systematic Bacteriology* 34: 77-79.

Manicom, B. Q. and Wood, R. 1981. Attempts to control bacterial black spot of mangoes. *Subtropica* 2: 12-14.

Pooler, M. R., Ritchie, D. F. and Hartung, J. S. 1996. Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindomic PCR, data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Applied and Environmental Microbiology* 62: 3421-3427.

Pruvost, O., Couteau, A. and Luisetti, J. 1990. Development of bacterial black spot of mangoes and epiphytic populations of the pathogen (*Xanthomonas campestris* pv. *mangiferaeindicae*) under natural conditions in Reunion Island. *Fruits* 45: 125 -140.

Pruvost, O. and Luisetti, J. 1991. Effect of time of inoculation with *Xanthomonas campestris* pv. *mangiferaeindicae* on mango fruits susceptibility. Epiphytic survival of *X. c.* pv. *mangiferaeindicae* on mango fruits in relation to disease development. *Journal of Phytopathology* 133: 139-151.

Pruvost, O. and Manicom, B. Q. 1993. *Xanthomonas campestris* pv. *mangiferaeindicae:* Cause of bacterial black spot of mangoes. Pages 91-119. In: Swings, J. G. and Civerolo, E. L. (eds*). Xanthomonas*. Chapman & Hall, London.

Pruvost, O., Couteau, A., Vernieré, C. and Luisetti, J. 1993. Epiphytic survival of *Xanthomonas campestris* pv. *mangiferaeindicae* on mango buds. *Acta Horticulturae* 341: 337-344.

Pruvost, O., Couteau, A., Luisetti, J. and Vernieré, C. 1995. Biology and epidemiology of the casual agent of mango black spot disease. *Fruits* 50: 183.

Pruvost, O., Couteau, A. and Luisetti, J. 1998. Phenotypic diversity of *Xanthomonas* sp. *mangiferaeindicae. Journal of Applied Microbiology* 84: 115-124.

Sanders, G. M. 1993. Detection of *Xanthomonas campestris* pv. *mangiferaeindicae* in mango plants. M.Sc. Thesis. University of Pretoria, Pretoria.

Schroeder, W. 1991a. Origin of the mango. Page A1. In: Mango. Institute for Tropical and Subtropical Crops. Agricultural Research Council, Pretoria.

Schroeder, W. 1991b. Botanical aspects of the mango. Page A2. In: Mango. Institute for Tropical and Subtropical Crops. Agricultural Research Council, Pretoria.

Some, A. and Samson, R. 1996. Isoenzyme diversity in *Xanthomonas campestris* pv. *mangiferaeindicae. Plant Pathology* 45: 426-431.

Stall, R. E., Loschke, D. C. and Jones, J. B. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* 76: 240-243.

Van Zyl, E. 1990. A numerical approach to the taxonomy of *Xanthomonas* and some phytopathogenic *Pseudomonas* spp. as determined by phenotypic properties and protein gel electrophoresis. Ph.D. Thesis. University of Pretoria, Pretoria.

Van Zyl, D. L. 1998. Introduction. Pages vi-vii. In: E. A. de Villiers (ed.). The cultivation of mangoes. Institute for Tropical and Subtropical Crops. Agricultural Research Council, Nelspruit.

Visser, A. A. 1995. Effect of bactericides and windbreaks on bacterial black spot of mangoes. M.Sc. Thesis, University of Pretoria, Pretoria.

CHAPTER TWO

LITERATURE REVIEW

THE HOST: MANGO

History, distribution and cultivars

Mango (*Mangifera indica* L.) belongs to the family *Anarcardiaceae*, also known as the cashew family (Nakasone and Paull, 1998). This family also includes other commercially important tree species, for example, the cashew (*Anacardium occidentale*, L.), the pistachio (*Pistachio vera,* L.) and the marula (*Sclerocarya birrea* (A. Rich.) Hochst subs. caffra, Sonder) (Fivaz, 1998).

The genus *Mangifera* consists of 69 species of Asian origin most of which produce edible fruits (Nakasone and Paull, 1998). Of these, *M. indica* is the only species that is grown commercially on a large scale in South Africa (Fivaz, 1998). The mango originated from the India/Burmese border region (Kwee and Chang, 1985). It was one of the first fruits to be commercially cultivated and has been grown in India for more than 4 000 years (Snyman, 1998a). Mangoes are now grown in nearly all suitable tropical and subtropical regions of the world (Schroeder, 1991a). It is not known exactly how the mango was distributed from India to the rest of the tropical and subtropical regions of the world (Snyman. 1998a). This fruit is intimately associated with the Hindu religion (Nakasone and Paull, 1998) and it is thought that Indian traders and Buddhist priests introduced the mango into Malaysia and east Asian countries (Nakasone and Paull, 1998). Mangoes were reported from east Somalia as early as 1331 and reached Southern Africa from east Africa before the 17th century (Schroeder, 1991a).

There are hundreds of named mango cultivars throughout the tropics and subtropics (Nakasone and Paull, 1998). Each country of origin has its own selected cultivars. Mango cultivars can be divided into three main groups, namely unimproved and improved tropical, and improved subtropical cultivars (Human and Snyman, 1998). The unimproved group of cultivars cultivated in South Africa includes Peach, Sabre, Kidney (Sugar) and Long green and the so-called Bombay types of India (Human and Snyman, 1998). Typical of this group is the high fibre content, undesirable external colour, susceptibility to disease, poor shelf life and turpentine flavour. The seed is mostly polyembryonic and seedling progeny is therefore usually genetically identical to the relevant parent (Human and Snyman, 1998). Improved tropical cultivars generally produce fibreless fruit with outstanding eating qualities. Most of these cultivars are monoembryonic and have to be propagated by grafting. The improved subtropical cultivars produce attractive fruit of good quality, but yield and resistance to disease is unsatisfactory (Human and Snyman, 1998).

The South African mango industry currently relies mainly on improved subtropical cultivars imported from Florida and on some local fibrous cultivars (Human and Snyman, 1998).

Biology

The mango is a perennial, branching, evergreen tree and can be 30-40m tall (Litz, 1994). The tree is deep-rooted and seedlings can develop into huge trees, especially in deep soils (Fivaz, 1998). Mango leaves are simple, entire, leathery, short-pointed and oblong to lanceolate (Schroeder, 1991b). Growth occurs in vegetative flushes of the terminal, with 10-12 new leaves per flush (Nakasone and Paull, 1998). In mature trees, the first flush during the dry season is usually transformed into a flower flush (Schroeder, 1991b).

The fruit of the mango is a drupe of variable size and shape, ranging in weight from a few grams to more than 1 kg (Nakasone and Paull, 1998). It is fleshy, flattened, rounded or elongated in shape. The quality of the fruit is based on the scarcity of fiber and lack of the turpentine taste (Mango Fruit Facts, www.crfg.org 1999). Fibers are more pronounced in fruits grown with hard water and chemical fertilisers (Mango Fruit Facts, www.crfg.org 1999). The seed may either have a single embryo, producing one seedling, or polyembryonic producing several seedlings that are identical but not always true to the parent type (Mango Fruit Facts, www.crfg.org 1999).

Marketing and international trade

The South African mango industry has grown rapidly since 1984/1985 where production has increased to 43 752 tonnes (Personal Communication, SAMGA). It is now ranked the fifth most important subtropical export fruit crop after citrus, banana, avocado and pineapple (Snyman, 1998b). The 2000 crop was 41 839 tonnes of which 12 239 tonnes were exported, 20 045 tonnes were sold on the local markets as fresh fruit, 6 480 tonnes were processed (Personal Communication, SAMGA).

Export revenue is important to the South African mango industry and competition from South American and central African countries for the European market is fierce (Swart, 1999). India produces 70% of the world's mangoes, although Mexico is the largest exporter of fresh fruit (Ploetz *et al.,* 1994).

Mango diseases

The mango tree is prone to attacks by a number of pathogens resulting in major losses in the industry (Korsten *et al.*, 1992). The important diseases of mangoes in South Africa are powdery mildew, blossom blight, bacterial black spot (BBS), anthracnose and soft brown rot (SBR)

(Manicom, 1992). Powdery mildew is caused by *Oidium mangifera* (Berthet) Burchill, blossom blight and SBR caused by *Nattrassia mangiferae* (Nattras) Sutton & Dyko (the current taxonomic status of this pathogen is being re-evaluated), anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. and BBS caused by *Xanthomonas campestris* pv. *mangiferaeindicae* (Manicom and Frean, 1992). Any one of these pathogens can cause total crop failure (Pruvost and Manicom, 1993).

Most of these diseases are prominent with ineffective disease control programs (Sanders, 1993). Bacterial black spot appears to be a relatively serious disease in South Africa, the islands of the Indian Ocean, south east Asia and Australia (Nakasone and Paull, 1998; Pruvost and Luisetti, 1991). Bacterial black spot is a preharvest disease, and is a serious necrotic threat to mango crops with recorded losses of up to 50 % in untreated orchards (Manicom and Wallis, 1984; Pruvost *et al.*, 1995).

Bacterial black spot of mango

The BBS causal agent, *X. c.* pv. *mangiferaeindicae* can infect all aerial parts of mango, with leaf and fruit lesions being the most common (Pruvost *et al.*, 1998). Symptoms can be seen on fruit, leaves and sometimes on stems.

Early stages of fruit and leaf lesions appear as small necrotic lesions (2–3 mm in diameter) with a surrounding water soaked area (Chand and Kishun, 1995; Sanders *et al.*, 1994). Mature symptoms on leaves are small angular, black and raised, releasing gum. Bacteria enter the plant through wounds, stomata, or lenticels (Gagnevin *et al.*, 1997). On fruit, lesions develop around lenticels or wounds and are irregular in shape (Manicom, 1992). Lesions eventually dry out, turn black and crack open (Manicom and Frean, 1992). Raised black spots with greasy margins can also later develop on leaves. Stem lesions often appear, as blackened cankers crack longitudinally, also releasing gum.

Disease incidence is high when rainfall with strong winds and high temperatures occur at the same time (Manicom, 1986). Disease incidence is also greater on exposed and abraded leaf and fruit surfaces or where fruits touch each other (Pruvost and Luisetti, 1991). The pathogen survives mostly in lesions and on aerial organs as epiphytes, and enter the plant through plant wounds, stomata and lenticels (Pruvost *et al.*, 1993). The pathogen can also be transmitted through irrigation water, insects, and mechanical transfer of infected planting material (Pruvost and Manicom, 1993).

Differences in cultivar susceptibility to BBS has been reported, but no cultivars were found to be entirely resistant (Sanders, 1993). Of South African cultivars commercially used, the cultivar Sensation is less susceptible than Zill and Kent, while Haden, Tommy Atkins and Keitt is more susceptible (Manicom, 1992).

THE PATHOGEN: *XANTHOMONAS CAMPESTRIS* PATHOVAR *MANGIFERAEINDICAE*

Taxonomy

The current classification of *X. c.* pv. *mangiferaeindicae* is as follows (Agrios, 1997; Dye *et al.*, 1980):

Kingdom:	Bacteria
Division:	Gracilicutes
Class:	Proteobacteria
Family:	Pseudomonadaceae
Genus:	*Xanthomonas*
Species:	*campestris*
Pathovar:	*mangiferaeindicae*

The causal organism of BBS was first reported in South Africa in 1915 by Doidge who described the pathogen as *Bacillus mangiferae* n. sp. (Pruvost and Manicom, 1993). The first description of the causal agent, then designated *Pseudomonas mangiferae-indicae*, was recorded in India by Patel *et al.* (1948). The causal organism was later re-classified as *Xanthomonas campestris* pv. *mangiferaeindicae* (Dye *et al.*, 1980).

Patel *et al.* (1948) reported that the host range for *X. c.* pv. *mangiferaeindicae* included plant species of the family *Anacardiaceae* i.e. cashew (*Anacardium occidentale* L.) and imra, or emrah (*Spondias mangifera* Willd = *S. pinnata* (l. f.) Kurz). Later other host species were reported i.e. mombin (*Spondias mombin* L.) (Robbs *et al.*, 1982) and the pepper tree (syn. Brazilian pepper), *Schinus terebenthifolius* Raddi (Pruvost *et al.*, 1992). In the French West Indies, Rott and Frossard (1986) isolated a xanthomonad which caused a severe dieback of ambarella (*Spondias cytherea* Sonnerat). They noticed phenotypic similarities between the organism and *X. campestris mangiferaeindicae*. However no lesions were present in mango groves implying that the organism was an epiphytic colonist. Pruvost and Luisetti (1989), obtained raised black lesions after inoculating the *Xanthomonas* spp. originally isolated from ambarella, into mango and

cashew. However, reciprocal inoculation with strains isolated from mango did not result in lesion development on ambarella (Pruvost and Luisetti, 1989). Pruvost and Luisetti (1989) could simulate lesion development on mombin with strains from ambarella, but not from strains obtained from mango and the pepper tree.

The etiological agent of BBS was described as a Gram negative organism with single, straight rods, (0.4 – 0.7 × 0.7 – 1.6 μm) being motile with a single flagellum (Kwee and Chang, 1985). On nutrient agar, it forms smooth, circular and butyrous or viscid, usually yellow colonies although many are non-pigmented strains (Harworth and Spiers, 1992). The organism is chemo-organotrophic, obligatory aerobic and does not denitrify or reduce nitrate (Kreig and Holt, 1984). The organism requires organic substances for growth e.g. methionine, glutamic acid, nicotinic acid, or a combination of two or more of these substances which are highly stimulatory for growth (Kreig and Holt, 1984).

Molecular differentiation

Until recently, the relatedness of *X. c.* pv. *mangiferaeindicae* isolates has been determined solely by testing one or several phenotypic markers. Studies using physiological and biochemical tests, sensitivity to antibiotics, heavy metals and bacteriophages, serological grouping, plasmid profiles, and multilocus isoenzyme analysis have shown that strains of the pathovar *mangiferaeindicae* display intrapathovar diversity (Some and Samson, 1996; Pruvost *et al.*, 1998).

However, these phenotype-based methods, are limited in their ability to group strains at the pathovar level (Gagnevin *et al.*, 1997). Furthermore, techniques such as serotyping can be labour intensive and costly (Farber, 1996). To clarify the relationships between strains belonging to the pathovar *mangiferaeindicae,* techniques that assess variation in genomic DNA can provide additional reliable tools to evaluate strain variability (Gagnevin *et al.*, 1997).

Molecular typing procedures can be broadly defined as methods used to differentiate bacteria, based on the composition of biological molecules such as proteins, fatty acids, carbohydrates, etc. or nucleic acids (Farber, 1996). The latter can also be more specifically defined as genotyping. In general, genomic variability is independent of external factors, and the techniques involved are adaptable to studies involving large numbers of strains (Gagnevin *et al.*, 1997). Furthermore, since these techniques allow for the measurement of many markers, they usually result in more comprehensive information than other methods (Gagnevin *et al.*, 1997).

Molecular approaches are being used to the larger extent in the taxonomy and epidemiology of *Xanthomonas* spp. (Gagnevin *et al.*, 1997). DNA-DNA hybridisation has demonstrated the

heterogeneity of the genus (Hildebrand *et al.*, 1990). Restriction fragment length polymorphism (RFLP) analysis of plasmid DNAs (Lazo and Gabriel, 1987) and genomic DNA, based on hybridisation with different probes, have been used to differentiate *X. campestris* pathovars (Lazo *et al.*, 1987). By using 16S and 23S rDNA probes from *Escherichia coli*, Berthier *et al.* (1993) characterised different *Xanthomonas campestris* pathovars. They found that variability within different pathovars was related to both plant and pathogenicity factors.

Genotyping, which involves direct DNA-based analysis of chromosomal (e.g., plasmid) genetic material, has many advantages over traditional typing procedures (Farber, 1996). The major advantage lies in its increased discriminatory power, i.e., in its ability to distinguish between two closely related strains (Farber, 1996). Other advantages of genotyping include: (i) DNA can always be extracted from bacteria so that all strains are typeable; (ii) analytical strategies for the genotypic methods are similar and can be applied to DNA of any source; (iii) in general, genomic DNA is a stable characteristic and its composition is independent of cultural conditions or methods of preparation; and (iv) it allows for statistical data analysis and is amenable to automation (Farber, 1996).

The most common molecular typing methods that are being used include chromosomal DNA restriction analysis, plasmid typing, ribotyping, pulse-field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) based typing systems.

Control

Worldwide, copper-based products are the only useful chemicals which can be used for control of BBS. These products are applied at fortnightly intervals during wet weather or during monthly intervals (Manicom, 1980; Visser, 1992). Copper ions are essential for bacteria but can cause a number of toxic cellular effects if levels of free ions are not controlled (Cooksey, 1993). Chemical compounds including copper oxychloride, copper sulphate and copper hydroxide have proven to be very economical and convenient, but pathogens have been reported to develop resistance after repeated use (Gunasekaran and Weber, 1996). Due to the fact that chemical control is only partially successful, plants with low susceptibility to the disease must be used as part of the control strategy (Pruvost *et al.*, 1990).

Disease can be managed either by removing the source of inoculum, or delaying and reducing the spread and development of the pathogen from the resting stage to an infective stage (Mew and Natural, 1993). For field planting, a site protected from strong winds must be selected (Visser, 1992). Alternatively, a field with adequate wind protection in the form of windbreaks around or within the orchard must be selected (Visser, 1992).

Biological control has been used since the early days of agriculture (Gunasekaran and Weber, 1996). Biological control of plant disease involves the use of antagonistic microorganisms that interact with the pathogen to reduce disease development. Korsten *et al.* (1992), showed that a natural mango phylloplane inhabitant, *Bacillus licheniformis* (Burger and Korsten, 1988), could effectively inhibit *X. c.* pv. *mangiferaeindicae* *in-vitro* and *in-vivo*. Visser *et al.* (1990) subsequently showed that *B. licheniformis* could control the BBS pathogen in four prehaverst spray applications. Similarly, effective control of BBS with *B. licheniformis* in field sprays has been shown (Pruvost and Manicom, 1993).

Generally, the best BBS control strategy is not to rely on a single method but to adopt an integrated approach comprising several measures (Sanders, 1993; Agrios, 1997). Exclusion, eradication, protection, resistance, therapy and avoidance are some of the fundamental principles that can be used for control of BBS (Kwee and Chang, 1985).

REFERENCES

Agrios, G. N. 1997. Plant Pathology. 4th Edition. Academic Press, USA.

Berthier, Y., Verdier, V., Guesdon, J., Chevrier, D., Denis, J., Decoux, G. and Lemattre, M. 1993. Characterization of *Xanthomonas campestris* pathovars by rRNA gene restriction patterns. *Applied and Environmental Microbiology* 59: 851-859.

Burger, R. and Korsten, L. 1988. Isolation of antagonistic bacteria against *Xanthomonas campestris* pv. *mangiferaeindicae.* *South African Mango Growers' Association Yearbook* 8: 9-10.

Chand, R. and Kishun, R. 1995. A semi-selective medium for the isolation of the bacterial pathogen *Xanthomonas campestris* pv. *mangiferaeindicae.* *African Plant Protection* 1: 45-48.

Cooksey, D. A. 1993. Copper uptake and resistance in bacteria. *Molecular Microbiology* 7: 1-5.

Dye, D. W., Bradbury, J. F., Goto, M. S., Hayward, A. C., Lelliot, R. A. and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Review of Plant Pathology* 59: 153-168.

Farber, J. M. 1996. An introduction to the hows and whys of molecular typing. *Journal of Food Protection* 59: 1091-1101.

Fivaz, J. 1998. Botanical aspects. Pages 9-13. In: E. A. de Villiers (ed.). The Cultivation of Mangoes. Institute for Tropical and Subtropical Crops, Agricultural Research Council, Nelspruit.

Gagnevin, L., Leach, J. E. and Pruvost, O. 1997. Genomic variability of *Xanthomonas* pathovar *mangiferaeindicae*, agent of mango bacterial black spot. *Applied and Environmental Microbiology* 63: 246-253.

Gunasekaran, M. and Weber, D. J. 1996. Molecular Biology of the Biological Control of Pests and Diseases of Plants. CRC Press, Inc.

Harworth, R. H. and Spiers, A. G. 1992. Isolation of *Xanthomonas campestris* pv. *populi* from stem lesions on *Salix matsudana* × alba 'Aokautere' in New Zealand. *European Journal of Plant Pathology* 22: 247-251.

Hildebrand, D. C., Palleroni, N. J. and Schroth, M. N. 1990. Deoxyribonucleic acid relatedness of 24 Xanthomonad strains representing 23 *Xanthomonas campestris* pathovars and *Xanthomonas fragariae*. *Journal of Applied Bacteriology* 68: 263-269.

Human, C. F. and Snyman, J. C. 1998. Cultivars. Pages 46-50. In: E. A. de Villiers (ed.). The Cultivation of Mangoes. Institute for Tropical and Subtropical Crops, Agricultural Research Council, Nelspruit.

Korsten, L., Lonsdale, J. H., de Villiers, E. E. and de Jager, E. S. 1992. Preharvest biological control of mango diseases. *South African Mango Growers' Association Yearbook* 12: 72-78.

Kreig, N. R. and Holt, J. G. 1984. Bergey's Manual of Systematic Bacteriology. William's and Wilkins, Baltimore.

Kwee, L. T. and Chang, K. K. 1985. Diseases and Disorders of Mango in Malaysia. Tropical Press, Kuala Lumpur.

Lazo, G. R. and Gabriel, D. W. 1987. Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris*. *Phytopathology* 77: 448-453.

Lazo, G. R., Roffey, R. and Gabriel, D. W. 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment-length polymorphism. *International Journal of Systematic Bacteriology* 37: 214-221.

Litz, R. E. 1994. Part III: Mango. Pages 33-34. In: Ploetz, R. C., Zentmeyer, G. A., Nishijima, W. T., Rohrbach, K. G. and Ohr, H. D. (eds). Compendium of Tropical Fruit Diseases. APS Press, Florida, USA.

Mango Fruits Facts@www.crfg.org 1999.

Manicom, B. Q. 1980. The effect of postharvest and enhanced copper sprays on the control of mango black spot. Institute for Tropical and Subtropical Crops, Nelspruit. Agricultural Research Council, Pretoria.

Manicom, B. Q. 1986. Factors affecting bacterial black spot of mangoes caused by *Xanthomonas campestris* pv. *mangiferaeindicae*. *Annals of Applied Biology* 109: 129-135.

Manicom, B. Q. 1992. Mango diseases. Pages 350-353. In: Trench, T. N., Wilkison, D. J. and Esterhuysen, S. P. (eds). South African Plant Disease Control Handbook. Farmer Support Group. University of Natal, Pietermaritzburg.

Manicom, B. Q. and Frean, R. T. 1992. Mango diseases. In: Mango. Institute for Tropical and Subtropical Crops Nelspruit. Agricultural Research Council, Pretoria.

Manicom, B. Q. and Wallis, F. M. 1984. Further characterization of *Xanthomonas campestris* pv. *mangiferaeindicae*. *International Journal of Systematic Bacteriology* 34: 77-79.

Mew, T. W. and Natural, M. P. 1993. Pages 341-362. Management of *Xanthomonas* diseases. In: Swings, J. G. and Civerolo, E. L. (eds). *Xanthomonas*. Chapman & Hall, London.

Nakasone, H. Y. and Paull, R. E. 1998. Mango. Pages 208-238. In: Tropical Fruits. CAB International, UK.

Patel, M. K., Kulkarni, Y. S. and Moniz, L. 1948. *Pseudomonas mangifera-indicae*, pathogenic on mango. *Indian Phytopathology* 1: 147-152.

Ploetz, R. C., Zentmeyer, G. A., Nishijima, W. T., Rohrbach, K. G. and Ohr, H. D. 1994. Part III. Mango. In: Compedium of Tropical Fruit Diseases. APS Press, Florida, USA.

Pruvost, O. and Luisetti, J. 1989. Strains of *Xanthomonas campestris* isolates from Ambarella (*Spondias cytherea* Sonn.) in the French West Indies are probably a new pathogenic form of *Xanthomonas campestris* pv. *mangiferaeindicae*. *Fruits* 44: 539-542.

Pruvost, O. and Luisetti, J. 1991. Effect of time of inoculation with *Xanthomonas campestris* pv. *mangiferaeindicae* on mango fruits susceptibility. Epiphytic survival of *X. c.* pv. *mangiferaeindicae* on mango fruits in relation to disease development. *Journal of Phytopathology* 133: 139-151.

Pruvost, O. and Manicom, B. Q. 1993. *Xanthomonas campestris* pv. *mangiferaeindicae:* Cause of bacterial black spot of mangoes. Pages 91-119. In: Swings, J. G. and Civerolo, E. L. (eds*). Xanthomonas*. Chapman & Hall, London.

Pruvost, O., Couteau, A. and Luisetti, J. 1990. Development of bacterial black spot of mangoes and epiphytic populations of the pathogen (*Xanthomonas campestris* pv. *mangiferaeindicae*) under natural conditions in Reunion Island. *Fruits* 45: 125-140.

Pruvost, O., Couteau, A. and Luisetti, J. 1992. Pepper tree (*Schinus terebenthifolius* Raddi), a new host plant for *Xanthomonas campestris* pv. *mangiferaeindicae*. *Journal of Phytopathology* 135: 289-298.

Pruvost, O., Couteau, A. and Luisetti, J. 1998. Phenotypic diversity of *Xanthomonas* sp. *mangiferaeindicae. Journal of Applied Microbiology* 84: 115-124.

Pruvost, O., Couteau, A., Luisetti, J. and Vernieré, C. 1995. Biology and epidemiology of the casual agent of mango black spot disease. *Fruits* 50: 183.

Robbs, C. F., Neto, J. R., Ribeiro, R. L. and Kimura, O. 1982. Annotated list of bacterial plant pathogens in Brazil. Pages 601-613. In: Lozano, J. C. and Gwin, P. (eds). Proceedings of the 5th International Conference on Plant Pathogenic Bacteria.

Rott, P. and Frossard, P. 1986. Un chancre bacterien du Prunier de Cythere (*Spondias cytherea* Sonn.) en Martinique. *Fruits* 41: 605-613.

Sanders, G. M. 1993. Detection of *Xanthomonas campestris* pv. *mangiferaeindicae* in mango plants. M.Sc. Thesis. University of Pretoria, Pretoria.

Sanders, G. M., Verschoor, J. A., van Wyngaard, S., Korsten, L. and Kotzé, J. M. 1994. Production of monoclonal antibodies against *Xanthomonas campestris* pv. *mangiferaeindicae* and their use to investigate differences in virulence. *Journal of Applied Bacteriology* 77: 509-518.

Schroeder, W. 1991a. Origin of the mango. Page A1. In: Mango. Institute for Tropical and Subtropical Crops Nelspruit. Agricultural Research Council, Pretoria.

Schroeder, W. 1991b. Botanical aspects of the mango. Page A2. In: Mango. Institute for Tropical and Subtropical Crops Nelspruit. Agricultural Research Council, Pretoria.

Snyman, J. C. 1998a. Origin and history of the mango. Pages 1-3. In: E. A. de Villiers (ed.). The cultivation of mangoes. Institute for Tropical and Subtropical Crops, Agricultural Research Council, Nelspruit.

Snyman, J. C. 1998b. Production areas of the World and in South Africa. Pages 4-8. In: E. A. de Villiers (ed.). The cultivation of mangoes. Institute for Tropical and Subtropical Crops, Agricultural Research Council, Nelspruit.

Some, A. and Samson, R. 1996. Isoenzyme diversity in *Xanthomonas campestris* pv. *mangiferaeindicae. Plant Pathology* 45: 426-431.

Swart, G. M. 1999. Comparative study of *Colletotrichum gloeosporioides* from avocado and mango. Ph.D. Thesis. University of Pretoria, Pretoria.

Visser, A. A. 1992. Evaluation of copper compounds, bacterial chemicals and windbreaks for the control of bacterial black spot of mangoes. *South African Mango Growers' Association Yearbook* 12: 23-24.

Visser, A. A., Kotzé, J. M. and Lonsdale, J. 1990. Chemical and biological control of bacterial black spot of mangoes. *South African Mango Growers' Association Yearbook* 10: 8-10.

CHAPTER THREE

COMPARISON OF ISOLATES OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* USING BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS

ABSTRACT

Xanthomonas campestris pv. *mangiferaeindicae,* the bacterial black spot pathogen of mango isolated from two different production areas in South Africa were compared using phenotypic characteristics. Thirty-two representative pathogenic isolates were selected and examined to assess the variability of the taxon. All isolates were nonpigmented. The results obtained did not show any differences amongst isolates and data was consistent with those reported in other studies of phenotypic diversity. All isolates were pathogenic.

INTRODUCTION

Bacterial black spot (BBS) caused by *Xanthomonas campestris* pv. *mangiferaeindicae* (Dye *et al.*, 1980), is one of the most important mango (*Mangifera indica*, L.) diseases in South Africa, Australia, Brazil, India and Reunion Island (Gagnevin *et al.,* 1997; Pruvost and Manicom, 1993). When wet conditions and high temperatures prevail simultaneously, the incidence of BBS increases causing significant yield losses due to premature abscission (Pruvost *et al.,* 1998). Typically BBS symptoms are characterised by small black angular lesions that release a gummy exudate observed on leaves, twigs or fruit (Pruvost and Luisetti, 1991). Bacterial cells survive mostly in older lesions and on aerial organs as epiphytes (Gagnevin *et al.,* 1997). Wounds and lenticels are effective entry sites for the bacterium which is mainly disseminated by wind (Manicom, 1986; Pruvost *et al.*, 1993).

Bacterial black spot is a preharvest disease that affects all cultivars although different levels of susceptibility occur (Sanders, 1993). Cultivars such as Haden, Kent, Irwin, Keitt, Tommy Atkins and Smith are highly, and Fascell and Sensation less susceptible (Smith, 1982). The disease can successfully be controlled by integrated control measures that involve planting of more resistant cultivars, timely copper sprays and windbreaks (Sanders, 1993).

Most strains isolated from mango are non-pigmented; however, yellow strains with low aggressiveness on mango have been described (Pruvost *et al.,* 1998). Sanders (1993) developed monoclonal antibodies (MAbs) against different virulent strains of *X. c.* pv. *mangiferaeindicae*. These MAbs were useful for identification and differentiation between strains (Sanders *et al.*, 1992). Alvarez *et al.* (1985), also used MAbs to identify xanthomonads and to group *X. campestris* pv. *campestris.* Monoclonal antibodies have also been used to characterise *X. campestris* pv. *dieffenbachiae* (Bonner *et al.*, 1987).

The purpose of this study was to compare phenotypes based on biochemical and physiological characteristics of strains of *X. c.* pv. *mangiferaeindicae* isolated from two cultivars and different mango-producing areas in South Africa. Monoclonal antibodies directed against *X. c.* pv. *mangiferaeindicae* were used to confirm identification.

MATERIALS AND METHODS

Bacterial isolates

Symptomatic fruit, leaf and stem samples were randomly collected from six year old Kent and Keitt orchards at Bavaria and Constantia Estates. Samples were placed in plastic bags in insulated, cooled containers and transported to the laboratory for immediate processing.

Plant material was surface sterilized with 3 % (v/v) sodium hypochlorite and rinsed three times with sterile distilled water (sdw). Small pieces of tissue were aseptically excised from the edges of the lesions with a sterile scalpel. Each excised piece was teased apart in a drop of sdw. The resultant suspensions were individually streaked onto Standard 1 Nutrient Agar (Biolab), and incubated at 28 °C for up to four days with daily inspection of growth. Single colonies of suspected *X. c.* pv. *mangiferaeindicae* were restreaked once on boost agar (BA) plates 2.3 % (w/v) [nutrient agar (Biolab), 2 % (w/v) sucrose, 1 % (w/v) yeast extract (Biolab)] (Manicom, 1986), to obtain pure cultures. Pure cultures were immediately preserved in 15 % glycerol in sterile quarter strength Ringer's solution (Oxoid) and stored at -70 °C. Isolates from Zebediela Estates were obtained from Gina Swart from the Department of Microbiology and Plant Pathology, University of Pretoria and were included for comparative purposes. All these isolates were obtained from symptomatic Sensation fruit. Cultures (48h-old) grown on BA plates at 28 °C where used in all tests except where stated otherwise.

The identity of the organisms was confirmed by physiological and biochemical tests routinely used to confirm identity of *Xanthomonas* spp. i.e: oxidase, catalase, nitrate reductase, urease, indole and acetoin production, arginine dihydrolase, starch hydrolysis, gelatin hydrolysis, Tween-80 hydrolysis and H_2S production tests (Harrigan and McCance, 1966). Identity was confirmed by enzyme-linked immunosorbent assay (ELISA) using MAbs (Clone 2C3G1) (Sanders *et al.*, 1994). Whole bacterial cells cultured on BA was used as a solid phase antigen. Four duplicate horizontal rows on microtitre plates (Cooke Microtiter system M29A, Sterilin products, Middlesex, UK) were layered with 100µl of each of the 32 isolates (Table 1) of *X. c.* pv. *mangiferaeindicae* at a concentration of 10^7 cells/ml water. The plates were incubated at 4 °C overnight and fixed with 100µl/well 70% (v/v) methanol for 30 min. The ELISA was carried out as described by Sanders *et al.* (1994). Colour development was measured at 450 nm on an Anthos 2001 ELISA plate reader. Sterile distilled water instead of bacteria was used as a negative control and three known positive isolates (1F2, 1F5 and 1F7) were included as positive controls. Signal-to-background ratios were determined for each antibody-antigen combination.

Biochemical and physiological characteristics

To compare all selected isolates (Table 1) biochemical and physiological tests listed in Table 2 were performed as described by Harrigan and McCance (1966), except for motility, sulfide formation and indole production, which were performed as described in the Merck microbiology manual (1990). All tests were performed at least twice and positive controls were included in all tests (Table 2).

To standardise concentrations used, absorbance readings were taken on a Biochrom Ultra Spec Spectrophotometer and the log colony forming units (cfu) was plotted using a growth curve (Visser, 1995). The bacterial suspensions were adjusted to contain 2 x 10^7 cfu/ml. To finally confirm pathogenicity, leaves were inoculated with different isolates using a detached leaf assay method (Visser, 1995).

RESULTS

From the 100 original isolations, only 75 revealed typical *X. c.* pv. *mangiferaeindicae* colonies. These isolates represented typical *X. c.* pv. *mangiferaeindicae* colony morphology characteristics ascribed to the apigmented group. From these only 32 isolates were selected randomly for further characterisation. Four of these isolates were from symptomatic Keitt cultivar leaves collected from Bavaria Estate. Five isolates were from leaves, four from fruit and another four from stem cankers isolated from symptomatic Kent trees collected from Bavaria Estate. Five isolates were obtained from symptomatic Keitt cultivar fruit and five from leaves collected at Constantia Estate. Another five isolates were obtained from symptomatic Kent cultivar leaves collected from Constantia Estate.

The isolates were all Gram-negative, motile, and oxidative. The results of the physiological and biochemical tests presented in Table 2, confirmed identity of the isolates as *X. c.* pv. *mangiferaeindicae* and revealed no variability between isolates obtained from different cultivars or production areas of South Africa. Beta amylase and endoglucanase were present and all isolates tested catalase positive. Typical of *X. campestris,* all isolates were oxidase negative, nitrate reductase negative, the activity of urease was absent, indole production was not found, and acetoin was not formed. All isolates hydrolysed starch.

With the ELISA test, all isolates had a signal:background ratio greater than two, indicating a positive reaction (Table 3) thereby confirming that all isolates were indeed *X. c.* pv. *mangiferaeindicae*. All isolates were found to be pathogenic on leaves (Fig. 1).

DISCUSSION

The BBS pathogen, *X. c.* pv. *mangiferaeindicae* was successfully isolated from symptomatic mango leaf, fruit and stem tissues. The ease of isolating the BBS causal agent is consistent with previous studies (Sanders, 1993). The high recovery rate (75% positive isolations) found in this study is similar to the one previously described by Sanders (1993). All isolates were typically

apigmented. Thus far pigmented strains of *X. c.* pv. *mangiferaeidicae* have only been isolated in Brazil (Robbs *et al*., 1978), and Reunion Island (Pruvost *et al*., 1989).

The isolates randomly selected for further analysis fitted the typical *X. c.* pv. *mangiferaeindicae* physiological and biochemical profiles. Starch was hydrolysed in our study when using the selected isolates. This feature is not uniformly present in all species of the genus *Xanthomonas* and is reportedly found in at least 75% of the strains from the *X. campestris* complex (Swings *et al*., 1993).

All isolates obtained in this study hydrolysed Tween 80 which according to Van den Mooter and Swings (1990) was characteristic with neopathotype strains of *X. c.* pv. *mangiferaendicae*. From their findings, the neopathotype strain of 266 *X.* sp. *mangiferaendicae* isolates were included in phenon 9 (Van den Mooter and Swings, 1990). The majority of strains in phenon 9 hydrolysed Tween-80 (Swings *et al.,* 1993).

To further confirm identity of *X. c.* pv. *mangiferaeindicae* isolates, MAbs previously developed by Sanders *et al*. (1992) was found to cross react with all isolates tested.

This study confirmed that isolates obtained from the two different production areas of South Africa have the same phenotypic characteristics which is consistent with previous reports (Sanders, 1993). In the study of Sanders (1993), she found that isolates from different production areas had similar profiles. Pruvost *et al.* (1998) also found that *X. c.* pv. *mangiferaeindicae* isolates from Reunion Island have not evolved differently over time and exhibited a high degree of phenotypic similarity.

REFERENCES

Alvarez, A. M., Benedict, A. A. and Mizumoto, C. Y. 1985. Identification of xanthomonads and grouping of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. *Phytopathology* 75: 722-728.

Bonner, R. L., Alvarez, A. M., Berestecky, J. and Benedict, A. A. 1987. Monoclonal antibodies used to characterize *Xanthomonas campestris* pv. *dieffenbachiae*. *Phytopathology* 75: 1352.

Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliott, R. A. and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Review of Plant Pathology* 59: 153-168.

Gagnevin, L., Leach, J. E. and Pruvost, O. 1997. Genomic variability of *Xanthomonas* pathovar *mangiferaeindicae*, agent of mango bacterial black spot. *Applied and Environmental Microbiology* 63: 246-253.

Harrigan, W. F. and McCance, M. 1966. Laboratory methods in Microbiology. Academic Press, London.

Manicom, B. Q. 1986. Factors affecting bacterial black spot of mangoes caused by *Xanthomonas campestris* pv. *mangiferaeindicae. Annals of Applied Biology* 109: 129-135.

Pruvost, O. and Luisetti, J. 1991. Effect of time of inoculation with *Xanthomonas campestris* pv. *mangiferaeindicae:* on mango fruits susceptibility. Epiphytic survival of *X. c.* pv. *mangiferaeindicae* on mango fruits in relation to disease development. *Journal of Phytopathology* 133: 139-151.

Pruvost, O. and Manicom, B. Q. 1993. *Xanthomonas campestris* pv. *mangiferaeindicae:* Cause of bacterial black spot of mangoes. Pages. 91-119. In: Swings, J. G. and Civerolo, E. L. (eds*). Xanthomonas*. Chapman & Hall, London.

Pruvost, O., Couteau, A. and Luisetti, J. 1989. Efficacite de differentes for-mulations chimiques pour lutter contre la maladie des taches noires de la mague (*Xanthomonas campestris* pv. *mangiferaeindicae*). Abstract in English. *Fruits* 44: 343-350.

Pruvost, O., Couteau, A. and Luisetti, J. 1998. Phenotypic diversity of *Xanthomonas* sp. *mangiferaeindicae. Journal of Applied Microbiology* 84: 115-124.

Pruvost, O., Couteau, A., Vernieré, C. and Luisetti, J. 1993. Epiphytic survival of *Xanthomonas campestris* pv. *mangiferaeindicae:* on mango buds. *Acta Horticulturae* 341: 337-344.

Robbs, C. F., Da Ponte, J. J. and Da Gloria Sales, M. 1978. Nota sobre *Xanthomonas mangiferaeindicae*no nordeste do Brasil. Abstract in English. *Fitopatologia Brasileira* 3: 215-217.

Sanders, G. M. 1993. Detection of *Xanthomonas campestris* pv. *mangiferaeindicae* in mango plants. M.Sc. Thesis. University of Pretoria, Pretoria.

Sanders, G. M. Korsten, L., Van Rooyen, E., Verschoor, J. A. and Kotzé, J. M. 1992. Production of monoclonal antibodies against different strains of *Xanthomonas campestris* pv. *mangiferaeindicae* and detection methods. *South African Mango Growers' Association Yearbook* 12: 66-70.

Sanders, G. M., Verschoor, J. A., van Wyngaard, S., Korsten, L. and Kotzé, J. M. 1994. Production of monoclonal antibodies against *Xanthomonas campestris* pv. *mangiferaeindicae* and their use to investigate difference in virulence. *Journal of Applied Bacteriology* 77: 509-518.

Smith, J. H. E. 1982. Mangokultivars. *South African Mango Growers' Association Yearbook* 2: 55-58.

Swings, J., Vauterin, L. and Kersters, K. 1993. The bacterium *Xanthomonas.* Pages. 121-156. In: Swings, J. G. and Civerolo, E. L. (eds). *Xanthomonas.* Chapman & Hall, London.

Van Den Mooter, M. and Swings, J. 1990. Numerical analysis of 295 phenotypic features of 266 *Xanthomonas* strains and related strains and an improved taxonomy of the genus. *International Journal of Systematic Bacteriology* 40: 348-369.

Visser, A. A. 1995. Effect of bactericides and windbreaks on bacterial black spot of mangoes. M.Sc. Thesis, University of Pretoria, Pretoria.

Table 1 *Xanthomonas campestris* pv. *mangiferaeindicae* isolates used in this study

Isolate code	Cultivar	Source[a]	Tissue of origin	Origin no. of isolations	Number of Isolates used[c]
BIL	Keitt	Bavaria	Leaves	8	4
BKF	Kent	Bavaria	Fruit	10	4
BKL	Kent	Bavaria	Leaves	13	5
BKS	Kent	Bavaria	Stem	17	4
CIF	Keitt	Constantia	Fruit	15	5
CIL	Keitt	Constantia	Leaves	17	5
CKL	Kent	Constantia	Leaves	20	5
1F	Sensation	Zebediela[b]	Fruit	-	3
Total number of isolations				100	35

All isolates were collected in 1999 except for Zebediela isolates which were isolated in 1992

[a] Estate from where materials were collected

[b] Isolates obtained from Gina Swart from the Dept. of Microbiology and Plant Pathology

[c] Isolates used in this Chapter.

Table 2 Biochemical and physiological characteristics of *Xanthomonas campestris* pv. *mangiferaeindicae* isolates

Physiological tests	Apigmented strains from Bavaria n=17	Apigmented strains from Constantia n=15	Apigmented strains from Zebediela n=3	Positive control
Oxidase	-	-	-	*P. fluorescens*
Catalase	+	+	+	*E.coli*
Nitrate reductase	-	-	-	*B. cereus*
Urease	-	-	-	*Proteus vulgaris*
Indole production	-	-	-	*E. coli*
Acetoin production	-	-	-	*Klebsiella pneumoniae*
Arginine dihydrolase	-	-	-	*P. aeruginosa*
Starch hydrolysis	+	+	+	*B. subtilis*
Gelatin hydrolysis	+	+	+	*B. subtilis*
Tween-80 hydrolysis	+	+	+	*P. stutzeri*
H_2S production	+	+	+	*Proteus vulgaris*

+ = positive reaction for all strains tested

- = negative reaction for all strains tested

Table 3 ELISA signal to background ratios to confirm identity of *Xanthomonas campestris* pv. *mangiferaeindicae* isolates used throughout this study

Isolate	Signal:Background
BIL1	2.99
BIL2	3.11
BIL3	4.56
BIL4	2.44
BKF1	2.41
BKF2	2.22
BKF3	2.11
BKF4	2.23
BKL1	2.10
BKL2	2.22
BKL3	2.13
BKL4	2.00
BKL5	2.06
BKS1	2.64
BKS2	3.11
BKS3	2.84
BKS4	2.73
CIF1	4.05
CIF2	2.66
CIF3	5.95
CIF4	3.64
CIF5	4.08
CIL1	2.26
CIL2	2.47
CIL3	3.59
CIL4	2.84
CIL5	3.38
CKL1	3.07
CKL2	2.99
CKL3	2.67
CKL4	3.14
CKL5	3.10
1F2	2.52
1F5	2.14
1F7	2.40

Fig. 1 Bacterial black spot symptoms on detached mango leaves of the cultivar Kent after six weeks.

CHAPTER FOUR

VARIATION IN VIRULENCE AND COPPER-RESISTANCE BETWEEN DIFFERENT ISOLATES OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE*

ABSTRACT

Lesion development was studied to determine differences in virulence between *Xanthomonas campestris* pv. *mangiferaeindicae* isolated from bacterial black spot infected mango fruit, leaves and stem cankers. Significant differences in disease severity between isolates were observed after six to eight weeks with the attached leaf bioassay test. Copper sensitivity was evaluated on the basis of viability of cells after exposure to different copper solutions. Isolates sensitive to copper were inactivated by all solutions but copper resistant isolates were inactivated only by the higher concentrations of soluble copper. Eleven isolates were found to be totally resistant and 13 were susceptible to copper. The attached leaf bioassay model previously developed could be used to assist the detection of resistance of mango cultivars to bacterial black spot or the prediction of virulence of *X. c.* pv. *mangiferaeindicae.*

INTRODUCTION

One of the most important diseases of mango (*Mangifera indica*, L.) is bacterial black spot (BBS) caused by *Xanthomonas campestris* pv. *mangiferaeindicae* (Dye *et al.*, 1980, Kwee and Chang, 1985). The disease causes serious annual losses in South Africa and the rest of the world where it infects twigs, leaves and fruit (Korsten *et al.*, 1992). The pathogen host range includes other plant species of the family *Anarcardiaceae* (Pruvost and Manicom, 1993). Mango cultivars differ in susceptibility (Sanders, 1993), with cultivar such as Tommy Atkins being highly susceptible, and cultivars such as Heidi, Sensation, Keitt and Kent showing a high degree of resistance (Kotzé and Visser, 1997). Differences in pathogen virulence has also been reported (Sanders, 1993).

Disease control of bacterial pathogens is difficult and relies mainly on preventative and integrated disease control programs (Sanders, 1993). Once established, *X. c.* pv. *mangiferaeindicae* is difficult to eradicate and control measures are mainly aimed at reducing the inoculum (Pruvost and Manicom, 1993). Chemical control provides limited scope with the use of mainly copper based compounds which can provide effective control if applied at the correct intervals (Pruvost and Manicom, 1993). Rainfall, high relative humidity (>90%), high temperatures and windy conditions favour disease development (Oosthuyse, 1997).

Effectiveness of copper compounds to control BBS depends on inoculum levels in the orchard (Pruvost and Manicom, 1993). Control varies widely from season to season, area to area and even within an orchard. Reasons for these discrepancies are largely unknown. Build-up of pathogen resistance due to continued use of copper based compounds are well known and have been described for strains of *Xanthomonas campestris* pv. *vesicatoria* (Stall *et al.*, 1986), and for *Pseudomonas syringae* pv. *syringae* (Sundin *et al.*, 1989). In these cases, copper resistance was found to be controlled by a self-transmissible plasmid (Stall *et al.*, 1986; Sundin *et al.*, 1989). Control of the disease by chemicals in the regions where the disease is endemic is limited since the pathogens have already developed resistance to the chemicals used (Gagnevin *et al.*, 1997). Recently, copper resistance on mango BBS pathogen was speculated by Boshoff *et al.* (1999).

The purpose of this study was to determine differences in virulence and copper resistance between *X. c.* pv. *mangiferaeindicae* isolates obtained from different sources, cultivars and production areas. Differences in pathogenicity were correlated with differences in symptom expression and copper resistance which was tested *in vitro*.

MATERIALS AND METHODS

Comparison of virulence

Bacterial isolates and preparation of inoculum

Isolates of *X. c.* pv. *mangiferaeindicae*, previously described in Chapter 3 (Table 1) were used in this study. Inoculum for the experiments was prepared by suspending 48-hour-old cultures of each isolate in sterile distilled water (sdw). The bacterial suspensions were adjusted to contain 2×10^7 colony forming units/ml using absorbance readings on a Biochrom Ultra Spec Spectrophotometer and the standard growth curve determined by Visser (1995).

Plant material

To investigate differences in virulence between isolates of *X. c.* pv. *mangiferaeindicae*, healthy one-year-old Kent mango trees maintained in the greenhouse, were used. The trees were grown in five liter plastic bags containing sand, bark and compost (1:1:2 v/v). Leaves of each tree were artificially inoculated with each of the 32 isolates as described below. Temperatures in the greenhouse ranged between 25-27 °C and relative humidity between 40-70 % for the duration of the experiment.

Leaf inoculation

Randomisation of leaves to be used was done by putting numbers in a separate bag and randomly selecting them and placing it on the leaves to be used. The trees were left covered with plastic bags for 24 hours prior to inoculation in accordance with Schaad and Stall (1988). Each leaf was surface disinfested by spraying with 70 % (v/v) ethanol and wiping it dry before inoculation. Care was taken not to damage the leaf surface. The leaves were inoculated by making pricks on both sides of the main veins (eight-pricks per leaf) (Visser, 1995). Ten microlitres of the 2×10^7 cfu/ml inoculum suspension was placed on each wound on the adaxial side of the leaf. Three leaves on different trees were inoculated with each isolate and sdw was included as a control. Eight pricks were thus made on three leaves on three different trees. Each inoculation using 32 isolates was repeated on three trees to eliminate all variation.

Infection was recorded after four to five days, two to three weeks and eight to ten weeks and was rated as the percentage of inoculated tissue showing disease symptoms. Lesion development rate and severity were also recorded. Severity was rated by counting the number of pricks that showed disease symptom development. Data were analysed statistically using analysis of variance and Duncan's multiple range test and Pearson's product moment correlation coefficient (r). Numerical classification of the inoculation data was carried out using the Statistica Statistical analysis package.

Data were also subjected to Ward's agglomerative hierarchical clustering method based on the sum-of-squares criteria. The resulting hierarchies were plotted as a dendogram.

Evaluation of copper-resistance

Bacterial Isolates

Isolates of *X. c.* pv. *mangiferaeindicae*, previously described in Chapter 3 (Table 1) were used to confirm copper resistance. To prepare the isolates for the copper resistance assay, isolates were transferred to boost agar (BA) plates, (2.3 % (w/v) nutrient agar (Biolab), 2 % (w/v) sucrose, 1 % (w/v) yeast extract (Biolab) (Manicom, 1986).

Evaluation of copper-resistance

The following technical grade copper sulphate ($CuSO_4.5H_2O$) (Merck) were dissolved in sdw to achieve a final concentration of 600, 1 200 and 1 800 ppm for analysis. Sterile distilled water was used as a control (Adaskaveg and Hine, 1985).

To each 0.9 ml treatment solution, 0.1 ml bacterial suspension (1 x 10^8 cfu/ml) was added and the mixture was incubated at room temperature. Incubation was stopped after 20 min by adding 0.1 ml of the treatment solution to 0.9 ml phosphate buffer (0.01M KH_2PO_4 and 0.01M Na_2HPO_4) to inhibit the action of copper sulphate (Visser, 1995). After 25 min incubation at 28 °C 100 µl of the suspension was spread on standard 1 nutrient agar plates. After 48 hours, the plates were examined and the surviving bacteria were enumerated. Data were analysed statistically using Pearson's product moment correlation coefficient (r).

RESULTS

Comparison of virulence

Hierarchial comparison of percentage virulence on Kent mango leaves showed that at a linkage distance of 41.8, the water control could be separated from all isolates (Fig. 1). At a linkage distance of 17.5, the isolates could be grouped into two groups, with two isolates being significantly more virulent than the remaining thirty.

Small necrotic lesions (2-2,5 mm in diameter) developed within 24 hours after inoculation (Fig.3). Symptoms initially appeared as water-soaked areas on both sides of leaf veins. The leaf surface became raised and a distinct yellow halo developed around the lesion. After six to eight weeks,

water-soaked lesions developed into typical black spot lesions (Fig. 4), thereby confirming pathogenicity of all tested isolates. No lesions developed at the control inoculation sites infiltrated with sdw (Fig. 3). After two to three weeks, no differences in lesion development between the isolates inoculated on the leaf main veins could be seen. Some symptoms were more severe and others were moderate (Table 1). A strong correlation between surface area affected and virulence was found in the virulence test ($P = 0.000$; $r^2 = 1.000$).

Upon re-isolation, pure cultures of *X. c.* pv. *mangiferaeindicae* were obtained and identity was confirmed as described in Chapter 3 thereby confirming Koch's postulates. Isolates were assigned into three groups based on virulence, i.e. severe, less severe and moderate (Table 1).

Evaluation of copper-resistance

Hierarchial comparison of isolates based on copper resistance showed that at a linkage distance of 1.80, isolates could be grouped into four groups (Fig. 2). Most of the isolates were within one of these groups, which split into two distinct groups at a linkage distance of 1.78. The differences in the linkage distance between percentage virulence and copper resistance clearly show that the isolates are much more closely related in terms of copper resistance as compared to virulence.

Bacterial growth could not be detected after 20 min incubation in copper with Bavaria isolates BIL 2, BKF 3, BKL 2, BKS 3, BIL 1, BKL 3, with isolate IF 7 from Zebediela and with Constantia isolate CIF 1, CIF 2, CIF 4, CIF 5, CIL 3 and CIL 5 (Table 2).

Some isolates (BIL 3, BKF 3, BKL 5, BKS 4, CIL 4, CKL 1, CKL 2, CKL 5) could only tolerate concentrations of 600 ppm (Table 2). Isolates BKF 2, BKS 1 could tolerate concentrations up to 1 200 ppm, while BKF 1, BKL1, BKL4, BKS 2, CIF 3, CIL 1, CIL2, CKL3, CKL 4, 1F 2 and 1F 5 could tolerate concentrations as high as 1800 ppm. No correlation was found with the copper-resistance studies on the basis of origin ($P = 0.11417$; $r^2 = 0.5137$) and cultivar ($P = 0.07318$; $r^2 = 0.6761$) from which isolates were obtained. No correlation was found between copper-resistance and virulence ($P = 0.06063$; $r^2 = 0.4898$) in all isolates.

DISCUSSION

In this investigation, mango trees inoculated with different *X. c.* pv. *mangiferaeindicae* isolates did not show significant differences in black spot lesion development after two to three weeks. However, after six to eight weeks, the water soaked lesions had developed into typical black spot lesions and a significant difference in severity between isolates was evident. This correlates with previous findings

where mango trees were inoculated with different *X. c.* pv. *mangiferaeindicae* isolates which subsequently developed typical black spot lesions after six to eight weeks showing significant differences in severity between isolates (Wachters *et al.*, 1991).

The method used to inoculate leaves by making 5-12 small wounds with a syringe needle (2mm in diameter) on both sides of the main vein have proven effective to determine pathogenicity or host resistance (Visser, 1995). Using this method on greenhouse trees has distinct advantages i.e. known concentrations of inoculum at the point of inoculation and an accurate incubation period and more effective manipulation of environmental conditions to favour infection, can be used.

In contrast to the report by Manicom (1980), these results showed that *X. c.* pv. *mangiferaeindicae* exists as strains differing in virulence. However, these results were in accordance with the results of Sanders (1994) which showed differences in virulence among strains. Not all inoculated sites evaluated developed typical disease symptoms. Tsai and Frasch (1982) suggested that variations in the chemical composition of LPS, might influence the manner of host response towards the particular pathogen invasion. However, Wachters *et al.* (1991) did find differences in virulence and also differences in LPS profiles between various strains of *X. c.* pv. *mangiferaeindicae* isolates.

Copper sulphate solutions effectively inhibited some of the pathogenic isolates after 20 minutes. The toxicity of copper compounds to bacteria is related to the amount of available or free copper ions (Cooksey, 1993). Although some isolates which were the most virulent showed high resistance to copper sulphate at 1 800 ppm, no significant correlation was found between virulence and copper resistance for all isolates. According to Marius Boshoff (Personal communication), trees from Constantia have been sprayed with less copper compounds than at Bavaria and Zebediela Estates. This could explain why some of the isolates from Bavaria and Zebediela were more resistant to copper i.e. 1F2, 1F5, BKL1, BKL4, BKF1, BKS1, BKS2, BKF2 and BKF4. However, there were some isolates from Constantia Estate that showed some resistance to copper, i.e. CIF3, CKL4, CIL1 and CIL2.

At 1 200 ppm, Zebediela isolates showed 66%, isolates from Bavaria showed 36% and isolates from Constantia showed 26% of resistance. At 600 ppm, Zebediela isolates showed 66%, isolates from Bavaria showed 70% and isolates from Constantia showed 55% of resistance. This was mainly due to the fact that Bavaria starts spraying copper bactericides twice monthly from harvest till flush thereafter, Constantia trees are sprayed only once a month till harvest compared to the other Estates i.e. Bavaria and Zebediela which spray every second week from fruit set up to two weeks before harvest.

Previous reports showed transmissible plasmids carrying virulence and copper resistance at the same loci (Stall *et al.*, 1986; Sundin *et al.*, 1989). This could explain why some isolates have a higher level of copper resistance and virulence. Therefore, this could be an indication that copper resistance could potentially be linked to virulence and this aspect should be further investigated in the future studies.

Boshoff *et al.* (1999), speculated that *X. c.* pv. *mangiferaeindicae* could be resistant to copper-based sprays in Mpumalanga Province. This study is in accordance with their preliminary results that certain isolates have developed resistance to copper-based sprays that are currently applied for control of bacterial black spot.

REFERENCES

Adaskaveg, J. E. and Hine, R. B. 1985. Copper tolerance and zinc sensitivity of Mexican strains of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. *Plant Disease* 69: 993-996.

Boshoff, M., Kotzé, J. M. and Korsten, L. 1999. Control of bacterial black spot of mango. *South African Mango Growers' Association* 18: 35-38.

Chand, R. and Kishun, R. 1995. A semi-selective medium for the isolation of bacterial pathogen *Xanthomonas campestris* pv. *mangiferaeindicae*. *African Plant Protection* 1:45-48.

Cooksey, D. a. 1993. Copper uptake and resistance in bacteria. *Molecular Microbiology* 7: 1-5.

Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliott, R. A. and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Review of Plant Pathology* 59: 153-168.

Gagnevin, L., Leach, J. E. and Pruvost, O. 1997. Genomic variability of *Xanthomonas* pathovar *mangiferaeindicae*, agent of mango bacterial black spot. *Applied and Environmental Microbiology* 63: 246 – 253.

Kotzé, J. M. and Visser, A. A. 1997. Observations on the Epidemiology and control of bacterial black spot in mango. *South African Mango Growers' Association Yearbook* 17: 66-70.

Korsten, L., Lonsdale, J. H., de Villiers, E. E. and de Jager, E. S. 1992. Preharvest biological control of mango diseases. *South African Mango Growers' Association Yearbook* 12: 72-78.

Kwee, L. T. and Chang, K. K. 1985. Diseases and Disorders of Mango in Malaysia. Tropical press, Kuala Lumpur.

Manicom, B. Q. 1980. Studies on bacterial black spot. M. Sc. Thesis, University of Natal, Pietermaritzburg.

Manicom, B. Q. 1986. Factors affecting bacterial black spot of mangoes caused by *Xanthomonas campestris* pv. *mangiferaeindicae. Annals of Applied Biology* 109:129-135.

Oosthuyse, S. A. 1997. Bacterial black spot in mango: A Mini Review. *South African Mango Growers' Association Yearbook* 17: 63-65.

Pruvost, O. and Manicom, B. Q. 1993. *Xanthomonas campestris* pv. *mangiferaeindicae:* Cause of bacterial black spot of mangoes. Pages. 91-119. In: Swings, J. G. and Civerolo, E. L. (eds*). Xanthomonas*. Chapman & Hall, London.

Pruvost, O., Couteau, A. and Luisetti, J. 1998. Phenotypic diversity of *Xanthomonas* sp. *mangiferaeindicae. Journal of Applied Microbiology 84: 115 –124.*

Sanders, G. M. 1993. Detection of *Xanthamonas campestris* pv. *mangiferaeindicae* in mango plants. M. Sc. Thesis. University of Pretoria, Pretoria.

Sanders, G. M. and Korsten, L. 1995. A rapid method for differentiation of *Xanthomonas campestris* pv. *mangiferaeindicae* from other Xanthomonads and mango phylloplane inhabitants. *Journal of Basic Microbiology* 35: 337-347.

Schaad, N. W. and Stall, R. E. 1988. *Xanthomonas*. Pages 81-94. In: Schaad, N. W. (ed.). Laboratory guide for identification of plant pathogenic bacteria. APS Press, the American Phytopathology Society, St. Paul, Minnesota.

Stall, R. E., Loschke, D. C. and Jones, J. B. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* 76: 240-243.

Sundin, G. W., Jones, A. I. and Fulbright D. W. 1989. Copper resistance in *Pseudomonas syringae* pv. *syringae* from cherry orchards and its associated transfer *in-vitro* and planta with a plasmid. *Phytopathology* 79: 861-865.

Tsai, C. M. and Frasch, C. E. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Annals of Biochemistry* 119: 115-119.

Visser, A. A. 1995. Effect of bactericides and windbreaks on bacterial black spot of mangoes. MSc. Thesis, University of Pretoria, Pretoria.

Wachters, A. E., Korsten, L. and Kotzé, J. M. 1991. Systemicity and variation in pathogenecity between strains of *Xanthomonas campestris* pv. *mangiferaeindicae*. *South African Mango Growers' Association Yearbook* 11: 22-24.

Table 1 Comparison of mango bacterial black spot disease symptoms when inoculated on Kent mango leaves, inoculated with different bacterial isolates at concentrations of 10^7cfu/ml

Isolate Code	Percentage infection[a]		Lesion severity[b]
Control	0	h	No symptoms
CKL1	93.75	abc	Severe
BKF4	100	a	Severe
CKL3	93.75	abc	Severe
BIL1	96.88	ab	Severe
CIL4	93.75	abc	Severe
CIF5	84.38	bcde	Severe/ Less severe
CIF2	93.75	abc	Severe
BIL2	93.75	abc	Severe
CIF1	93.75	abc	Severe
CKL2	78.13	de	Less severe
BKF1	93.75	abc	Severe
CIL3	100	a	Severe
BIL3	81.25	cde	Severe/ Less severe
CIL2	78.13	de	Less severe
BKF5	71.88	e	Less severe
CKL5	90.63	abcd	Severe/ Less severe
CIL1	84.38	bcde	Severe/ Less severe
CIF4	100	a	Severe
CIL5	96.88	ab	Severe
CIF3	96.88	ab	Severe
BKF3	96.88	ab	Severe
CKL4	96.88	ab	Severe
BKF2	78.13	de	Severe/ Less severe
BKL2	78.13	de	Less severe
BKL1	93.75	abc	Severe
BKS3	40.63	g	Moderate
BKS4	56.25	f	Moderate
BKS2	90.63	abcd	Severe/ Less severe
BKS1	93.75	abc	Severe
BKL3	90.63	abcd	Severe/ Less severe
BKL4	96.88	ab	Severe
BKL5	100	a	Severe
1F2	88.7	bcde	Severe
1F5	42.5	g	Moderate
1F7	60.4	f	Less severe
Pr > F	0.0001		

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

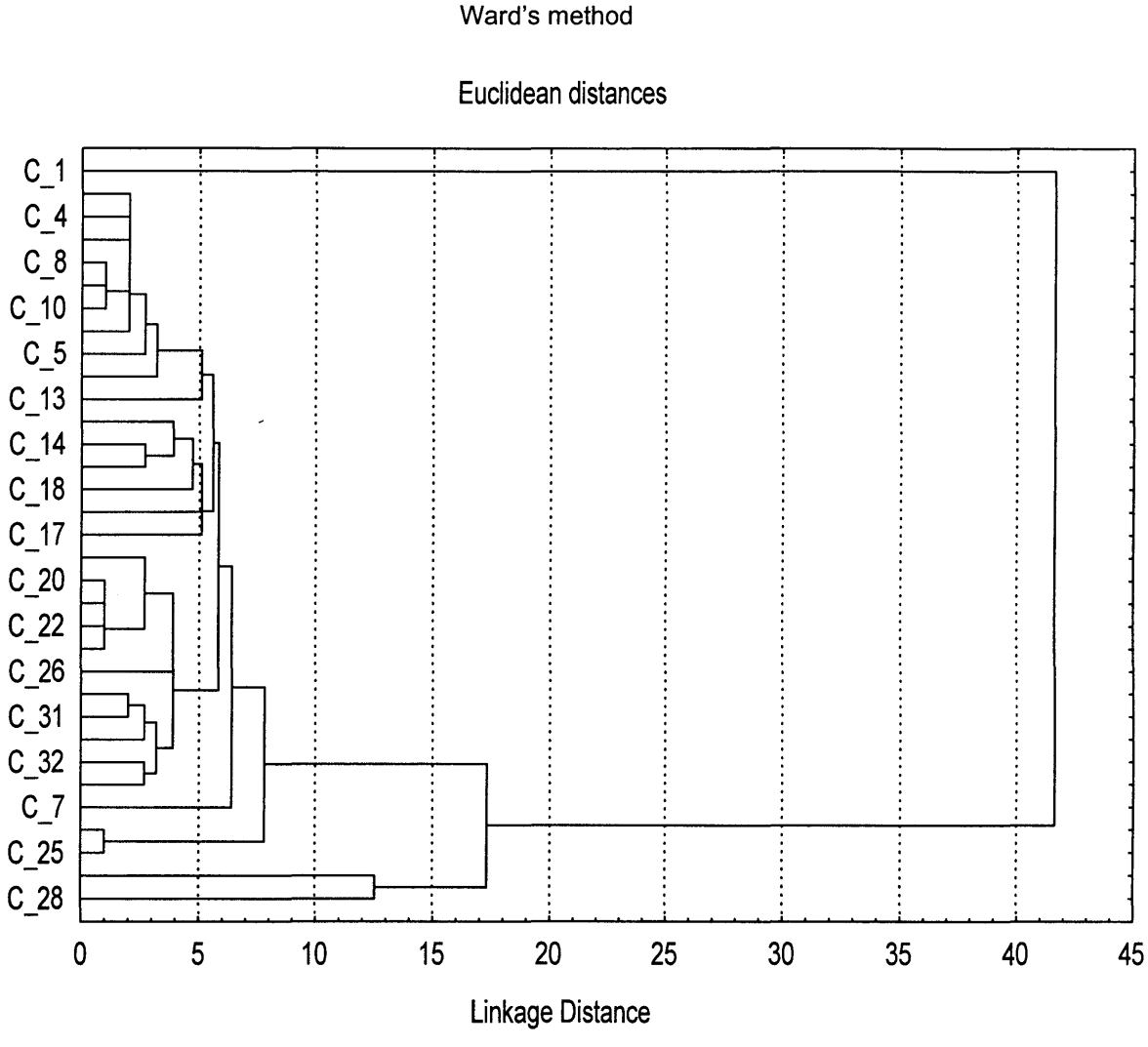
Means with the same letter are not significantly different according to Duncan's multiple range test (P = 0.0001).

Table 2 Viability of *Xanthomonas campestris* pv. *mangiferaeindicae* isolates after a 25 min exposure to copper sulphate in an aqueous suspension

Isolate code	Number of surving cfu Cu sulphate conconcentration (ppm)		
	600	1 200	1 800
CIF2	-	-	-
BKF4	+	+	-
BIL2	-	-	-
CKL4	++	++	+
BKL1	+	+	+
BKL4	++	++	+
CIL4	+	-	-
1F5	+	+	+
1F2	+	+	+
CIL1	++	++	+
BKF1	++	++	++
BKS2	++	+	+
CIF3	+	+	+
BKF2	++	+	-
BKS1	+	+	-
BKL5	+	-	-
BIL1	-	-	-
BKS3	-	-	-
CIL2	++	+	-
CIF5	-	-	-
CKL5	++	-	-
BKF3	+	-	-
BKS4	+	-	-
BKL2	-	-	-
BKF3	-	-	-
BKL3	+	-	-
BIL3	+	-	-
CKL3	++	-	-
CKL1	-	-	-
CIF1	++	-	-
CKL2	-	-	-
CIL3	-	-	-
CIF4	-	-	-
CIL5	-	-	-
1F7	-	-	-
Control	-	-	-
Pr > F			0.0001

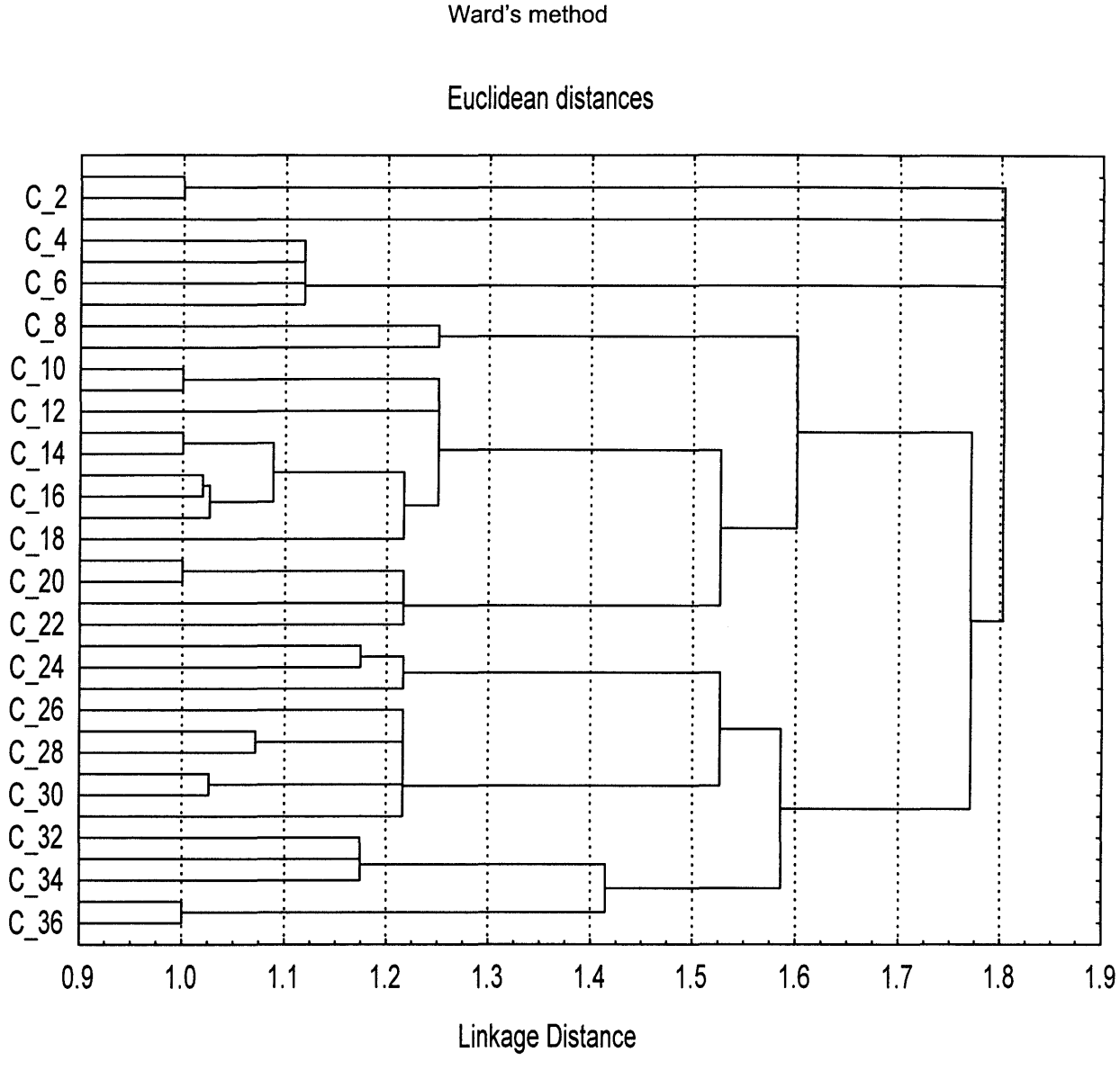
Codes for strains: BIL - Bavaria Keitt leaves, BKF - Bavaria Kent fruit, BKL - Bavaria Kent leaves, BKS - Bavaria Kent Stem Canker, CIF - Constantia Keitt fruit, CIL - Constantia Keitt leaves, CKL - Constantia Kent leaves, 1F – Zebediela Sensation Fruit

Codes for surviving cfu: ++ - >30, + - <30, - means no growth



C-values are isolate case number designations assigned by Statistica

Fig. 1 Hierarchical comparison of percentage virulence on Kent mango leaves produced by mango isolates of (Appendix 1) *Xanthomonas campestris* pv. *mangiferaeindicae* using Ward's method of agglomerative clustering according to Euclidean distances.



C-values are isolate case number designations assigned by Statistica

Fig. 2 Hierarchical comparison of copper resistance on mango isolates of (Appendix 1) *Xanthomonas campestris* pv. *mangiferaeindicae* using Ward's method of agglomerative clustering according to Euclidean distances.

Fig. 3 Six week old bacterial black spot symptoms on detached Kent cv. mango leaves and a control.

Fig. 4 Six week old bacterial black spot symptoms on detached Kent cv. mango leaves.

CHAPTER FIVE

DIFFERENTIATION OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* ISOLATES BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

ABSTRACT

The applicability of numerical analysis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis of whole cell protein extracts for classification of *Xanthomonas campestris* pv. *mangiferaeindicae,* was investigated on 28 isolates. It was found that all isolates were related to each other at similarity levels of more than 60 %, indicating that they all belong to one group. It was concluded that electrophoresis of the total soluble proteins of the bacterial cell is a powerful, rapid, and relatively easy method for differentiation of xanthomonads up to sub-species level of *X. c. pv. mangeferaeindicae*.

INTRODUCTION

The causal organism of bacterial black spot (BBS) of mango (*Mangifera indica*, L) was described in 1915 as *Bacillus mangifera* n. sp. (Pruvost and Manicom, 1993). In 1948, it was re-classified as *Pseudomonas mangiferaeindicae* (Patel *et al.*, 1948). With the introduction of the pathovar system in 1980, the pathogenic agent of BBS disease of mango was re-classified as *Xanthomonas campestris* pv *mangiferaeindicae* (Dye *et al.*, 1980).

The bacterial genus *Xanthomonas* consists of numerous plant pathogens occurring worldwide and causing diseases on diverse economically important crops (Vauterin *et al.*, 1991). Until recently, the taxonomy of bacterial plant pathogens relied mainly on physiological and biochemical tests and was categorised within the pathovar system established by Dye *et al.* (1980). These pathovars, were defined by host or symptom specificity. Alternative techniques such as antibiotic sensitivity, heavy metal bacteriophages, serological grouping, plasmid profiles and multilocus isoenzyme analysis have been used for grouping strains for genus and species classification (Gagnevin *et al.*, 1997). However, these techniques were found to be limited in their ability to group strains at the pathovar level.

Development of a rapid high-resolution fingerprinting technique such as protein electrophoresis in combination with computer-assisted processing of profiles made it possible to characterise and compare large numbers of strains at the infrasubspecific level (Jackman, 1987). El-Sharkaway and Huisingh (1971), first demonstrated the usefulness of protein electrophoresis in taxonomic studies of *Xanthomonas*. Their results clearly illustrated that protein electrophoresis may provide a valuable tool in taxonomic studies to identify *Xanthomonas.* Later, protein electrophoretic studies were used to differentiate between different pathovars of *Xanthomonas* (Vera Cruz *et al.*, 1984; Van Den Mooter *et al.*, 1987a; Van Den Mooter *et al.*, 1987b).

The method of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is relatively simple and inexpensive (Van Zyl, 1990). This technique can be used to classify and identify bacterial strains at a molecular level (Van Zyl and Steyn, 1990). Van Zyl (1990) concluded that electrophoresis of total soluble proteins of the bacterial cell can be applied to differentiate and identify phytopathogenic pseudomonads and xanthomonads up to the infrasubspecific level. Electrophoresis of SDS-solubilised whole-cell proteins has contributed to the elucidation of the natural relationship between currently defined *X. campestris* pathovars (Vauterin *et al.*, 1993). At present, protein electrophoresis analysis has been extended to all known *X. campestris* pathovars (Vauterin *et al.*, 1993).

In this study, isolates of *X. c.* pv. *mangiferaeindicae* were numerically compared using SDS-PAGE patterns of cellular proteins.

MATERIALS AND METHODS

Preparation of whole-cell extracts

The bacterial isolates used in this study and their sources of origin are listed in Table 1 (Chapter 3). Only 28 of the previous isolates in Chapter 3 (Appendix 2) were used in this study due to the loss of five of the isolates. All bacterial isolates to be compared were grown on nutrient agar (Biolab) at 28 °C for 48 hours. The cells were harvested by scraping them off the agar surface with a sterile spatula (0.08 g wet weight). The cells were washed by suspending the cells in 700 µl 0.2 M sodium phosphate buffer (NaPB) pH 6.88 [22.5 ml of 0.2 M $NaH_2PO_4.2H_2O$ (Merck), 27.5 ml of 0.2 M $Na_2HPO_4.2H_2O$ (Merck) made up to 100 ml with distilled water], vortexed and centrifuged at 15 000 g for 8 min. The washing procedure was repeated twice. The supernatant was discarded and 270 µl sample treatment buffer (STB) pH 6.8 [0.75 g Tris (Boehringer Mannheim), 5 ml 2-β-mercaptoethanol (Merck), 10 ml Glycerol (Merck) made up to 100 ml with distilled water], without sodium dodecyl sulphate (SDS) (Merck), was added to the cell pellet before mixing and vortexing of the sample. Thirty microliters of 20 % (m/v) SDS was added to the mixture, before being mixed and vortexed. The mixture was heated to 95 °C for three min and cooled on ice.

The ice-cooled suspension was treated with an ultrasonic homogenizer (Series 4710) (Cole-Parmer Instrument Co., Chicago), with 50 % maximum output (40 Watt). Two hundred and seventy microliters of STB and 30 µl 20 % SDS were added to the suspension and manually mixed before centrifugation at 15 000 g for 8 min. The supernatant (protein extract) was placed into a clean Eppendorf tube, and stored at -20 °C until required.

Protein concentration determination

Prior to use, the previously prepared protein extracts obtained from the different isolates were defrosted and the protein concentration determined. Five microliters of each protein extract was blotted on Whatman no. 1 filter paper by using a Hamilton microsyringe. A Coomassie Brilliant Blue R solution [10 g Coomassie Brilliant Blue R stock solution (Merck) made up to 500 ml with distilled water] was poured onto the filterpaper and staining was allowed for 5 to 10 min. The filterpaper was destained by using a solution containing 10 ml 10 % (v/v) acetic acid (Merck) and 15 ml 95 % (v/v)

ethanol. The intensity of the colour of each blot was used as indication of the protein concentration - the darker the blot, the higher the protein concentration.

Electrophoresis of SDS-solubilised proteins

After determining the protein concentrations, the extracts were loaded into each slot of the vertical gel with a Hamilton microsyringe. The volume to be applied depended upon the concentration of the protein extract and varied between 10 to 20µl. Reference proteins already prepared in the Department of Microbiology, University of Pretoria enabled accurate comparison between protein patterns on different gels. A bromophenol blue (Merck) solution containing 50% (v/v) glycerol was added as a tracking dye during electrophoresis. Two gels were run simultaneously, *Cycrobacter immobilis* LMG 1125 was used as a maker.

Electrophoresis was performed in a 12 % (w/v) gel slab, run vertically at a constant current of 30 mA through the stacking gel and 60 mA through the separation gel. A constant temperature (10 °C) was maintained during electrophoresis which was completed within four hours.

Gels were stained for one hour in a staining solution containing 150 ml Coomassie Brilliant Blue R stock solution and destained in a solution containing 250 ml methanol (Merck), 100 ml acetic acid, made up to 1 L with distilled water. All buffers and stock solutions were stored at 4°C but were used at room temperature. Gels were cast one day prior to the electrophoresis run and left overnight at room temperature to ensure complete polymerisation. Differences among isolates were assessed on the basis of migration patterns of protein profiles using Gel-Compar 4.0 programme (Applied Maths, Kortrijk, Belgium). Dendrograms were generated using the Pearson product-moment correlation coefficient (r) for calculating similarities between strains, and clustered using the unweighted pair group method of arithmetic averages (UPGMA).

RESULTS

The dendogram obtained after numerical analysis of the protein electrograms of *X. c.* pv. *mangiferaeindicae* isolates is shown in Fig. 1. The results of the numerical analysis of the phenotypic features correspond with a high degree to the present taxonomic grouping in this genera. At 67.5 % similarity, isolates could be grouped into two clusters, designated cluster I and cluster II. Cluster I could be divided into two groups at 79.5 %, namely 1 and 2 (Fig. 1). Group 1 could be separated into two subgroups at 85 % similarity. The first sub-group 1a consisted of four isolates of which three

were originally obtained from Constantia and the other from Bavaria Estates. The second sub-group 1b consisted of only one isolate originally obtained from Zebediela Estate.

At 89 % similarity group 2 could be divided into two subgroups (Fig. 1). Sub-group 2a consisted of ten isolates (eight from leaf lesions), half of these isolates were from Constantia and the other half from Bavaria Estates. The second sub-group 2b consisted of one isolate originally obtained from Bavaria Estate.

Cluster II could be divided into two groups at 80 % similarity, namely group 1 and 2. Group 1 could be separated into two sub-groups at 82.5 % similarity. The first sub-group 1a consisted of three isolates of which two are from Zebediela and one from Constantia Estates. The second sub-group 1b comprised six isolates from Bavaria and one from Constantia Estate. Group 2 consisted of a single isolate originally obtained from Constantia Estate.

DISCUSSION

Cluster analysis of BBS isolates obtained from different cultivars and production areas showed that they were closely related. These results are in agreement with results obtained by Van Zyl (1990), who found that all strains of *X. c.* pv. *mangiferaeindicae* clustered in a single subgroup at 67. 5 %.

No differences were observed between *X. c.* pv. *mangiferaeindicae* isolates both with the virulence and copper-resistance tests as described in the previous chapter. Groupings mainly contained isolates obtained from mostly one production area e.g. the second sub-group 1b comprised six isolates from Bavaria and one from Constantia. This may be due to the fact that these isolates exhibit close DNA homology.

Isolates that were more virulent and more copper resistant from the previous findings (Chapter 4), tended to mostly cluster under one group (Cluster I) i.e. BKS 2, BKF 1, CIF 3, CKL 4 and 1F 5 in the protein profile analysis. The work done by Van Zyl (1990), showed no correlation between virulence and protein electrophoretic (or phenotypic) clustering in the case of the avirulent *X. c.* pv. *mangiferaeindicae* strains used. Knowledge of the homogeneity of a pathovar grouping is important for both its classification and practical diagnosis. From the results presented in this study it is clear that *X. c.* pv. *mangiferaeindicae* is a remarkably distinct and homogeneous pathovar but that differences in virulence could be detected although at low similarity index. All isolates grouped together at 67.5%, which clearly indicate that all pathogenic xanthomonads isolated from mango and used in this study belong to one species (Gagnevin *et al.*, 1997).

From the results presented here, it is clear that the SDS-PAGE database of whole-cell protein patterns established in highly standardised conditions can be of great value to identify *Xanthomonas* strains. The *X. c.* pv. *mangiferaeindicae* showed a common basic SDS-PAGE protein pattern. Vauterin *et al.* ((1991), has conducted a study for analyzing *X. campestris* strains from citrus using SDS-PAGE of proteins, fatty acids and DNA hybridization. The results of the three methods correlated well but it was found that SDS-PAGE of proteins was more reliable than the other two methods (Vauterin *et al.*, 1991). The protein proteins clearly showed more variation.

REFERENCES

Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliott, R. A. and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Review of Plant Pathology* 59: 153-168.

El-Sharkaway, T. A. and Huisingh, D. 1971. Electrophoretic analysis of esterases and other soluble proteins from representatives of phytopathogenic bacterial genera. *Journal of General Microbiology* 68: 149-154.

Gagnevin, L., Leach, J. E. and Pruvost, O. 1997. Genomic variability of *Xanthomonas* pathovar *mangiferaeindicae*, agent of mango bacterial black spot. *Applied and Environmental Microbiology* 63: 246 – 253.

Jackman, P. J. H. 1987. Microbial systematics based on electrophoretic whole-cell protein patterns. *Methods in Microbiology* 19: 209-225.

Patel, M. K., Kulkarni, Y. S. and Moniz, L. 1948. *Pseudomonas mangifera-indicae*, pathogenic on mango. *Indian Phytopathology* 1: 147-152.

Pruvost, O. and Manicom, B. Q. 1993. *Xanthomonas campestris* pv. *mangiferaeindicae*: Cause of bacterial black spot of mangoes. Pages 91-119. In: Swings, J. G. and Civerolo, E. L. (eds). *Xanthomonas*. Chapman & Hall, London.

Van Den Mooter, M., Mariaite, H., Meiresonne, L., Swings, J., Gillis, M., Kersters, K. and De Ley, J. 1987a. Comparison between *Xanthomonas campestris* pv. *manihotis* (ISPP List1980) and *X. campestris* pv. *cassavae* (ISPP List 1980) by means of phenotypic, protein electrophoretic,

DNA hybridization and phytopathological techniques. *Journal of General Microbiology* 133: 57-71.

Van Den Mooter, M., Steenackers, M., Maertens, C., Gossele, F., De Vos, P., Swings, J., Kersters, K. and De Ley, J. 1987b. Differentiation between *Xanthomonas campestris* pv. *graminis* (ISPP List 1980), pv. *phleipratensis* (ISPP List 1980) emend, pv. *poae* Egli and Schmidt 1982 and pv. *arrhenatheri* Egli and Schmidt 1982 by numerical analysis of phenotypic features and protein gel electrograms. *Phytopathology* 118: 135-156.

Van Zyl, E. 1990. A numerical approach to the taxonomy of *Xanthomonas* and some phytopathogenic *Pseudomonas* spp. as determined by phenotypic properties and protein gel electrophoresis. Ph.D. Thesis. University of Pretoria, Pretoria.

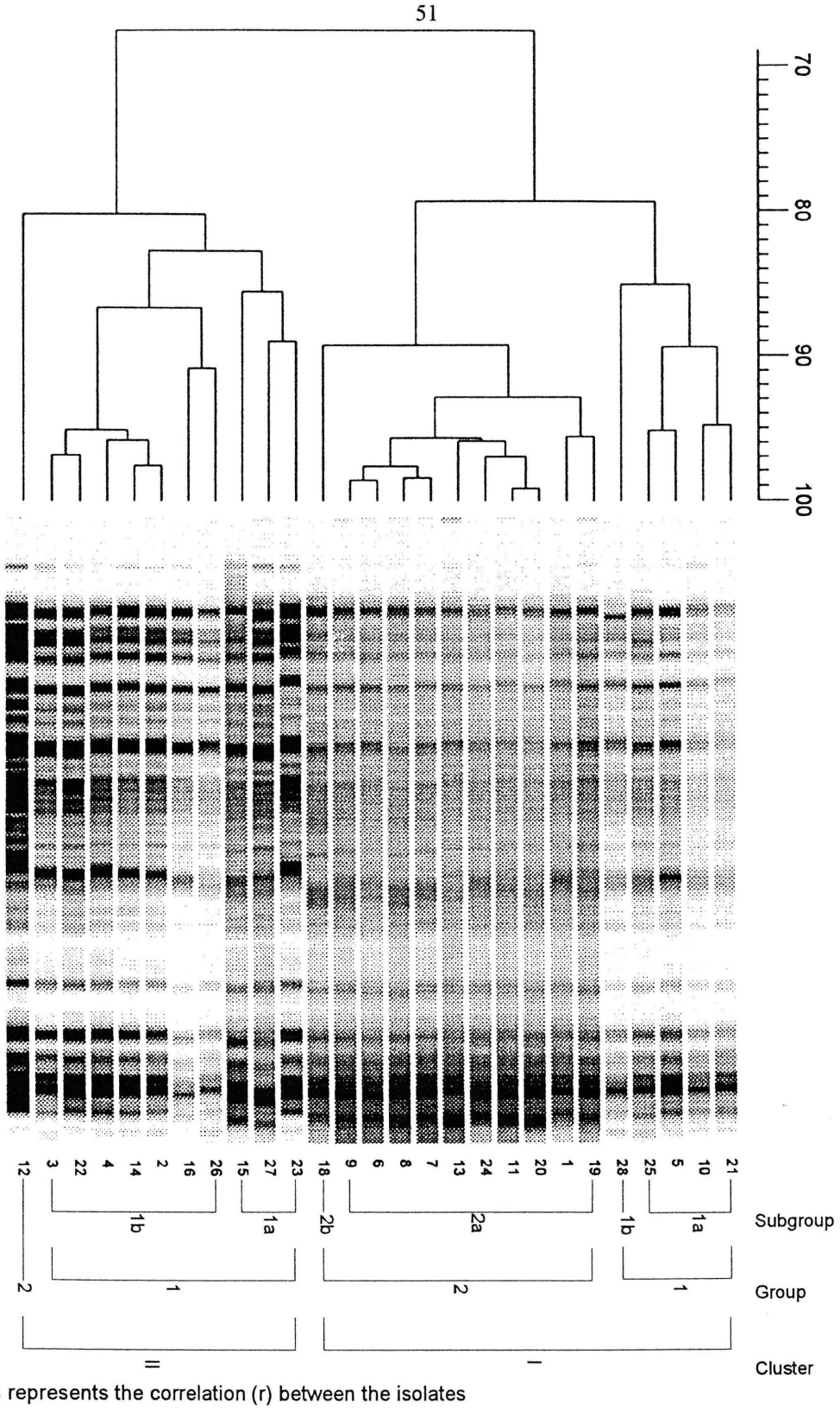
Van Zyl, E. and Steyn, P. L. 1990. Differentiation of phytopathogenic *Pseudomonas* and *Xanthomonas* species and pathovars by numerical taxonomy and protein gel electropherograms. *Systematic and Applied Microbiology* 13: 60-71.

Vauterin, L., Hoste, B., Yang, P., Alvarez, A., Kersters, K. and Swings, J. 1993. Taxonomy of the genus *Xanthomonas*. Pages 157-185. In: Swings, J. G., and Civerolo, E. L. (eds). *Xanthomonas*. Chapman & Hall, London.

Vauterin, L., Swings, J. and Kersters, K. 1991. Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. *Journal of General Microbiology* 137: 1677-1687.

Vauterin, L., Yang, P., Hoste, B., *et al.* 1991. Differentiation of *Xanthomonas campestris* pv. *Citri* strains by sodium dodecyl sulphate-polyacrylamide gel electrophoresis of proteins, fatty acid analysis, and DNA-DNA hybridization. *International Journal of Systematic Bacteriology* 41: 535-542.

Vera Cruz, C. M., Gossele, F., Kersters, K., Seegers, P., Van Den Mooter, M, Swings, J. and De Ley, J. 1984. Differentiation between *Xanthomonas campestris* pv. *oryzae*, *X. campestris* pv. *orzicola* and bacterial 'brown blotch' pathogen on rice by numerical analysis of phenotypic features and protein gel electropherograms. *Journal of General Microbiology* 130: 2983-2999.



The x-axis represents the correlation (r) between the isolates

Fig. 1 SDS-PAGE protein patterns and dendogram showing the relationship between 28 isolates of *Xanthomonas campestris* pv. *mangiferaeindicae* (Appendix 3).

CHAPTER SIX

USE OF POLYMERASE CHAIN REACTION TO FINGERPRINT GENOMES OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* ISOLATES

ABSTRACT

Genetic comparison of 28 isolates of *Xanthomonas campestris* pv. *mangiferaeindicae* obtained from different geographic regions and cultivars was done using polymerase chain reaction (PCR) methods. These PCR methods rely on different amplification priming strategies:i.e. enterobacterial repetitive intergenic consensus (ERIC) PCR, BOX-PCR and ribosomal intergenic spacer analysis (RISA). The results of these assays are mutually consistent and indicate that pathogenic strains are closely related to each other. Isolates which had been postulated to be closely related by protein profile analysis also revealed similar ERIC-, BOX-PCR, and RISA patterns. These results suggest that the ERIC-, BOX-PCR, and RISA methods are useful for the identification and classification of bacterial strains.

INTRODUCTION

Detailed characterisation of the genetic variability among strains of *Xanthomonas campestris* pv. *mangiferaeindicae* has not been reported despite the prevalence, survival and economic importance of the bacterium (Pruvost *et al.*, 1998). Such characterisation would be useful for pathogenicity studies and to trace the spread of the disease amongst continents. In addition to physiological tests, there are several alternative methods in use to identify and compare isolates of bacterial pathogens. These techniques include serological testing (Benedict *et al.*,1989; Benedict *et al.*, 1990), fatty acid profiling (Stead, 1992; Vauterin *et al.*, 1992), genomic and plasmid DNA analysis (Lazo and Gabriel, 1987; Hildebrand *et al.*, 1990; Berthier *et al.*, 1993) and protein analysis (Van Zyl and Steyn, 1990; Vauterin *et al.*, 1991). However, these techniques are often time-consuming, too expensive, or too insensitive for use in routine diagnosis (Louws *et al.*, 1994) and is often ineffective in distinguishing between closely related isolates.

Analysis of genomic DNA using PCR-based methods has recently proven to be a fast, sensitive, and reliable method for determining genetic relationships amongst pathogenic organisms (Pooler *et al.*, 1996). Families of repetitive DNA sequences are dispersed throughout the genome of diverse bacterial species (Sharples and Lloyd, 1990; Hulton *et al.*, 1991). These sequences contain highly conserved central inverted repeats and can be divided into two classes that do not share significant homology (De Bruijn, 1992). These conserved regions have been called repetitive extragenic palindromic (REP) sequences (Stern *et al.*, 1984), enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton *et al.*, 1991), and the BOX element (Martin *et al.*, 1992). The REP, ERIC, and BOX elements have the potential to form stem-loop structures and may play an important role in the organization of the bacterial genome (Krawiec and Riley, 1990). Genome organization is thought to be shaped by selection. The dispersion of the REP, ERIC, and BOX sequences may thus be indicative of the structure and evolution of the bacterial genome (Krawiec and Riley, 1990).

Ribosomal intergenic spacer analysis (RISA) (Fischer and Triplett, 1999) is another genotyping method used which is also based on the PCR reaction (Jensen *et al.*, 1993). Ribosomal DNA (rDNA) is composed of namely, 23S, 16S and 5S rRNA genes, which are separated by spacer regions, which may show a large degree of sequence and length of variation at both the genus and species level (Campbell *et al.*, 1993). The method is focused on the heterogeneity found in the spacer regions which exist between genes coding for rRNA in microorganisms (Farber, 1996). Most bacterial genera contain multiple copies of the operon for rRNA, so that the spacer regions within a single strain may differ in their length and/or sequence (Farber, 1996).

The objective of this study was to assess genetic variability among strains of *X. c.* pv. *mangiferaeindicae* isolated from different production areas and cultivars by using ERIC- and, BOX-PCR, and RISA.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction

Bacterial isolates (28) used in this study (Appendix 2) and their source of origin are listed in Table 1 (Chapter 3). Four isolates from the original set could not be included due to loss of viability during storage and loss of other characteristics due to sub-cultiring. Template DNA was extracted from *X. c.* pv. *mangiferaeindicae* isolates using a modified rapid lysis method of Maniatis *et al.* (1982). A single colony was transferred to Luria Bertani (LB) broth (Maniatis *et al.*, 1982) and incubated for 48 hours at 28 °C. Briefly, one ml of the cells were centrifuged (14,000 g for 10 min), the supernatant was removed and the pellet was washed twice in one ml sterile physiological buffered saline solution pH 8.0 by centrifuging the cells (14,000 g for 10 min). The pellet was then resuspended in 100 µl sterile milli Q water and heated for 10 min at 95 °C. The cell lysate was immediately placed on ice. Outgroups that were included were two *Erwinia* spp. for BOX -, RISA and ERIC – PCR obtained from the culture collection of Lise Korsten from the Department of Microbiology and Plant Pathology, University of Pretoria.

Amplification and separation of DNA bands

Primers corresponding to ERIC elements (ERIC1R: 5'-ATG TAA GCT CCT GGG GAT TCA C-3' and ERIC2: 5'-AAG TAA GTG ACT GGG GTG AGC G-3') (De Bruijn, 1992) were used in this study and BOXA, a subunit of the BOX element (BOXA1R: 5'-CTA CGG CAA GGC GAC GCT GAC G-3') (Louws *et al.*, 1994), 16 s rDNA: 5'-TTG TAC ACA CCG CCC GTC A-3' and 23s rDNA 5'-GGT ACC TTA GAT GTT TCA GTT C-3' (RISA) (Mc Manus and Jones, 1996) were synthesized by MWG Biotech, Germany. A volume of, 1.5 µl DNA was added to the reaction mixture containing 1 x reaction buffer (100 mM NaCl; 50 mM Tris-HCl pH 8.0; 0.1 mM DDT; 50 % glycerol and 1 % Triton X-100), 50 pmoles each of the oligonucleotide primers, 0.625 mM of each dNTP, 2.5 mM $MgCl_2$, milli Q water and 0.2 U of *Taq* DNA polymerase (Promega). Thermal cycling was conducted with a Perkin Elmer 2400 thermocycler.

ERIC-, and BOX - PCR was performed using the following conditions: one initial denaturation cycle for seven min, followed by 30 cycles of denaturation i.e. one min at 94 °C, annealing for one min at

52 °C and extension for three min at 72 °C. The reaction was completed with a final 10 min extension at 72 °C.

Amplification products (10 µl) were separated on a 1.5% agarose gel in Tris-Acetate EDTA (Merck, Johannesburg) buffer (TAE) and visualised by ethidium bromide staining (Maniatis *et al.*, 1982) and UV transillumination. The data obtained was analysed with the Gel-Compar 4.0 programme (Applied Maths, Kortrijk, Belgium). Dendrograms were generated using the Pearson product-moment correlation coefficient (r) for calculating similarities between strains, and clustered using the unweighted pair group method of arithmetic averages (UPGMA).

RESULTS

The outgroups of *Erwinia spp.* separated out clearly at 41% similarity in RISA patterns (Fig. 1) but only at 88.5% similarity in the BOX-PCR patterns (Fig. 2).

Primers corresponding to conserved DNA sequences of ERIC sequences, BOXA subunits of BOX elements and RISA sequences annealed to genomic DNA and yielded multiple distinct DNA products. The ERIC-, BOX-PCR, and RISA yielded five to more than 10 distinct PCR products. Common bands (PCR products of analogous mobility) could be identified between the isolates. The ERIC-PCR patterns were found to be highly similar with almost no polymorphic bands. Due to these similarities the ERIC-PCR profiles and its dendogram was not included in this report. The RISA analysis revealed a relatively simple pattern for all isolates (Fig. 1). However, the BOX-PCR analysis revealed clear differences for all *X. c.* pv. *mangiferaeindicae* isolates (Fig. 2).

For RISA at 75% similarity, isolates could be grouped into two clusters (Fig. 1), designated cluster I and II. Cluster I could be divided into two groups at 86% similarity, namely Ia and Ib. Group Ia has 22 isolates; 12 isolates from Bavaria, eight from Constantia and two from Zebediela Estates. Group 1a could be separated into two sub-groups at 87 % similarity. The first sub-group i consisted of three isolates of which two were from Bavaria and one from Constantia Estates. The second sub-group ii consisted of 19 isolates of which 11 isolates were isolated from Bavaria, six from Constantia and two from Zebediela Estates. Group Ib consisted of only one isolate, obtained from Bavaria Estate.

Cluster II could be divided into two groups at a 87% similarity, namely group IIa and IIb. Group IIa had two isolates from Constantia and Zebediela Estates which separated at 91% similarity. Group IIb could be separated into two sub-groups at a 98% similarity. The first sub-group i consisted of two

isolates, one from Bavaria and the second from Constantia Estates. The second sub-group ii consisted of only one isolate originally obtained from stem canker symptoms at Bavaria Estate.

For BOX-PCR (Fig. 2) at 69% similarity, isolates could be grouped into two clusters, designated cluster I and cluster II. Cluster I could be divided into two groups at a similarity of 72%, namely group Ia and Ib. Group Ia consisted of the two *Erwinia* outgroups. Group Ib consisted of 13 isolates and could be separated into two sub-groups at a similarity of 94%. The first sub-group i consisted of six isolates of which four were from Bavaria, one from Constantia and one from Zebediela Estates. The second sub-group ii consisted of seven isolates, of which three were from Bavaria, three from Constantia and one from Zebediela Estates.

Cluster II could be divided into two groups at a 75.5% similarity, namely group IIa and group IIb. Group IIa consisted of 15 isolates and could be separated into two sub-groups at a similarity of 85.5%. The first sub-group ia consisted of six isolates, of which three were from Bavaria, two from Constantia and one from Zebediela Estates. The second sub-group iib consisted of nine isolates, of which five were from Bavaria and four from Constantia Estates.

The ERIC-, BOX-PCR, and RISA protocols provided similar data about the apparent relatedness amongst isolates. Each set of primers (BOX, and RISA) was effective in distinguishing different isolates.

DISCUSSION

Genomic fingerprinting using PCR-based techniques are sensitive, and relatively easy to use to identify relationships between *X. c.* pv. *mangiferaeindicae* isolates. In this study, it has been demonstrated that repetitive extragenic ERIC- and BOX-PCR elements and differences in 16S and 23S rDNA, are present in the genome of *X. c.* pv. *mangiferaeindicae* isolates. This confirms and extend the conclusion of Louws *et al.* (1994), and Pooler *et al.* (1996), that these sequences are virtually ubiquitous.

The results presented here also support the suggestion of Louws *et al.* (1994), that rep-PCR could be a useful tool for diagnostic purposes in plant pathology. These findings suggest that the genomic fingerprint patterns generated by ERIC-, and BOX-PCR, and RISA are stable over a distance. In this study it was found that isolates obtained from Constantia and Bavaria Estates (Appendix 4) have mostly similar fingerprint profiles. The BOX-PCR technique was able to discriminate among related but distinct bacterial isolates with sufficient resolution. This means that BOX-PCR is reliable in differentiating *X. c.* pv. *mangiferaeindicae* isolates.

It has been demonstrated that closely related isolates, as determined by protein profiles can have divergent PCR profiles and that bacteria associated with the same disease can easily be differentiated because of the complexity of DNA amplification products generated by using the three different primer sets. By using three different primer sets, a broader survey of the DNA structure was possible and more specific conclusions concerning diversity or similarity among isolates were achieved. The isolates from the same area were grouped together and the more resistant and virulent isolates tend to group together. No significant differences among the cultivars could be detected in the grouping of isolates. In the RISA dendogram, group Ia consisted mainly of isolates from Bavaria Estate that were grouped as being more virulent (Chapter 4). In the BOX-PCR dendogram group IIa consisted mainly of isolates previously shown to be virulent (Chapter 4) and ERIC-, BOX-PCR, and RISA suggested that *X. c.* pv. *mangiferaeindicae* isolates were closely related. The ERIC-PCR and RISA fingerprints of isolates of *X. c.* pv. *mangiferaeindicae* were not diverse, but analogous profiles were more obvious when BOX-PCR was used. No significant differences in lesion size could be detected among the cultivars evaluated.

In conclusion, it was found that ERIC-, BOX-PCR, and RISA techniques can be used in generating genomic fingerprints of isolates, facilitating molecular analysis. From these results it can be concluded that the copper resistant and virulent isolates tend to group together which shows that there is a link between these too.

REFERENCES

Benedict, A. A., Alvarez, A. M., Berestecky, J., Imanaka, W., Mizumoto, C. Y., Pollard, L. W., Mew, T. W. and Gonzalez, C. F. 1989. Pathovar specific monoclonal antibodies for *Xanthomonas campestris* pv. *oryzae* and for *Xanthomonas campestris* pv. *oryzicola*. *Phytopathology* 79: 322-328.

Benedict, A. A., Alvarez, A. M. and Pollard, L. W. 1990. Pathovar specific antigens of *Xanthomonas campestris* pv. *begoniae* and *Xanthomonas campestris* pv. *pelargonii* detected with monoclonal antibodies. *Applied and Environmental Microbiology* 56: 572-574.

Berthier, Y., Verdier, V., Guesdon, J., Chevrier, D., Denis, J., Decoux, G. and Lemattre, M. 1993. Characterization of *Xanthomonas campestris* pathovars by rRNA gene restriction patterns. *Applied and Environmental Microbiology* 59: 851-859.

Campbell, K. D., East, A. K., Thompson, D. E. and Collins, M. D. 1993. Studies on the large subunit ribosomal RNA genes and intergenic spacer regions of non-proteolytic *Clostridium botulinum* types B, E and E. *Research in Microbiology* 144: 171-180.

De Bruijn, F. J. 1992. Use of repetitive (repetitive extrageneric palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology* 58: 2180-2187.

Farber, J. M. 1996. An introduction to the hows and whys of molecular typing. *Journal of Food Protection* 59: 1091-1101.

Fischer, M. M. and Triplett, E. W. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to fresh water bacterial communities. *Applied and Environmental Microbiology* 65: 4630-4636.

Hildebrand, D. C., Palleroni, N. J. and Schroth, M. N. 1990. Deoxyribonucleic acid relatedness of 24 xanthomonad strains representing 23 *Xanthomonas campestris* pathovars and *Xanthomonas fragariae*. *Journal of Applied Bacteriology* 68: 263-269.

Hulton, C. S. J., Higgins, C. F. and Sharp, P. M. 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other enteric bacteria. *Molecular Microbiology* 5: 825-834.

Jensen, M. A., Webster, J. A. and Strauss, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Applied and Environmental Microbiology* 59: 945-952.

Krawiec, S. and Riley, M. 1990. Organisation of the bacterial chromosome. *Microbiological Reviews* 54: 502-539.

Lazo, G. R. and Gabriel, D. W. 1987. Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris*. *Phytopathology* 77: 448-453.

Louws, F. J., Fulbright, D. W., Stephens, C. T. and De Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains

generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* 60: 2286-2295.

Maniatis, T., Fritsch, E. F. and Sambrook, J. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory. Cold Spring Harbour.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbech, R., Morrison, D. A., Boulnois, G. J. and Claverys, J. P. 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research* 20: 3479-3483.

Mc Manus, P. S. and Jones, A. L. 1996. Genetic fingerprinting of *Erwinia amylova* by repetitive sequence-PCR and PCR-ribotyping. *Acta Horticulturae* 411: 281-283.

Pooler, M. R., Ritchie, D. F. and Hartung, J. S. 1996. Genetic relationships among strains of *Xanthomonas frariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Applied and Environmental Microbiology* 62: 3121-3127.

Pruvost, O., Couteau, A. and Luisetti, J. 1998. Phenotypic diversity of *Xanthomonas* sp. *mangiferaeindicae*. *Journal of Applied Microbiology* 84: 115-124.

Sharples, G. J. and Lloyd, R. G. 1990. A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. *Nucleic Acids Research* 18: 6503-6508.

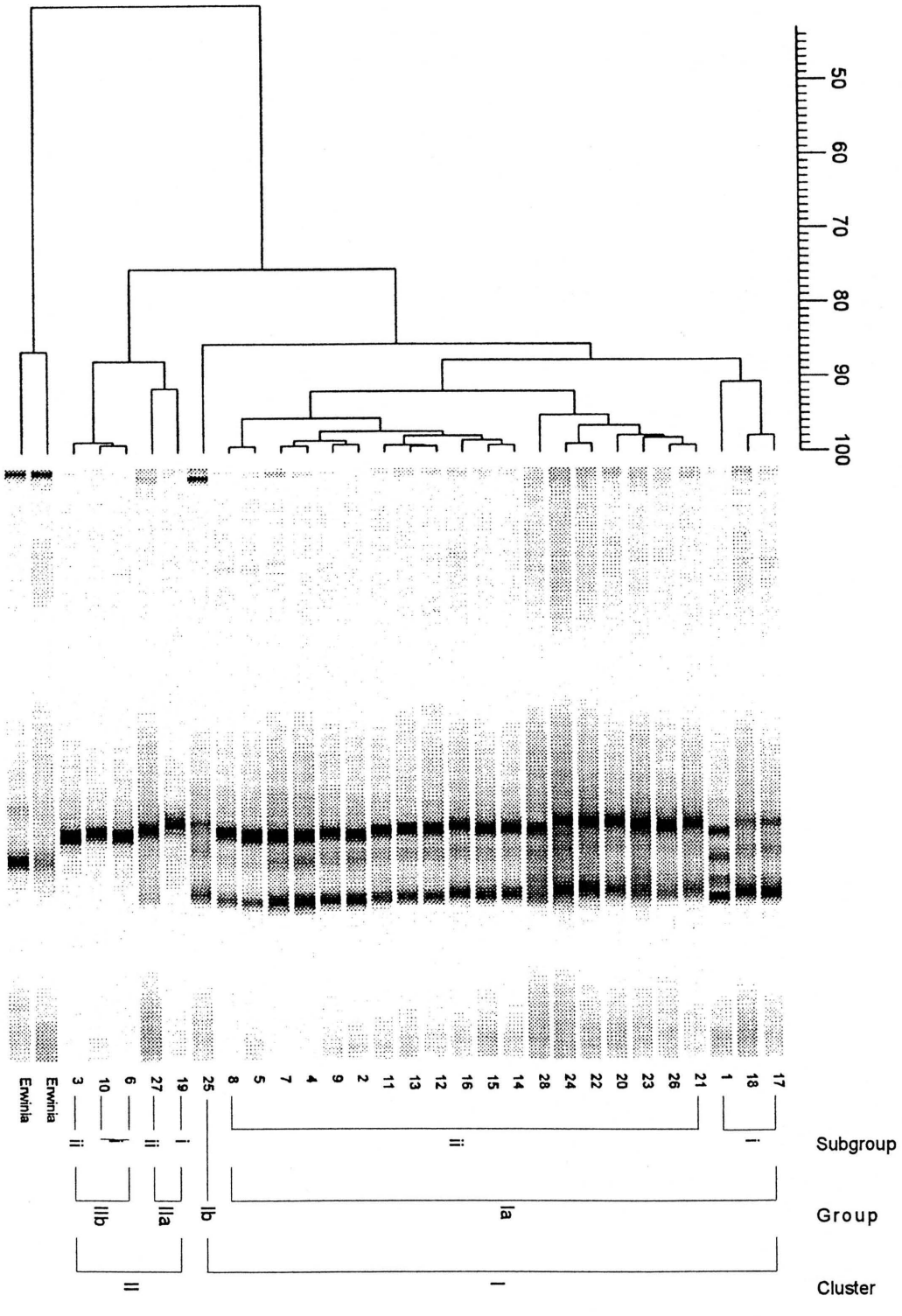
Stead, D. E. 1992. Grouping of plant-pathogenic and other *Pseudomonas* species by using cellular fatty acid profiles. *International Journal of Systematic Bacteriology* 42: 281-295.

Stern, M. J., Ames, G. F.-L., Smith, N. H., Robinson, E. C. and Higgins, C. F. 1984. Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* 37: 1015-1026.

Van Zyl, E. and Steyn, P. L. 1990. Differentiation of phytopathogenic *Pseudomonas* and *Xanthomonas* species and pathovars by numerical taxonomy and protein gel electrophoregrams. *Systematic and Applied Microbiology* 13: 60-71.

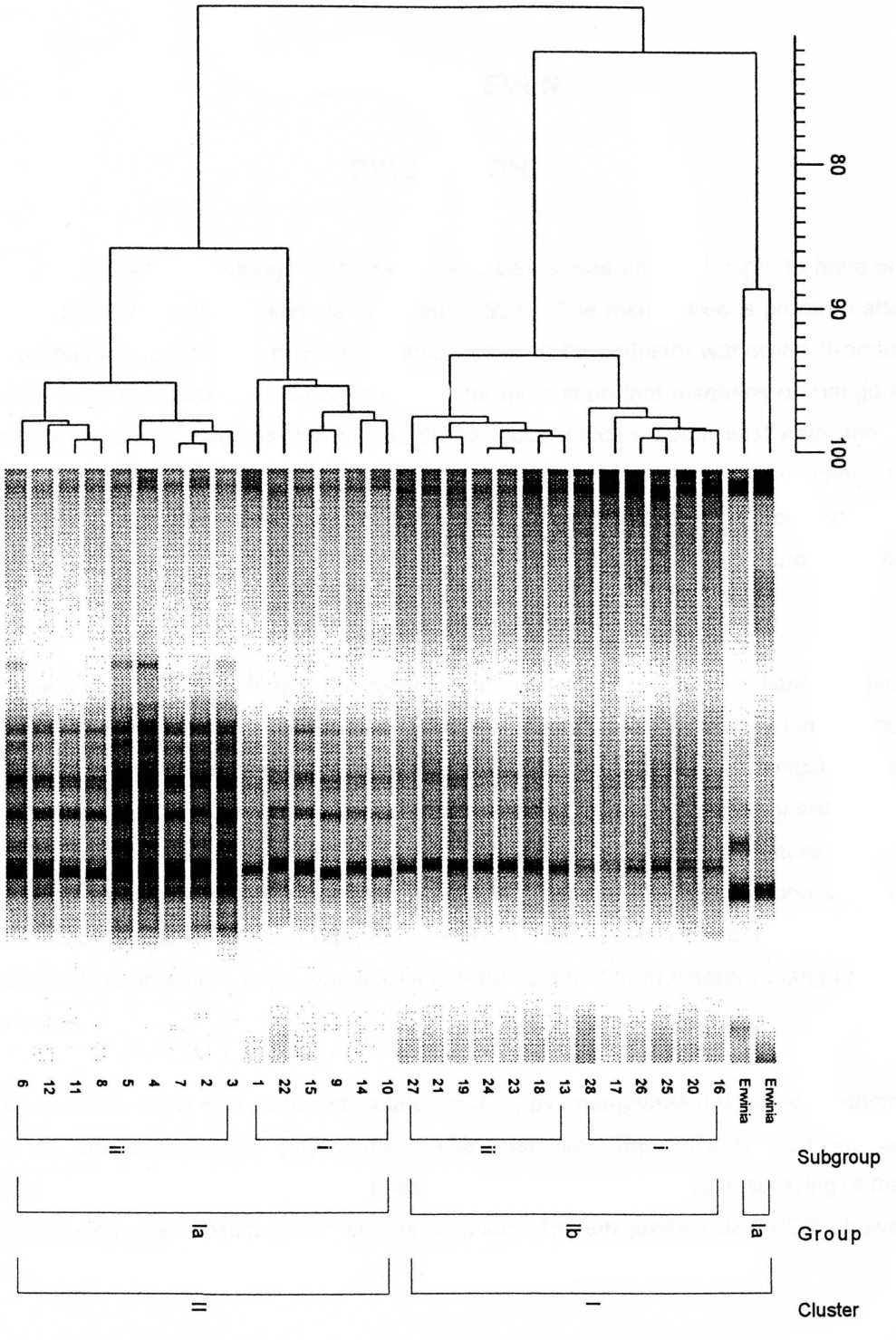
Vauterin, L., Swings, J. and Kersters, K. 1991. Grouping *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. *General Microbiology* 137: 1677-1687.

Vauterin, L., Yang, P., Hoste, B., Pot, B., Swings, J. and Kersters, K. 1992. Taxonomy of the xanthomonads from cereals and grasses based on SDS-PAGE of proteins, fatty acid analysis and DNA hybridization. *Journal of General Microbiology* 138: 1467-1477.



The x-axis represents the correlation (r) between the isolates

Fig. 1 RISA patterns and dendogram showing relationships between 28 isolates of *Xanthomonas campestris* pv. *mangiferaeindicae* (Appendix 4).



The x-axis represents the correlation (r) between the isolates

Fig. 2 BOX-PCR patterns and dendogram showing relationships between 28 isolates of *Xanthomonas campestris* pv. *mangiferaeindicae* (Appendix 4).

CHAPTER SEVEN

DISCUSSION

Mango (*Mangifera indica* L.) belongs to the family *Anarcardiaceae* and is thought to have originated from the India/Burnese border (Nakasone and Paull, 1998). The mango tree is prone to attack by a number of pathogens resulting in major economic losses to the industry worldwide (Korsten *et al.*, 1992). Today bacterial black spot (BBS) is one of the most important diseases of mango in South Africa and other countries such as the Islands of the Indian Ocean, south east Asia and Australia (Korsten *et al.*, 1992). The causal organism of BBS is *Xanthomonas campestris* pv. M*angiferaeindicae*, a Gram negative bacterium which can also infect other hosts such as *Anacardiaceae* i.e. cashew (*Anacardium occidentale*, L.) and imra, or emrah (*Spondias mangifera* Wild = *S. pinnata* (I. F.) Kurz) (Patel *et al.*, 1948).

In 1996, Some and Samson evaluated relationships among strains of *X. c.* pv. *mangiferaeindicae* by using isoenzyme analysis. Their results showed that the strains could be classified into eight groups. Gagnevin *et al.* (1997) compared genetic diversity of strains of *X. c.* pv. *mangiferaeindicae* by restriction fragment length polymorphism (RFLP) analysis and obtained groups consistent with those described by Some and Samson (1996). However, comparative studies of this nature have not been conducted in South Africa and detailed characterisation of genetic variability among strains of *X. c.* pv. *mangiferaeindicae* have not been reported. In view of the above, this study was undertaken to compare different isolates of *X. c.* pv. *mangiferaeindicae* obtained from different mango cultivars and production areas.

The aim of the of this study was achieved isolates of *X. c.* pv. *mangiferaeindicae* were compared on the bases of biochemical and physiological characteristics, the isolates had an ununiform characteristic of hydrolyzing starch. The isolates from Estates with continuous spraying of the copper compounds showed more resistance to copper solution. Protein profiles and PCR showed a link between copper resistant and virulent isolates, they grouped together.

The pathogen *X. c.* pv. *mangiferaeindicae* was successfully isolated from symptomatic leaf, fruit and stem tissues. All isolates were apigmented. The randomly selected isolates (32) fitted the *X. c.* pv. *mangiferaeindicae* profile according to standard physiological and biochemical tests. All isolates hydrolysed starch. This is a characteristic that is not present in all *Xanthomonas* spp. but only in 75 % of the *X. c.* pv. *mangiferaeindicae* strains (Swings *et al.*, 1993). The results presented here showed that all isolates studied hydrolysed Tween-80, which is in accordance with the findings of Van Den

Mooter and Swings (1990). This study also confirmed that isolates obtained from two different estates in South Africa have the same phenotypic characteristics, as previously reported (Sanders, 1993).

When mango trees were inoculated in the Greenhouse with different isolates, not all inoculation sites developed typical disease symptoms. The results of this study indicated that isolate BKF4, CIL3, CIF4 and BKL5 were the most virulent while isolate BKS3 was the least virulent. In contrast to the report by Manicom (1980), these results showed that *X. c.* pv. *mangiferaeindicae* exists as strains differing in virulence which was in accordance with the work of Wachters *et al.* (1991) and Sanders (1993).

Copper sulphate solutions were effective in eradicating some of the pathogenic isolates when tested *in-vitro*. Copper-sensitive and copper-resistant isolates of *X. c.* pv. *mangiferaeindicae* were obtained from both Bavaria (12 sensitive and five resistant isolates), and Constantia (10 sensitive and five resistant isolates) and from comparative isolates from Zebediela Estate (one sensitive and two resistant isolates). Cluster analysis of copper-resistance data and SDS-PAGE of soluble proteins clearly showed that the isolates were related to each other. No significant differences were observed between the isolates both with the virulence and copper-resistance tests. The correlation was low between copper-resistance and virulence meaning that the virulence factor was not necessarily related to copper resistance. With copper-resistance, correlation was found on the basis of origin, but no correlation was found on the basis of plant material and cultivar from which the isolates were obtained. However, there was a strong correlation between production areas and virulence when using the virulence data, leading to speculation that the development of a virulence factor can possibly be related to certain production areas. No significant differences in lesion size could be detected among the cultivars evaluated.

On comparison of current and previous chemical spray programs from the different Estates included in this investigation it was evident that Constantia Estate sprayed less copper over the years which reflected the lower level of resistance detected in isolates from that Estate compared to Bavaria Estate. Seven isolates from Bavaria were more resistant compared to four isolates from Constantia that showed resistance. The majority of isolates that were more virulent and more copper resistant tended to cluster together. All isolates grouped together at 67.5 %, which confirmed that they belong to one species. According to the results obtained in this study *X. c.* pv. *mangiferaeindicae* is remarkably distinct and a homogeneous pathovar. The SDS-PAGE database of whole-cell protein patterns used under highly standardised conditions can be of great value to identify new and unknown *Xanthomonas* strains.

It was demonstrated that repetitive extragenic elements such as ERIC, BOX, and differences of the 16S and 23S rDNA are present in the genome of *X. c.* pv. *mangiferaeindicae* isolates. This confirms and extends the conclusion of Louws *et al.* (1994), and Pooler *et al.* (1996), that these sequences are virtually ubiquitous. From the results obtained with the BOX-PCR, and RISA it can be deducted that *X. c.* pv. *mangiferaeindicae* isolates are from one pathovar. The RISA fingerprints of isolates of *X. c.* pv. *mangiferaeindicae* did show differences, but analogous profiles were more obvious when BOX-PCR was used. In BOX-PCR all isolates clustered together at 70% and in RISA they clustered together at 75%. In this study, it was shown that it is not necessary to use purified genomic DNA template for ERIC-, BOX-PCR, and RISA. It was also found that bacterial cells derived from liquid cultures could directly be used for PCR tests instead of purified DNA template. These techniques can in future be used to generate genomic fingerprints of isolates, facilitating molecular analysis.

When comparing the patterns obtained from SDS-PAGE and those obtained from PCR and RISA, isolates clustered in almost the same groups when using the different techniques. However, isolates CIL1 and CKL2 which have been shown to be closely related in SDS-PAGE, were not closely related in PCR and RISA. These isolates were clustered in two different groups in PCR and RISA. This proves that PCR and RISA techniques are more sensitive and informative than SDS-PAGE in differentiating between closely related isolates.

In conclusion, it has been demonstrated that closely related isolates, as demonstrated with the protein profiles can have divergent PCR profiles. Bacteria associated with the same disease can easily be differentiated because of the complexity of DNA amplification products generated by using three different primer sets. Future studies should focus on determining the presence of plasmids in *X. c.* pv. *mangiferaeindicae* and the possible link between copper-resistance and enhanced virulence.

REFERENCES

Gagnevin, L., Leach, J. E. and Pruvost, O. 1997. Genomic variability of *Xanthomonas* pathovar *mangiferaeindicae*, agent of mango bacterial black spot. *Applied and Environmental Microbiology* 63: 246-253.

Korsten, L., Lonsdale, J. H., de Villiers, E. E. and de Jager, E. S. 1992. Preharvest biological control of mango diseases. *South African Mango Growers' Association Yearbook* 12: 72-78.

Louws, F. J., Fulbright, D. W., Stephens, C. T. and de Bruin, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains

generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* 60: 2286-2295.

Manicom, B. Q. 1980. Studies on bacterial black spot. M. Sc. Thesis, University of Natal, Pietermaritzburg.

Nakasone, H. Y. and Paull, R. E. 1998. Mango. Pages 208-238. In: Tropical Fruits. CAB International, UK.

Patel, M. K., Kulkarni, Y. S. and Moniz, L. 1948. *Pseudomonas mangifera-indicae*, pathogenic on mango. *Indian Phytopathology* 1: 147-152.

Pooler, M. R., Ritchie, D. F. and Hartung, J. S. 1996. Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Applied and Environmental Microbiology* 62: 3421-3427.

Sanders, G. M. 1993. Detection of *Xanthomonas campestris* pv. *mangiferaeindicae* in mango plants. M.Sc. Thesis. University of Pretoria, Pretoria.

Some, A. and Samson, R. 1996. Isoenzyme diversity in *Xanthomonas campestris* pv. *mangiferaeindicae. Plant Pathology* 45: 426-431.

Swings, J., Vauterin, L. and Kersters, K. 1993. The bacterium *Xanthomonas.* Pages. 121-156. In: Swings, J. G. and Civerolo, E. L. (eds). *Xanthomonas.* Chapman & Hall, London.

Van Den Mooter, M. and Swings, J. 1990. Numerical analysis of 295 phenotypic features of 266 *Xanthomonas* strains and related strains and an improved taxonomy of the genus. *International Journal of Systematic Bacteriology* 40: 348-369.

Wachters, A. E., Korsten, L. and Kotzé, J. M. 1991. Systemicity and variation in pathogenicity between strains of *Xanthomonas campestris* pv. *mangiferaeindicae*. *South African Mango Growers' Association Yearbook* 11: 22-24.

SUMMARY

COMPARISON OF *XANTHOMONAS CAMPESTRIS* PV. *MANGIFERAEINDICAE* FROM MANGO PRODUCING AREAS IN SOUTH AFRICA

PROMOTER : PROF. L. KORSTEN
CO-PROMOTER: DR. G. M. SWART

Xanthomonas campestris pv. *mangiferaeindicae*, the bacterial black spot pathogen of mango isolated from two different production areas in South Africa were compared using phenotypic characteristics. Thirty-two representative pathogenic isolates were selected and compared to assess variability within the taxon. Comparison of isolates was based on: biochemical and physiological characteristics, virulence and copper-resistance, protein profiles and polymerase chain reaction. Analysis of data obtained was consistent with those reported in other phenotypic diversity studies. All isolates were apigmented. Significant differences in disease severity between isolates were observed after six to eight weeks. Some isolates were sensitive and others showed resistance to copper sulphate in *in-vitro* tests. Some of the most virulent isolates were also more resistant to copper sulphate. Based on the SDS-PAGE of soluble proteins, groups contained isolates predominantly from a specific production area. Isolates that were more virulent and more copper resistant tended to cluster together. According to the ERIC-, BOX-PCR, and RISA analysis, isolates of *X. c.* pv. *mangiferaeindicae* were closely related. This study has demonstrated that PCR and RISA analysis are more sensitive than SDS-PAGE to differentiate closely related isolates and that there is a correlation between virulence and copper-resistance. Future, studies should focus on the presence and role of *X. c.* pv. *mangiferaeidicae* plasmids and its possible role in copper-resistance and virulence.

Appendix 1 *Xanthomonas campestris* pv. *mangiferaeindicae* isolates used in hierachical comparison of percentage virulence

Isolate code	Number[a]
Control	C_1
CKL1	C_2
CKL3	C_3
CKL5	C_4
BKF4	C_5
BIL1	C_6
CIL4	C_7
CIF5	C_8
CIF2	C_9
BIL2	C_10
CIF1	C_11
CKL2	C_12
CIL3	C_13
BKF1	C_14
BIL3	C_15
CIL2	C_16
BKF3	C_17
CIL1	C_18
CIF4	C_19
CIL5	C_20
CIF3	C_21
BKF3	C_22
CKL4	C_23
BKF2	C_24
BKL2	C_25
BKL1	C_26
BKS3	C_27
BKS4	C_28
BKS1	C_29
BKL3	C-30
BKL4	C_31
BKL5	C_32

Codes for strains: BIL - Bavaria Keitt cv. leaves; BKF - Bavaria Kent cv. fruit; BKL - Bavaria Kent cv. leaves; BKS - Bavaria Kent cv. Stem Canker; CIF - Constantia Keitt cv. fruit, CIL - Constantia Keitt cv. leaves, CKL - Constantia Kent cv. leaves.

[a] - represents the number designating a particular isolate code in Ward's method for percentage virulence.

Appendix 2 *Xanthomonas campestris* pv. *mangiferaeindicae* isolates used in hierachical comparison of copper resistance

Isolate code	Number[a]
Control	C_1
CIF2	C_2
CKL4	C_3
BKF4	C_4
BKL1	C_5
BKL4	C_6
BKF1	C_7
CKL4	C_8
CIL4	C_9
1F5	C_10
1F2	C_11
CIL1	C_12
BKS2	C_13
CIF3	C_14
BKF2	C_15
BKS1	C_16
BKL5	C_17
BIL1	C_18
BKS3	C_19
CIL2	C_20
CIF5	C_21
CKL5	C_22
BKF3	C_23
BKS4	C_24
BKL2	C_25
BKF3	C_26
BKL3	C_27
BIL3	C_28
CKL3	C_29
CKL1	C-30
CIF1	C_31
CKL1	C_32
CIF1	C_33
CKL2	C_34
CIL3	C_35
CIL5	C_35

Codes for strains: BIL - Bavaria Keitt cv. leaves; BKF - Bavaria Kent cv. fruit; BKL - Bavaria Kent cv. leaves; BKS - Bavaria Kent cv. Stem Canker; CIF - Constantia Keitt cv. fruit, CIL - Constantia Keitt cv. leaves, CKL - Constantia Kent cv. leaves; all 1F - Zebediela Estates Sensation cv. fruit

[a] - represents the number designating a particular isolate code in the Ward's method for copper resistance.

Appendix 3 *Xanthomonas campestris* pv. *mangiferaeindicae* isolates used in SDS-PAGE

Isolate code	SDS-PAGE number[a]
CIL5	1
CIL1	2
CKL5	3
CKL2	4
CKL1	5
BKS2	6
BKL5	7
BKL3	8
BKL2	9
BKF5	10
BKF3	11
BKF2	12
1F5	13
1F2	14
1F7	15
BKS4	16
BIL3	18
CKL4	19
CIL4	20
CIF3	21
CIF1	22
CIL3	23
CIF2	24
BKF1	25
BKF4	26
BKS3	27
BIL1	28

Codes for strains: BIL - Bavaria Keitt cv. leaves; BKF - Bavaria Kent cv. fruit; BKL - Bavaria Kent cv. leaves; BKS - Bavaria Kent cv. Stem Canker; CIF - Constantia Keitt cv. fruit, CIL - Constantia Keitt cv. leaves, CKL - Constantia Kent cv. leaves; all 1F - Zebediela Estates Sensation cv. fruit

[a] - represents the number designating a particular isolate code in the SDS-PAGE dendogram.

Appendix 4 *Xanthomonas campestris* pv. *mangiferaeindicae* isolates used in PCR and RISA

Isolate code	PCR and RISA number[a]
CKL2	1
BKL4	2
BKF4	3
CIF3	4
CIF1	5
BKL5	6
BKL2	7
BKL1	8
BKL3	9
CIF2	10
CKL1	11
CKL4	12
BKF2	13
BIL3	14
1F5	15
BIL1	16
BKF5	17
BKF3	18
CIL1	19
CIL5	20
CIL3	21
BKF1	22
CIL4	23
BKS2	24
BKS3	25
BKS4	26
IF2	27
IF7	28

Codes for strains: BIL - Bavaria Keitt cv. leaves; BKF - Bavaria Kent cv. fruit; BKL - Bavaria Kent cv. leaves; BKS - Bavaria Kent cv. Stem Canker; CIF - Constantia Keitt cv. fruit, CIL - Constantia Keitt cv. leaves, CKL - Constantia Kent cv. leaves; all 1F - Zebediela Estates Sensation cv. fruit

[a] - represents the number designating a particular isolate code in the dendogram for the PCR and RISA.