Developing a sensitive, high-throughput tool for rapid detection of agronomically important seed-borne pathogens of tomato

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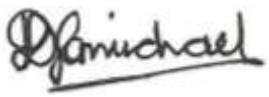
I declare that the content presented in this dissertation is my own, unaided work. This report is being submitted for the degree of Master of Science to the University of Witwatersrand, Johannesburg. It has not been submitted previously to any other institution or in fulfilment of another degree.

Signed:



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Abstract

The limited specificity, sensitivity and multiplex capacity of detection techniques currently available for important seed-borne pathogens of tomato is a significant risk for the global tomato trade and production industry. These pathogens can be associated with seed at low concentrations but, due to their highly virulent nature, these low levels can be sufficient to infect germinating seedlings and spread to neighbouring plants and fields, potentially causing epidemics and economic losses. In this study, detection techniques currently available for phytodiagnostics were evaluated for the capacity to accurately detect and identify five agronomically important seed-borne pathogens of tomato: *Pepino mosaic virus* (PepMV), *Tomato mosaic virus* (ToMV), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato*. A prototype diagnostic microarray was also designed in an attempt to develop a tool that could simultaneously detect these five seed-borne pathogens from a single sample. Viral detection based on serological techniques was rapid, accurate and reliable but only detected a single pathogen per assay and required supplementary bioassays to indicate the viability of detected viral pathogens. Selective media plating for bacterial detection demonstrated unreliable recovery of targeted bacteria from infected seed and leaf samples and required supplementary tests to validate the identity of presumptive positives. Assays were lengthy, laborious and sometimes too ambiguous for accurate diagnosis of bacterial pathogens. Nucleic acid-based technologies demonstrated improved sensitivity and specificity for detection of targets from pure culture, leaf and seed extracts, compared to conventional and serological methods, yet also required supplementary bioassays or media assays to validate the viability of detected pathogens. Amplification efficiency however, was affected by the presence of PCR inhibitors and despite positive detection, variable banding intensity in electrophoretic analysis of amplified products necessitated the use of reference cultures to validate diagnosis. The developed microarray incorporated 152 pathogen-specific and control probes to facilitate diagnosis and taxonomic classification of detected pathogens. The array was challenged with pure culture extracts of the five target pathogens, selected related and non-target, unrelated pathogens of tomato. Positive detection of each of the pathogens was demonstrated but the production of hybridisation signals was highly variable and extremely sensitive to minor technical differences. Each of the five pathogens were successfully detected in combination proving that different classes of seed-borne pathogens could be detected from a single sample using the developed microarray. This prototype microarray has good potential for phytodiagnostic screening of the five targeted pathogens, and further validation, optimisation and extension for testing tomato seed samples may facilitate incorporation of this array into standard diagnostic protocols.

Research Outputs

Publications

Carmichael, D. J., Rey, M. E. C., Naidoo, S., Cook, G., and Van Heerden, S.W. (2011). First Report of *Pepino mosaic virus* Infecting Tomato in South Africa. Plant Disease *95*, 767.

Conference contributions

Carmichael, D. J. (2011). Poster: First report of *Pepino mosaic virus* infecting Tomato in South Africa. *South African Society for Plant Pathology Bienniel Conference,* 23 January, Mpumalanga, South Africa.

Carmichael, D. J. (2010) Poster: Developing a sensitive, high-throughput tool for rapid detection of agronomically important seed-borne pathogens of tomato. *University of Witwatersrand: Postgraduate Symposium*, 26 October, Gauteng, South Africa.

This dissertation is dedicated to my parents, and my fiancé, Dario Matteucci, whose unfailing love, support and encouragement inspired me to complete this work and persevere despite impossible challenges. To my loving and supportive siblings, Timothy, Jennifer and Susan.

‘When you walk through the waters, I will be with you. And through the rivers, they will not overwhelm you. When you walk through fire, you will not be burned; and the flame will not consume you. For I am the Lord, your God, the Holy One of Israel, your Saviour.’

Isaiah 43:2-4

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Contents

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Abbreviations

β-ME – Beta-Mercaptothanol

Aa-dUTP – Amino-allyl deoxyuridine triphosphate

Ab – Antibody

ARC – Agricultural Research Council

Amp - Ampicillin

BLAST – Basic Local Alignment Search Tool

BLASTN – Standard nucleotide BLAST

Bp – Base-pair

BSA – Bovine Serum Albumin

cDNA – Complimentary DNA

Cfu/mℓ - Colony forming units per millilitre

CH1/2 – Chilean isolates of PepMV

Cmm – *Clavibacter michiganensis* subsp. *michiganensis*

CP – Capsid/Coat protein

CTAB - Hexadecyltrimethyl ammonium bromide

Cv. – Cultivar

CV – Co-efficient of variation

Cy3/Cy5 – Cyanine dye

CymMV - *Cymbidium mosaic virus*

DAS-ELISA – Double-Antibody Sandwich ELISA

DH_2O – Distilled water

DIG – Digoxygenin

DMSO – Dimethyl Sulfoxide

dNTP – Deoxyribonucleotide triphosphate

Dpi – Days post inoculation/infection

DsDNA – double-stranded DNA

EACMV – *East African cassava mosaic virus*

ELISA – Enzyme-linked Immunosorbent Assay

EM – Electron microscopy

EPS - Exopolysaccharide

EPPO – European and Mediterranean Plant Protection Organisation

EtBr – Ethidium Bromide

EtOH – Ethanol

EU – European Union/ European isolate of PepMV

FDR – False discovery rate

FRET – Fluorescent resonance energy transfer probes

ΔG – Gibb's free enery

GAL – Gene Array List

GAPS – Gamma Amino Propyl Silane

GUS – Beta-glucoronidase

GyrB – DNA gyrase subunit B

Hrp – Hypersensitive reponse/pathogenicity gene

IC-PCR – Immunocapture PCR

IEM – Immunosorbent electron microscopy

IF – Immunofluorescence

IgG – Immunoglobulin G

IMB – Immunomagnetic beads

IMS – Immunomagnetic separation

IPTG - Isopropyl-β-thio-galactopyranoside

ISF – International Seed Federation

ISTA – International Seed Testing Association

ITS – Intergenic transcribed sequence

KB – King's B media

KBC – King's B media with cycloheximide and cephalexin

LATE-PCR – Linear after exponential PCR

LB broth – Luria-Bertani broth

LD – Ligation-dependent array system

LNA – Locked nucleic acid

LP – Peruvian genotype of PepMV

Min – minutes

MCS – multiple cloning site

MP – Maximum parsimony phylogenetic tree/movement protein

MSA – Multiple sequence alignment

MT – Milk-tween agar

MUSCLE – Multiple Sequence Comparison by Log Expectation

NA – nutrient agar

NB – nutrient broth

NMV - *Narcissus mosaic virus*

Nt - nucleotide

ORF – Open reading frame

PBS – Phosphate buffered saline

P-value – Probability value

PBS – Phosphate buffered saline

PBST – Phosphate buffered saline with Tween 20

PCR – Polymerase chain reaction

PdN9 – Random nonamer primer

PepMV – *Pepino mosaic virus*

PLP – Padlock probe

PMMoV – *Pepper Mild Mottle Virus*

PMT – Photomultiplier tool power

PNP - p-Nitrophenyl phosphate substrate

Poly(A) tail – 3’ poly-adenaline tail

Propn - proportion

Pss - *Pseudomonas syringae* pv. *syringae*

Pst – *Pseudomonas syringae* pv. *tomato*

PTA-ELISA – Plate-trapped antigen ELISA

Pv. – Pathovar

PVX - *Potato virus x*

rDNA/RNA – Ribosomal DNA/RNA

RdRp – RNA-dependent RNA polymerase

Rep - Replicase

Rep-PCR – repetitive-sequence-based PCR

RFLP – Random fragment length polymorphism

Rpm – Revolutions per minute

RpoB – RNA polymerase subunit B

RpoD – sigma factor 70 for RNA polymerase

RT-PCR – reverse-transcription PCR

SA – South African PepMV isolate

SACC – South African Culture Collection

SDS - Sodium Dodecyl Sulfate

SEB – Seed extraction buffer

SI – Similarity index

SNP – Single nucleotide polymorphism

SNR – Signal to noise ratio

SPIEM – Solid phase IEM

SPS – Sanitary and Phytosanitary agreement

SSC - Saline-sodium Citrate buffer

Subsp. - Subspecies

SVR – Sakata Vegenetics RSA (Pty) Ltd.

T_a – Annealing temperature

T_m – Melting temperature

TE – Tris-EDTA

TGA – Tryptone glucose agar

TGB – Triple gene block

THR – Tobacco hypersensitive response assay

TMV – *Tobacco mosaic virus*

ToMV – *Tomato mosaic virus*

TTSS – Type three secretion system

TZC – Tetrazolium chloride media

US1/2 – United States' isolates of PepMV

UTR – Untranslated region

Xcv – *Xanthomonas campestris* pv. *vesicatoria*

X-gal - 5-bromo-4-chloro-indoyl-β-D-galactopyranoside

Xv – *Xanthomonas vesicatoria*

Rationale and Research Question

Tomato is one of the most important vegetable crops globally, with an annual market value of over 55 billion US Dollars (FAOSTAT, 2009). The high value of tomato can be attributed to various uses of the fruit for fresh consumption and processing into pastes, sauces, powder or soup. For producers, the tomato is highly attractive for cultivation as it is a low cost, short-duration crop (Naika et al., 2005), produces high yields and can be grown year round in greenhouses or in fields of countries with warmer climates. Global cultivation of tomato has thus rapidly increased in the last 100 years to yield on average, 152 million metric tons of tomato fruit per year. Major producers include the USA and China, though five European countries appear in the top 20 global producers. In South Africa, tomato is considered one of the most important vegetables along with potato, onion and cabbage, with an annual production of over 530 000 metric tons (FAOSTAT, 2009). Grown mainly by subsistence farmers, the majority of tomato trade in South Africa occurs in the informal sector, although a third of the total national production is supplied by the tomato-processing industry.

To obtain maximum yields from sown tomato seed, farmers utilise hybrid seeds which have been specially bred using marker-assisted selection to demonstrate higher nutritional content, higher yield, longer shelf life, improved tolerance to abiotic stresses and improved resistance to diseases and pests (Bai and Lindhout, 2007; Barone and Frusciante, 2007). Over 200 diseases have been reported to occur on tomato which are caused by a range of nematodes, fungi, bacteria, viruses, viroids and phytoplasmas (Jones et al., 1997; Lukyanenko, 1991). These drastically affect the growth and yield of cultivated tomato, translating into significant losses in the tomato industry. Production areas should be monitored continuously for symptom development on plants (Njambere et al., 2011). Once identified, rapid implementation of control measures for a detected pathogen may reduce or eliminate its impact on neighbouring plants or fields.

The growing demand for tomato fruit on a global scale has led to an exponential increase in international trade of hybrid seed and seedlings to produce fruit yields of high quality and quantity within a short period of time. The high volumes of seed traded, however, has facilitated cross-border transport of numerous seed-borne pathogens. These can pass undetected between countries and continents, causing severe outbreaks and epidemics in destination countries, that can result in mass spoilage and/or loss of fruit yields and substantial economic losses (Da Silva et al., 2008; Hanssen et al., 2010a). Of the many seed-borne pathogens that have been reported to infect tomato, five pathogens have been identified that are of high risk to the global tomato industry owing to their

destructive impact on fruit yields and value (Abdalla, 2000; Cuppels et al., 2006; EPPO/CABI, 2005; Hadas et al., 2004; Hanssen and Thomma, 2010; Hanssen et al., 2010a; Kritzman, 1991; Ozdemir, 2009). These include: *Pepino mosaic virus*, *Tomato mosaic virus*, *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato*.

Current control measures have proven to be insufficient to curb the impact and spread of these pathogens, and thus, strict phytosanitary standards have been implemented to prevent the trade of infected seed (Deyong et al., 2005; EPPO/CABI, 2005; Fanelli et al., 2007; GSPP, 2009; Nome et al., n.d.; Xu, 2010). Before trade is permitted, seed must be certified for the absence of all quarantine pathogens by internationally accepted detection methods. Protocols and reports, published by seed trade organisations, detail an appropriate diagnostic process for the detection of each target pathogen during screening of host plants or seed (EPPO/CABI, 2005; ISF, 2008a, 2009a, 2009b, 2009c). Protocols usually incorporate conventional detection techniques like selective media assays, pathogenicity tests and serological assays. These are characteristically inexpensive, technically simple and enable straightforward interpretation of results, making them suitable for routine testing in even poorly equipped facilities (Abdalla, 2000; Harris-Baldwin and Gudmestad, 1996; Hadas et al., 2004; Kritzman, 1991; Ward et al., 2004). Techniques included in standard detection protocols are modified and optimised to favour the recovery of the target pathogen from host samples and are extensively validated against relatives and non-target organisms that may be present in plant hosts.

Current seed-borne pathogen detection protocols demonstrate varying sensitivity and specificity for reliable identification of target pathogens (De Leon et al., 2008; Schaad and Frederick, 2002). Some processes are lengthy, often requiring a few weeks before the presence or absence of the target pathogen can be confirmed. Incorporation of molecular-based methods into standard protocols recently has drastically improved the specificity of pathogen detection as well as the labour and time required to process numerous samples (Asma, 2005; EPPO/CABI, 2005). Even these methods, however, are limited to detecting a single pathogen per assay, making screening of hybrid seed-lots for all potential biotic threats, expensive and arduous. Research into more sensitive, multiplex detection methods have reported improved diagnostic potential, but only array technology has shown superior high-throughput potential, with sensitive detection of different classes of pathogens (Wilson et al., 2002). Assays using arrays for the detection of either viral (Tiberini et al., 2010) or bacterial (Pelludat et al., 2009) seed-borne pathogens of tomato have been developed, but none combine detection of the most important pathogens of different classes. The development of a

broad spectrum, multiplex detection tool for viral and bacterial seed-borne pathogens, is thus, desperately lacking in the tomato industry.

The objective of the current study was, therefore, to develop an alternative detection technique, based on microarray technology, for simultaneous, high-throughput and sensitive detection of multiple viral and bacterial seed-borne pathogens of tomato. The specific aims were:

1. ***To evaluate the efficiency, accuracy and consistency of standard detection methods currently used for five viral and bacterial seed-borne pathogens of tomato***

   Many of these methods are used in standard detection protocols for seed-borne pathogens. To analyse the capacity of these methods for routine detection of the five selected pathogens, inoculated host plants and the seed were subjected to a specific diagnostic process incorporating the commonly used techniques for phytopathogen detection.
2. ***To detect target pathogens from infected host samples using an improved, molecular-based approach***

   For more specific and sensitive detection of seed-borne pathogens, we sought to improve previously developed diagnostic protocols based on conventional PCR. Assays were performed on pure cultures and infected leaf and seed samples using automated extraction systems and robust amplification methods. Assays were evaluated for improved diagnostic potential and discrimination capacity for reliable identification and classification of target pathogens.
3. ***To characterize an unknown isolate of Pepino mosaic virus detected from tomato production areas in South Africa***

   The presence of Pepino mosaic virus in tomato has previously not been reported in South Africa. Confirmation by conventional and serological methods proved that particles belonging to this species were detected from a leaf sample of a symptomatic plant isolated from the Mooketsi region of the Limpopo Province, South Africa. Sub-species characterization of this isolate was, therefore, necessary to determine the potential source and risk of spread of this infection.
4. ***To develop a diagnostic microarray for simultaneous detection and identification of five viral and bacterial seed-borne pathogens of tomato***

   Potential diagnostic regions of DNA or RNA for the five selected pathogens had to be identified from literature or sequence analysis to facilitate detection and taxonomic classification, and only those classified as unique and specific to each targeted pathogen, were used to develop novel diagnostic probes. Designed probes were compiled into a microarray and challenged using pure cultures of target pathogens and non-target relatives.

Chapter 1 Literature Review

1.1. Tomato, the plant

The tomato, *Solanum lycopersicum*, is an herbaceous, perennial plant forming part of the *Solanaceae* or nightshade family. Containing over 3000 species, this group is one of the top three most valuable plant families as it includes other agronomically important vegetables like potato, pepper, pepino, eggplant and the research model species, tobacco (Diez and Nuez, 2006; Mueller et al., 2005). Tomato plants grow in optimal conditions of cool, dry climates with temperatures between 21 and 24°C though plants have adapted to grow within a range of 10 to 38°C (Bai and Lindhout, 2007; Diez and Nuez, 2006; Naika et al., 2005). Plants reach up to 2 m, supported by strong taproots, and develop flowers in clusters at every three internodes. Tomato plants develop fruit, which are mainly red or yellow, of different shapes and sizes depending on the cultivar (Naika et al., 2005). Numerous cultivated varieties of tomato exist, and for cultivation, selection is based on various factors including disease resistance, harvest period, adaptability to climate conditions, yield and demand.

Tomato fruit boast a high nutritional value and, therefore, represent a major source of vitamins and minerals (Abushita et al., 1997; Diez and Nuez, 2006). Levels of nutrients in tomato and tomato-based products depend on the type of cultivated variety used, climatic conditions and the amount of processing involved which may make nutrients more or less available for metabolism. All cultivars produce fruit with moderate to high levels of ascorbic acid (vitamin C), tocopherols (vitamin E), phenolic compounds, chromium, folate, potassium and fibre which function in the prevention of chronic diseases or neutralisation of harmful compounds (Abushita et al., 1997; Frusciante et al., 2007; Giovannucci, 1999; Seybold et al., 2004). Tomatoes also contain a range of carotenoids, such as lycopene and β-carotene (pro-vitamin A), which play important roles as anti-oxidants, anti-mutagens and anti-carcinogens (Abushita et al., 1997; Frusciante et al., 2007; Giovannucci, 1999; Rao and Agarwal, 2000). Lycopene, responsible for the red pigment of mature tomato fruit, makes up 90% of the total carotenoid content of tomato. When consumed, this carotenoid functions in scavenging free radicals, protecting vital biomolecules and modulating cellular signalling or metabolic pathways (Abushita et al., 1997; Frusciante et al., 2007; Rao and Agarwal, 2000). Lycopene is directly involved in the prevention of cardiovascular diseases as well as prostate, lung and stomach cancers (Giovannucci, 1999; Rao and Agarwal, 2000).

The tomato plant originated in the tropical and subtropical climates of the South American Andean region (Bai and Lindhout, 2007; Diez and Nuez, 2006; Naika et al., 2005) and was introduced into Europe as an ornamental plant in the 16th century. Only at the end of the 17th century was its value in fresh fruit consumption realised, prompting cultivation of tomato plants. Domestification of tomato led to the development of various cultivars demonstrating higher yield and better fruit quality than their wild relatives (Bai and Lindhout, 2007). From Europe, the tomato was distributed to Africa, Asia and the New World by trading companies and explorers and today tomato is one of the top 10 most valuable agricultural commodities globally (FAOSTAT, 2009). Tomatoes form the basis of diets in many cultures and, thus, the fruit are constantly in demand. To obtain the maximum yield from cultivation and, therefore, meet demand, farmers and producers must decrease the impact of other factors affecting fruit yields such as abiotic and biotic stresses.

1.2. Pathogens of tomato

The increase in tomato cultivation in the last 100 years has exposed the number of pathogens capable of causing disease in tomatoes. Some of the pathogens causing the most significant impact on the tomato industry are listed in Table 1. 1. These pathogens drastically affect the yield of fruit from diseased tomato plants, in severe cases causing yield losses of 70 – 95% (Rude et al., 1982). The impact of pathogens depends on favourable environmental conditions as well as the genetic composition of both the pathogen population and host. The easy spread of pathogens is one of the major reasons for the destructive impact of biotic diseases on global yield. Fungi and bacteria can spread readily in soil (Agrois, 2005), or produce spores which may be transmitted by insects, wind or water. Many pathogens are spread via wind, rain water or irrigation systems and thereby colonise plantations, greenhouses and fields (Agrois, 2005; Rude et al., 1982). Viroid and viral pathogens are often spread via insect vectors, infected pollen or by mechanical contact. These methods of transmission, however, primarily explain the local spread of pathogens, but a number of diseases have emerged in continents and countries where the pathogens were not known to occur previously. Cross-border spread can be attributed to increasing international trade and the transport of contaminated seedlings, fruit and seed (Abdalla, 2000; Hanssen et al., 2010b; Kritzman, 1991; Lievens et al., 2003; Njambere et al., 2011).

Global seed trade represents an important aspect of sustainable food production and provides a valuable source of income for exporting companies and countries. The gross value of imported seed was recorded as over 2 billion US Dollars in 2008 alone (ISF, 2008b). Increased international trade of

Table 1. 1 A selection of some of the most detrimental pathogens of tomato according to literature and diagnostic handbooks (Blancard, 2012; Hanssen et al., 2010a; Jones et al., 1997, 2004)**. Pathogens highlighted in bold are the focus of this study.**

Nematodes	Fungi	Bacteria	Viruses	Viroids/Phytoplasma
Belonolaimus longicaudatus	*Alternaria alternata*	***Clavibacter michiganensis* subsp. *michiganensis***	*Alfalfa mosaic virus*	*Tomato chlorotic dwarf viroid*
Meloidoyne arenaria	*A. solani*	*Enterobacter cloacae*	*Beet curly top virus*	*Tomato bunchy top viroid*
M. brasiliensis	*Botrytis cinerea*	*Pectobacterium carotovorum* subsp. *carotovorum*	*Cucumber mosaic virus*	*Tomato planta macho viroid*
M. exigua	*Colletotrichum* spp.		***Pepino mosaic virus***	*Tomato apical stunt viroid*
M. hapla	*Fusarium oxysporum* f. sp.	***Pseudomonas syringae* pv. *tomato***	*Potato virus V*	*Potato spindle tuber viroid*
M. incognita	*Leveillula taurica*	*Ps* pv. *syringae*	*Potexvirus X*	*Candidatus* Phytoplasma asteris
M. javanica	*Phytophthora* spp.	*P. corrugata*	*Tobacco etch virus*	*Candidatus* Phytoplasma lycopersici
M. mayanguense	*Pythium* spp.	*P. viridiflava*	*Tobacco mosaic virus*	
M. paranaensis	*Rhizoctonia solani*	*Ralstonia solanacearum*	*Tomato bushy stunt virus*	
Nacobbus aberrans	*Sclerotinia sclerotiorum*	***Xanthomonas euvesicatoria***	*Tomato chlorotic spot virus*	
Paratrichodorus	*Sclerotium rolfsii*	***X. gardneri***	***Tomato mosaic virus***	
Trichodorus	*Septoria lycopersici*	***X. perforans***	*Tomato mottle geminivirus*	
	Stemphylium spp.	***X. vesicatoria***	*Tomato spotted wilt virus*	
	Thielaviopsis basicola		*Tomato yellow leaf curl virus*	
	Verticillium albo-atrum		*Tomato yellow top virus*	
	V. dahlia			

tomato seed, however, facilitates the cross-border transport of seed-borne pathogens (Abdalla, 2000; Córdoba-Sellés et al., 2007; De Leon et al., 2006; Hadas et al., 2004; Hanssen et al., 2010b; Kritzman, 1991; Leite et al., 1995; Ling, 2008; Milijašević et al., 2007; Nome et al., n.d.). These pathogens may establish a systemic infection in the seed prior to germination, via wounds, penetration or infected pollen (Nome et al., n.d.), and thus, usually demonstrate high transmission rates from infected plants to neighbouring plants and production areas. Other seed-borne pathogens contaminate/inhabit the surfaces of seed or seed coats and can be transmitted to hosts during the germination process. Although these pathogens characteristically demonstrate low transmission rates (Chitra et al., 1999; Hadas et al., 2004; Hanssen et al., 2010b; Ling, 2008; Sevİk and Kose-tohumcu, 2011; Xu, 2010), pathogens that inhabit seed coats are usually highly virulent and stable, with efficient mechanisms of secondary dissemination to neighbouring plants and, therefore, pose a significant threat to the tomato industry. Infected seed from diseased plants can also serve as an inoculum source for the next growing season (Sevİk and Kose-tohumcu, 2011).

To reduce the risk of importing seed-borne pathogens and to improve international trade and food security, the Sanitary and Phytosanitary (SPS) agreement was introduced in 1995 (World Trade Organisation, 1995). The SPS describes a list of standards to be met by exporting companies before trade is permitted, thereby, improving the health and safety risks of international trade. Amongst other objectives, this agreement led to the publication of a range of seed health tests to validate the absence of the most important seed-borne pathogens. Five of the most destructive tomato seed-borne pathogens have been selected as the focus of the current study, namely *Pepino mosaic virus* (PepMV), *Tomato mosaic virus* (ToMV), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Xanthomonas campestris* pv. *vesicatoria* (Xcv) and *Pseudomonas syringae* pv. *tomato* (Pst). These particular pathogens have proven to be difficult to control and, thus, the industry relies on phytosanitary measures to reduce their spread on a global scale.

1.2.1. *Pepino mosaic virus*

Pepino mosaic virus (PepMV) is a rod-shaped, single-stranded, positive sense RNA virus of the genus *Potexviridae*. The genome of this pathogen spans approximately 6400 nt in length (Aguilar et al., 2002; Jones et al., 1980; Van der Vlugt et al., 2002) with a 5’ guanine cap and a 3’ polyadenylated tail. The genome contains 5 open reading frames (ORFs) encoding: a RNA-dependent RNA polymerase (RdRp), a triple gene block (TGB) and a capsid protein (CP). The virus was first isolated from Peruvian pepino crops in 1974 (Jones et al., 1980), that displayed an intense yellow mosaic on young leaves. More severe symptoms were associated with the detection of PepMV in infected

tomato plants in the Netherlands in 1999 (Van der Vlugt et al., 2000), from which the virus spread rapidly across Europe, North America, South America and Asia (Diez and Nuez, 2006; Hanssen and Thomma, 2010; Hanssen et al., 2010a; Mansilla et al., 2003; Spence et al., 2006; Van der Vlugt et al., 2002). The exact economic impact of PepMV is not quantifiable as the disease affects the quality of fruit instead of overall yield. Spence et al. (2006) estimated that in UK glasshouses, the downgrade of fruit quality as a result of PepMV infection was associated with a loss of approximately 24.33 million US dollars per season. Ling (2008) predicted that annual greenhouse losses in the USA reached approximately 400 million US dollars due to the disease, thus indicating the destructive capacity of this disease on tomato production.

Pepino mosaic virus is known to induce a variety of symptoms on tomato hosts (Hanssen et al., 2009, 2010b; Ling, 2008; Soler-Aleixandre et al., 2005). These range in severity according to the viral isolate/genotype as well as environmental conditions. Typical vegetative symptoms associated with PepMV infection include leaf mosaic/mottling, distortion, bending, dwarfing, nettlehead-formation, scorching, leaf bubbling, premature senescence and plant collapse (Figure 1. 1 A – C) (Hanssen et al., 2009; Soler-Aleixandre et al., 2005; Van der Vlugt et al., 2002). Symptom development on fruit is responsible for the significant economic impact of this virus (Hanssen et al., 2009; Mansilla et al., 2003; Spence et al., 2006) and symptoms include mild 'flaming', uneven ripening or marbling and the more severe open fruit symptom, where fruit surfaces split open (Figure 1. 1 D – F).

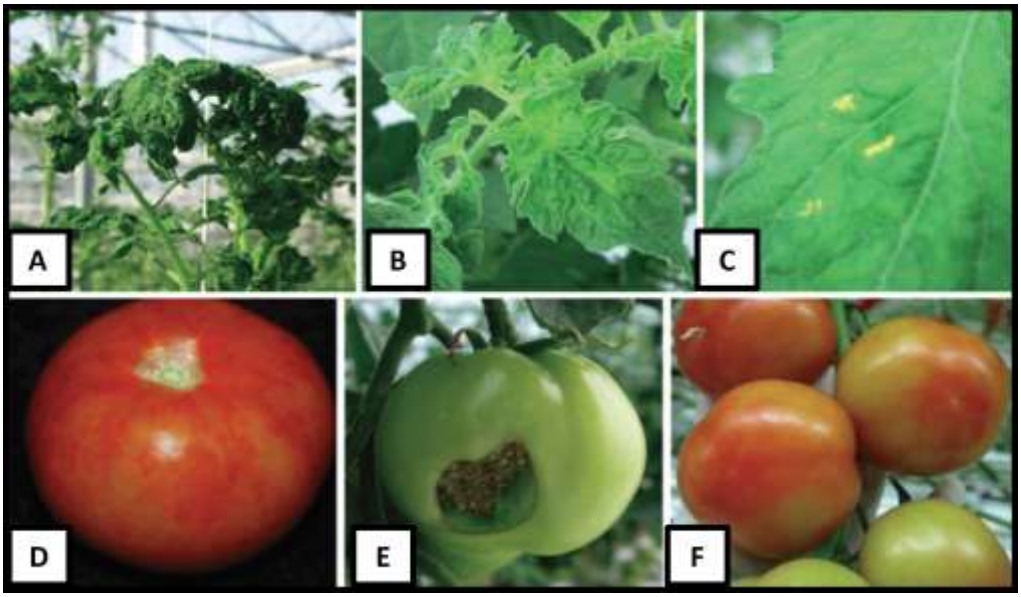

Figure 1. 1 Typical symptoms of PepMV infection - nettle-head formation (A); mild mosaic and severe leaf bubbling (B); yellow lesion development (C); marbling of fruit (D); open fruit (E) and discolouration or flaming of fruit (F) (Hanssen and Thomma, 2010)**.**

Five major genotypes of PepMV have been characterized to date (Aguilar et al., 2002; Hanssen et al., 2008, 2009; Ling, 2007, 2008). These include the LP genotype, the original strain isolated from pepino plants; the EU genotype which are typically found in European tomato production areas; and US1/CH1, CH2 and US2, typically prominent in the Americas. Along with the different origins, PepMV isolates also demonstrate different levels of virulence and symptom induction, despite minimal sequence variation (Alfaro-Fernandez et al., 2009). The CH2 genotype has been described as the most aggressive PepMV isolate (Hanssen et al., 2009), accumulating rapidly in infected plants and producing the most severe symptoms. This isolate has become the predominant isolate in Europe since its emergence on that continent in 2006 (Hanssen et al., 2008), rapidly colonising tomato fields and overcoming the incidence of the EU genotype. Evidence of mixed infections of PepMV strains in tomato plants have also been reported (Alfaro-Fernandez et al., 2009; Hanssen et al., 2008; Hanssen and Thomma, 2010; Ling, 2007), which have become more common than single infections in Europe (Alfaro-Fernandez et al., 2009), with affected plants displaying an earlier onset of more severe symptoms.

The rapid spread of PepMV world-wide can be attributed to its highly infectious nature (Gutierrez-Aguirre et al., 2009; Hanssen and Thomma, 2010; Ling, 2008). The virus is transmitted mechanically to healthy plants via contact with infected plants, on contaminated clothing or equipment of field-workers. It is a quarantine virus in many countries (Gutierrez-Aguirre et al., 2009), especially in Europe and, therefore, strict hygienic and phytosanitary measures have been implemented in attempt to contain infected material. Seed transmission of PepMV has been established as an important means of viral dissemination (Córdoba-Sellés et al., 2007; Hanssen et al., 2010b; Ling, 2008), and is thought to be responsible for the introduction of PepMV from South America into Europe and the USA. Although PepMV was not detectable in the embryos of tomato seeds, Hanssen et al. (2010) demonstrated the presence of PepMV particles on tomato seed coats. Reported seed transmission rates have not exceeded 2% in various trials (Córdoba-Sellés et al., 2007; Hanssen et al., 2010b; Ling, 2008), but even low transmission rates are sufficient to affect entire fields of tomato due to the virulent nature of PepMV. Other explanations for the quick spread of PepMV are the increasing populations of PepMV vectors, for example, bumblebees and the root-parasite *Olpidium virulentus* (reviewed by Hanssen and Thomma (2010).

1.2.2. *Tomato mosaic virus*

The genus *Tobamovirus* contains a number of significant phytopathogens which are divided into three major sub-groups of species based on the genomic position of the origin of viral assembly

(Dovas et al., 2004; Letschert et al., 2002; Lewandowski, 2000). Sub-group 1 contains viruses that mainly infect solanaceous plants, and includes *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV). Both viruses can cause moderate to severe losses in tomato yields worldwide, with some reports indicating losses of 90% of total yield due to infection with one of these viruses (Chitra et al., 1999; Lukyanenko, 1991; Vinayarani et al., 2011). Despite close similarities, ToMV is reportedly more prevalent than TMV and is, therefore, the focus of this study. All species of *Tobamovirus* are characterized by rigid, rod-shaped virions containing a positive sense, single-stranded RNA genome of approximately 6.4 kb (Hagiwara-Komoda et al., 2008; Lewandowski, 2000). The genome is divided into four coding regions including: 130 kDa and 180 kDa proteins, with putative roles in viral replication and suppression of host silencing mechanisms; a coat protein; and a movement protein for cell-to-cell movement of viral particles. Both ToMV and TMV infect vegetative organs as well as flowers. The development of disease symptoms on fruit are the most economically destructive symptoms, resulting in the greatest losses with viral infection at early tomato growth stages (Agrois, 2005; Chitra et al., 1999; Jones et al., 1997; Vinayarani et al., 2011).

The *Tomato mosaic virus* induces a range of symptoms on infected tomato plants, dependent on environmental conditions as well as host/pathogen genetic factors (Hollings, 1977). Symptoms include leaf mottling (light green or yellow), chlorosis, leaf dwarfing, distortion, stunting, leaf narrowing and leaf curling (Figure 1. 2 A) (Agrois, 2005; Ganoo and Saumtally, 1998; Hollings, 1977; Jones et al., 1997). Severely infected plants may demonstrate tendril or leaf malformation, where

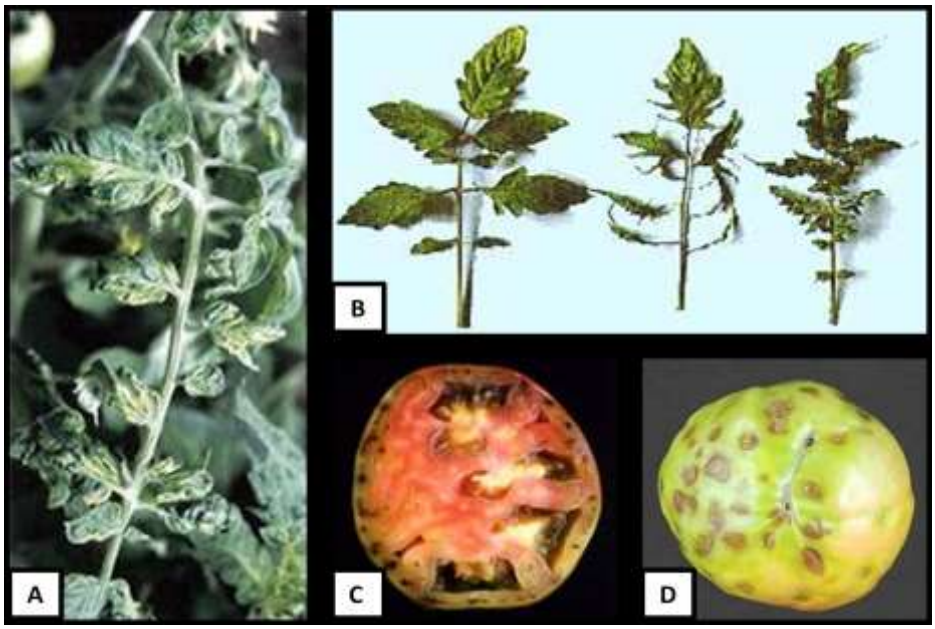

Figure 1. 2 Symptoms of ToMV infection of tomatoes: leaf distortion and mottling (A); a branch from a healthy tomato plant (left) compared to infected leaves with tendril formation (centre) and fern-like appearance (right) (B); browning of fruit walls (C); and necrotic blotches on the surface of fruit (D) (Ganoo and Saumtally, 1998; Lamey, 2010)

leaves become string- like or develop a fern-like appearance (Figure 1. 2 B). The fruit yield of tomato plants infected with the virus can be reduced up to 75% (Eraslan et al., 2007; Hollings, 1977; Vinayarani et al., 2011) with overall loss in fruit weight of up to 59%. Symptomatic fruit often display blemishes, necrotic blotches, russeting, internal necrosis and browning of fruit walls (Figure 1. 2 C and D) which severely affect the marketable value of fruit (Hollings, 1977; Jones et al., 1997).

Primary spread of ToMV is via seed, but viral particles are also spread by wind, guttation fluid, water and weed hosts or are mechanically transmitted by plant contact and on contaminated equipment (Chitra et al., 1999; Hadas et al., 2004; Jones et al., 1997; Lewandowski, 2000). The virus is highly virulent and particles are viable for up to 2 years on plant debris, increasing the risk of spread as a result of latent infections (Ganoo and Saumtally, 1998; Jones et al., 1997). Tomato plants are susceptible to infection at all growth stages, though the greatest concentrations of virus in leaves and seeds are linked to infections at earlier stages of plant growth.

1.2.3. *Clavibacter michiganensis* subsp. *michiganensis*

Bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), is the most important bacterial disease of tomato and is responsible for at least 80% of diseased tomatoes (Burokienė, 2006). The disease was first reported in USA tomato fields in 1910, and has since spread world-wide, causing substantial economic losses (EPPO/CABI, 2005; Gartemann, 2003; Werner et al., 2002). This organism forms one of five subspecies of *Clavibacter michiganensis* which are all known to be important plant pathogens, but have different host ranges (Bach et al., 2003; Louws et al., 1998). Although primarily infecting tomatoes, a number of other solanaceous hosts, including some weed species, have been shown to be alternate hosts for Cmm, and act as reservoirs for over-wintering of the pathogen. Losses due to Cmm infection can be variable due to the sporadic nature of the disease, often emerging suddenly after extended periods of absence, and resulting in devastating epidemics (Chang et al., 1992; De Leon et al., 2006; EPPO/CABI, 2005; Louws et al., 1998). Losses have ranged from 50 to 80% of total fruit yield in the USA, Canada and Kenya (Chang et al., 1992; EPPO/CABI 1999). As a result, Cmm has been listed as a quarantine pest in North America and the European Union (EU) (Bach et al., 2003; EPPO/CABI, 2005; Kaneshiro et al., 2006) to control distribution of infected material.

This pathogen is characterized as a Gram positive, aerobic, rod-shaped bacterium with a circular chromosome of approximately 3.3 Mb (Gartemann, 2003; Gartemann et al., 2008). Its pathogenicity is dependent on two circular plasmids, pCM1 and pCM2, encoding β-1,4-endocellulase and a

putative serine protease, respectively. A number of chromosomal determinants are also involved in the invasion and colonization of the host as well as the evasion of host immune responses. Within the Cmm subspecies, non-virulent and hypovirulent strains have been isolated from hosts and often obscure accurate detection of pathogenic isolates (Kaneshiro et al., 2006; Louws et al., 1998).

Primary spread of Cmm is via infected seed where bacterial cells reside on the surface of seed coats following infection of fruit pulp (EPPO/CABI, 2005; Hadas et al., 2005; Milijašević et al., 2007). Reported transmission rates seldom exceed 1% (EPPO/CABI, 1999) and, thus, the rapid spread of bacterial canker is usually attributed to secondary means of transmission (Burokienė, 2006; Milijašević et al., 2007; Werner et al., 2002). This is achieved via irrigation, rain, contaminated equipment and cultural practices. Cells of Cmm are also stable for up to a year in soil in association with plant debris. Non-seed transmitted entry into the host occurs through natural wounds in roots and stems, or via hydathodes and leaf trichomes (Carlton et al., 1998; EPPO/CABI, 2005; Gartemann, 2003; Louws et al., 1998). Bacteria target and colonize the xylem tissue of the plant using a suite of extracellular enzymes and exopolysacchirides which degrade host components and prevent recognition by host factors (EPPO/CABI, 2005; Gartemann, 2003; Gartemann et al., 2008). Accumulation of bacterial cells and associated metabolites blocks the xylem, thereby, reducing water transport in diseased hosts.

Symptom development on hosts thus only occurs after a latent period (Burokienė, 2006; EPPO/CABI, 1999, 2005), while bacteria accumulate in xylem tissue. Initial symptoms appear after 7 days under optimal conditions of between 25 and 30°C (EPPO/CABI, 2005), and are characterized by unilateral wilting of a few leaves which rapidly spreads to all leaves (Figure 1. 3 A). In severe systemic infections, entire branches may dessicate, wilt and die. Lesions develop on leaves as dark, oily spots in interveinal regions, becoming brown and necrotic over time (Figure 1. 3 B) and giving leaves a scorched appearance (EPPO/CABI, 1999, 2005; Gartemann, 2003; Jones et al., 1997). Later in the infection cycle, brownish-yellow stripes may develop on stems due to vascular discolouration, which crack, especially at nodal regions, to expose reddish-brown cavities or cankers (Figure 1. 3C). Symptoms on fruit of infected plants are determined by whether *Cmm* infection is systemic or localised. Fruit from systemic infections ripen unevenly, displaying a marbled appearance, while internal symptoms include bleaching and discolouration of vascular tissue (EPPO/CABI, 1999, 2005; Jones et al., 1997). Late infections at the anthesis stage of floral development (Medina-Mora et al., 2001), lead to the development of localised symptoms of small, raised, necrotic spots with brownish centres and white halos, referred to as bird's eye lesions (Figure 1. 3D). Both systemic and localised

symptoms reduce fruit marketability and seed trade (Chang et al., 1992; Medina-Mora et al., 2001), resulting in substantial economic losses.

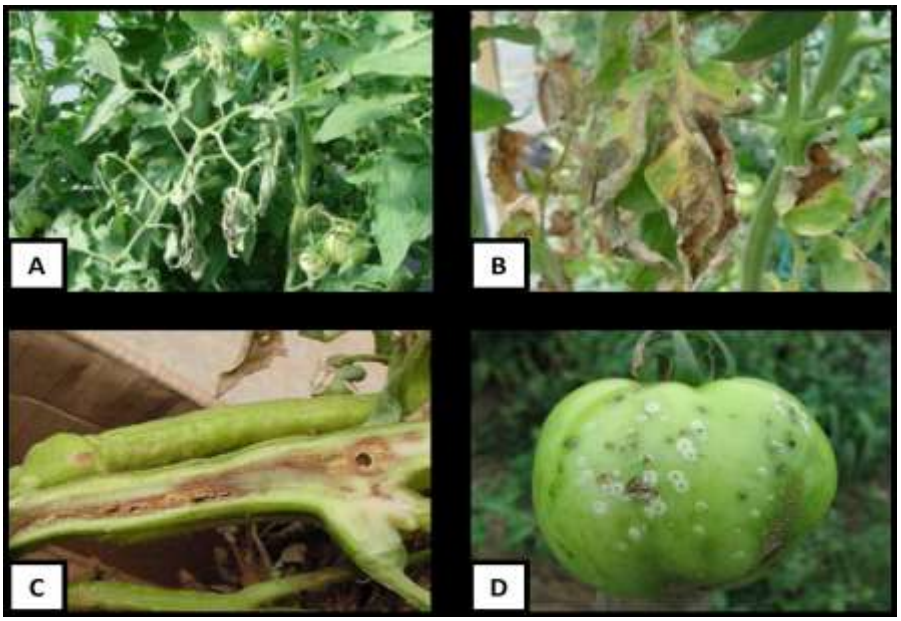

Figure 1. 3 Symptoms of bacterial canker: wilting of infected leaves and branches (A); yellowing and necrosis/scorching of older leaves (B); vascular browning and necrosis (C); birds-eye lesions and necrotic spots on infected fruit (D) (Bachi, UKREC, Bugwood.org, 2008; Mississippi Agricultural and Forestry Experimental Station, 2009; Cornell, 2010)

1.2.4. *Xanthomonas campestris* pv. *vesicatoria*

The genus *Xanthomonas* contains over 200 species that are known phytopathogens of dicotyledonous plants (Leite et al., 1994; Maes, 1993). Bacterial spot of solanaceous plants, including pepper (*Capsicum annuum*) and tomato, is specifically caused by a group of four species/pathovars, including 11 races affecting pepper and 3 races affecting tomato (Bouzar et al., 1999; Jones et al., 2004; Obradovic et al., 2004). Originally characterized as *Xanthomonas campestris* pv. *vesicatoria* (Xcv) by E.M. Doige in 1921, reports of divergent strains, races and pathovars causing bacterial spot in tomato and pepper have complicated taxonomic classification of this group of pathogens. Jones et al. (2004) presented a detailed study into characterization of the different strains and proposed designation of four species: *X. euvesicatoria*, *X. vesicatoria* (Xv), *X. perforans* and *X. gardneri*. Recharacterizing previously isolated strains is a difficult and arduous task (Jones et al., 2000; Leite et al., 1994; Jones et al., 2004) and, therefore, for the purpose of this study, isolates will be referred to as Xcv or Xv, when appropriate.

Although Xcv affects tomato production areas worldwide, it is prominent in tropical and sub-tropical regions (EPPO/CABI, 1995; Jones et al., 1997, 2005; Thieme et al., 2005). Infection is especially detrimental in optimal conditions of heavy rainfall, high humidity and temperatures of 24 - 30°C.

This pathogen is seed-borne, but the bacteria can also infect a number of solanaceous weeds which facilitates overwintering of the pathogen (Agrois, 2005; EPPO/CABI, 1995). Secondary spread is achieved via rain, irrigation, wind, infected debris, direct contact between plants or contaminated equipment. The economic impact of Xcv results from damage of the quality and size of fruit by the disease, causing severe economic losses for fresh fruit industries of between 35 and 50% (Jones et al., 1997, 2005; Lukyanenko, 1991; Obradovic et al., 2004; Shenge et al., 2007). Thus, Xcv has been designated quarantine status to restrict trade to pathogen-free seed (EPPO/CABI, 1995; ISF, 2009c).

This pathogen is characterized as a motile, Gram negative, rod-shaped bacterium with a circular genome of 5.17 MB and four plasmids (Thieme et al., 2005). Pathogenicity of Xcv is conferred by the expression of a Type III protein secretion system (TTSS) that encodes and translocates effector proteins such as cell wall-degrading enzymes, detoxification determinants and adhesion proteins into host cells to initiate disease (El-Hendaway et al., 2005; EPPO/CABI, 1995; Thieme et al., 2005). The pathogen targets all vegetative organs of tomato plants including stems, leaves and petioles as well as flowers and fruit.

Symptoms characteristic of Xcv infection develop as localised, small, circular, water-soaked lesions approximately 3 mm in diameter (Abdalla, 2000; EPPO/CABI, 1995; Jones et al., 1997). These become brown and necrotic, disintegrating in dry conditions to give a ‘shot-hole’ appearance (Cox et al., 1956; Obradovic et al., 2008). Numerous spots may develop on a single leaf, which can enlarge with leaf expansion (Figure 1. 4 A), and coalesce to cover entire leaf surfaces, causing yellowing or chlorosis of leaves (Jones et al., 1997; Obradovic et al., 2004; Shenge et al., 2007).

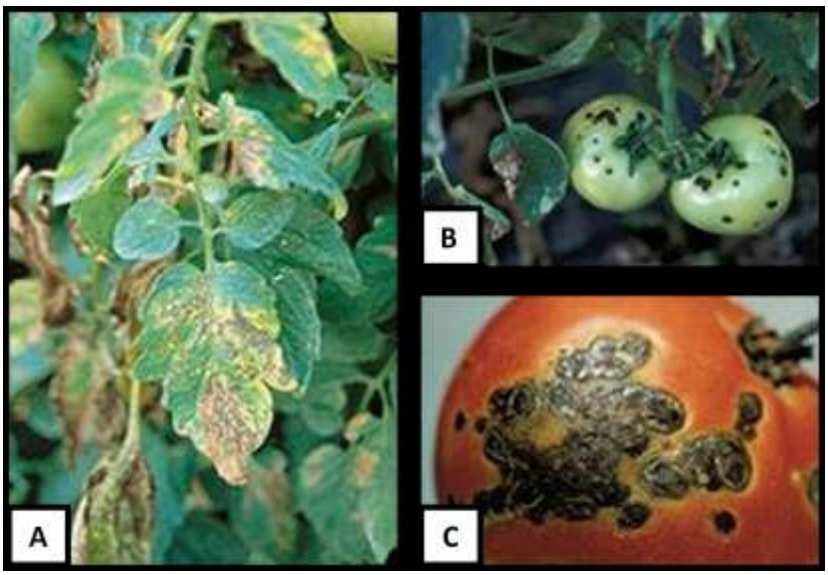

Figure 1. 4 Symptoms of bacterial spot on tomato: leaves with brown necrotic lesions, blight and chlorosis (A); sunken lesions on immature fruit (B); raised black scabs on mature fruit (C) (Dept of Crop Science, University of Illinois, 2009)

Symptomatic fruit arise from systemic infections or as a result of late infections of blossoms and young fruit. Spots develop on the surfaces of infected fruit as raised, water-soaked or brown lesions which develop into dark, sunken lesions/scabs (Figure 1. 4 B and C) (Cox et al., 1956; Jones et al., 1997; Obradovic et al., 2004). Bacterial spot also reduces the weight and size of tomato fruit. Although most Xcv infections do not have a severe impact on the overall growth of the host plant, the development of scabby lesions on fruit surfaces drastically decreases the quality and market value of fruit.

1.2.5. *Pseudomonas syringae* pv. *tomato*

The Gram negative, flagellate *Pseudomonas syringae* contains fifty pathovars of significant phytobacteria responsible for substantial economical losses for a variety of important crops (Buell et al., 2003; Feil et al., 2005). One of these pathovars, *Pseudomonas syringae* pv. *tomato* (Pst) is specifically pathogenic to tomato and arabidopsis plants, causing foliar spot called bacterial speck. First reported in 1933, bacterial speck has been found in tomato fields and greenhouses world-wide, and is particularly detrimental in optimal conditions of high relative humidities and temperatures between 18 and 25°C (Jones et al., 1997). The disease gained significance in the late 1970's when massive outbreaks in Californian production areas caused large-scale downgrade in fruit quality resulting in severe economic losses (Schneider and Grogan, 1977). Numerous outbreaks have since been reported, in one case resulting in yield losses of up to 20% (Sahin, 2001). Primary spread of Pst is via infected seed on which bacterial cells survive in the cavities of seed surfaces for extended periods (Devash et al., 1980; Uppalapati et al., 2008). The pathogen is also spread via water or contaminated equipment and can survive as an epiphyte on the surface of plants due to tolerance of environmental stresses (Bashan, 1986; Cuppels and Elmhirst, 1999; Fanelli et al., 2007; Jones et al., 1997; Uppalapati et al., 2008).

The Pst genome consists of a 6.5 Mb circular chromosome encoding approximately 300 putative virulence genes (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005; Zhao et al. 2003). Pathogenicity, similar to that of Xcv, is conferred by the expression of a TTSS system that translocates effector proteins into the host environment to facilitate host infection (Zaccardelli et al., 2005). One such important virulence factor is the phytotoxin coronatine (Cuppels and Ainsworth, 1995; Cuppels et al., 2006; Uppalapati et al., 2008). Unique to certain pathovars of *Pseudomonas syringae*, this toxin provides a selective advantage for virulence by suppressing stomatal closure and host immune response pathways, thereby facilitating infection.

Symptoms appear as water-soaked lesions on leaves of infected plants, which develop into raised, dark-brown or black necrotic specks (Cuppels and Elmhirst, 1999; Jones et al., 1997). Bacterial speck is distinguishable from bacterial spot as lesions are smaller, between 1 and 2 mm in diameter, and are usually surrounded by yellow chlorotic halos (Figure 1. 5 A) due to production of coronatine (Cuppels and Ainsworth, 1995; Cuppels and Elmhirst, 1999; Uppalapati et al., 2008; Zhao et al., 2003). Numerous lesions developing on a single leaf may coalesce to form larger lesions, eventually resulting in chlorosis of leaves and premature leaf abscission. Symptoms develop on stems, petioles and peduncles as dark, elongated lesions (Figure 1. 5 B) (Jones et al., 1997). Besides systemic infections established in early developmental stages of the host, infected fruit may also arise from late Pst infections via secondary modes of transmission. Fruit have been reported to be the most susceptible to secondary or late infection between the late corolla stage of flowering or before young fruit have reached 3 cm in diameter (Getz et al., 1983). Both systemic and late infections of Pst induce the development of numerous small, black, necrotic lesions across the fruit surfaces (Figure 1. 5 C and D), making fruit unmarketable. The impact of bacterial speck on the tomato industry is considerably lower than that of other seed-borne pathogens; however, Pst can still cause a significant impact on the tomato industry if spread of the pathogen is not contained.

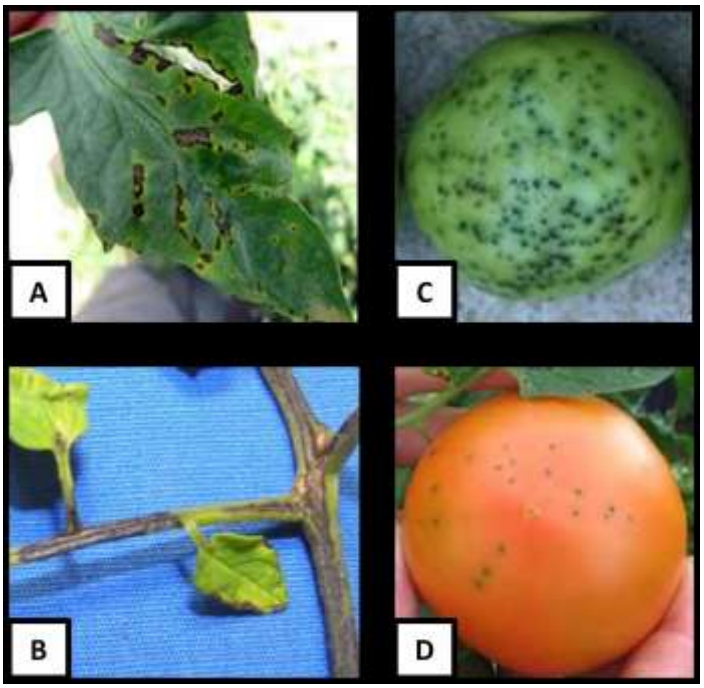

Figure 1. 5 Typical symptoms of bacterial speck. Dark necrotic lesions surrounded by a chlorotic halo (A) (Michigan State University); elongated necrotic lesions on stems and petioles (B) (University of Arkansas); severe infection of Pst on immature fruit (C) (University of Illinois) and mature fruit (D) (D. Maeso, INIA, Uraguay).

1.2.6. Control strategies

Growth in global tomato production has been accompanied by intensive breeding for economically attractive traits; however, this has consequently decreased the natural genetic basis for disease resistance in tomato (Hanssen et al., 2010a). Breeding efforts have, therefore, been expanded to develop commercial cultivars that are resistant or tolerant to economically significant pathogens by targeting appropriate genes identified in wild relatives or non-hosts. The process is lengthy, however, and dependent on the knowledge and discovery of new target genes (Da Silva et al., 2008; Ganoo and Saumtally, 1998; Hanssen and Thomma, 2010; Obradovic et al., 2004). Sub-species variation and the high mutation rate of some pathogen genomes affect the development of durable resistance in cultivated varieties (Cuppels et al., 2006; Da Silva et al., 2008; Ganoo and Saumtally, 1998; Obradovic et al., 2004; Shenge et al., 2007; Xu, 2010). Methods for in-field control of diseases that have been implemented include crop rotation, rouging, calcium sprays and applications of aphicides for viral vectors (Eraslan et al., 2007), applications of copper-based bactericides (Hausbeck et al., 1999; Obradovic et al., 2004, 2008), biological control (Bashan and De Bashan, 2002; El-Hendaway et al., 2005) and seed treatment strategies (Bashan and De Bashan, 2002; Córdoba-Sellés et al., 2007; Hadas et al., 2004; Xu, 2010). Although these strategies are sometimes efficient for immediate control, few are adequate for stable control over multiple seasons.

Current control strategies rather focus on preventative and phytosanitary measures by disinfection of farm equipment, decontaminating or sterilising production areas and implementing strict hygienic measures for workers (GSPP, 2009; EPPO/CABI, 2005). The use of pathogen-free seed and healthy transplants in carefully maintained, sanitary production areas is extremely important to limit the spread of these diseases. Phytosanitary standards are developed and monitored by organisations like ISTA (International Seed Testing Association), ISF (International Seed Federation) and EPPO (European and Mediterranean Plant Protection Organisation). These organisations publish standardised, thoroughly validated, detection protocols to prove the absence of a pathogen of interest in a host sample. Detection protocols provide all the information necessary to detect, identify and classify regulated pathogens as well as suggested curative methods (Etter et al., 2011; EPPO/CABI, 2005). By 2005, EPPO had developed and published 41 standard protocols for significant pathogens from a range of important crops and ornamentals and currently standard protocols exist for 4 out of the 5 pathogens discussed in this study (EPPO/CABI, 1999, 1995; ISF, 2008a, 2009a, 2009b). This form of regulation, if carefully managed, will reduce the incidence of epidemics and outbreaks caused by seed-borne pathogens.

1.3. Tools for the detection of seed-borne pathogens of tomato

To limit the spread and impact of important seed-borne pathogens, stringent trade conditions have been introduced (Deyong et al., 2005), whereby only seed-lots certified as "pathogen-free" or "clean" by international standards may be exported (reviewed by Fanelli et al., 2007). Evidence must be provided to prove that seed-lots have been assessed by standard pathogen detection tests for major seed-borne pathogens (Kritzman, 1991), and that contamination levels are lower than a predetermined threshold value for disease safety, before trade is permitted. The importance of these standards is evident from a study conducted by Abdalla (2000) prior to the implementation of phytosanitary control measures in Egypt. Two major seed-borne pathogens of tomato were detected in 20 out of 75 seed samples imported from different sources which could have lead to severe economic and yield losses.

1.3.1. The importance of pathogen detection

In order to limit the spread of destructive diseases, especially via trade of seed, accurate detection, identification and implementation of curative strategies is vital for regulated pathogens (Lievens et al., 2003; Njambere et al., 2011; Rajeshwari et al., 1998). Even low levels of pathogen populations on host plant or seed material may be sufficient to cause or spread disease in production areas (Abdalla, 2000; Lievens et al., 2005; Milijašević et al., 2007). Correct diagnosis of infected host material is essential, in some cases, incorrect identification may cause an even greater impact on plants than disease because of the implementation of inappropriate control measures, such as harsh anti-microbial chemical sprays, which may cause severe crop damage and yield reduction (Chitarra et al., 2000; Njambere et al., 2011; Rajeshwari et al., 1998). The determination of the causal agent of a disease was first described by Robert Koch (1887) who outlined four criteria to prove whether an isolated organism was responsible for disease of a host:

1. The suspected agent should be present in all symptomatic, diseased hosts.
2. The agent should be subsequently isolated and propagated in pure culture.
3. This pure culture should then be inoculated onto a susceptible host plant and produce similar symptoms to those originally observed.
4. This same agent should be isolated from the symptomatic, inoculated hosts and demonstrate similar cultural and morphological characteristics as the original isolate.

Detection of pathogens based on symptom display alone does not guarantee that an asymptomatic host is not infected. Infected plants do not always display symptoms due to a range of factors, for example, unfavourable environmental conditions for disease development, yet, the pathogen may still be transmitted to seed (Da Silva et al., 2008; Kritzman, 1991; Njambere et al., 2011; Rajeshwari et al., 1998; Van der Vlugt et al., 2002). A study conducted in the USA (Du Toit et al., 2005), found that *Xanthomonas campestris* pv. *carotae* (also known as *X hortorum* pv. *carotae*), could be detected on carrot seedlings a few months after planting, whereas symptoms were only visible on foliage after a further 2 months. Harvests of large amounts of fruit from tomato production areas, also makes the detection of individual symptomatic plants difficult and sometimes impractical. Even seed that has been treated, for example by acid or heat (Chitarra et al., 2000), may still contain viable phytopathogens if the treatment was not completely effective. Furthermore, saprophytic organisms inhabiting seed or plant material do not cause visible symptoms, but some may become opportunistic pathogens in favourable conditions (Abdalla, 2000; Kaneshiro et al., 2006). Plant protection is, therefore, dependent on access to tools or processes capable of accurately detecting potential pathogens on plant and seed material without relying on characteristic symptom development.

The ideal detection tool should satisfy a set of criteria that ensure results are reliable, accurate and meet international standards for certification. This detection tool should be specific to a target pathogen and be able to distinguish between different classes of phytopathogens as well as different strains or races (Rajeshwari et al., 1998; Zhang et al., 2007). Some strains or races may be more severe and aggressive than others, therefore, representing more of a threat than small concentrations of related strains or races. Distinction between pathogens and morphologically or genetically similar saprophytes is also a necessary requirement, as this can obscure identification of target pathogens (Abdalla, 2000; Goszczynska and Serfontein, 1998; Kaneshiro et al., 2006; Milijašević et al., 2007; Sijam et al., 1991). The tool should be highly sensitive or able to detect targeted pathogens at very low concentrations within a host sample without the risk of false positive or negative results (Lievens and Thomma, 2005; Lievens et al., 2005; Milijašević et al., 2007; Njambere et al., 2011; Rajeshwari et al., 1998; Schaad and Frederick, 2002; Zhang et al., 2007). The ability to quantify pathogen infections on seed-lots is important to determine whether levels of seed-borne pathogens are sufficient to cause disease in destination countries. Detection of only viable pathogenic particles or cells is also a beneficial characteristic of a detection tool, to guarantee a true reflection of the potential risk associated with disease-causing agents in seed-lots (Chitarra et al., 2000).

A wide range of classes of phytopathogenic agents may infect seed (Boonham et al., 2007; Kritzman, 1991; Pelludat et al., 2009), many of which are capable of co-existing in mixed infections. An ideal detection tool should facilitate screening of seed samples simultaneously for the presence of multiple pathogens as well as any new, emerging species, strains or races. Using such a tool, pathogens should be detectable from all relevant inoculum sources such as plant leaf and seed samples, soil, water and air traps (Lievens and Thomma, 2005; Njambere et al., 2011). This would facilitate maintenance of a pathogen-free, fruit production environment. Diagnosis should also be robust, i.e., highly reproducible results which are not susceptible to technical errors. Results should be attainable in a short time with limited technical expertise and only require small sample sizes of the expensive, hybrid seed for accurate detection (Kritzman, 1991; Lievens et al., 2003; Lievens and Thomma, 2005). The material and equipment costs for the ideal detection process should be economical and practical for monitoring host plants as well as large-scale screening of seed-lots.

For use in seed-health certification, potential diagnostic tools should undergo intense validation using various sources of seed samples and tested in different labs before the tools are accepted as international standards (De Leon et al., 2008; Lievens and Thomma, 2005; Njambere et al., 2011). Although a number of tests have been adopted as standard detection tests, no single tool has been developed to date that demonstrates all the necessary characteristics discussed above for pathogen detection for tomato seed. Detection techniques developed in the past mainly employed conventional methods or serological tests, but more recently developed tools have expanded to include molecular-based approaches.

1.3.2. Conventional detection tools

Conventional strategies have been used for phytopathogen detection for decades and, thus, have become widely accepted as standard detection techniques. These methods are characteristically inexpensive and simple, and are routinely used in industry for identification of phytopathogens or to validate newly developed detection strategies. These methods facilitate detection and broad taxonomic classification of individual pathogens and, thus, tests must be repeated in order to detect multiple pathogens from a sample.

1.3.2.1. Microscopy

Microscopy of infected plant samples enables visualisation of the presence and morphological structure of pathogens within a host or on plant and seed surfaces. The two major types are light

and electron microscopy (EM) (Figure 1. 6). Although light microscopy is relatively inexpensive, the resolution is typically limited and only larger organisms like fungi and bacteria are usually detectable (Biel and Gelderblom, 1999; Hamilton et al., 1981; Rubio-Huertos and Bos, 1973). All forms of EM enable high resolution for detecting even viral pathogens, for which the exact size and morphology of particles may be accurately determined. As the morphology of bacterial cells and viral particles is typically similar within taxonomic groups, EM can be used to broadly classify pathogens and, therefore, can sometimes distinguish between unrelated pathogens in mixed infections (Bos, 1975; Bos and Rubio-Huertos, 1972; Da Silva et al., 2008; Van der Vlugt et al., 2002). Sample preparation and evaluation for EM requires a maximum of 240 minutes, and, due to the 'catch all' characteristic of this technique, EM enables unbiased visualisation of organisms present in a plant sample (Biel and Gelderblom, 1999). Drawbacks, however, include the expense of the microscope and materials for sample preparation. The microscope is also sensitive and requires technical expertise for operation (Biel and Gelderblom, 1999; Boonham et al., 2007; Bos, 1975; Hamilton et al., 1981). Microscopy has, thus, been replaced with other detection methods, but can still be valuable for analysis of host-pathogen interactions and evaluating mechanisms of pathogen infection and transmission.

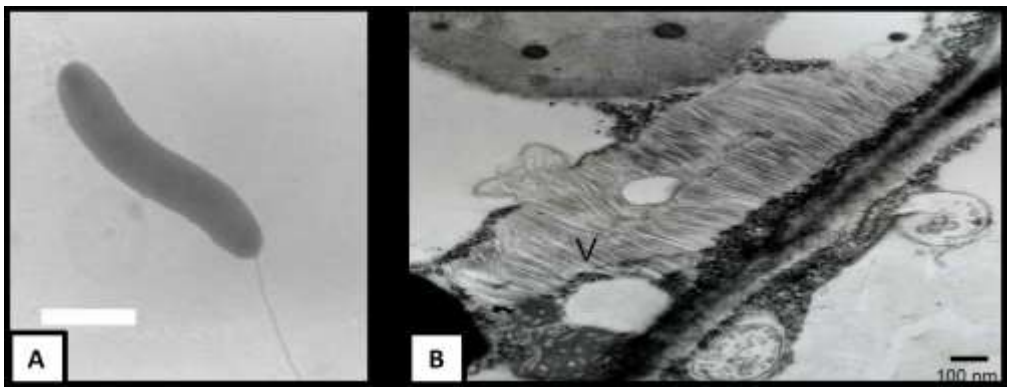

Figure 1. 6 Transmission electron micrographs of: polar flagellate *Herbaspirilium* bacterium (A) (Obradovic et al. 2007)**; section of tomato leaf showing viral particles (V) aggregated in the cytoplasm of an infected cell (B)** (Da Silva et al., 2008)**.**

1.3.2.2. Biological assays

Bioassays or biological assays indicate the presence of infectious or viable agents in seed or plant samples based on the reaction induced on inoculated, susceptible host plants. Pathogens may induce similar or different symptoms on a range of plant species or cultivars, referred to as indicator hosts, and this interaction is, thus, informative about the nature of the pathogen (Adams et al., 2009; Hamilton et al., 1981; Kaneshiro et al., 2006; Sijam et al., 1991; Van der Vlugt et al., 2002). A popular host species for bioassays of tomato pathogens is *Nicotiana tabacum* (Tobacco) (Adams et

al., 2009; Hadas et al., 2004), as this host demonstrates visible reactions rapidly, with compatible and incompatible pathogens (Figure 1. 7). Bioassays discriminate between saprophytic and infectious organisms present in inoculum, while also indicating the viability and pathogenicity of detected organisms (Abdalla, 2000; Kritzman, 1991; Kaneshiro et al., 2006). Bioassays can be lengthy, however, and are dependent on the rate of plant growth and symptom development on inoculated indicator plants (Hadas et al., 2004; Hamilton et al., 1981; Harris-Baldwin and Gudmestad, 1996; Sijam et al., 1991). As no single plant species is a host or informative for all pathogens of interest, tests need to be repeated on various indicator host plants and, even then, classification may be limited to a genus or species level (Boonham et al., 2007; Da Silva et al., 2008; Hadidi et al., 2004; Lee et al., 2003). Despite these disadvantages, bioassays are technically simple, cost-effective and informative especially when used in conjunction with other, more specific, detection tools.

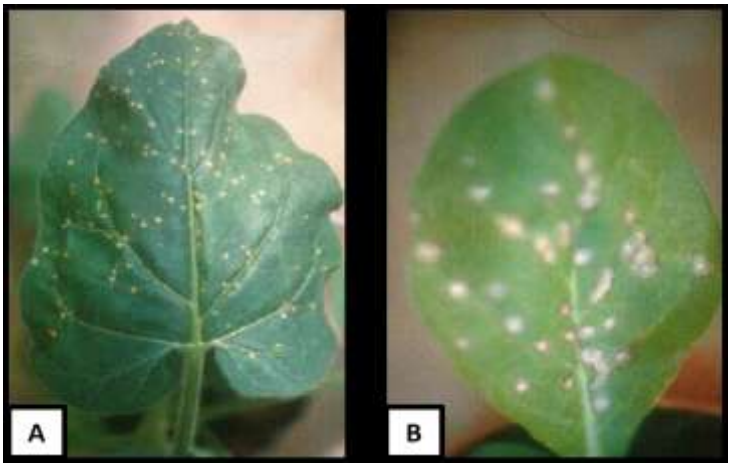

Figure 1. 7 Local lesion assay of Tomato mosaic virus on tobacco hosts: Nicotiana glucose (A) and Nicotiana tabacum cv. Xanthi (B) (ISF, 2009b)

1.3.2.3. Identification by nutrient-utilisation assays

Though phytopathogens are armed to establish infection within host plants, most bacteria and fungi can be isolated from infected material and propagated outside of the host. This is unlike obligate pathogens such as some fungi, oomycetes and all viruses, viroids and phytoplasmas, which must be associated with a host or a vector to replicate. As a result, the biochemical and physiological properties of bacterial and fungal pathogens can be investigated by exposing organisms to various chemicals, nutrients or antibiotics during propagation. By exploiting differentially expressed metabolic pathways, enzyme activity and cell structure, such organisms can be classified into groups, genera, species, subspecies or pathovars based on their morphological or physiological reactions to the applied components (Harris-Baldwin and Gudmestad, 1996; Kritzman, 1991; McGuire et al.,

1986; Obradovic et al., 2004). Nutrient-utilisation analysis has, thus, become an important technique for detection, isolation and identification of some phytopathogens from host samples (Abdalla, 2000; Fatmi and Schaad, 1988; Kritzman, 1991; Kuflu and Cuppels, 1997). Combinations of these tests have become routine for diagnosis in most laboratories, especially in industry, as results can be highly informative, assays are relatively easy to prepare and evaluate, results are well defined for most pathogens and associated costs are relatively inexpensive as limited equipment is required.

To detect and isolate bacteria from host plant samples, a set of these tests is performed to provide preliminary identification and characterization (Abdalla, 2000; Klingler et al., 1992; Obradovic et al., 2000; Shenge et al., 2007). Diagnosticians can refer to references like Bergey's manual (Bergey et al., 2005), to facilitate identification of a range of bacteria based on reactions observed in standard diagnostic tests. Gram stains and potassium hydroxide (KOH) solubility tests, for example, classify bacteria as Gram positive or negative based on their cell wall structure as indicated by retention of the Gram stain or reaction with KOH. Oxidase, catalase and nitrate reduction tests give an indication of the metabolic properties of a bacterium, while other assays expose isolates to particular chemical components during propagation on a nutrient-supplemented growth medium (Fatmi and Schaad, 1988; Shenge et al., 2007). Bacteria may be classified based on their utilisation of essential nutrient sources like carbon and nitrogen, as these are available in different isoforms, but not all organisms metabolise these forms equally. Some bacterial genera, species and pathovars demonstrate conserved utilisation patterns of carbohydrates like glucose, sucrose, mannose, lactose, erythitol and sorbitol (Goszczynska et al., 2000). By performing assays on general growth media supplemented with different carbohydrates, differential growth patterns may enable taxonomic characterization of an isolated bacterial pathogen.

Although general characterization of pathogens is important for disease identification and management, the presence of saprophytes and non-target organisms in host material often obscures accurate detection of a pathogen, or can lead to false positive detection of a related pathogen (Goszczynska and Serfontein, 1998; Sijam et al., 1991). Selective media assays promote selective growth of organisms from a plant sample based on specially formulated growth media (McGuire et al., 1986; Sijam et al., 1991). The addition of specific carbon and nitrogen sources, antibiotics, indicators, chemicals, detergents and co-factors support differential morphological growth and recovery of target bacteria while reducing growth of contaminating organisms. By supporting the growth of only viable cultures, media assays also indicate the potential of recovered pathogens to cause disease on hosts (Maes, 1993; Schaad et al., 1995).

The formulation of selective media for diagnostic assays is challenging (Fatmi and Schaad, 1988; Goszczynska and Serfontein, 1998; McGuire et al., 1986). If a medium incorporates too many inhibitory compounds to eliminate contaminants, the growth of the target organism may also be impacted negatively, resulting in an unreliable estimation of pathogen concentration in host samples. This could lead to the trade of seed-lots with an unsafe level of pathogen contamination. Numerous media have been developed that successfully distinguish genera, species or pathovars of pathogens that are isolated from seed, soil and plant material (Bouzar et al., 1999; Fatmi and Schaad, 1988; Goszczynska and Serfontein, 1998; McGuire et al., 1986; Sijam et al., 1991). A study conducted by Kritzman (1991) analysed the capacity of a range of selective media to detect seed-borne pathogens of tomato. The authors were able to detect one cell of Pst in one million seeds, and at least one cell of Xcv and Cmm per gram of seed, thus demonstrating suitable recovery and isolation of viable target pathogens from host seed.

Despite the attractive features of selective medium plating, this technique is extremely labour intensive and time-consuming (Chitarra et al., 2000; Harris-Baldwin and Gudmestad, 1996; Kuflu and Cuppels, 1997; Lievens et al., 2003) and, therefore, unattractive for large-scale testing. Although most medium components are cheap and easy to use, some of the antibiotics used to limit growth of contaminating organisms are costly (De Leon et al., 2006). Selective media assays are also limited to the detection of culturable organisms (Lievens et al. 2003) and are unfavourable for slow-growing bacteria like *Clavibacter* spp. which may require between 7 and 14 days for colonies to reach a detectable size (De Leon et al., 2006; Harris-Baldwin and Gudmestad, 1996). Media must be screened extensively using target and non-target organisms, as cultural morphology may vary even among strains of the same pathogen. Currently, available selective media are limited to isolating individual pathogens, thus, assays must be repeated to get reliable results for multiple pathogens.

1.3.2.4. Biolog

Over the past few decades, multisystems have been developed for commercial phytodiagnostic applications which incorporate the principles of standard detection techniques into rapid, simplified and automated detection tests. The most popular of these is the Biolog system (Biolog Inc., CA, USA), which exploits differential carbohydrate utilisation patterns of aerobic bacteria to generate a metabolic fingerprint for each bacterial genus, species or pathovar for identification (Abdalla, 2000; Bouzar et al., 1999; Harris-Baldwin and Gudmestad, 1996; Klingler et al., 1992; Truu et al., 1999). The system features 95 different carbon sources in independent wells of a microplate, including sugars, alcohols, polymers and organic acids, coupled with a redox dye, tetrazolium. Suspensions of a pure

culture of the test bacterium are applied to the microplate and incubated for 4 – 24 hours. The optical density of each well is then recorded using a turbidimeter and results are interpreted by specialised computer software (Klingler et al., 1992; Truu et al., 1999). Changes in optical density result from electron release by bacteria during respiration/utilisation of the carbon source, which reduce the dye to the optically dense, violet formazan. Reactions with the 95 carbon sources are compiled into a profile and compared to a database containing carbon utilisation profiles of over 2000 different bacteria, to yield a list of the top 10 species with the closest statistical match to the output profile/fingerprint (Biolog Inc., 2009; Harris-Baldwin and Gudmestad, 1996). Microplates are available for both Gram negative and Gram positive bacteria, or may be customized to suit the needs of the application. Biolog is a rapid and simple test for straightforward identification of bacteria. The database is also regularly updated, re-evaluated and expanded to include more species and pathovars (Harris-Baldwin and Gudmestad, 1996; Klingler et al., 1992). In phytopathology, Biolog has been successfully utilised for rapid identification and classification of bacterial pathogens isolated from host samples (Abdalla, 2000; Bouzar et al., 1999; Harris-Baldwin and Gudmestad, 1996; Kaneshiro et al., 2006; Shenge et al., 2007). The Biolog system cannot be used to detect pathogens directly from host samples however, and requires isolation of bacteria into pure culture and propagation on standard growth media. Pathogens can also only be screened individually as the presence of multiple bacteria would obscure readings and likely cause misidentifications.

Conventional detection methods for plant pathogens are relatively simple, cost-effective and require limited technical expertise. They are, however, often laborious and time-consuming (Kuflu and Cuppels, 1997) and assays may take a number of weeks before results can be obtained. Only a single pathogen is detectable in most of these assays and, thus, tests must be repeated for all potential pathogens when validating a single seed-lot or plant sample. Despite limitations, techniques like selective media assays remain the standard method for screening seed-lots for important pathogens by organisations like EPPO and ISTA. This is mainly due to a lack of suitable alternatives, however, and the development of more reliable, specific and multiplex tools is expected to replace or augment these assays in the near future.

1.3.3. Serological detection techniques

Serology involves the detection and isolation of targeted proteins from a sample. The process is based on the specific binding of complimentary protein-based antibodies and foreign proteins (antigens) during immune responses in humans and animals. To develop these antibodies, a purified sample of the protein of interest is injected into a mammalian host, for example rats, mice, rabbits

or sheep, stimulating the mass production of complimentary antibodies which collect in the blood serum of the host. Antibodies are isolated from extracted serum, purified and then used during protein-targeted studies. In phytodiagnostics, antibodies are raised to target surface proteins of pathogens, thereby facilitating the detection of the respective pathogens from a host sample (Voller et al., 1976). Commonly targeted proteins included exopolysaccharides, glycoproteins and viral coat proteins which are largely conserved between groups of pathogens and are, therefore, suitable to detect and identify pathogens. Because pathogen detection is based on a highly specific interaction between complimentary proteins rather than on a morphological or physiological response, serological techniques are typically more sensitive and often more reliable than conventional methods, depending on the class of pathogen targeted and the inclusion of a stringent control to avoid false positive reactions (Boscia and Myrta, 1998; Hadas et al., 2004; Hamilton et al., 1981; Rajeshwari et al., 1998). Associated costs are moderate, making mass screening of samples feasible. Applications of serology have thus been adopted into standard protocols for certification of seed-lots or plant samples.

1.3.3.1. Immunofluorescence

Detection of pathogenic organisms by immunofluorescence (IF) involves target-specific binding of antibodies to a target pathogen protein, for example the 70S ribosome in bacteria (Schaad, 1978). Antibody:target complexes are then visualised using fluorescent staining and a fluorescent microscope. The simplicity, specificity and total assay time of approximately 30 minutes make this technique extremely attractive for quick validations, including direct assays on bacterial colonies (Kaneshiro et al., 2006; Veena and Van Vuurde, 2002). These features supported inclusion of IF into the latest revision of standard detection protocols for Cmm from tomato seed samples (EPPO/CABI, 2005). Immunofluorescence diagnostic assays do not indicate pathogen viability however (Chitarra et al., 2000; De Leon et al., 2008), and must be coupled with pathogenicity tests or media assays to establish the risk of detected cells in infected samples (Veena and Van Vuurde, 2002). High levels of background signal associated with fluorescent staining of complexes (Fatmi and Schaad, 1988) also affect the sensitivity of pathogen detection by this technique.

1.3.3.2. Enzyme-linked immunosorbent assay

First used in phytopathology for the detection of plant viruses (Voller et al., 1976), enzyme-linked immunosorbent assays (ELISA) have become routine diagnostic tests, especially for viral detection from plant and seed samples. The principle of an ELISA is based on the complimentary binding of a

targeted pathogenic protein within a sample to an applied enzyme-conjugated antibody (Clark and Adams, 1977; Voller et al., 1976). Assays are performed in light-permeable plastic wells that contain and bind antibody:antigen complexes. The presence of complexes is then determined by a colorimetric reaction produced by the hydrolysis of an applied chromogenic substrate by the immobilised, complex-conjugated enzyme (Boscia and Myrta, 1998; Clark and Adams, 1977). Alkaline phosphatase or horseradish peroxidase are enzymes commonly used in these assays as these metabolise particular substrates to produce coloured by-products. Higher levels of metabolism thus cause an increase in the concentration of coloured by-products and consequently an increase in optical density where target-antibody complexes have formed. A change in the optical density of a sample, therefore, indicates the presence of a target protein and, thus, the pathogen in that sample.

Assays are defined as direct or indirect according to the type of antibody to which the enzyme is conjugated (Van Regenmortel and Burckard, 1980). Primary antibodies, used in direct assays, are complimentary to the targeted, pathogenic protein and are also conjugated directly to the substrate-utilising enzyme. Indirect assays utilise both primary and secondary antibodies, but in these assays, enzymes are conjugated to secondary antibodies which are complimentary to the non-binding end of the primary antibody. An example of an indirect assay is depicted in Figure 1. 8, the plate-trapped antigen ELISA (PTA-ELISA), whereby samples or pathogen particles are applied to the surfaces of wells and trapped by a complex of primary and secondary antibodies for detection (Kaneshiro et al., 2006; Van Regenmortel and Burckard, 1980; Ward et al., 2004). In phytodiagnostics, the double antibody sandwich ELISA (DAS-ELISA) is the preferred serological

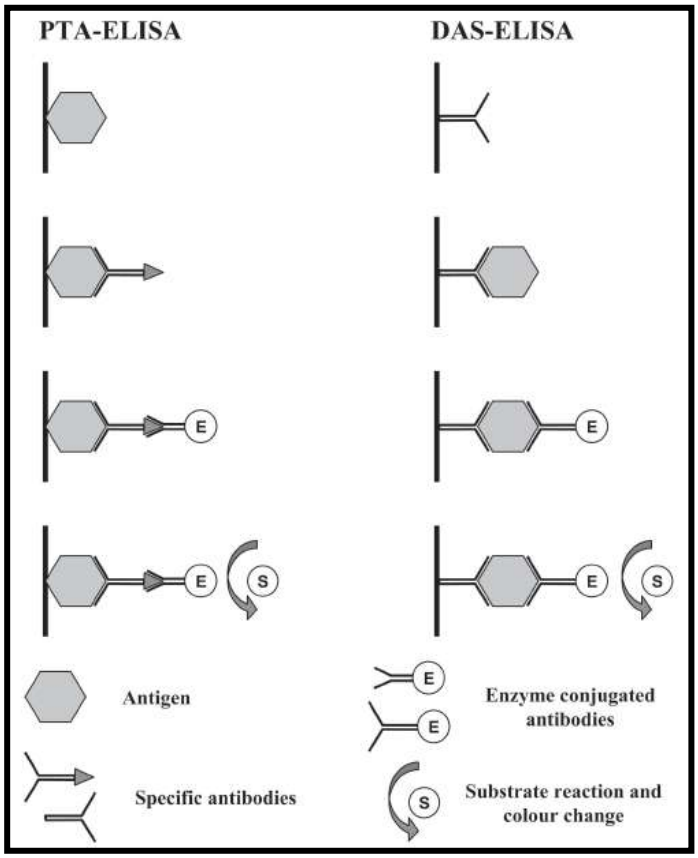

Figure 1. 8 Schematic representation of the plate-trapped antigen ELISA (PTA-ELISA) and double antibody sandwich ELISA (DAS-ELISA) (Ward et al., 2004)**. Antigens represent pathogen proteins within a sample that are targeted by complimentary antibodies. Immobilisation of enzyme-conjugated antibodies on a target allow for metabolism of an applied chromogenic substrate to produce a coloured by-product.**

technique, especially for viral detection from host samples (Hadas et al., 2004). This technique involves coating wells with target-specific antibodies to capture any complimentary pathogenic proteins in a sample (Figure 1. 8) (Clark and Adams, 1977; Voller et al., 1976). Bound complexes are then detected by applying another target-specific antibody, which is conjugated to an enzyme, to enable colorimetric detection of the antibody:target complex.

Successful detection of tomato pathogens from plant and seed samples using ELISAs has been reported previously (Bar-Joseph and Salomon, 1980; De Leon et al., 2008; Hadas et al., 2004; Kaneshiro et al., 2006; Rajeshwari et al., 1998). The specificity of this technique for bacterial detection can be variable, however, as target proteins are largely conserved between groups of bacteria (De Leon et al., 2008; Rajeshwari et al., 1998) and, therefore, may bind to irrelevant saprophytes. Low specificity has been attributed to the presence of seed debris and saprophytes within samples, which cause non-specific cross-reactions with antibodies, thus producing false positive results. The use of ELISAs for viral detection from plant and seed samples, on the other hand, has been shown to be superior to other available detection techniques. Surface proteins of viruses are more specific to targeted pathogens than bacteria and, therefore, this technique demonstrates higher sensitivity and specificity for viral detection than bacterial detection. Hadas et al. (2004) demonstrated reliable detection of low titres of ToMV, even in seed samples, using DAS-ELISA, reporting detection thresholds of 10 ng/mℓ from host samples. This technique also facilitated characterization of a new strain of PepMV infecting tomato based on serological homology with the pepino isolate (Van der Vlugt et al., 2000, 2002).

Antibodies for detection of viral pathoens are usually developed to target viral coat proteins and are, therefore, usually capable of differentiating between various morphologies and sizes of viruses, from both purified and crude extracts of infected samples within 48 hours (Clark and Adams, 1977; Voller et al., 1976). With the development of solid-surface microplates (Clark and Adams, 1977), this technique has been converted into easy-to use kits for robust and sensitive screening of numerous field samples or seed-lots for a target pathogen. Reagents are fairly inexpensive and limited specialised equipment is required for the assays, making ELISAs extremely economical (Vinayarani et al., 2011; Ward et al., 2004). Mixed infections in seed or plant samples are detectable using ELISAs by including antibodies specific to each targeted pathogen in the assay. Sample assays cannot be performed in a single well, however, as competing antibodies may obscure results or result in cross-reactions between serologically related pathogens (Engel et al., 2010; Hadidi et al., 2004; Vinayarani et al., 2011). Detection of multiple pathogens from a sample using this technique can, thus, be

laborious and impractical. High specificity is not always advantageous in pathogen detection, as this limits diagnosis to known or expected pathogens (Boscia and Myrta, 1998), reducing the chance of detecting emerging or unknown viruses. This technique does not discriminate between proteins of viable viral particles and those of fragmented or dead pathogens, potentially resulting in false positive classifications especially for treated seed. Assays are, thus, usually combined with other diagnostic techniques such as pathogenicity tests or bioassays (Hadas et al., 2004) to obtain an accurate indication of the presence of viable pathogens within a sample.

1.3.3.3. Immunosorbent electron microscopy

To extend the detection capacity of electron microscopy, antibody-targeted binding was introduced that preferentially binds targeted pathogens to EM grids in a technique referred to as immunosorbent electron microscopy (IEM) (Nicolaieff and Van Regenmortel, 1980). The most popular application of this is, solid phase IEM (SPIEM), where microscope grids were coated with capture antibodies, was found to increase detection sensitivity of EM by 100 fold (Biel and Gelderblom, 1999), as antibodies enable stringent discrimination between target and non-target particles. By forming a double sandwich of antibodies as utilised in DAS-ELISA (known as double decoration IEM), sensitivity and specificity of detection is comparable to that of DAS-ELISA (Biel and Gelderblom, 1999; Kerlan et al., 1982) with the added advantage of visual validation of pathogen presence. Antibodies for use in IEM are less expensive as they are unconjugated, and needn't be purified, as a pathogen of interest can be observed visually. For accurate results, however, control samples should be incorporated into assays as the binding of particles in the presence of electrons may be erratic (Nicolaieff and Van Regenmortel, 1980). Nevertheless, dependence on expensive and technical equipment still limit the use of this technique for mass screening of leaf and seed samples.

1.3.3.4. Immunomagnetic separation

A serological technique with good diagnostic potential for phytobacterial detection is immunomagnetic separation (IMS) (De Leon et al., 2006, 2008; Güven and Mutlu, 2000; Hartung et al., 1996; Walcott and Gitaitis, 2000). This technique employs spherical, magnetisable beads (known as IMBs), which are coated with pathogen-specific antibodies that selectively trap and concentrate pathogenic cells or particles present within samples. Target pathogens or particles are then isolated from extracts through application of a strong magnetic field and recovered on non-selective or semi-selective growth media (De Leon et al., 2006, 2008; Güven and Mutlu, 2000). Specific antibodies are developed against highly conserved bacterial surface proteins by challenging mammalian hosts with

entire cells of the target pathogen (Güven and Mutlu, 2000). This technique is attractive as it combines the specificity and sensitivity of serology with the isolation and propagative capacity of media plating. Most contaminating saprophytes or non-target bacteria are removed from extracts prior to plating and any viable cultures that remain can be distinguished from target bacterial pathogens by morphological differences on the agar media (De Leon et al., 2006, 2008). By isolating and concentrating targets before media assays, the presence of seed debris, leaf tissue and saprophytes have less adverse effect on the specificity of detection (De Leon et al., 2006, 2008; Güven and Mutlu, 2000; Hartung et al., 1996; Walcott and Gitaitis, 2000). Thus IMS is highly efficient and sensitive, while not requiring complex equipment or technical expertise. Immunomagnetic separation has been used for the specific detection of pathogens from plant seeds including those of tomato (De Leon et al., 2006, 2008; Walcott and Gitaitis, 2000). Detection limits range from 10 to 10^3 CFU/ml with a 50 – 90% recovery of bacteria from host samples (De Leon et al., 2006, 2008; Güven and Mutlu, 2000). These results make IMS superior to other serological techniques and media assays for bacterial pathogens boasting up to 100 fold improvement in detection sensitivity. Assays may be combined with other detection techniques such as molecular strategies for increased specificity and sensitivity or for validation of detection (Hartung et al., 1996; Walcott and Gitaitis, 2000). However, a large portion of target bacteria may be lost during buffer washes and, therefore, detection may not be entirely reliable.

Serological techniques demonstrate greater specificity and sensitivity compared to conventional detection methods. Though good specificity is crucial for accurate diagnostics and classification, it also limits detection of emerging strains or related pathogens within a sample, especially if an infection is not suspected in a plant sample (Adams et al., 2009; Boscia and Myrta, 1998). The high degree of conservation in surface and coat proteins also facilitates cross-reactions of antisera with non-pathogenic relatives and, potentially, seed extracts, causing misdiagnosis (Bar-Joseph and Salomon, 1980; Leite et al., 1995; Letschert et al., 2002; Van Regenmortel and Burckard, 1980; Vinayarani et al., 2011). Serological methods cannot detect very low titres of pathogen infection and are limited to organisms with detectable surface or coat proteins, therefore, excluding detection of viroids and phytoplasmas (Boscia and Myrta, 1998; Hadidi et al., 2004). Even exposed and/or surface antigens of pathogen particles or cells, can be risky targets for antibody development as these constantly evolve to evade recognition by host immune systems (Wang et al., 2002). New antisera should therefore be under continuous evaluation but this requires intensive screening and optimisation assays (Clark and Adams, 1977; Engel et al., 2010; Van Regenmortel and Burckard, 1980). Although ELISA and IF techniques form part of many seed certification protocols (ISF 2009a,

2009b), more sensitive methods are needed for accurate detection of multiple pathogens from a large number samples.

1.3.4. Molecular-based strategies

Detection tools based on the molecular characteristics of a pathogen target their genomic nucleic-acid sequences. Portions of the genome form genetic elements that encode functional proteins facilitating replication and pathogenicity of the pathogen within a host. Certain elements of pathogen genomes are specific to groups or species of organisms, making these ideal targets for detection. Targeting nucleic acid, as opposed to exposed proteins, enables molecular-based detection strategies to be more specific for detection of all pathogens, including pathogens without cell walls, such as phytoplasmas and viroids (Boonham et al., 2000; Mumford et al., 2006; Njambere et al., 2011). Pathogens can be detected directly from DNA or RNA extracts of infected host samples and, therefore, the tests do not rely on culturing pathogens on supplemented media, enabling detection of even non-culturable organisms in a much shorter time than other diagnostic tools (Lievens and Thomma, 2005; Lievens et al., 2005; Nicolaisen and Bertaccini, 2007; Sholberg et al., 2005; Van Doorn et al., 2009). Total extraction of both host and pathogen nucleic acid is performed using a range of chemicals to remove contaminating cellular components, proteins, polysaccharides and polyphenols. Although methods can be laborious and technical (Boonham et al., 2008; Dovas et al., 2004; Lievens et al., 2003; Mumford et al., 2006; Sholberg et al., 2005), these have been improved by the development of partially automated, column-based purification procedures that facilitate efficient, high quality recovery of DNA and RNA from a variety of host samples including leaves, roots, seed and fruit.

Targeting pathogen nucleic acid has exponentially improved sensitivity and specificity of detecting a particular pathogen or group of pathogens from leaf and seed samples (Gutierrez-Aguirre et al., 2009; Mansilla et al., 2003). These techniques facilitate detection of even low titres of pathogenic particles which was not possible by serological and conventional methods. This is crucial for detection of seed-borne pathogens like PepMV (Córdoba-Sellés et al., 2007; Gutierrez-Aguirre et al., 2009; Hanssen et al., 2010b; Ling, 2008), where a single infected seed amongst hundreds or thousands of healthy seeds can cause devastating epidemics. Molecular techniques also demonstrate improved capacity to detect new, unknown or emerging pathogens (Adams et al., 2009; Deyong et al., 2005; Mansilla et al., 2003; Van der Vlugt et al., 2000). The emergence of PepMV in tomato plants in 1999, though broadly characterized by electron microscopy, was reliably characterized using nucleic-acid based techniques that targeted a conserved region within the

Potexvirus genus (Mansilla et al., 2003; Van der Vlugt and Berendsen, 2002; Van der Vlugt et al., 2002).

Targeting particular pathogens or groups of pathogens is achieved by exploiting the mechanism of complimentary binding between nucleic acids. By generating a sequence of nucleic acids that is specifically complimentary to a portion of the target pathogen genome, a probe or primer will preferentially detect and bind to a complimentary sequence if it is present in a plant sample. Binding, therefore, indicates the presence of a target pathogen within a sample. Synthetic primers/probes are developed using a pre-determined sequence of nucleic acids which are designed based on *in silico* analysis of pathogen genome sequences and facilitate identification of suitable diagnostic regions (Schaad and Frederick, 2002). The advent of various methods of sequencing has led to an exponential increase in available sequence data for various organisms, which has facilitated the design of highly specific primers/probes to enable diagnosis of target pathogens (Adams et al., 2009; Leite et al., 1995; Ling et al., 2007; Schaad and Frederick, 2002). By aligning more sequences of the target group of pathogens, primers and probes can be designed that will definitely bind to targeted families, species or pathovars of a pathogen.

A wide range of techniques targeting pathogen nucleic acid has been developed over the past few decades. Although few of these have been incorporated into standard detection protocols, numerous applications have demonstrated improved capacity for rapid, specific and sensitive detection compared to other available detection methods. Template preparation, associated costs and machinery requirements of each of these methods however, affect the practicality of adoption for large-scale diagnosis of tomato pathogens from plants and seed.

1.3.4.1. Blotting of diagnostic probes

The use of DNA probes in detection was an early method of molecular-based phytodiagnostics and has been used in a number of studies for detection of bacterial, and some viral, pathogens of tomato (Alfaro-Fernandez et al., 2009; Fanelli et al., 2007; Kuflu and Cuppels, 1997; Leite et al., 1994; McManus and Jones, 1995; Thompson et al., 1989). In these assays, pathogen-specific DNA probes are immobilised or blotted onto a support substrate, such as a nylon filter or membrane, and host plant DNA extracts are applied to the membrane to hybridise to the probes. Any non-bound sample fragments are removed by buffer washes such that only probe-target complexes remain on the substrate. In order to visualise the bound complexes, probes or sample extracts are labelled prior to hybridisation using radioactive labels (Thompson et al., 1989), or fluorescent dyes such as

digoxygenin (DIG) (Fanelli et al., 2007; Kuflu and Cuppels, 1997; Leite et al., 1994). By exposing the substrate to X-ray or fluorescent light, the presence of a complex and, thus, the presence of a target pathogen within a sample, is indicated by the emission of a detectable signal. By using *in silico* alignments and BLAST searches for evaluation of sequence homology, synthetic probes can be designed to detect, identify and even classify detected organisms from a sample by genus, species or even subspecies. Diagnostic probes for pathovars of bacterial pathogens of tomato have been reported for Cmm (Thompson et al., 1989) as well as Xv and Xav (Kuflu and Cuppels 1997). A dot blot assay with potential for seed health testing for Pst was developed by Fanelli et al. (2007), who designed a probe that bound only Pst cells from both pure cultures and seed wash samples, and no other related, non-target or host DNA (Figure 1. 9). The technical nature of blotting has made this technique limited for high-throughput testing of host samples, as assays can take up to 5 days to obtain reliable results (Cuppels and Elmhirst, 1999; McManus and Jones, 1995; Park et al., 2009; Schaad and Frederick, 2002). However, the high specificity demonstrated by this technique makes it ideal for validating binding specificity and sensitivity of primers or probes designed for other molecular strategies (Alfaro-Fernandez et al., 2009; Cuppels et al., 2006; Fanelli et al., 2007; McManus and Jones, 1995; Park et al., 2009). The development of premade, preblotted membranes has improved the capacity of this tool for use in seed pathology testing.

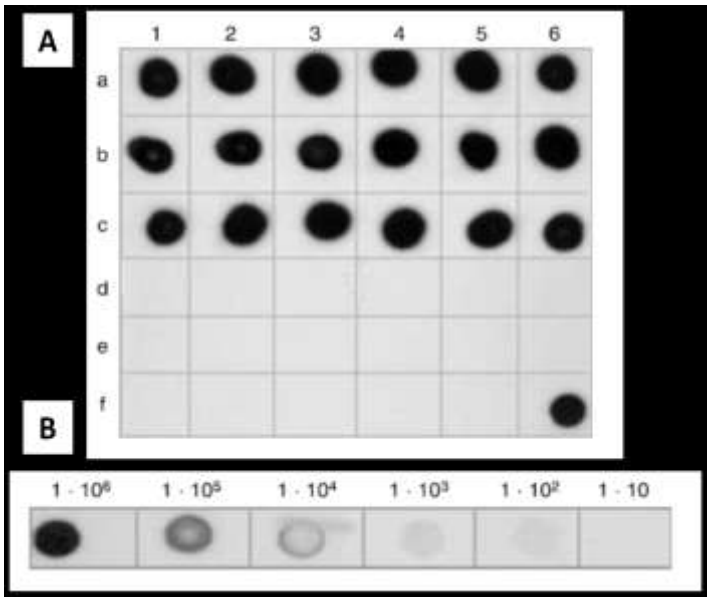

Figure 1. 9 Dot-blot of a diagnostic probe designed for the detection of Pst. A - Blocks 1a – 6c represent hybridisations between the probe and Pst isolates where 6f is a positive control. The remaining blocks demonstrate incompatible reactions with a range of other phytopathogens and related pathovars of Pst. B – Blots demonstrate the sensitivity of the diagnostic probe at a range of dilutions of pure culture Pst DNA extracts. (Fanelli et al., 2007)**.**

1.3.4.2. Detection by PCR and PCR applications

The development of the polymerase chain reaction (PCR) by Kary Mullis in 1984 revolutionised molecular biology. It became instrumental in diverse applications due to its high sensitivity and versatility for nucleic acid amplification from any organism, including human, animal and plant pathogens (reviewed by Schaad and Frederick, 2002; Wang et al., 2002). The value of PCR in phytodiagnostics was first described by Henson and French (1993), and has since been utilised for the detection of nematodes, fungi, bacteria, viruses, viroids and phytoplasmas, including pathogens of tomato (Cuppels et al., 2006; Da Silva et al., 2008; Dovas et al., 2004; Leite et al., 1994, 1995; Kaneshiro et al., 2006; Maes, 1993; Obradovic et al., 2004; Palacio-Bielsa et al., 2009; Park et al., 2009; Van der Vlugt and Berendsen, 2002; Van der Vlugt et al., 2002; Vinayarani et al., 2011). Briefly, PCR in diagnostics involves directed amplification of a specific fragment of a pathogenic genome by targeted binding of a pair of complimentary primers on opposite ends of the target region. Subsequent polymerization to copy the sequence fragment occurs by exploiting the function of replication and transcription enzymes - DNA or RNA polymerase. If the pathogen of interest is present within a plant sample, primer binding and subsequent cycles of amplification result in the production of thousands of copies of the target fragment which can then be detected and visualised by using down-stream techniques like electrophoresis and southern blotting (Alfaro-Fernandez et al., 2009; McManus and Jones, 1995; Schaad and Frederick, 2002). Pathogens like viruses, viroids and phytoplasmas, which contain RNA genomes, are amplified by reverse-transcription PCR (RT-PCR) (Van der Vlugt and Berendsen, 2002; Vinayarani et al., 2011). This form of PCR converts RNA into the more stable, single stranded complimentary DNA (cDNA) and then into double-stranded DNA (dsDNA) in either a single RT-PCR reaction (one-step) (Dovas et al., 2004; Jacobi et al., 1998; Ling, 2008; Pagán et al., 2006), or two separate reactions using two sets of primers (two-step) (Gutierrez-Aguirre et al., 2009; Vinayarani et al., 2011).

The degree of specificity of pathogen detection from infected samples is dependent on meticulous primer design such that primers bind only to pre-determined regions in the pathogen genome, thereby producing copies of only a single amplified fragment when a pathogen is present (Alfaro-Fernandez et al., 2009; Park et al., 2009; Schaad and Frederick, 2002). Cross-hybridisations, where primers bind to more than one site in either the host or pathogen genome, lead to production of more than one amplified fragment, which decreases amplification efficiency (McManus and Jones, 1995; Wang et al., 2011) and reduces the accuracy of pathogen diagnosis. Diagnostic primers are designed to target certain regions based on the taxonomic level of detection that is required. Subspecies and pathovar classification is not always necessary, especially when detection of both

characterized and unknown or emerging strains of the same genus or species is required (Boonham et al., 2007; Dovas et al., 2004; Van der Vlugt and Berendsen, 2002). Genus- and species-specific primers are generally designed for highly conserved portions of pathogen genomes like the 16S rDNA region for bacterial classification (Kaneshiro et al., 2006; Lievens and Thomma, 2005; Maes, 1993) and the coat protein or replicase genomic regions for viral detection (Deyong et al., 2005; Van der Vlugt and Berendsen, 2002; Van der Vlugt et al., 2002). Incorporating degenerate primers is especially valuable for genus-specific amplification where a conserved consensus sequence is difficult to find among related species, for example in viral diagnosis. Degenerate primers contain a few universal nucleic acids and decrease the stringency of primer binding by allowing for more flexible nucleotide complimentarity (Dovas et al. 2004; Wang et al. 2002). These, thus, facilitate detection of known and novel strains of a genus and are valuable when more than one species of a genus is known to infect a host, for example, PVX and PepMV *Potexviruses* (Mansilla et al., 2003; Van der Vlugt and Berendsen, 2002) or bacterial spot of tomato (Cuppels et al., 2006; Jones et al., 2004, 2005; Maes, 1993). These primers are insufficient for identification of a particular pathogen though (Deyong et al., 2005), and may require a subsequent assay for more informative characterization.

For subspecies, pathovar or isolate detection, primer design is usually based on regions that are unique to the targeted pathogen or demonstrate sufficient sequence variability from related strains, non-target saprophytes and host genomes to enable stringent discrimination and, therefore, specific detection (Da Silva et al., 2008; Lievens and Thomma, 2005; Park et al., 2009). Two independent studies (Leite et al., 1994; Obradovic et al., 2004) both developed primers targeting the *Hrp* pathogenicity island for detection of Xcv from infected tomato and pepper samples. In both assays, developed primers did not cross-hybridise to other tomato pathogens, and binding to saprophytes, opportunistic pathogens and host DNA usually present in leaf and seed samples was reliably prevented. A few mismatched nucleotides between the primer and target sequence may be sufficient to prevent binding to target regions that demonstrate as little as one single nucleotide polymorphism (SNP) (Boonham et al., 2000; Cuppels et al., 2006). This feature supports the high discrimination capacity of the PCR technique for specific diagnosis but can also result in false negative results or poor amplification of strains and isolates with spontaneous mutations and random sequence divergence (Alfaro-Fernandez et al., 2009; Ling et al., 2007; Lee et al., 2003). Besides target sequence regions, stringent primer binding is also dependent on a range of thermodynamic conditions which can be manipulated to favour binding to specific regions of a pathogen genome (Widmer et al., 1998).

The use of conventional PCR for diagnosis of tomato pathogens is far more sensitive than ELISA and selective plating, and interpretation of results is less complicated (Fanelli et al., 2007; Kuflu and Cuppels, 1997; Lievens and Thomma, 2005; Park et al., 2009). Assays are capable of reliably detecting as little as 30 CFU from pure cultures of phytobacteria (Cuppels et al., 2006) and between 10^2 and 10^3 CFU/mℓ from seed and leaf samples (Leite et al., 1994, 1995; Maes, 1993; McManus and Jones, 1995; Meng et al., 2004), even in the presence of higher concentrations of competing non-target DNA than target pathogen DNA. Detection by conventional PCR is, therefore, highly specific, requires limited technical expertise and results can be generated in a few hours once nucleic acid has been extracted from host samples (Da Silva et al., 2008; Lievens and Thomma, 2005; Maes, 1993; Park et al., 2009). Testing large numbers of samples for a single pathogen has also become practical with the development of 96 well plates that are compatible with most PCR thermocycler machines. Environmental samples, however, often contain high concentrations of saprophytes, non-targets and non-virulent relatives of the target pathogen, which may obscure detection of the pathogen of interest or result in misdiagnosis (Leite et al., 1995; Lievens and Thomma, 2005; Kaneshiro et al., 2006; Wang et al., 2002; Wilson et al., 2002). Dead cells and free nucleic acid fragments are also detectable by primers and, thus, PCR is incapable of distinguishing between DNA from viable and non-viable pathogens within a host sample (Kuflu and Cuppels, 1997; Lievens et al., 2005; Lievens and Thomma, 2005; McManus and Jones, 1995; Meng et al., 2004). Quantification of pathogen infection by this technique is limited, relying on comparisons with calibration curves which can be difficult to develop accurately. To improve the short-comings of conventional PCR a number of complimentary or independent applications of PCR have been developed over the past few decades. Only a few of these, however, have proven to be useful in phytodiagnostics and still require optimisation before they can be successfully incorporated into certification protocols for tomato seed.

Direct PCR and bio-PCR

The technical and laborious process of nucleic acid extraction is removed in the method of direct PCR as detection assays are performed directly on leaf or seed extracts. This method has been used successfully for the detection of bacterial pathogens in tomato seed extracts (De Leon et al., 2006, 2008; Maes, 1993; Milijašević et al., 2007; Schaad et al., 1995). Direct detection from host samples enables quicker generation of results than conventional PCR but the sensitivity of this technique is often reduced due to the effect of host cell residues on amplification efficiency (De Leon et al., 2008; McManus and Jones, 1995; Milijašević et al., 2007). Determining the viability of detected pathogens is also not possible using direct PCR.

Schaad et al. (1995) described the technique, bio-PCR, that incorporated a pathogen culturing step into direct PCR protocols for the detection of phytobacteria from seed extracts. This was shown to be successful for detection of Xanthomonad species from host samples by Maes (1993) prior to the study conducted by Schaad et al. (1995). The added step essentially enriches all culturable organisms present on the seed, therefore enabling detection of pathogens present in low concentrations (Milijašević et al., 2007; Schaad and Frederick, 2002; Schaad et al., 1995). In the assays, seed are homogenized in buffer and the extracts are plated onto a selective (Maes, 1993), or general growth medium (Schaad et al., 1995), recovered colonies are then washed off the media after incubation, and the plate washes are used directly in PCR reactions. This technique is simple, recovers only viable organisms and reduces the effect of PCR inhibitors, thereby reducing the risk of false positive and false negative results (Schaad et al., 1995; Schaad and Frederick, 2002). The specificity of PCR detection also enables distinction between morphologically similar saprophytes or other non-target organisms (Maes, 1993), making identification more reliable than selective plating. Improved sensitivity of the technique compared to ELISA and conventional PCR has been indicated by a study where bio-PCR facilitated the detection of one seed infected with Cmm in a sample of 2000 non-infected seed (Milijašević et al., 2007). Dependence on growth media again restricts detection to culturable pathogens with this technique and delays generation of results compared to conventional PCR. Nevertheless, bio-PCR is ideal for phytobacterial detection in facilities with limited equipment and, when coupled with positive controls, reliable and accurate diagnosis should be possible.

Nested PCR

To increase the sensitivity of conventional PCR, nested PCR was developed which incorporated a second round of amplification using primers targeting regions internal to those used in the primary PCR reaction (McManus and Jones, 1995; Schaad et al., 1995). Low concentrations of pathogen nucleic acid are augmented in this secondary round of amplification, which reduces the incidence of false negative results and improves the sensitivity of detection. By incorporating species-specific or pathovar-specific primers in the second round of amplification, pathogens of the same genus can also be distinguished reliably, thereby facilitating discrimination among related species/strains in a mixed infection, for example ToMV and TMV in tomato (Dovas et al., 2004; Letschert et al., 2002). Pathogen-specific primers used in the second round of amplification have also increased the specificity of detection by excluding amplification of any false positive fragments potentially generated during the initial PCR amplification (McManus and Jones, 1995). Detection of both bacterial and viral pathogens have been reported using nested PCR (Dovas et al., 2004; Hartung et al., 1996; Letschert et al., 2002; McManus and Jones, 1995; Schaad et al., 1995), but application in

standard diagnostics is limited due to the high risk of cross contamination when processing large numbers of samples. Relatively simple sub-species discrimination by this method however, makes it favourable for further optimisation of high-throughput capacity.

Genomic fingerprinting

Repetitive-sequence-based PCR (rep-PCR) or genomic fingerprinting methods were developed to standardise PCR protocols for the detection and differentiation of pathovars of prokaryotic pathogens from various substrates (Louws et al., 1995, 1998). Three conserved elements, BOX, REP and ERIC are randomly repeated throughout bacterial genomes and, when used as primers, generate numerous amplicons of various lengths that form a characteristic pattern or fingerprint when separated by gel electrophoresis. Although these elements are highly conserved within a number of genera, fingerprints are usually unique to isolates and strains of even closely related species, making this tool ideal for detection and characterization of bacterial isolates (Bouzar et al., 1999; Louws et al., 1998). Genomic fingerprinting is one of the few methods that can reliably distinguish pathovars causing bacterial spot of tomato (Bouzar et al., 1999; Kuflu and Cuppels, 1997; Louws et al., 1995) and has also been used to differentiate subspecies of Cm (Figure 1. 10) (Louws et al., 1998) and pathovars of *Pseudomonas syringae* (Cuppels et al., 2006). This technique is, however, susceptible to variations associated with spontaneous mutations among bacterial strains, and is dependent on visual interpretation of results which may affect accurate and consistent classification.

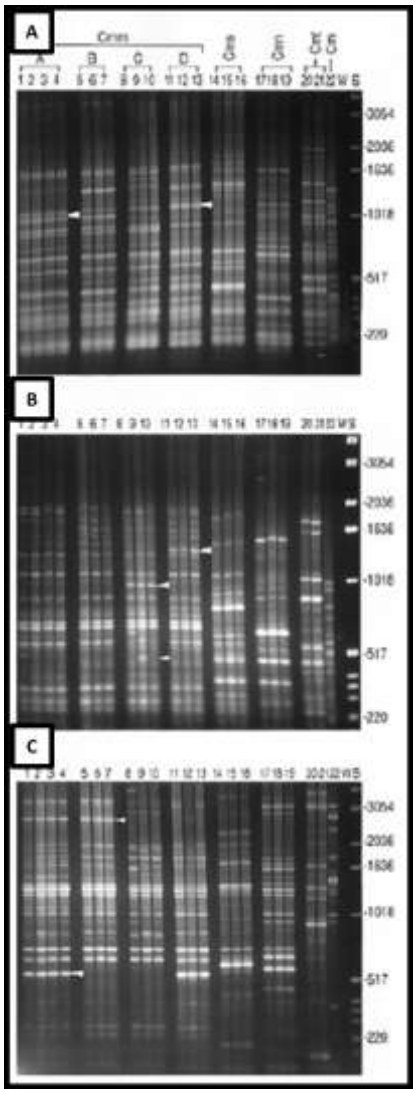

Figure 1. 10 Genomic fingerprint of subspecies of *Clavibacter michiganensis* generated by rep-PCR using the BOX (A), ERIC (B) and REP (C) genetic elements as primers (Louws et al., 1998).

Immunocapture PCR

Serological and molecular methods have been combined in immunocapture (IC)-PCR techniques where pathogens are detected directly from host samples without the use of nucleic acid extraction procedures (Jacobi et al., 1998; Ling et al., 2007; Mansilla et al., 2003). This technique, in contrast to

bio-PCR, is favoured for detection of viral particles from host samples as antisera for bacterial detection typically have limited discrimination capacity. In IC-PCR, viral particles are isolated and concentrated from samples by coating PCR tubes with complimentary antibodies (Jacobi et al., 1998). After an incubation period, non-bound samples are removed from tubes by buffer washes and bound viral particles are detected by RT-PCR using pathogen-specific primers. Combining serological and molecular techniques drastically improves the sensitivity and specificity of detection compared to each technique individually and eliminates the need for complex RNA extraction procedures (Mansilla et al., 2003; Jacobi et al., 1998). For example, a diagnostic assay for PepMV from tomato seed using IC-RT-PCR (Ling et al., 2007), demonstrated a detection limit of 100 fg/mℓ of purified viral particles, and detection of one infected seed in 1000 healthy seeds. Quantification and high-throughput detection of pathogen infection by this technique is limited, however (Schaad and Frederick, 2002), and detection of non-viable viral particles can still occur.

Multiplex PCR

Detection of more than one pathogen in a host sample by conventional PCR is possible only if the target pathogens fall within the same taxonomic group (Szemes et al., 2005; Van der Vlugt and Berendsen, 2002). Tomato seed may host a number of diverse pathogens simultaneously and, thus, seed certification tests using PCR to prove the absence of all potential threats must be done individually, which is arduous and often impractical (Alfaro-Fernandez et al., 2009; Kritzman, 1991). Multiplex PCR assays are, therefore, highly relevant in the field of tomato seed pathology, which, by incorporating multiple primer pairs into a single PCR assay, facilitate the detection of more than one pathogen from a host sample (Lievens and Thomma, 2005; Ozdemir, 2009; Vinayarani et al., 2011; Wang et al., 2002). This drastically reduces the time, labour and cost of reagents required to validate the presence or absence of individual or groups of pathogens, i.e., in mixed infections (Alfaro-Fernandez et al., 2009; Boonham et al., 2000).

With careful design of diagnostic primers for each target pathogen, different genera (Ozdemir, 2009), species (Vinayarani et al., 2011), strains and isolates (Alfaro-Fernandez et al., 2009) can be detected and distinguished using multiplex PCR. Results are visually concluded by size-dependent gel electrophoresis and, thus, primers are designed to amplify fragments of different lengths from targets to facilitate adequate resolution, thereby facilitating detection and differentiation of pathogens (Lievens and Thomma, 2005; Vinayarani et al., 2011). Detection and discrimination of up to nine different pathogens in a single reaction has been reported (Alfaro-Fernandez et al., 2009; Mortimer-Jones et al., 2009), but including too many primer sets in a single reaction becomes

difficult technically and may impact the efficiency of amplification. Designing primers to be highly discriminatory for target pathogen sequences is possible, but extremely challenging (Agindotan and Perry, 2007; Boonham et al., 2007; Engel et al., 2010; Lievens and Thomma, 2005; Ozdemir, 2009). Competition, inter-primer homology and poor target binding efficiency are all potential drawbacks which may cause false negative results. Primers must amplify each target efficiently under the same reaction conditions (Ozdemir, 2009) and, thus, amplification of each primer set must be optimised carefully, which can be labour-intensive and time-consuming.

Despite this, three seed-borne pathogens of tomato, Cmm, Xav and Pst, have been detected in an artificial sample using multiplex PCR with three pathogen-specific primer pairs (Ozdemir, 2009), but a significant decrease in the sensitivity of detection of the three pathogens, compared to conventional PCR assays run independently for each pathogen, was observed. The detection limit of multiplex PCR is highly variable however, and dependent on the nature of the target pathogen. Alfaro-Fernandez et al. (2009) developed a multiplex PCR system to characterize five isolates of PepMV using 3 primer pairs and an internal control sample. They claimed that the assay demonstrated a 3.125x higher sensitivity than both DAS-ELISA and conventional PCR for detection of the five isolates. Disadvantages of multiplex PCR currently discourage routine use of this technique in phytodiagnostics, but combining multiplex with other techniques should improve the detection and discrimination capacity of multiplex PCR for accurate, sensitive and specific detection of multiple pathogens from host samples (Boonham et al. 2007).

Real-time PCR

The development of real-time PCR in the late 1990s sparked interest in a range of fields, including plant pathology, in which assays demonstrated the capacity to detect bacterial or viral pathogens from pure cultures, leaves and seed extracts with a much higher accuracy and sensitivity compared to conventional PCR (Bach et al., 2003; Boonham et al., 2000; Fanelli et al., 2007; Gutierrez-Aguirre et al., 2009; Ling et al., 2007; Schaad and Frederick, 2002; Zhao et al., 2007). In real-time PCR, a fluorescently labelled probe specifically targets a complimentary region within a primer-amplified fragment of the pathogen genome, emitting a fluorescent signal upon binding. Because amplification of the target fragments by pathogen-specific primers increases template concentration, higher incidences of probe binding causes a proportional increase in signal emission (Fanelli et al., 2007; Schaad and Frederick, 2002). The emitted signal is detected by software that compiles signal readings into a readable curve for the entire reaction cycle. Formation of a fluorescent curve indicates both primer and probe binding and, thus, the detection of a target template or pathogen

sequence within a sample. Dependence on both primer and probe binding in real-time PCR increases both the specificity and accuracy of detecting a complimentary pathogen target, and enables quantification of pathogen concentration within the sample (Deyong et al., 2005; Gutierrez-Aguirre et al., 2009; Lievens and Thomma, 2005; Ling et al., 2007; Schaad and Frederick, 2002; Zhao et al., 2007). Amplification curves are also informative of weak hybridisations, false positives or the presence of PCR inhibitors as these deviate from standard curves of expected results for primer/probe-target binding (Bach et al., 2003; Schaad and Frederick, 2002).

With this technique, results are generated in real-time, therefore eliminating the need for post-assay confirmation techniques like southern-blotting and electrophoresis (Boonham et al., 2000). The fast cycling time of reactions also drastically reduces amplification assays to between 20 minutes and five hours (Fanelli et al., 2007; Gutierrez-Aguirre et al., 2009; Ling et al., 2007; Schaad and Frederick, 2002; Zhao et al., 2007). Improved sensitivity of detection using real-time PCR is approximately 10 – 1000 fold higher than with conventional PCR and ELISA, respectively (Fanelli et al., 2007; Gutierrez-Aguirre et al., 2009; Schaad and Frederick, 2002), with detection limits for seed-borne pathogens ranging from 1 infected seed in 1000 healthy seed (Ling et al., 2007) to 1 in 5000 healthy seed (Gutierrez-Aguirre et al., 2009). The increased availability of multiple fluorescent dyes stimulated the development of multiplex real-time PCR. For multiple pathogen detection from a sample using this technique, target-specific primers and probes differentiate among pathogens from different genera, species (Boonham et al., 2000), subspecies (Figure 1. 11) (Bach et al., 2003), strains or isolates (Ling, 2007) in a single reaction. The wavelengths of the different dyes form discrete amplification curves for each probe, therefore eliminating the need for resolution by amplicon size (Lievens and Thomma, 2005). Only a limited number of dyes are currently available however, and too many fluorescent signals in a single reaction obscures resolution among targets. Currently, multiplex detection is limited to six pathogens or six different primer/probe sets per reaction. Fluorescent dyes are costly and, thus, impractical for use outside of research (Lievens et al., 2005; Schaad and

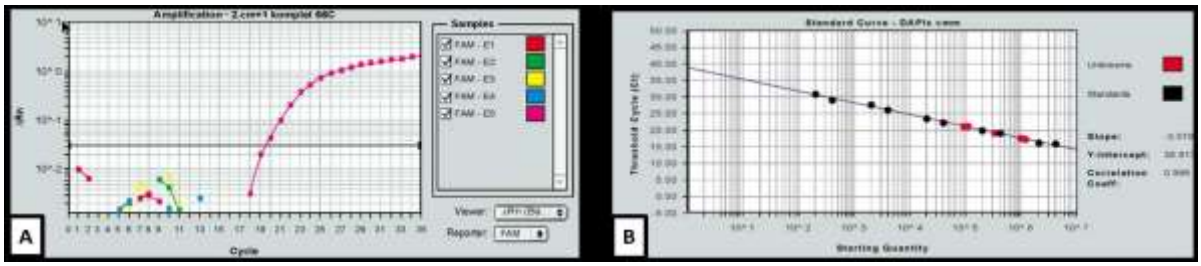

Figure 1. 11 Real-time PCR assay for the detection of subspecies of *Clavibacter michiganensis* (Bach et al., 2003)**. Differentiation of Cmm from other subspecies – Cmm (pink), Cms (red), Cmt (green), Cmn (yellow), Cmi (blue) (A). Standard curve for quantification of pathogen concentration (B) – the regression line indicates the relative concentration expected after various amplification cycles using a range of concentrations of target DNA.**

Frederick, 2002). Design and optimisation of primers and probes for detection is as complicated for real-time PCR as multiplex PCR detection assays, which must be highly specific for targets and not related organisms or host DNA/RNA.

Conventional PCR and the many applications of this technology have demonstrated an exponential improvement in specificity and sensitivity of multiple pathogen detection from a variety of samples. Quantification of pathogen infection within host samples is achievable with some of these techniques. Accurate detection, classification and differentiation among members of the same species is, however, dependent on meticulous primer design and, even then, the risk of incorrect characterization, especially of divergent isolates, can still be a cause for concern. Assays should, thus, be combined with other applications to enable reliable detection and identification.

1.3.4.3. Restriction fragment length polymorphism

A similar principle to genomic fingerprinting, restriction fragment length polymorphism (RFLP) assays generate a unique DNA profile or fingerprint based on restriction digests of a genomic fragment or PCR product. Single (Alfaro-Fernandez et al., 2009), or multiple restriction enzymes (Dovas et al., 2004; Hanssen et al., 2008; Leite et al., 1994; Letschert et al., 2002; Obradovic et al., 2004), are used to digest the template DNA or cDNA at conserved recognition sequences. The variation in position, and the absence or presence of these recognition sites determines the pattern of fragments formed when digested templates are resolved by electrophoresis. In phytodiagnositics, RFLP assays have been coupled with PCR applications to characterize detected pathogens and, specifically, to differentiate closely related species, strains or isolates. This technique was shown to be valuable for differentiation among isolates of the species complex causing bacterial spot of tomato and pepper (Obradovic et al., 2004). Using three restriction enzymes, four discrete patterns were resolved, indicating four groups of races or pathovars. Diagnosis and classification of viral pathogens of tomato was also reported, where authors used an RT-PCR–RFLP approach to separate highly homologous species of *Tobamovirus* (Dovas et al., 2004; Letschert et al., 2002) and genetically similar isolates of PepMV (Alfaro-Fernandez et al., 2009; Hanssen et al., 2008). Unexpected RFLP patterns are also informative for mixed infections, divergent strains or breakdown of genetic resistance, where a pathogen has evolved to overcome a cultivar's resistance to infection (Letschert et al., 2002). Although improved characterization of pathovars and strains is possible by RFLP analysis, discrimination from relatives is complicated (Alfaro-Fernandez et al., 2009; Leite et al., 1995) and characterization should rather be performed by more informative techniques like sequencing.

1.3.4.4. Nucleic acid sequencing

The advent of nucleic acid sequencing by Sanger in 1976 gave insight into the complexity of functioning of organisms by generating the exact DNA or RNA genomic sequences (reviewed by Studholme et al., 2010). As the technique became automated, associated costs and turn-around time decreased, and more organisms have been sequenced. Sequences are compiled and uploaded onto comprehensive databases like GenBank (www.ncbi.nlm.nih.gov) where they can be viewed, annotated and manipulated for use in diverse molecular applications. In phytopathology, sequencing pathogen genomes has given incredible insight into mechanisms of pathogenicity as well as host-pathogen interactions (Almeida et al., 2009; O'Brien et al., 2011). An arsenal of sequences for all significant pathogens, related organisms and hosts has, thus, become available over the last decade. Because a genetic sequence of a detected pathogen can be compared directly to others on databases, closely related organisms like strains, isolates and races of a pathogen may be characterized readily by sequencing (Dovas et al., 2004; Pagán et al., 2006; Schaad and Frederick, 2002; Studholme et al., 2010; Van der Vlugt and Berendsen, 2002).

The high cost of current sequencing methods and complicated template preparation processes limit the use of sequencing in plant diagnostics (Adams et al., 2009; Dovas et al., 2004). Partial genome sequencing of amplified PCR fragments is sufficient for diagnosis and characterization however, provided that the target region is adequately informative for the pathogen. Van der Vlugt et al. (2002) sequenced a region of the replicase gene to identify an unknown *Potexvirus* isolated from tomato as PepMV. Other studies have also utilised sequencing to confirm pathogen detection (Alfaro-Fernandez et al., 2009; Da Silva et al., 2008; Pagán et al., 2006) or to differentiate between non-specific fragments and unexpected truncated amplicons generated during PCR applications (Cuppels et al., 2006). Sequencing is not viable for detecting new or emerging pathogens however, as the time taken to generate sequences, compare to relatives and design diagnostic primers severely delays the implementation of control strategies therefore risking the spread of mass epidemics, as was observed with PepMV in Europe (Mansilla et al., 2003).

Advancements in sequencing technology led to the development of the Next-generation sequencing platform in recent years (Studholme et al., 2010). This has resulted in an exponential increase in high-throughput sequencing capacity with rapid turn-around and simultaneous generation of over 100 copies of a single genome, ensuring reliable coverage with minimal errors (Adams et al., 2009; Studholme et al., 2010). This format of sequencing does not rely on prior predictions of the potential causal agent and no pathogen-specific primers or antibodies are required for detection, making the

technique applicable for all types of pathogens. Adams et al. (2009) used next-generation sequencing successfully to isolate and characterize a new virus from an infected ornamental host demonstrating unfamiliar symptoms.

Characterization of sub-species of pathogens is highly accurate and reliable using sequencing, especially because repeat copies produced eliminate potential sequencing errors (Adams et al., 2009; Coetzee et al., 2010; Studholme et al., 2010). Associated costs and labour, however, are enough reason to avoid sequencing in standard phytodiagnostics and favour tools with lower-resolution unless sub-species levels of characterization of a detected pathogen is required (Adams et al., 2009; Dovas et al., 2004). In many cases, certification protocols just require species-specific diagnosis of a pathogen and exact sequence information is unnecessary. Sequencing produces masses of data and sifting through this to construct genome maps or find diagnostic regions can be arduous for routine phytodiagnostic application.

Molecular-based pathogen detection techniques have resulted in a drastic improvement in sensitivity and specificity for single and multiple pathogen detection compared to serological and conventional methods (Alfaro-Fernandez et al., 2009; Cuppels et al., 2006; De Leon et al., 2008; Dovas et al., 2004; Fanelli et al., 2007; Gutierrez-Aguirre et al., 2009; Ling et al., 2007; Obradovic et al., 2004; Schaad and Frederick, 2002; Schaad et al., 1995). Although adoption of such methods in industry is currently limited, PCR and PCR applications have been described as a 'golden standard' for phytodiagnostics (Schaad and Frederick, 2002) and recent updates to some standard detection protocols now include well validated molecular-based assays (Asma, 2005; EPPO/CABI, 2005). Dependence on nucleic acid extraction procedures can limit the use of assays for processing large numbers of samples (McManus and Jones, 1995; Meng et al., 2004; Schaad and Frederick, 2002). An excellent claim was made by Dovas et al. (2004), though, who suggested that less complex extraction procedures were necessary if a detection assay still demonstrates sufficient sensitivity. Automated extraction processes and applications like IMS and bio-PCR have improved this major disadvantage of molecular techniques. Actual assay times only require a few hours compared to the days and weeks of conventional tests (Da Silva et al., 2008; Maes, 1993; Park et al., 2009; Lievens and Thomma, 2005), but template preparation and post-PCR confirmation methods can diminish this advantage. Molecular-based techniques generally require a reasonable prediction of the nature of the target pathogen (Adams et al., 2009; Mansilla et al., 2003), and attempting to detect and characterize unknown pathogens requires extensive trial and error analysis with available primers or probes. Despite the development of multiplex assays, detection of multiple pathogens from a single

sample is still limited to three or four pathogens, and the reactions should be repeated for all pathogens of interest to declare the absence or presence of each target pathogen in host samples.

1.3.5. The use of array technology for pathogen detection

1.3.5.1. The structure of arrays

The limitations of multiplex PCR for multiple target detection led to the development of array technology (Schena et al., 1995; Zhang et al., 1991). This was especially necessary for gene expression studies which require simultaneous analysis of hundreds of target genes at specific sampling time points. Due to the high-throughput nature, arrays have since been adopted in diverse applications including genomics (Figure 1. 12), sequencing, drug discovery, genetic disorders, clinical diagnostics and the detection of animal and plant pathogens (Kyselková et al., 2008, 2009; Nicolaisen and Bertaccini, 2007; Rajendhran and Gunasekaran, 2011; Wilson et al., 2002; Zhang et al., 2007).

The structure of an array is essentially similar to a reverse dot-blot, consisting of a solid base substrate, approximately 1 cm^2, to which thousands of probes are covalently bound (Boonham et al., 2007; Fessehaie et al., 2003; Kumar, 2009; Lee et al., 2003; Lievens et al., 2003; Schena et al., 1995). These probes are organised into pico-mole collections, or spots, of identical sequence fragments that are immobilised onto the array substrate in designated positions. Array technology exploits the specificity of complimentary base-pairing of nucleotides or amino acids in order to detect specific targets (Hadidi et al., 2004). Probes are able to bind a variety of organic molecules by this principle (Kumar, 2009), including DNA, cDNA, mRNA and various proteins. In preparation for nucleic acid-based arrays, DNA or RNA is extracted from a sample and amplified by conventional PCR using universal, random or target-specific primers. The amplification step augments extracted nucleic acid templates, thereby improving detection of target nucleic acid present at low concentrations within a sample (Agindotan and Perry, 2007; Hadidi et al., 2004; Lievens et al., 2003; Lievens and Thomma, 2005). Converting RNA to cDNA also enables more stable detection in array assays, instead of detection based on transient, unstable RNA. Single-stranded or denatured dsDNA is labelled with radioactive or fluorescent dyes and hybridised to an array of complimentary probes under stringent experimental conditions. These conditions facilitate irreversible binding of labelled PCR products to complimentary probes, while unbound, non-target DNA is removed by subsequent buffer washes. Bound probe:target complexes are visualised by X-ray or UV-illuminating scanners which produce a readable image for statistical analysis. By designing arrays so that specific probes are spotted at

particular positions on an array (Boonham et al., 2007; Lee et al., 2003), images may be used to infer the presence, absence or expression level of a target gene within a sample according to the position of the detected fluorescent signal (Figure 1. 12).

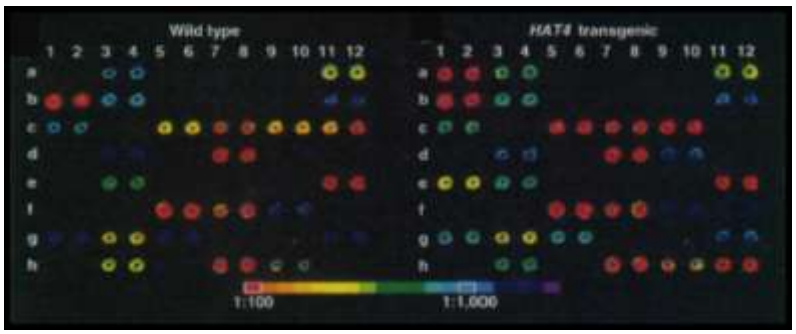

Figure 1. 12 Analysis of gene expression variation by array technology (Schena et al., 1995)**. Arrays consisted of 45 probes derived from random cDNA clones from the *Arabidopsis* cDNA library. Scans show the different hybridisation patterns of fluorescein-labelled cDNA prepared from mRNA transcripts of wild-type *Arabidopsis* and *HAT4* mutants.**

Probe spots, or features, on an array are usually between 100 and 400 µm in diameter and, therefore, a single array has the capacity to bind thousands of different features (Boonham et al., 2007; Hadidi et al., 2004; Nicolaisen and Bertaccini, 2007; Pelludat et al., 2009; Tiberini et al., 2010). This exponentially increases the number of potential gene targets that may be analysed simultaneously compared to PCR applications (Lievens et al., 2006; Schena et al., 1995). Hence, array technology was attractive for diagnostic applications. This technique was first used to detect multiple viral pathogens implicated in human respiratory diseases (Wang et al. 2002) and have since been instrumental in the detection and identification of other significant human pathogens like hepatitis C, polio (reviewed by Kumar, 2009) and SARS coronavirus (Wang et al., 2003).

1.3.5.2. The use of array technology in plant pathogen detection

In phytodiagnostics, array technology has drastically improved pathogen detection compared to other available techniques. Plant pathogens frequently occur in mixed infections, which are generally more severe than single infections, and makes correct diagnosis and differentiation among pathogens challenging. By including pathogen-specific probes at designated positions, arrays allow for the simultaneous detection, discrimination and identification of a range of pathogens in single or mixed infections from tested samples (Boonham et al., 2007; Nicolaisen and Bertaccini, 2007; Pelludat et al., 2009; Zhang et al., 2007). Thus, hundreds of different pathogens may be detected in a single array (Tiberini et al., 2010), without the limiting dependence on differential dyes and complex PCR mixes.

Research into the use of arrays has rapidly become the focus of phytodiagnostic studies. Tiberini et al. (2010) reported that over 230 papers had been published prior to 2010 detailing array-based detection of plant viruses alone. Arrays have been used to detect all classes of plant pathogens including fungi, oomycetes, bacteria, viruses, phytoplasmas and viroids from a variety of environmental and host samples (Agindotan and Perry, 2007; Deyong et al., 2005; Engel et al., 2010; Fessehaie et al., 2003; Lievens et al., 2003, 2007; Lee et al., 2003; Nicolaisen and Bertaccini, 2007; Pelludat et al., 2009; Sholberg et al., 2005; Szemes et al., 2005; Tiberini et al., 2010; Van Doorn et al., 2007, 2009; Zhang et al., 2007). Some of these studies have also reported successful detection of more than one class of pathogen in a single assay, which is favourable for broad spectrum disease surveys of important crops (Pelludat et al., 2009; Sholberg et al., 2005; Van Doorn et al., 2007, 2009; Wilson et al., 2002; Zhang et al., 2007). Arrays have also been used successfully in other plant-pathogen applications such as host-pathogen interactions, biological control studies and epidemiological studies of pathogens (Kyselková et al., 2008, 2009; Lee et al., 1997).

Besides the high-throughput, highly specific capacity of this technique, arrays also reduce the time required to test samples for a range of pathogens (Lievens et al., 2003; Tiberini et al., 2010). The process is culture-independent, rapid and less labour intensive than other detection techniques, as the process is largely automated with sample extraction and labelling preparations only requiring a few hours. As each array slide contains section repeats of probes and several slides may be hybridised simultaneously, as many as 16 samples can be analysed concurrently (Nicolaisen and Bertaccini, 2007). Thus tens, hundreds or thousands of pathogens may be detected from more samples in less than 72 hours compared to other detection techniques (Lievens et al., 2003, 2005; Njambere et al., 2011). Results are usually extremely reliable due to spot repetition, and the high specificity and sensitivity of capture probes eliminates the need for repeat tests. Multiple probes may be included for a single pathogen, referred to as pathogen redundancy, which also ensures higher accuracy and confidence in results than other molecular techniques. Even at low levels of infection, the signal produced from probe-target complexes is easily visible and detectable by scanners compared to faint bands observed in gel electrophoresis analysis of PCR products (Nicolaisen and Bertaccini, 2007). Indeed, the sensitivity of arrays has been likened to DAS-ELISA tests and recent optimisations have further improved detection limits to be comparable to real-time PCR (Agindotan and Perry, 2007; Kumar, 2009; Van Doorn et al., 2007, 2009). Exploiting genome conservation and variability in probe design has since allowed arrays to be designed to not only identify, but also classify detected pathogens by genus, species or subspecies according to the number and sets of probes to which the detected pathogen binds.

1.3.5.3. Types of arrays

Two major types of arrays exist with different capacities for target detection, namely macro and microarrays. Macroarrays are low-density arrays generally based on nylon membrane substrates (Hadidi et al., 2004; Sholberg et al., 2005), which have a high affinity for DNA binding. These arrays characteristically have 10 - 100 probe spots per cm^2, each with a diameter of approximately 300 µm or larger. Microarrays, or chips, are high-density arrays based on glass slide substrates, though in most cases the glass is coated with silicon or poly-L-lysine to increase DNA binding capacity (Deyong et al., 2005; Hadidi et al., 2004). Between 1000 and 100 000 spots/cm^2 may be arrayed onto chips and spots are 200 µm or less in diameter. Macroarray substrates are much larger, averaging between 10 and 15 cm^2 while microarray slides are only 2 – 3 cm^2. Each type of array is suited to different applications in phytodiagnostics and, to date, numerous studies have reported successful pathogen detection from diseased plants using both types of arrays (Agindotan and Perry, 2007; Boonham et al., 2003; Engel et al., 2010; Lievens et al., 2003, 2005; Sholberg et al., 2005; Tiberini et al., 2010).

1.3.5.4. Macroarrays

Membrane-based macroarrays were first used for genetic profiling of the 60 point mutations known to occur at the cystic fibrosis locus (Zhang et al., 1991), and have since been utilised in diverse fields including phytodiagnostics (Agindotan and Perry, 2007; Lievens et al., 2005; Sholberg et al., 2005; Zhang et al., 2007). The use of membranes is attractive as the components are less expensive than most other substrates and membranes may be reused up to six times without any effects on binding efficiency (Agindotan and Perry, 2007). Membranes have a greater binding strength than other substrates, as the components form strong, electrostatic covalent bonds with probes which translate into better signal strength (Fessehaie et al., 2003; Lievens et al., 2003). The size of membranes used in macroarray-based assays may be altered easily according to the aims of a study, which may or may not necessitate the use of hundreds of probes. These arrays are useful for small pilot studies to test specificity and binding efficiency of designed probes before incorporation into larger diagnostic assays (Agindotan and Perry, 2007).

Nucleic acid probes are spotted in higher concentrations on macroarrays than microarrays, and greater concentrations of capture probes increases the sensitivity of detection of targets (Agindotan and Perry, 2007; Lievens and Thomma, 2005). Probe:target complexes are visualised by resulting luminescence, based on digoxygenin-dUTP incorporation, or X-ray scanning, based on radioactive

labelling, of the array after hybridisation reactions with targets present in samples (Figure 1. 13) (Agindotan and Perry, 2007; Fessehaie et al., 2003; Lievens et al., 2003; Sholberg et al., 2005). Results can be visualised with or without complex analytical equipment, making this tool ideal for laboratories with limited equipment (Njambere et al., 2011). Dependence on radioactive or DIG-labelling, however, reportedly limits the resolution of detectable complexes (Naidoo, S 2010, pers. comm.[1]). This may be improved by incorporating more costly fluorescent dyes but this can compromise the economy of macroarrays (Agindotan and Perry, 2007; Njambere et al., 2011).

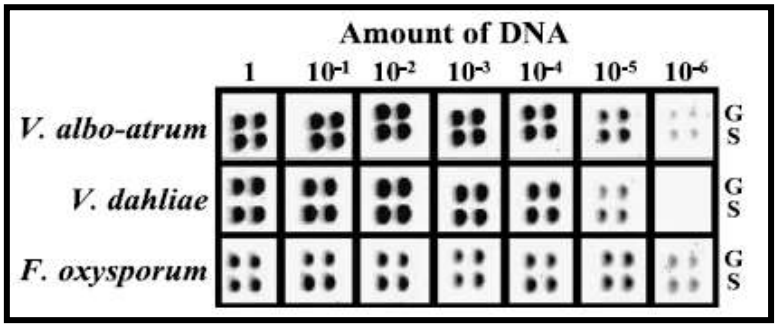

Figure 1. 13 Detection and quantification of fungal wilt pathogens of tomato by macroarray. Undiluted pathogen DNA between 250 and 500 ng was detected at various dilutions by genus-specific (G) and species-specific (S) probes. The degree of chemiluminescent signal produced by probe-target complex conjugated enzymes correlated to the concentration of pathogen nucleic acid in samples (Lievens et al. 2003)**.**

Macroarrays have been used in a number of phytodiagnostic studies for detection and classification of multiple pathogens in single and mixed infections (Agindotan and Perry, 2007; Lievens et al., 2005; Sholberg et al., 2005). A three- to five-fold increase in sensitivity was reported using macroarray-based detection compared to ELISA and PCR applications (Njambere et al., 2011; Zhang et al., 2007), with detection limits as low as 0.01 fg. Quantification of pathogen infection using macroarrays has been investigated by analysing serial dilutions of positive control samples (Figure 1. 13) and utilising standard curves to infer the concentration of target pathogens in test samples (Lievens et al., 2005; Sholberg et al., 2005). But the inter-spot variability that is associated with crude spotting methods used for macroarray assays limits the accuracy of pathogen quantification. The large number of probes that may be included in a single macroarray assay makes this technique cost-effective compared to other detection techniques. The lower density of spots used in a macroarray, as well as the lower cost of reagents, materials and equipment involved, make these arrays less expensive than microarrays which require sophisticated spotting, hybridisation and scanning machinery (Agindotan and Perry, 2007; Lievens and Thomma, 2005; Sholberg et al., 2005).

[1] S. Naidoo, Department of Genetics in 2010 at the University of Pretoria, Pretoria, South Africa

The thousands of probes that may be included in a microarray, however, is associated with a much greater detection capacity and, thus, the average cost per unit tested using microarrays is comparable with that of macroarrays.

1.3.5.5. Microarrays

Since the first application in gene expression profiling of mutated *Arabidopsis* plants (Schena et al., 1995), the high-density, high-throughput nature of microarrays has seen this type of array become instrumental in various applications. In phytodiagnostics, microarrays have been used to analyse host-pathogen interactions, epidemiological relationships, disease control and pathogen discovery (Kyselková et al., 2009; Marathe et al., 2004; Pelludat et al., 2009; Tiberini et al., 2010; Wang et al., 2002). Pathogens infecting a range of agronomically important fruit, vegetable and field crops have also been successfully and simultaneously detected using microarrays, including potatoes, grapes, cucurbits, plums and tomatoes (Boonham et al., 2003; Deyong et al., 2005; Engel et al., 2010; Lee et al., 2003; Pelludat et al., 2009; Tiberini et al., 2010). The success of microarrays as a detection tool is attributed not only to the thousands of target sequences that may be detected in a single assay, but also the ease of extending arrays to add more probes which, therefore, facilitates the detection of more pathogens simultaneously (Chou et al., 2006; Deyong et al., 2005; Engel et al., 2010). By increasing the number of pathogens detectable in a single assay, the time and cost of testing a single sample is reduced as tests need not be repeated. The threshold limit of detection of microarrays has been reported to be between 0.01 and 5 pg of target DNA/RNA in the presence of significant concentrations of non-target host DNA (Lievens et al., 2003, 2005; Szemes et al., 2005; Wilson et al., 2002). Lee et al. (2003) reported discrimination among four species of cucurbit viruses up to a dilution of 1:200 of total extracted RNA, which was far more sensitive than the standard detection tools for these viruses.

Identification and differentiation of taxonomically related pathogens is possible using microarrays by careful probe design (Chou et al., 2006; Pelludat et al., 2009). Synthetic probes may be designed specifically to facilitate classification of groups of similar pathogens along with their detection. Discrimination efficiency of a microarray is, however, not only dependent on probe design; but temperature and hybridisation conditions are also critical factors for preventing false positive binding to non-target sequences (Call, 2005; Kyselková et al., 2009; Zhang et al., 2007).

Probe Design

A diagnostic microarray usually incorporates synthetic probes of between 18 and 70 bp which are designed using published sequence data for the pathogens of interest (Boonham et al., 2007; Bystricka et al., 2005; Chou et al., 2006; Letowski et al., 2004). Synthetic probes are manufactured with high quality and purity, and are ready for hybridisation to the substrate without further processing. These probes may be altered chemically during or after production to increase binding efficiency, for example, by including a desthiobiotin moiety for chemically-targeted binding to complimentary sequences (Van Doorn et al., 2009).

The design of probes is facilitated by various algorithms and software that have been developed for different applications of target detection (Chou et al., 2006). A stringent microarray assay requires uniform thermodynamic conditions which should, thus, be standardised during probe design, such as GC content, secondary structure formation, cross-hybridisation, steric hindrance patterns and melting temperatures (T_m), which all affect the binding capability of probes (Chou et al., 2006; Letowski et al., 2004). The length of detector probes has a significant effect on the sensitivity of pathogen detection. Short probes (15 – 30 nt), are better for discrimination among closely related species, strains or sub-types which contain few nucleotide differences between targeted sequences (Boonham et al., 2007; Chou et al., 2006; Deyong et al., 2005; Engel et al., 2010; Wilson et al., 2002). Longer probes (50 – 70 nt) demonstrate increased specificity and sensitivity for targeted pathogen detection than shorter probes, but are less discriminatory against small mismatches. Recently, novel modified bases called locked nucleic acids (LNAs) have been included in probe design, specifically around SNPs, for improved discrimination among highly related target sequences (Stewart, 2006; Veedu et al., 2007). These have a greater affinity for a specific complimentary nucleic acid compared to normal bases and, thus, LNAs also demonstrate higher stability and mismatch discrimination than normal bases.

Probe sequences are either derived from conserved portions of genes and regulatory regions or from divergent diagnostic regions identified by sequence alignments (Chou et al., 2006; Engel et al., 2010; Lievens and Thomma, 2005; Pelludat et al., 2009). Chosen regions must be specific for the target pathogens and not contain any significant homology to host genomes, other pathogens and saprophytes potentially present in the tested samples. The percentage conservation of these regions determines the level of taxonomic classification that the designed probe is capable of achieving, though design may be limited by the uniformity of the genome of a taxon (Fessehaie et al., 2003). The large sizes of bacterial and fungal genomes necessitate the use of previously characterised or

well-studied genes as targets for diagnostic probe design as opposed to entire genome scans. Ribosomal DNA (rDNA) is commonly targeted for the design of diagnostic probes for bacteria and fungi (Engel et al., 2010; Fessehaie et al., 2003; Kyselková et al., 2008; Lievens et al., 2005; Lievens and Thomma, 2005; Nicolaisen and Bertaccini, 2007; Sholberg et al., 2005; Zhang et al., 2007). This gene is highly conserved and expressed at high levels in organisms due to crucial roles in protein synthesis, therefore, facilitating accurate and sensitive detection of the target when incorporated as a probe in an array. A large amount of sequence data is available for the rDNA region for thousands of organisms, as this region has been shown to be extremely informative for molecular characterization studies. Other genes which have been targeted are, therefore, well-characterized house-keeping genes, genetic markers and important virulence factors (Pelludat et al., 2009; Wilson et al., 2002). Plant viruses, however, have smaller, dynamic genomes and, thus, no conserved regions exist for sufficient comparison among different classes of viruses (Boonham et al., 2007; Dovas et al., 2004). This makes techniques such as PCR applications unsuitable for the detection of multiple unrelated viruses without including a number of primer sets in complex PCR mixes (Boonham et al., 2007). Microarrays eliminate this problem as numerous probes for each class, genus, species and strain of virus may be included in a single array.

Pathogen redundancy in microarrays, or the inclusion of multiple probes per pathogen on various taxonomic levels, has been shown to drastically increase the accuracy, reliability and sensitivity of detection (Lievens and Thomma 2005; Wang et al. 2002). From the results of previous research it can be concluded that a combination of synthetic probes for conserved and divergent regions of a number of genes per pathogen ensures greater accuracy of detection. A major concern, however, is that highly target-specific probes incorporated into microarrays might increase the risk of false negatives or result in a lower signal production due to inter-strain variation (Njambere et al., 2011). While high stringency is necessary to classify and disregard non-target species, sequencing errors and silent point mutations may result in detection inaccuracy (Call, 2005). For this reason, probe design cannot rely solely on sequence data as a reference for diagnostic potential, and the efficiency of a chosen probe should be analysed and optimised experimentally before use in pathogen diagnosis.

Structure and strategy of diagnostic microarrays

The microarray system is composed of a number of components which must be assembled before samples may be tested. The most common support used in microarray studies is a glass slide (Deyong et al., 2005; Fessehaie et al., 2003) though recent developments utilising electronic beads

with sequence tags may soon replace the use of slides. Glass slides have advantages over membranes as they are easier to use, readily and cheaply available, because they are used in many other applications, and glass exhibits low internal fluorescence influences on signal readings. Plain glass, however, has a poor affinity for nucleic acids and other charged molecules and, thus, bind probes poorly resulting in weak signals (Lee et al., 2003). Coating slides with substances like agarose, poly-L-lysine and acrylimide, that attract charged molecules like DNA to form strong electrostatic bonds (Boonham et al., 2007; Bystricka et al., 2005; Deyong et al., 2005), improves not only the binding capacity of glass slides but also increases signal intensity by reducing the loss of probe:target complexes during washing. The coated slide is spotted with a few nano-litres of solutions of diagnostic probes. This is usually done robotically to ensure uniform spot size, shape and concentration, which are essential for accurate interpretation of microarrays for pathogen detection (Boonham et al., 2007; Lievens et al., 2005). Various mechanisms of spotting have been employed, such as thermal inkjet printing, photolithographical-based printing, electrocapture, contact dip-deposition and the affymetrix linker system which are reviewed by Boonham et al. (2007) and Hadidi et al. 2004).

Although only a small amount (5 – 20 µg) of extracted nucleic acid is required for array hybridisations (Boonham et al., 2007), a preceding PCR amplification step is usually performed to augment low levels of pathogenic nucleic acid in samples to a detectable concentration. Some studies have attempted to eliminate this step as pathogen-specific primers for amplification may introduce a bias for expected pathogens over unknown organisms (Boonham et al. 2007; Szemes et al. 2005; Van Doorn et al. 2007; Van Doorn et al. 2009; Wang et al. 2011), but this is usually accompanied by poorer signal production. Including too many pathogen-specific primers in a single assay can complicate the formation of compatible PCR mixes or result in inter-template interference (Wang et al., 2011). By using random or conserved primers for amplification of groups of pathogens, these problems can usually be resolved.

For visualisation of probe-target complexes, extracted nucleic acid is labelled with a fluorescent dye before hybridisation to the array (Figure 1. 14). Labelling can either be performed during or after amplification, though dye molecules are usually large and incorporation during amplification may reduce the efficiency and final concentration of DNA (Engel et al., 2010; Hadidi et al., 2004; Lievens et al., 2003; Szemes et al., 2005). Post-labelling or LATE-PCR (linear after exponential PCR) of DNA involves targeted labelling of modified bases incorporated during initial amplification steps. These generally yield greater cDNA concentrations and, subsequently, greater signal production. Many

dyes and modified linker sequences are available for template labelling, including amino-alkyl bases, dendrimeric molecules, Klenow fragments, biotin-16-dUTP, digoxygenin-11-dUTP, and Cy3 and Cy5 dyes (Boonham et al., 2007; Hadidi et al., 2004; Lievens and Thomma, 2005; Van Doorn et al., 2007; Wilson et al., 2002). Dyes which fluoresce at different wavelengths may be combined in a single assay, for example the red and green of Cy3 and Cy5 dyes, respectively. This enables more than one sample to be labelled, processed and visualised simultaneously for the presence of targeted pathogens (Figure 1. 14).

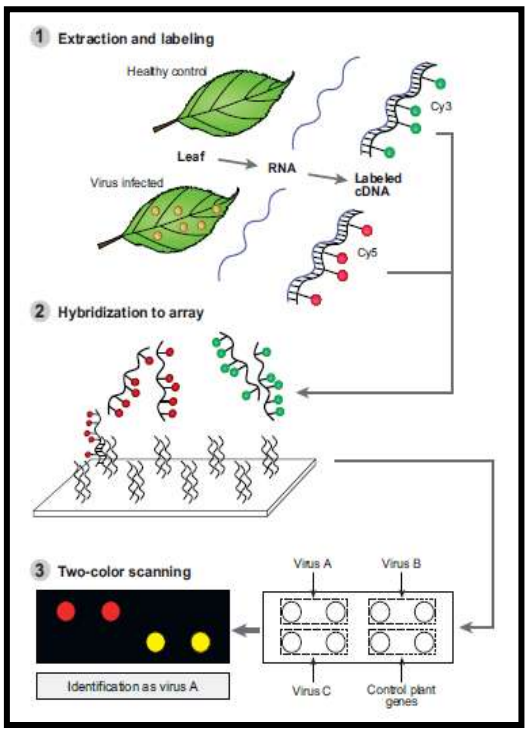

Figure 1. 14 Strategy of a viral diagnostic array. Total RNA is extracted from infected or healthy plant samples and labelled with fluorescent dyes. Probe-target complexes form between gene-derived probes and complimentary labelled nucleic acid which are then visualised by laser scanning. Site-specific illumination of probe spots allow for accurate diagnosis (Boonham et al. 2007)**.**

The maintenance of an even temperature distribution during hybridisation is extremely essential to binding specificity and discrimination among target and non-target sequences. Higher temperatures have been associated with reduced incidences of non-specific hybridisations, but lower temperatures are easier to maintain and produce greater signal intensities (Zhang et al., 2007). Hybridised slides are analysed by laser scanners which illuminate the array and excite fluorescent dyes within any immobilised complexes remaining after buffer washes. Scanning may be direct, where the entire array is illuminated at once and the resulting image is captured and analysed, or indirect, where each spot is illuminated and captured individually. Indirect scanning usually results in better resolution of the fluorescing spots than direct scanning and, therefore, tends to be the favoured method (Hadidi et al., 2004; Sholberg et al., 2005). Although images are visible with the naked eye, scanned images are best interpreted by statistical software, which consider potential background signals and calculate statistically significant signals, and facilitate the formation of reliable conclusions for pathogen identification and classification. Although most of the microarray process is automated, there are still critical steps during template preparation and probe-target binding that can be variable and may limit the resolution and accuracy achievable with an assay. These aspects have been under optimisation, and over the last decade, new strategies have been developed to improve phytopathogen detection using microarrays.

Optimisation of microarrays for pathogen detection

One of the major drawbacks of microarrays and array technology compared to techniques like real-time PCR, is the inability or limited capacity for quantification of a detected pathogen within a sample (Lievens et al., 2005; Van Doorn et al., 2007). Successful quantification of pathogens within environmental or plant samples using arrays has been reported over the last few years (Lievens and Thomma, 2005; Sholberg et al., 2005; Van Doorn et al., 2007). These studies always utilize calibration curves for template DNA quantification, based on dilutions of samples infected with known concentrations of pure cultures of the target pathogen. It is apparent from these studies that accurate quantification of pathogens by microarrays requires a number of replications per sample, optimised thermodynamic conditions and identical spot morphology of probes on substrates.

Another limiting factor of array technology is the presence of background signal on hybridised arrays (Szemes et al., 2005; Van Doorn et al., 2009). Background signal is created by non-specific labelling and binding of non-target sequence fragments from the genomes of hosts or other organisms present in samples. Great or variable levels of background signal among assays can obscure readings and may result in false positive or false negative detection. Specialised capture probes called padlock-probes (PLPs) have been developed to solve this, which separate target-binding and array hybridisation procedures, thereby reducing the production of background signals (Szemes et al., 2005; Van Doorn et al., 2007, 2009; Wang et al., 2011). These capture probes are generally about 100 bp long and are specifically designed so that 5' and 3' ends of each probe are complimentary to adjacent target regions of a pathogen nucleic acid sequence (Figure 1. 15). Between these two ends, the probe contains a unique set of bases or zip-code, which is complimentary to a separate immobilised Zip-code probe on the array. Zip-code probes are standard array probes with predetermined sequences that are independent of those targeted. Therefore, taxonomically related and unrelated groups of pathogens may be detected in the same array. Array probes are premade with uniform length and thermodynamic characteristics, therefore standardising hybridisation

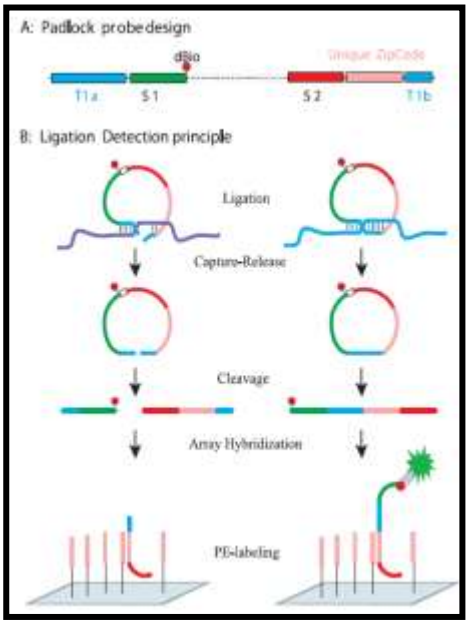

Figure 1. 15 An overview of the ligation-dependent (LD) array system. The Padlock Probe containing: asymmetrical 5' (T1a) and 3' (T1b) regions complimentary to target pathogen sequences, the unique Zip-code sequence (pink) and spacer regions (S1 and S2) – A. Ligation-dependent identification: binding of the PLP to target and non-target sequences, ligation-dependent circularisation and cleavage probes, hybridisation to the universal Zip-code array and fluorescence of probes based on differential cleavage – B. (van Doorn et al. 2009)

conditions for probe-target binding (Wang et al., 2011). When the target sequence is present in a sample, the PLP binds to the target and circularises by ligation of adjacent 3' and 5' ends (Figure 1. 15). Any non-bound or linear sequence fragments are then removed by exonuclease treatment. Probes are then decircularised and hybridised to the Zip-code array, producing a fluorescent signal upon illumination of probe-target complexes. The decircularised PLP, not the sample itself, is applied to the array and, as a result, the background signal is drastically reduced using this system (Szemes et al., 2005; Van Doorn et al., 2007, 2009). It is relatively simple to add new probes to this system to broaden its detection capacity and this is limited only by the number of unique zip-code probes available. Studies based on this system thus far have reported good levels of sensitive and specific detection of phytopathogens from infected samples with low background signal (Szemes et al., 2005; Van Doorn et al., 2007, 2009; Wang et al., 2011). Synthesis of PLPs is expensive and the design is complicated, however, which limits adoption of PLP arrays to commercial research laboratories.

1.3.5.6. Application of microarrays in industrial phytodiagnostics of tomato

The absence of a high-throughput tool that can rapidly detect all detrimental seed-borne pathogens of tomato can severely delay and restrict the trade of tomato seed and, thus, global fruit production. Microarrays facilitate rapid detection of numerous pathogens at various concentrations in a single assay, with relatively straightforward statistical analysis to interpret results. Repeating tests to include all pertinent pathogens is, thus, unnecessary, which reduces the labour and costs involved compared to other detection methods, especially when testing the large quantities of tomato seed that are exported regularly. The time required to certify seed-lots for the absence of important pathogens can be reduced to 72 hours using microarray-based detection (Lievens et al., 2003, 2005; Njambere et al., 2011), compared to current detection protocols, which may take a number of weeks to generate results. The high fidelity of pathogen-specific detector probes ensures accurate diagnosis of the absence or presence of target pathogens within a sample. With potential detection limits reported to be as low as 0.01 fg (Njambere et al., 2011; Van Doorn et al., 2009; Zhang et al., 2007), this tool also demonstrates superior potential for sensitive pathogen detection from seed compared to other diagnostic techniques. Results from analysed seed could, thus, be reliable and of an acceptable standard for certification in the seed trade industry.

Unlike macroarrays, microarrays or diagnostic chips can be mass-produced, and additional probes for any other significant pathogens, even of different classes, may be added easily to a premade chip to broaden the scope of use, without restarting the tedious design process (Boonham et al., 2007). Each manufactured array could include pre-spotted pathogen-specific probes in designated positions

and at optimised concentrations, ready for use in diagnostic assays. To date, a number of chips have been commercialised and marketed for targeted pathogen diagnosis. These include: DNA scan (Germany), Microbiometrix (Belgium), Relab Den Haan (Netherlands) and Blgg (Spain). Lievens et al. (2003) designed and developed a diagnostic array capable of detecting over 45 fungal and bacterial wilt pathogens of tomato, now commercially available as the DNA multiscan chip (www.dnamultiscan.com). This chip has since been expanded to include fungal and bacterial pathogens of a variety of economically important plants such as vegetables, fruit, ornamentals and turf grass. Pelludat et al. (2009) developed an array specifically for the detection of European quarantine bacterial pathogens which included some significant seed-borne pathogens of tomato. However, a microarray chip dedicated to the detection of a range of different classes of seed-borne pathogens of tomato is yet to be developed.

Conventional detection methods still play an important role in phytodiagnostics and seed-lot certification due to the low cost, equipment and technical requirements. Nucleic-acid and PCR based approaches have been included in recent updates of standard diagnostic protocols, because they demonstrate improved accuracy and specificity of detection compared to the classic detection methods. These tests also facilitate faster turn-around for sample diagnosis, and at a relatively low cost. The costs involved with microarray detection of plant pathogens, especially due to the complex machinery required for analysis, still limit the use of this tool to well-funded projects and laboratories. Certainly, future research should focus on ways of reducing costs associated with microarray assays to make the technique more practical for adoption in standard protocols and on-site/field applications. Further research into pathogen quantification using microarrays could also boost the potential use of this tool in standard diagnostic assays. Through collaborative efforts, the outstanding, high-throughput, sensitive and specific features of array technology for detection may become accessible to all spheres of disease diagnosis. Research should, therefore, focus on developing potential candidate diagnostic microarrays to be optimised for adoption into standard detection protocols.

Chapter 2 Detection of Seed-borne Pathogens of Tomato by Conventional and Serological Techniques

2.1. Abstract

Rapid detection of seed-borne pathogens of tomato from infected seed or plant hosts is crucial to limit severe economical losses in the tomato industry. The capacity and reliability of common conventional and serological methods for the detection of five of the most significant tomato pathogens found in South Africa and globally, were evaluated in this study. Leaf and seed samples used to challenge these methods were collected from susceptible tomato hosts inoculated with previously characterised, reference pathogen cultures. A leaf sample infected with an unknown viral pathogen, also collected in this study, was included in detection assays in an attempt to characterise the causal agent. This pathogen was confirmed as *Pepino mosaic virus* by characteristic symptom development on inoculated tomato and tobacco hosts, as well as positive reactions with PepMV-specific antibodies in enzyme-linked immunosorbent assays (ELISA). Detection and identification of viral pathogens from diseased tomato seed and leaf samples was accurate, sensitive and reliable using ELISA screening, but concurrent bioassay trials were necessary to prove the viability of detected pathogens. Selective media assays for detection of bacterial pathogens using leaf and seed homogenates demonstrated inconsistent and variable recovery of target pathogens. The presence of saprophytes and non-target organisms in host samples obscured accurate detection, and accurate identification was, thus, dependent on subsequent analysis using physiological and biochemical techniques. All methods demonstrated limited capacity for multiplex detection of tomato pathogens, and testing numerous host samples for the presence of all potential tomato pathogens would be arduous and time-consuming. Automation and further optimisation of these techniques to improve specificity, sensitivity and reliability of detection would support continued use of these methods for routine pathogen detection and seed health testing.

2.2. Introduction

Of the many important vegetables, *Solanum lycopersicum* (tomato) is one of the most valuable, with global demands increasing annually to yield an overall production valued at over 55 billion US dollars (FAOSTAT, 2009). Global seed trade facilitates sustainable food production and is a valuable source of income for exporting countries and cultivators. Expensive hybrid seed for glasshouse and field production are specially bred to demonstrate desirable crop qualities for farmers (Bai and Lindhout,

2007; Barone and Frusciante, 2007; Diez and Nuez, 2006), such as high yield, increased fruit mass, longer shelf life as well as improved tolerance to abiotic and biotic stresses. The increase in trade of tomato, however, has facilitated the accidental distribution of numerous seed-borne pathogens, including fungi, bacteria and viruses, into destination countries. These may cause disease outbreaks leading to severe fruit yield and economic losses (Rude et al., 1982) in the tomato industry.

Control methods to limit the impact of the most detrimental seed-borne tomato pathogens, have largely proven to be inefficient and unreliable (Lievens et al., 2003). Focus has, therefore, shifted to phytosanitary methods to limit the introduction of seed-borne pathogens into tomato production areas. International agencies like the ISF (International Seed Federation), ISTA (International Seed Transit Association) and EPPO (European and Mediterranean Plant Protection Organisation), have been established to regulate the trade of 'pathogen-free' seed (Etter et al., 2011). They provide information about quarantine pathogens for important vegetable, fruit and grain crops, and supply a set of validated standard procedures of seed health testing to detect these pathogens in seed-lots (EPPO/CABI, 2005; GSPP, 2009). Before the trade and export of seed-lots is permitted, seed companies must provide certification that seed lots have been evaluated according to these standards.

These agencies have identified five seed-borne pathogens of tomato that pose a significant threat to global tomato production and trade: *Pepino mosaic virus* (PepMV), *Tomato mosaic virus* (ToMV), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Xanthomonas campestris* pv. *vesicatoria* (Xcv) and *Pseudomonas syringae* pv. *tomato* (Pst) (Hanssen et al., 2010a; Jones et al., 1997; Kritzman, 1991; Pelludat et al., 2009; Tiberini et al., 2010). Standard detection protocols that boast international approval and application have been developed for each of these pathogens (EPPO/CABI 2005; ISF 2008, 2009a, 2009b, 2009c). These methods have been thoroughly validated in a variety of laboratories and are periodically updated to include newly developed and improved techniques or modifications. Chosen methods should have significant advantages over other available techniques. They should demonstrate sensitive and specific detection of a particular pathogen while only requiring testing of small samples of expensive hybrid seed for analysis (Hadidi et al., 2004; Kritzman, 1991). Current standard protocols are primarily based on conventional and serological methods of pathogen detection. These are well-suited to mass screening of seed samples from large seed-lots for trade, as the methods are economical (Fatmi and Schaad, 1988; Hadas et al., 2004; Hamilton et al., 1981; Kaneshiro et al., 2006; Kritzman, 1991), and limited technical expertise is required for preparation and evaluation of results. Dependence on limited, technically complex

equipment also allows for adoption of these techniques even in laboratories with limited equipment and, as a result, they have become routine in most seed testing facilities.

Conventional detection methods exploit morphological, structural, physiological and biochemical characteristics of a pathogen for detection and identification from host samples. These characteristics are conserved among groups, genera, species and sometimes pathovars, allowing for classification of detected bacterial pathogens and discrimination from saprophytes (Fatmi and Schaad, 1988; Harris-Baldwin and Gudmestad, 1996; McGuire et al., 1986; Sijam et al., 1991). Preliminary identification is achieved using general characterization tests such as Gram stain, potassium hydroxide solubility, Kovac's oxidase test, pathogenicity tests and carbohydrate utilisation assays for broad taxonomic classification (Goszczynska et al., 2000). Isolation and more specific identification, for example, to the species or sub-species level of a pathogen, are achieved by selective plating of leaf and stem extracts from diseased plants samples, whereby phytobacterial agents are identified based on differential morphological growth characteristics on a set of standard growth media. This may include a collection of up to 10 different media to support selective recovery and varying morphologies of bacterial pathogens known to infect a plant. Mass screening of seed samples, however, requires the use of fewer media to reduce laborious sample processing. Analysis of seed-lots for the presence of a regulated pathogen is, therefore, typically performed using two selective media, optimised for the recovery of the target pathogen, and a differential growth medium, to discriminate the target from morphologically similar saprophytes or non-virulent strains of the pathogen (Asma, 2005; Chun, 1982; Fatmi and Schaad, 1988; Kritzman, 1991; McGuire et al., 1986; Sijam et al., 1991).

As viruses cannot replicate outside of hosts or vectors, detection of viral phytopathogens from host samples is typically based on serological methods. These exploit specific complimentary binding between antibodies and accessible pathogenic proteins like viral coat proteins for detection (Boscia and Myrta, 1998; Voller et al., 1976). Many antibodies have been developed and validated extensively against viral targets, such that the best performing antibodies are now commercially available for use in standard DAS-ELISA (double antibody sandwich – enzyme-linked immunosorbent assay) systems for detection of the most significant viral pathogens (Hadas et al. 2004; ISF 2009a; 2009b). These assays, however, do not give an indication of the viability of detected viral particles. This is crucial for assessing the risk of disease development from exported seed and, thus, ELISAs are usually coupled with biological assays that are performed on indicator hosts. Some tomato viruses can establish infections in other solanaceous hosts like *Nicotiana tabacum* (Hamilton et al., 1981;

Hadas et al., 2004), inducing characteristic symptoms within a short time period, and providing a rapid indication of the presence of viable pathogen particles in a sample.

Despite the economy and simplicity of these methods, many conventional diagnostic techniques have been described as unreliable and limited for pathogen identification and classification (Boscia and Myrta, 1998; De Leon et al., 2008; Harris-Baldwin and Gudmestad, 1996; Van Regenmortel and Burckard, 1980). The aim of this study was, therefore, to analyse and confirm whether conventional and serological methods are capable of accurately and efficiently detecting and identifying seed-borne pathogens of tomato selected for this study, from both diseased plant leaf tissue and seed from diseased hosts. Tests for each pathogen were performed under the same conditions, in the same laboratory and using samples from either a pooled collection of leaves, or seed suspected to be infected with the respective pathogens. This was to ensure that results from each of the tested protocols or tools were comparable.

2.3. Materials and methods

2.3.1. Propagation of plant material

Seed of *S. lycopersicum* cv. Rooikhaki, *Nicotiana tabacum* cv. Xanthi and *N. benthamiana* were treated in sequential steps including: immersion in 0.5% sodium hypochlorite solution for 30 minutes, heat treatments in water at 37°C for 10 minutes and 50°C for 25minutes, and immersion in 10% trisodium phosphate solution for 20 minutes. The seed was then air-dried overnight and sown in pasteurized pine bark/peat moss mixture with a once-off addition of $CaCO_3$ and superphosphate fertilizer (Culterra, South Africa). Trays with sown seed were maintained in the dark for three days at room temperature and then transferred to greenhouses under controlled climatic conditions. These included regulated light intensity, watering for 5 x 8 minute cycles per day and a temperature range of 15 - 25°C. Tomato and tobacco plants were maintained under these conditions for the duration of the study unless otherwise stated. Three-week-old tomato and tobacco seedlings, approximately 10 cm tall, were transferred to a pot, 10 mm wide, filled with sterile Coir. Seedlings between two and four-weeks-old were used for pathogen inoculations.

2.3.2. Maintenance of pathogen cultures

Pathogen isolates used in this study were obtained from the Sakata Vegenetics RSA (Pty) Ltd. (SVR) culture collection (Table 2. 1). Viral cultures of ToMV, previously confirmed prior to this study at SVR, and suspected viral cultures of PepMV were maintained in infected *S. lycopersicum* leaf material and stored at -20°C (Table 2. 1). Leaf material suspected to be infected with PepMV was obtained from tomato production areas in the Limpopo Province, South Africa and reacted positively with PepMV antibodies in DAS-ELISA tests performed prior to this study (Carmichael et al., 2011). For confirmation of pathogenicity, isolates were inoculated onto tomato and tobacco seedlings to observe symptom development. Bacterial cultures of Cmm, Xcv and Pst (**Error! Reference source not ound.**) were identified by standard detection techniques prior to this study. Bacterial isolates used for inoculations in this study were stored in sterile milk glycerol solution (0.1% skimmed milk powder and 0.15% glycerol) (Goszczynska et al. 2000) at -20°C.

Table 2. 1 Origin of pathogen isolates used in this study

Pathogen	Origin	Date of Isolation	Storage
Pepino mosaic virus **(PepMV)**	Limpopo Province, RSA	28/08/2008	Infected leaves of *S. lycopersicum* cv. Monalbo at -20°C
Tomato mosaic virus **(ToMV)** **Strain 0**	Eastern Cape, RSA	01/11/2006	Infected leaves of *S. lycopersicum* cv. Santa at -20°C
Clavibacter michiganensis **subsp. *michiganensis*** **(Cmm)**	Western Cape, RSA	13/06/2008	Milk glycerol suspension at -20°C
Xanthomonas campestris **pv. *vesicatoria*** **(Xcv)**	SVR - Gauteng, RSA	30/04/2008	Milk glycerol suspension at -20°C
Pseudomonas syringae **pv. *Tomato*** **(Pst)**	SVR – Gauteng, RSA	08/02/2008	Milk glycerol suspension at -20°C

2.3.3. Inoculation of selected pathogens on susceptible tomato seedlings

Viral inoculum was prepared using 1 – 2 g of either ToMV-infected leaf material or putative PepMV-infected leaf material in 0.05M phosphate buffer (176mM KH_2PO_4, 0.031M K_2HPO_4, with added 0.01M Na_2SO_4, at ph 7.2) at a ratio of 1:10 (m:v) in a sterile 50 mℓ centrifuge tube. Leaf material was homogenized using the SilentCrusher M (Heidolph, Germany), sterilised between samples by sequential washes in 25% bleach (1% NaOCℓ, 16.5% NaCℓ), 0.25M HCℓ and 70% ethanol (EtOH). The homogenate was centrifuged in a 5810R centrifuge (Eppendorf, Germany) for six minutes at 10 000 rpm at 10°C, and the supernatant was placed on ice until inoculations were performed. Four-week-old susceptible tomato seedlings (cv. Rooikhaki) were used for inoculations. Trials for both PepMV and ToMV were performed using 10 negative control plants (inoculated with buffer only) and 10 seedlings inoculated with the respective virus. Prior to inoculation, true leaves of all seedlings were dusted with Celite powder (Sigma, USA) and lightly pricked with a sterile hypodermic needle (SURGI Plus, China) to form wounds to facilitate viral entry. Seedlings were inoculated by lightly rubbing cotton wool swabs soaked in buffer or inoculum, respectively, onto all adaxial leaf surfaces until the leaves were damp. Inoculated plants were then transferred to greenhouses (25°C) and symptom development was recorded from 14 days post inoculation (dpi).

Susceptible tomato seedlings were inoculated with Cmm by the stab inoculation method (ISF, 2008a). Tryptone glucose agar (TGA) was used for culturing Cmm and prepared by adding 24 g/ℓ TGA powder (Duchefa, Netherlands) to distilled water (dH_2O). Inoculum was prepared by streaking 100 µℓ of Cmm stock culture (Table 2. 1) onto TGA medium incubated for four - seven days at 28°C. Four-week-old tomato seedlings (cv. Rooikhahki) were used for the trials, which included 20 negative control plants (inoculated with buffer) and 30 test seedlings. Yellow, mucoid *Cmm* colonies from TGA medium were picked with sterile toothpicks, and stabbed into cotyledonary nodes of the stems of the test seedlings. Control seedlings were stab inoculated with sterile toothpicks in the same manner. Seedlings were then transferred to the greenhouse (25°C) and symptoms were evaluated after 14 dpi.

Bacterial suspensions of Xcv and Pst were prepared by inoculating 250 ml of 1.6% nutrient broth (Biolab, South Africa) with 500 µl of respective stock cultures (Table 2. 1). The inoculated broth was placed on a SPO-MP15 rotary shaker (Labcom, South Africa) set at 150 rpm and incubated overnight at 25°C. Cultures were transferred to sterile 50 mℓ centrifuge tubes and centrifuged at 10°C for 10 minutes at 10 000 rpm, and the pellets were then resuspended in 250 ml of sterile dH_2O.

Suspensions were concentrated to a minimum of 10^6 CFU/mℓ (colony forming units/mℓ), according to the McFarland Scale, for a further four – six hours at 150 rpm (Sijam et al., 1991). Susceptible four-week-old tomato seedlings (cv. Rooikhahki) were preconditioned overnight at 24 - 26°C in non-hydrated mist chambers to create high humidity. Prior to inoculations, true leaves of all seedlings were lightly pricked with a sterile dissecting needle to create wounds/entry sites for the pathogens. Of these seedlings, 20 were treated as negative control plants and were sprayed with sterile dH_2O on adaxial and abaxial surfaces of leaves until the surfaces were damp. Pathogen inoculations were performed in a similar manner using Xcv or Pst bacterial suspensions and 30 plants per pathogen. Inoculated seedlings were transferred to enclosed plastic humidity chambers (Figure 2.3.1) in greenhouses to maintain a relative humidity of 60 - 80% and temperature settings were adjusted to 22 - 25°C for optimal disease development. Symptom development on Xcv- and Pst-inoculated plants was evaluated after 14 days. Control and inoculated plants in the Xcv and Pst trials were maintained until floral development. Floral inoculations with Xcv and Pst bacterial suspensions were then performed to improve the incidence of pathogen transmission to seed harvested from the fruit. Bacterial suspensions were prepared as mentioned above but diluted to a final concentration of 1 x 10^6 cfu/mℓ (Sijam et al., 1991). Suspensions were sprayed on all flowers of Xcv- and Pst-inoculated tomato plants until the flowers were damp. All flowers of control plants were sprayed in a similar manner with sterile dH_2O.

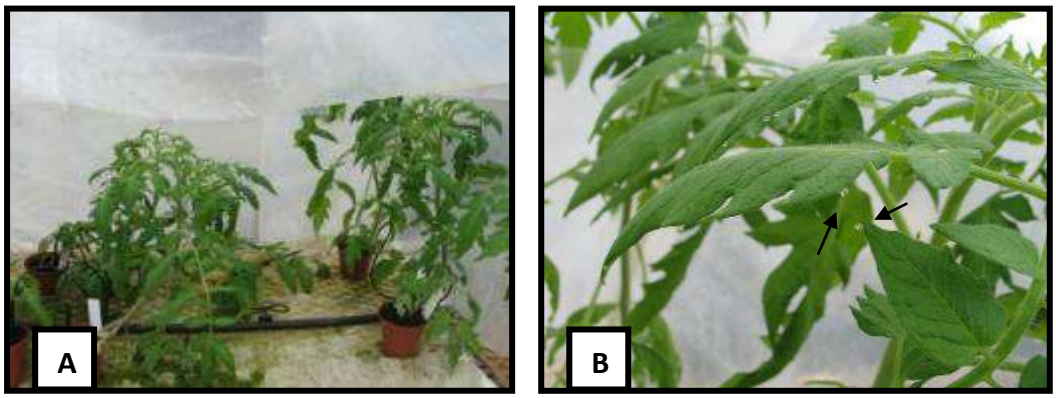

Figure 2.3.1. Plastic chambers created to increase humidity for maximum symptom development of bacterial spot and speck (A). Tomato leaves with guttation droplets (arrows) indicative of highly humid conditions (B).

Asymptomatic leaves on control plants and symptomatic leaves on inoculated plants, that were characteristic of bacterial and viral infection, were sampled throughout each of the trials and stored at -20°C. All infected and control plants were maintained for four – six months to develop fruit. Mature fruit were harvested from plants during this period. Seed were extracted from fruit by manually removing the seed/pulp mixture and incubating overnight at room temperature in 10%

soda ash solution (Kalloo, 1991). To separate seed from the pulp/ash mixture, seed were washed, sieved and then air dried at 26°C for 48 hours. Dried seed was stored at 10°C until used.

2.3.4. Confirmation of pathogen infection by standard methods of detection

2.3.4.1. Confirmation of the presence of viral particles

Leaf and seed samples from inoculated plants that demonstrated characteristic symptoms of PepMV and ToMV infection, were diagnosed according to the process outlined in Figure 2.3. 2. Samples were initially tested by DAS-ELISA to confirm the presence of suspected causal agents. Leaf samples tested in assays included: original, confirmed PepMV/ToMV-infected viral cultures (Table 2. 1), seedlings prior to inoculation (referred to as 'Clean' samples), buffer-inoculated plants (referred to as negative controls) and samples from the 10 symptomatic, viral-inoculated plants (referred to as test samples). Test samples were divided into four or five sub-samples and a pooled sample was also included in each assay. Between 0.5 and 1.0 g of leaves from clean, positive and negative controls as well as test samples were added to sterile 0.05M phosphate buffer at a ratio of 1 g: 10 mℓ of buffer in sterile stomacher bags (Bioreba, Switzerland). A general seed extraction buffer (SEB) (1X Phosphate buffered saline [PBS] at pH 7.4: 0.137M of NaCl, 8mM of $Na_2HPO_4.12H_2O$, 14mM of K-H_2PO_4, 27mM KCL with added 8mM Na_2SO_3) was used to extract viral particles from seed samples. Seed from fruit of PepMV- and ToMV-infected plants, were divided into six sub-samples of 250 seed per pathogen and soaked overnight in 10 mℓ of SEB at 4°C in sterile stomacher bags. Leaf and seed samples were homogenized for up to seven minutes using the SilentCrusher M (Heidolph, Germany) followed by centrifugation to remove excess plant debris.

Monoclonal antibodies complimentary for coat protein regions of ToMV or PepMV, were diluted at a ratio of 1:200 with 1X carbonate coating buffer according to the manufacturers' instructions (Agdia, EU). Test wells of 96-well Nunc-immuno ELISA plates (Thermo Fisher Scientific, Denmark) were coated with 100 µℓ/well of the appropriate, diluted antibody solution. Wells that were not designated for test samples or control samples were filled with 100 µℓ of dH_2O to maintain uniform temperature distribution during incubation of the plates for antibody coating. Plates were incubated in a humid box for three hours at 28°C followed by four, 350 µℓ/well washes with 1X PBST solution (PBS with 0.02% Tween 20). Duplicate wells were prepared for each sub-sample by dispensing 100 µℓ/well of seed/leaf homogenate, positive, negative and buffer control samples into designated wells. Plates were then incubated in a humid box overnight at 4°C for antibody:antigen binding.

After incubation, wells were emptied and washed with 350 µℓ/well of 1X PBST solution. Washes were repeated five times with a three minute interval between washes. PepMV/ToMV Immunoglobin G (IgG) conjugate antibodies (Agdia, EU) were diluted according to the manufacturers' instructions with 1X conjugate buffer and 100 µℓ/well was applied to test wells. Plates were incubated in a humid box at 28°C for three hours for antibody:antigen binding. Excess solution was removed after incubation and wells were washed with 1X PBST as described above. Test wells were filled with 100 µℓ/well of a 1 mg/mℓ solution of p-nitrophenyl phosphate (PNP) substrate (Sigma-Aldrich, USA) and incubated in a humid box in the dark at room temperature for 60 minutes for enzymatic substrate utilisation. The absorbance of test wells was determined using a Multiskan Ex ELISA reader (Thermo Electron Corporation, China) at dual wavelengths of 402 nm and 492 nm. Results were interpreted by Ascent ELISA Ver. 2.6 (Thermo Labsystems, China). Substrate utilisation led to colour development which resulted in an increase in optical density, therefore indicating a positive reaction; whereas the absence of colour represented a negative reaction. A positive reaction implied a reaction between antibodies and the components of a test sample and, thus, confirmed the presence of the relevant pathogen. Ascent ELISA software was programmed to implement a cut-off limit based on the absorbance of the negative control sample for computational discrimination between positive and negative results. Absorbance values of the test samples from healthy tomato leaves/seed that were greater than two times the mean absorbance of the negative control sample (Ling, 2008), were designated as positive for the presence of the target virus.

2.3.4.2. Validation of the viability of viral particles in infected material

A bioassay on indicator hosts was performed for all DAS-ELISA-positive samples to determine the viability of viral particles detected in leaf and seed homogenates (Figure 2.3. 2). Samples to be confirmed for the presence of PepMV following positive DAS-ELISA results were assayed on *N. benthamiana* indicator hosts (ISF, 2009a), and those for the presence of ToMV, on *N. tabacum* cv. Xanthi (ISF, 2009b; Hadas et al., 2004). Bioassays were performed on seedlings at the 4 – 5 leaf stage, with two plants inoculated per sub-sample. For both assays, two negative control plants inoculated with buffer only, were included. Samples were divided as described in section 2.3.4.1 and inoculum was prepared in the same manner for test samples of plant and seed. The expanded leaves of all the seedlings to be used in the ToMV bioassay were dusted with celite powder prior to inoculations. Leaves were then inoculated by lightly rubbing swabs of sterile cotton wool soaked in inoculum/buffer onto adaxial leaf surfaces until the surfaces were damp. The presence of viable ToMV particles in the inoculum was characterized by the development of local lesions on inoculated,

adaxial leaf surfaces after four to seven days (ISF, 2009b; Hadas et al., 2004). Development of a single characteristic lesion on inoculated leaves was considered a positive indication of the presence of viable ToMV particles in inoculum. Seedlings of the PepMV bioassay were inoculated in a similar manner to the ToMV bioassay, however, only two expanded leaves per plant were inoculated and labelled. This was done in order to observe the systemic progression of infection by the development of symptoms on non-inoculated leaves. Inoculated plants were transferred to greenhouses and evaluated for symptom development after 14 dpi. The viability of PepMV in *N. benthamiana* was characterized by the development of a systemic infection and not by local lesion production. Thus, host leaves other than those originally inoculated, were sampled and subjected to DAS-ELISA tests as described previously, to confirm that a systemic infection of PepMV had been established in indicator hosts (ISF, 2009a).

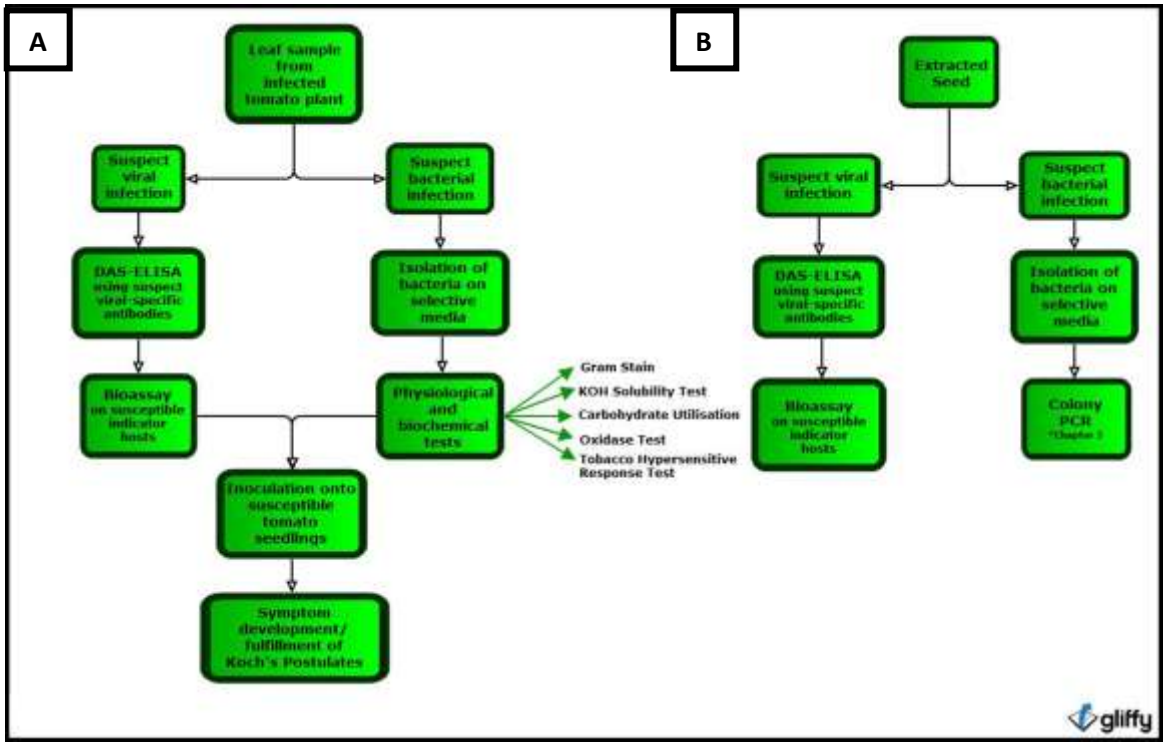

Figure 2.3. 2 Standard detection techniques used in this study to identify and characterize bacterial and viral pathogens from symptomatic leaf and stem samples (A), and seed from diseased hosts (B).

2.3.4.3. Confirmation of bacterial infection by selective plating

Isolation of bacteria from diseased plants

Regions of plants demonstrating symptoms characteristic of the bacterial diseases were sampled to isolate potential causal agents using the selective plating technique (Figure 2.3. 2 A). Media were

selected according to the ability of components of each medium to differentiate between expected bacterial pathogens and other pathogens (able to induce similar symptoms on this host) or saprophytes (inducing a secondary infection) based on colony growth, morphology and colour (Goszczynska et al., 2000). Selected media included: TGA, tetrazolium chloride (TZC) agar, King's B (KB) medium, KB medium supplemented with cycloheximide and cephalexin (KBC) and milk tween (MT) medium; prepared as outlined in (Table 2. 2). All media were autoclaved for 20 minutes at 120°C and cooled to 60°C before decantation into 10 cm diameter plastic petri dishes. Respective antibiotics or additional components (Table 2. 2) were filter-sterilised separately and added to the appropriate, cooled media.

Plants demonstrating wilt and canker symptoms characteristic of Cmm infection, were sampled from the lower part of stems and sections of leaves. A sterile scalpel was used to dissect stem samples and slice thin sections off the vascular tissue. Leaf samples with and without lesions, were taken from plants displaying leaf speck and spot symptoms characteristic of Pst- and Xcv-infections respectively. Stem and leaf samples were surface sterilised with 70% EtOH and then macerated in individual 600 – 800µℓ drops of sterile dH_2O in plastic petri dishes. Macerates were left to stand for 15 – 30 minutes in a laminar flow hood to allow for dissemination of bacterial cells in the solution. The five selective media were streak-inoculated in duplicate with 100µℓ of macerate from each of the isolated samples. Control plates for each of the expected pathogens were prepared on the five selective media using a loopful of reference culture (Table 2. 1). Inoculated media was incubated at 28°C for three - five days. Suspect colonies demonstrating Cmm, Xcv or Pst characteristic morphology were isolated and re-inoculated onto the five selective media to obtain pure cultures of suspect bacteria. Pure cultures were then used for further characterization studies.

Isolation of bacteria from seed samples

The presence of bacteria on tomato seed was determined using antibiotic supplemented selective media (Figure 2.3. 2B) recommended by standard seed-health protocols for the detection of regulated pathogens or as reported in literature (ISF, 2008a, 2009c; King et al., 1954; Kritzman, 1991). Seed obtained from the fruit of plants demonstrating symptoms characteristic of Cmm, Pst or Xcv infection were used for seed assays. In all cases, a starting sample of 4 – 8 g of seed was used to detect each of the expected pathogens.

Detection of Cmm on putatively infected seed was investigated according to the standard ISF protocol (ISF, 2008a) using D_2ANX (Duchefa, Germany) and SCM (Duchefa, Germany) selective media (Table 2. 2). Relevant antibiotics were dissolved separately according to manufacturer's instructions and filter-sterilised into D_2ANX and SCM media once cooled to 60°C. To extract bacteria, seed were placed in phosphate-tween buffer (55mM Na_2HPO_4, 12mM KH_2PO_4 with added 0.02% Tween 20 [pH7.4]) in a sterile stomacher bag at a ratio of 1g of seed:4 mℓ of SEB. To extract greater concentrations of bacteria from contaminated seed, seed was incubated in buffer overnight at 4°C (ISF, 2008a). Seed were homogenized in buffer for a minimum of seven minutes using the SilentCrusher M (Heidolph, Germany) and sterilised between samples by successive washes of 25% NaOCℓ, 0.25M HCℓ and 70% EtOH. Homogenate was incubated on ice until use. Positive control plates were prepared from reference cultures of Cmm (Table 2. 1). A loopful of stock culture was transferred to 1 mℓ of sterile dH_2O (referred to as dilution 10^0) and a ten-fold dilution series (up to 10^{-5}) was prepared from this positive control sample. A ten-fold dilution series (up to 10^{-4}) of the test sample was prepared in 1 mℓ of SEB using the concentrated seed homogenate (10^0). All samples were vortexed vigorously using a Vortex-2 Genie (Scientific Industries Incorporated, NY, USA). From each dilution of test and control samples, 100 µℓ of inoculum was spread in quadruplicate onto plates of SCM and D_2ANX media. Inoculated media were incubated at 28°C for a maximum of 10 days before colony growth and morphology were analysed.

The presence of Xcv on seed was investigated according to the ISF (ISF, 2009c) and the ISTA (Asma, 2005) standard protocols for *Xanthomonas* detection using the selective media: mTBM, CKTM and mKM-1 (Table 2. 2). Seed were placed in a sterile stomacher bag with 1X PBST (pH 7.4) at a ratio of 1g of seed:3 mℓ SEB. Seed were homogenized as in the Cmm seed assay and a dilution series of the homogenate was set up from 10^0 – 10^{-4}, each dilution in 1 mℓ of SEB. A control dilution series was prepared as in the Cmm seed assay using reference cultures of Xcv (Table 2. 1). Petri plates of the selective media: mTBM, CKTM and mKM, were each spread in quadruplicate with 100 µℓ of dilutions of both control and test samples, and incubated at 28°C for seven days before evaluation.

Colonies recovered from dilutions of seed extract which demonstrated similar growth patterns and morphological characteristics to positive control cultures of Xcv or Cmm were isolated for further characterization tests. Suspect colonies were streaked onto YDC and/or CDA selective media (Table 2. 2) along with colonies from the relevant reference control cultures. Inoculated media were incubated at 28°C for three days, after-which growth of suspect cultures was compared to positive

Table 2. 2 Composition of bacterial selective media used in this study

Medium	Isolation or Culture of:	Seed or Leaf Extracts	Basal Medium Composition/ ℓ of sterile dH_2O	Additional components	Antibiotics[1]	Reference
King's B media (KB)	Cmm, Xcv and Pst	Leaf Seed	20 g Proteose Peptone No.3 1.5 g K_2HPO_4 1.5 g $MgSO_4.7H_2O$ 1.5% Glycerol 15 g Bacteriological Agar	n/a	n/a	King et al. (1954)
King's B media with antibiotics (KBC/KBBC)	Cmm, Xcv and Pst	Leaf Seed	20 g Proteose Peptone No.3 1.5 g K_2HPO_4 1.5 g $MgSO_4.7H_2O$ 1.5% Glycerol 15 g Bacteriological Agar	1.5 g/ℓ Boric Acid[2]	10 mg/mℓ Cephalexin 100 mg/mℓ Cycloheximide	Mohan and Schaad (1987)
Milk Tween Agar (MT)	Cmm, Xcv and Pst	Leaf Seed	10 g Proteose Peptone No. 3 0.34 g $CaCl_2$ 0.5 g Tyrosine 15 g Bacteriological Agar	10 g/ℓ Skim Milk powder[2,3] 1% Tween 80[2]	10 mg/mℓ Cephalexin 100 mg/mℓ Cycloheximide 10 mg/mℓ Vancomycin	Goszczynska and Serfontein (1998)
Tetrazolium Chloride Agar (TZC)	Cmm, Xcv and Pst	Leaf Seed	10 g Proteose Peptone No.3 1 g Casein Hydrolysate (Difco) 0.5% Glycerol 15 g Bacteriological Agar	1.25% 2,3,5 –Triphenyl Tetrazolium Chloride (TZC)[2,4]	n/a	Kelman (1954)

[1] Dissolved in the appropriate solvent, filter-sterilised and added to the appropriate media once cooled to 60°C
[2] Autoclaved separately and added to appropriate media once cooled to 60°C
[3] Powder dissolved in 5 mℓ of sterile dH_2O
[4] 1.25 g of TZC dissolved in 100 mℓ of 75% EtOH

Medium	Isolation or Culture of:	Extracts from seed or leaf	Basal Medium Composition/ ℓ of sterile dH_2O	Additional components	Antibiotics[1]	Reference
D_2ANX	Cmm	Seed	37.35 g D_2ANX powder (Duchefa, Germany) 0.15 g $MgSO_4$. $7H_2O$	n/a	28 mg/ℓ Nalidixic Acid 10 mg/ℓ Polymyxin B Sulfate 100 mg/ℓ Cycloheximide	Chun (1982)
SCM	Cmm	Seed	32.22 SCM powder (Duchefa, Germany) 1 g KH_2PO_4	n/a	30 mg/ℓ Nalidixic Acid 100 mg/ℓ Nicotinic Acid 1% Potassium Tellurite 200 mg/ℓ Cycloheximide	Fatmi and Schaad (1988)
CKTM	Xcv	Seed	29 g CKTM powder (Duchefa, Germany) 0.2 g $MgSO_4$. $7H_2O$ 0.25 g $CaCl_2$	1% Tween 80[2]	65 mg/mℓ Cephalexin 12 mg/ℓ 5-Fluorouracil 0.4 mg/ℓ Tobramycin sulphate 100 mg/mℓ Bactricin 10 mg/ℓ Neomycin sulphate 35 mg/ℓ Nystatin	Sijam et al. (1991)
mTBM	*Xanthomonas*	Seed	35.3 g TBM powder (Duchefa, Germany)[5]	1% Tween 80[2] 10g/ℓ Skim Milk powder[2,3]	65 mg/ℓ Cephalexin 12 mg/ℓ 5-Fluorouracil 150 mg/ℓ Cycloheximide	Asma (2005); McGuire et al. (1986)
mKM-1	*Xanthomonas*	Seed	36.1 g KM powder (Duchefa, Germany)	n/a	2 mg/ℓ Tobramycin sulphate 50 mg/ℓ Bactricin 10 mg/ℓ Cephalexin 150 mg/ℓ Cycloheximide	Asma (2005); Kim et al. (1982)

[5] pH adjusted to 7.4 before sterilisation

Medium	Isolation or Culture of:	Extracts from seed or leaf	Basal Medium Composition/ ℓ of sterile dH_2O	Additional components	Antibiotics[1]	Reference
YDC	Xcv Cmm	Seed	10 g Yeast Extract 20 g $CaCO_3$ 20 g D-Glucose 17 g Bacteriological Agar	n/a	n/a	Schaad (1988)
CDA	Xcv Cmm	Seed	2 g Yeast Extract 10 g D-Glucose 15 g Bacteriological Agar	15 g/ℓ Skimmed Milk Powder	n/a	T. Day (1991, pers. com.)
KBZ	Pst	Seed	20 g Proteose peptone No.3 1.5 g K_2HPO_4 1.5 g Boric Acid 1.5 g $MgSO_4.7H_2O$ 1.8% Parosaline Base[6] 1.5% Glycerol 15 g Bacteriological agar[7]	1.4% 2,3,5-Triphenyl Tetrazolium Chloride[2,3]	160mg/ℓ Cephalexin 150mg/ℓ Cycloheximide	King et al. (1954)

[6] 1.8% Parosaline base solution prepared in 10 mℓ of 70% EtOH
[7] pH adjusted to 7.5 before sterilisation

control reference cultures. Those which demonstrated similar morphology to the control colonies were then characterized by Gram-staining (2.3.4.4 below) and the five-plate assay on TGA, TZC, MT, KB and KBC selective media as described above, in conjunction with positive control cultures. Media was incubated for five days at 28°C and comparative colony morphology was analysed.

Seed from fruit of diseased plants inoculated with Pst were tested to confirm the presence of Pst using KBZ and KBBC selective media (King et al. 1954; Mohan and Schaad 1987), prepared as outlined in (Table 2. 2). Seed were placed in phosphate-tween buffer at a ratio of 1 g of seed: 3 mℓ SEB and homogenized for a minimum of seven minutes in sterile stomacher bags. A dilution series of both the seed homogenate and a positive reference culture of Pst (Table 2. 1) were prepared as described in the Cmm seed assay. Dilutions were spread in quadruplicate onto KBZ and KBBC media, and incubated for seven days at 28°C before morphology of the resulting colonies was analysed. Colonies similar to Pst control colonies were streaked onto KB medium (Table 2. 2), in conjunction with positive cultures for further comparative studies. Streaked plates of KB medium were incubated for an additional seven days at 28°C before evaluation of culture morphology.

Detection of Xcv or Pst from seed was also analysed using a seed wash assay adapted from Du Toit et al. (2005). Seed samples of 5 g were each suspended in 50 mℓ of 12.5mM phosphate buffer in a sterile 250 mℓ glass flask and incubated at room temperature for 2 hours on a rotary shaker at 50 rpm. A few drops of Tween 20 were then added to the buffer and seed suspensions were shaken for an additional five minutes at 250 rpm to remove bacteria from surfaces of seed. Seed suspensions were filtered through four layers of sterile cheesecloth into sterile, plastic 50 mℓ centrifuge tubes (Plastpro, South Africa) and centrifuged at 10 000rpm at 4°C for 10 minutes. Pellets were resuspended in 5 mℓ of 12.5mM sterile phosphate buffer and used to inoculate selective media. Selective media chosen for the seed wash assay were mTBM and mKM-1 for Xcv testing, and for Pst, KBZ and KBC media. Serial dilutions of the seed wash suspension (10^0) were prepared to 10^{-3} and 100µℓ of each dilution was spread in triplicate on the respective selective media as well as Nutrient agar (NA) medium (1.6% w/v Nutrient Broth and 1.5% w/v Bacteriological Agar). The relevant positive Xcv or Pst reference cultures (Table 2. 1) were included in the assay for morphological comparison. Plates were incubated for seven days at 28°C after which growth characteristics of the recovered colonies were analysed. Further characterization of suspect colonies was performed as mentioned above for Xcv and Pst suspect cultures.

All seed assays were repeated in duplicate or until reliable, that is repeatable, and/or similar, conclusions for recovered bacteria could be made. From each assay the average number of cfu/mℓ of inoculum spread on the plates was determined for both suspect and non-target bacteria recovered from each type of selective medium for Cmm, Xcv and Pst assays. The recovery percentage for each medium was also calculated by dividing the cfu/mℓ of suspect or non-target bacteria by the total recovered cfu/mℓ. Suspect colonies with growth and morphology similar to that of positive control colonies of Cmm, Xcv or Pst were subjected to further confirmation testing by bio-PCR (Chapter 3).

2.3.4.4. Physiological and biochemical tests for the characterization of isolated bacteria

Suspect cultures of Cmm, Xcv and Pst, recovered from infected leaf and seed samples onto selective media, were subjected to a set of physiological and biochemical tests for genus, species, subspecies and/or pathovar characterization (Figure 2.3. 2). Colonies were cultured on nutrient agar (NA), TGA or KB media for three days at 28°C to obtain pure cultures to be used in subsequent tests.

The Gram stain technique (Gram, 1884) was performed on suspect bacterial colonies from leaf and seed homogenates to characterize the recovered bacteria (Figure 2.3. 2). A loopful of pure culture for each suspect isolate was diluted in a drop sterile dH_2O on a glass slide and heat-fixed to the slide. Colonies were stained with 0.5% crystal violet for one minute followed by application of 1% Gram's iodine, used as a dye fixative, for one minute. Cultures were destained with acetone for a few seconds and counterstained with 0.5% safrinin. Stained cultures were then examined under the 40X objective of an Axiostar Plus light microscope (Zeiss, Germany) to analyse dye retention patterns that enabled characterization of cell structures. Gram positive bacteria were characterized by the purple appearance of the bacterial cells, due to the retention of the crystal violet stain, whereas, Gram negative bacteria were pink, due to the counter stain of safrinin. Gram stain results were confirmed by the potassium hydroxide solubility test whereby a suspect colony was blended with a drop of 3% KOH solution on a glass slide using a sterile inoculation loop. After mixing the culture so that an even bacterial suspension was formed, the loop was lifted gently from the suspension (Goszczynska et al., 2000). Gram negative bacteria were expected to produce a stringy, mucoid thread from the suspension, which was absent for Gram positive bacteria.

The ability of the isolated bacteria to utilise various carbon sources and substrates was investigated (Figure 2.3. 2 A) using a range of selected carbohydrate-supplemented media (Goszczynska et al.,

2000). Tested media consisted of a basal medium (1 g/ℓ $NH_4H_2PO_4$, 0.2 g/ℓ KCℓ, 0.2 g/ℓ $MgSO_4.7H_2O$, 12 g/ℓ Bacteriological agar and 1.6% w/v bromothymol blue [pH7.2]) supplemented with 1 g/ℓ of one of the five carbohydrates: sucrose, sorbitol, erythritol, mannitol or inositol, dissolved according to the manufacturer's instructions (Sigma, USA). A loopful of inoculum from a pure culture of each test isolate was streaked in duplicate onto the five carbohydrate-supplemented media and incubated at 28°C for five days. Growth of bacterial colonies on each medium represented the ability to metabolise the supplemented carbohydrate. Levan medium (NA medium with 5% sucrose) was also streaked with suspect cultures in conjunction with appropriate positive or negative reference cultures to evaluate the ability of isolates to utilise high concentrations of sucrose as a carbohydrate source and produce the by-product levan. Media was incubated at 28°C and the bacterial growth was evaluated after three days. Test cultures that developed white, mucoid colonies on this medium were considered levan-positive.

To establish the oxidative properties of suspect bacteria recovered from leaf samples, isolates were subjected to the oxidase test (Figure 2.3. 2 A). A sterile cotton wool swab was drenched in Kovac's solution (1% NNN'N-tetramethyl-p-phenylene-diamine-dichloride solution – Sigma, USA) and used to isolate a colony from pure cultures of the appropriate test culture. After 30 seconds, appearance of a dark purple colour indicated the ability of the bacterial culture to metabolise and oxidise Kovac's solution.

2.3.4.5. Evaluation of the pathogenicity of bacteria isolated from infected plants

The tobacco hypersensitive response (THR) assay was performed on suspect isolates from infected plant samples (Figure 2.3. 2 A) that demonstrated similar results in selective media and other characterization tests to those expected results of Cmm, Xcv or Pst. The THR assay aimed to establish the pathogenicity of these cultures and, therefore, determine whether each isolate was the causal agent of the symptoms observed on diseased tomato plants. A loopful of culture from NA was diluted in 1 mℓ of sterile dH_2O and vortexed thoroughly to ensure even dissemination of bacterial cells throughout the suspension. The THR assay was performed on *N. tabacum* cv. Xanthi seedlings at the four – six true leaf stage. The abaxial surfaces of expanded tobacco leaves were inoculated by infiltrating bacterial inoculum, or dH_2O (negative control treatment), into the intracellular spaces of the leaf epidermis, using a sterile 2 mℓ syringe (Neomedic, UK) and light manual pressure. Each isolate was inoculated in six different regions of a single leaf, and inoculated plants were transferred to the greenhouse (25°C). After two – three days, inoculated leaves were evaluated for the

development of localised, water-soaked or brown, necrotic lesions indicative of an induced hypersensitive response from the host and, thus, pathogenicity of the tested culture.

2.3.4.6. Biolog classification of isolated bacteria

Of the bacterial cultures isolated from diseased leaf material, three were chosen that displayed the greatest similarity to control cultures of Cmm, Xcv and Pst in the above characterization tests. These were sent to the Agricultural Research Council (Plant Protection Research Institute -Gauteng, South Africa) for characterization using the Biolog microplate system (Biolog Inc., Hayward, USA). Presumptive Gram negative Xcv and Pst isolates were cultured on tryptic soy agar (TSA – Biolog, USA), and the presumptive Gram positive Cmm isolate was cultured on Biolog Universal Growth medium (BUG – Biolog, USA), to obtain pure cultures of bacteria prior to testing. A bacterial suspension was prepared for each pure culture which was then inoculated into each of the 95 wells of the GN2 (for Gram negative bacteria) or the GP2 (for Gram positive bacteria) microplate. Inoculated plates were incubated at 30°C for 24 hours. Wells contained a redox dye, tetrazolium, and 95 various carbon sources to facilitate classification of Gram negative and Gram positive bacteria. After incubation, wells were scored visually for colour development, which represented utilisation of each particular carbon source. A utilisation profile was constructed for each isolate which was compared to the GN or GP Database (Biolog, USA) for identification, using Bionumerics software Version 4.5 (Applied Maths, Belgium) to determine statistically significant matches.

2.3.4.7. Proving the pathogenicity of isolates on tomato

To prove Koch's third postulate that symptoms that developed on inoculated tomato plants could be attributed to the viruses and bacteria isolated from these diseased plants, isolates had to be re-inoculated onto healthy tomato plants and reproduce similar symptoms (Figure 2.3. 2 A). This would, therefore, prove that isolates were pathogenic on tomato plants. For each of the five original pathogens (Table 2. 1), a culture was isolated from host samples that was tentatively classified as PepMV, ToMV, Cmm, Xcv or Pst by conventional detection tests (section 2.3.4). These isolates were used for inoculations onto individual groups of 10, healthy, 4-week-old seedlings of susceptible *S. lycopersicum* cv. Rooikhaki as described in 2.3.3 above. In addition five negative control plants, inoculated with buffer only, were included in each pathogenicity trial. Inoculated and control plants were transferred to greenhouses and evaluated for symptom development after two - four weeks. Koch's third postulate for each pathogen was considered positive if inoculated plants developed symptoms similar to those observed in initial trials with the respective pathogens. The final step of

Koch's postulates includes re-isolation and re-characterization of the causal agent. This was omitted in this study, as the use of a range of identification and characterization tests, as well as the induction of similar symptoms on plants was sufficient to prove detection of the target pathogen.

2.4. Results

2.4.1. Symptom development on inoculated plants

2.4.1.1. Pepino mosaic virus

All of the 10 seedlings inoculated with suspect PepMV-inoculum developed similar symptoms with only slight variation in symptom severity after a minimum of 14 days. Older leaves developed a mild light/dark green mosaic which spread to all regions of each inoculated plant and became more distinct as the disease progressed, with dark green regions forming in strong contrast to lighter regions (Figure 2.4. 1 A and B). Interveinal regions of leaves became raised unevenly to form a bubble-like appearance, especially visible on younger leaves, which intensified over time, often leading to the distortion of overall leaf shape. At least 50% of leaves, both with and without bubbling symptoms, developed yellow spots about 3 – 4 mm in diameter which became necrotic and/or expanded over the surface of the leaf to give a scorched appearance approximately 12 weeks post inoculation (Figure 2.4. 1 C). Severe infections were associated with the curling of leaf margins which was generally accompanied by distortion, narrowing and upturning of leaves. Although the overall growth of diseased plants was similar to that of non-inoculated control plants, the upper branches of mature, infected plants were stunted and bent (Figure 2.4. 1 D). Fruit development on diseased plants was not delayed compared to the control plants, but a reduction in size and number, as well as a distortion of fruit shape, were observed. The surfaces of some immature fruit developed shallow furrows that disappeared with maturation, while others developed a permanently dented surface referred to as marbling (Figure 2.4. 1 E). Fruit also ripened unevenly, giving rise to uneven discoloration and/or the formation of dark red blotches called flaming (Figure 2.4. 1 F). Control plants did not demonstrate any of these symptoms and developed asymptomatically throughout the trials, producing healthy, mature fruit after approximately 12 weeks.

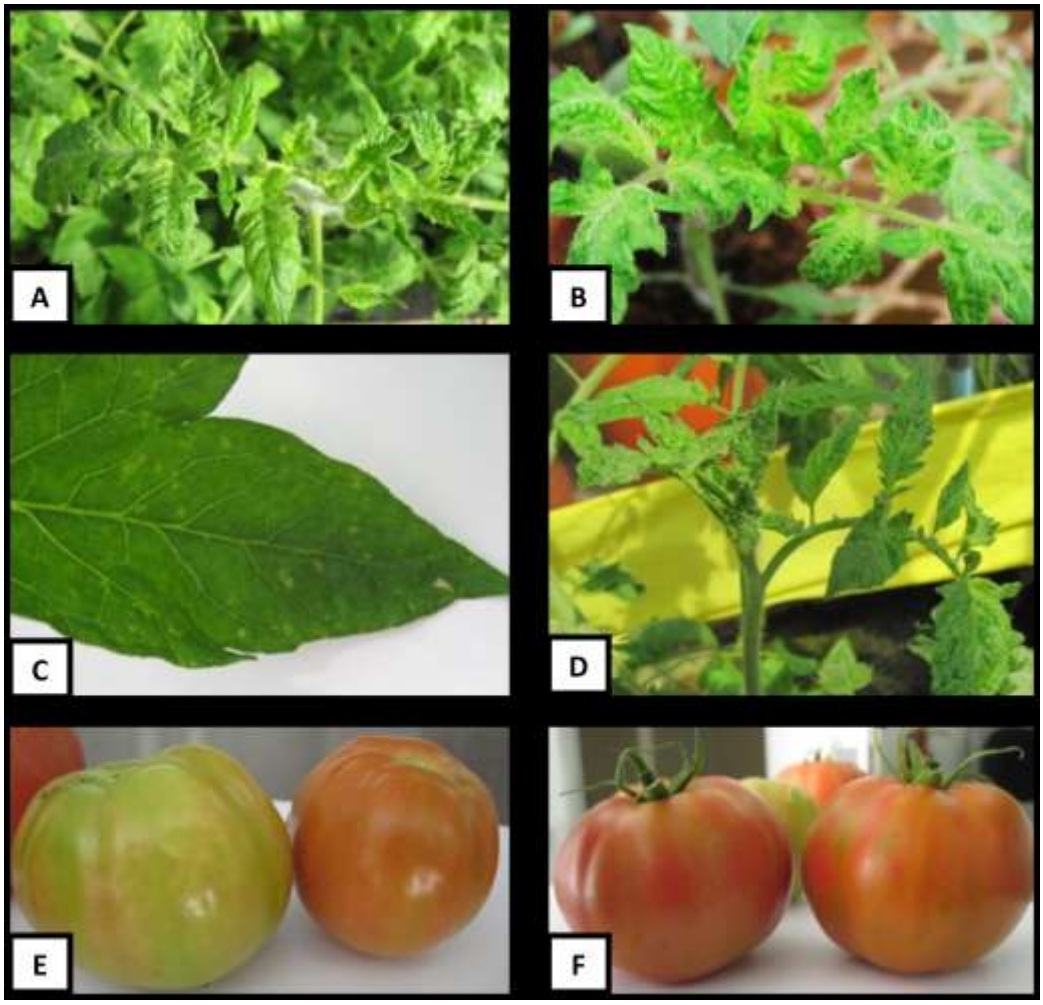

Figure 2.4. 1 Symptoms observed on plants inoculated with suspect PepMV-inoculum: lateral branches displaying severe leaf mosaic, leaf bubbling and leaf distortion (A and B), yellow spot development on leaves (C), stunting and bending of upper branches of mature plants (D), marbled surfaces of fruit (E), mature fruit demonstrating uneven discolouration and flaming (F).

2.4.1.2. Tomato mosaic virus

The onset of symptoms of seedlings inoculated with suspect ToMV-infected plant material occurred about 14 days after inoculation. Initially, only leaves in the apical region of inoculated plants were distorted, displaying a folded and curled or tapered phenotype in an upward orientation (Figure 2.4. 2 A). With progression of infection, symptoms spread to all regions of infected plants. Younger leaves developed a 'shoe-string' appearance whereby leaf surfaces were completely reduced to givea stringy appearance (Figure 2.4. 2 B). Other leaves expanded unevenly and developed serrated margins. Mild bubbling symptoms were visible on some leaves but were not as severe as those observed on suspect PepMV-infected plants. Distinct mosaic symptoms developed on older leaves which became more intense over time with entire portions of leaf surfaces appearing either dark or light green (Figure 2.4. 2 D). From early infection, emerging apical and lateral branches as well as individual leaves, demonstrated uneven and stunted growth compared to control plants. All

symptoms intensified with increasing duration after inoculation. Three weeks after inoculation, as many as 90% of the leaves of all plants were distorted coupled with various combinations of the above-mentioned symptoms, including uneven leaf expansion, upturning and curling of leaves, varying degrees of leaf bubbling, leaf split and tapering of leaf margins (Figure 2.4. 2 B and C). The incidence of tapered, folded and string-like leaves had increased to greater than 70% on each branch. After 3 weeks, a number of newly developed leaves were malformed demonstrating a fern-like appearance characterized by leaves splitting into smaller individual leaves (Figure 2.4. 2 E). The size of developed fruit was drastically reduced compared to healthy control plants, and some fruit were distorted. The onset of fruit ripening was noticeably delayed and subsequent ripening was uneven with fruit displaying discrete red and green regions (Figure 2.4. 2 F).

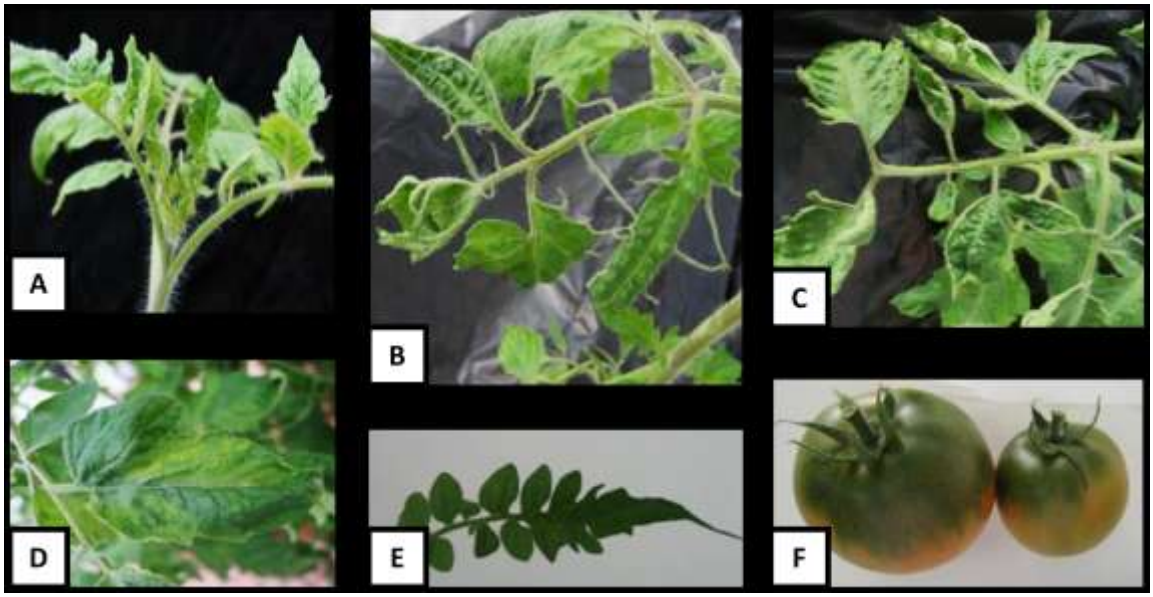

Figure 2.4. 2 Symptoms observed on plants infected with ToMV: apical regions of young plants displaying curled and stunted leaves distorted upwards (A), severely infected plants demonstrating various combinations of leaf tapering, bubbling, distortion, shoe-string formation, curling, upturning and serration of leaf margins (B and C), distinct dark/light green leaf mosaic (D), leaf split or fern-like formation of infected leaves (E), uneven ripening of stunted fruit (F).

2.4.1.3. Clavibacter michiganensis subsp. *michiganensis*

Stab inoculation of 30 tomato seedlings with a suspected Cmm culture resulted in the upturning or curling of leaf margins especially on leaves of branches closest to the inoculation site. At least one leaf per inoculated plant demonstrated loss in turgidity 14 dpi, indicated by unilateral wilting, which was generally accompanied by purple leaf discoloration (Figure 2.4. 3 A). Wilting intensified with disease progression and by 21 dpi, as many as 4 or 5 leaves per branch had curled inwards or wilted entirely to become brown and necrotic. Interveinal regions of other leaves developed small white

spots which expanded to form yellow, chlorotic regions and coalesced to cover the leaf surfaces of infected plants. The development of adventitious roots on the lower stems of some of the inoculated plants was also observed. Stems close to the inoculation site of all plants developed brown streaks at about 21 dpi and, upon dissection, revealed browning or necrosis of vascular tissue. Internodal regions of some of these stems split to form cankers along the brown streaks which deepened and elongated (Figure 2.4. 3 B). The loss in turgidity of stems combined with the apparent necrosis of vascular tissue eventually led to the collapse of branches of severely infected plants approximately 28 dpi (Figure 2.4. 3 D). Despite the wilting and collapse of numerous branches of inoculated plants, the overall growth of most of the inoculated plants was similar to that of the control plants. Several inoculated plants, however, were severely infected and wilted completely to yield dry, brittle and necrotic leaves, stems and roots (Figure 2.4. 3 E). Deterioration of the vascular system appeared to compromise the immune system of infected plants as various secondary infections developed, likely due to opportunistic pathogens, which hastened death of the host. Wilting also reduced floral (Figure 2.4. 3 C) and subsequent fruit development in severely infected plants. All fruit were small, most likely due to the lack of adequate water and food transport

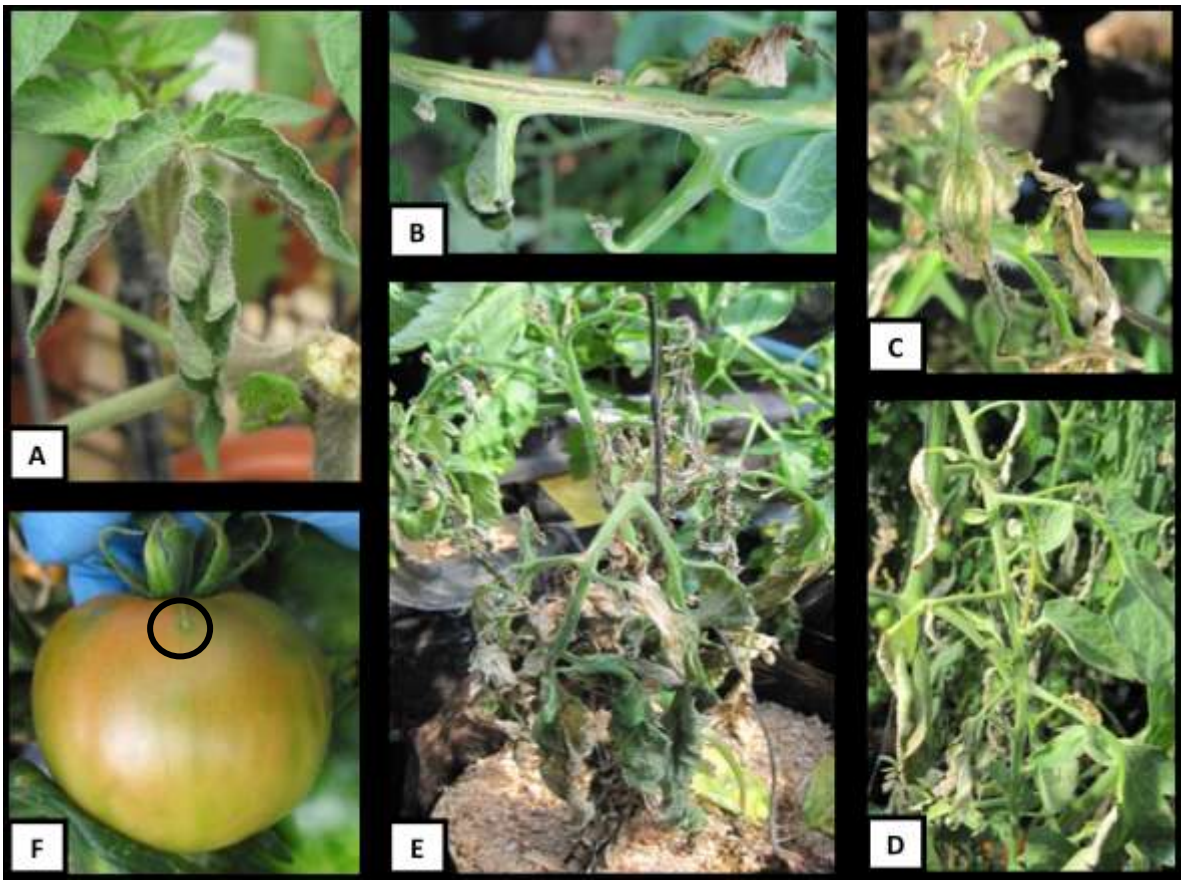

Figure 2.4. 3 Symptoms observed on plants inoculated with a suspect Cmm culture: purple discolouration and upturning of leaf margins (A), severe stem canker (B), wilting and necrosis of floral bundles (C), unilateral wilting of individual leaves on branches (D), wilting and collapse of branches and stems of a severely infected plant (E), white necrotic lesions on infected fruit (circle) (F).

in the plant. A flaccid vascular system also weakened fruit peduncles, causing premature fruit drop, especially of larger fruit. Fruit that developed from plants with less severe infection were a comparable size to healthy fruit but, some developed whitish lesions and spots (Figure 2.4. 3 F).

2.4.1.4. Xanthomonas campestris pv. *vesicatoria*

Initial symptom development on plants inoculated with the suspect Xcv suspension was mild with only one or two older leaves per plant displaying small, black lesions. These circular lesions were approximately 1 mm in diameter and generally situated in interveinal regions on or close to leaf margins (Figure 2.4. 4 A). As the disease progressed, more lesions became visible across the surfaces of leaves and stems, enlarging to between 3 and 5 mm in diameter (Figure 2.4. 4 A – C). Lesions varied in appearance on different plants and even between different leaves of the same plant, ranging from green, water-soaked lesions, to tan brown or black lesions. Many water-soaked or black lesions dried out to form necrotic holes with a black or brown halo, referred to as shot-gun lesions. Lesions that developed in close proximity on leaf surfaces often coalesced to form larger,

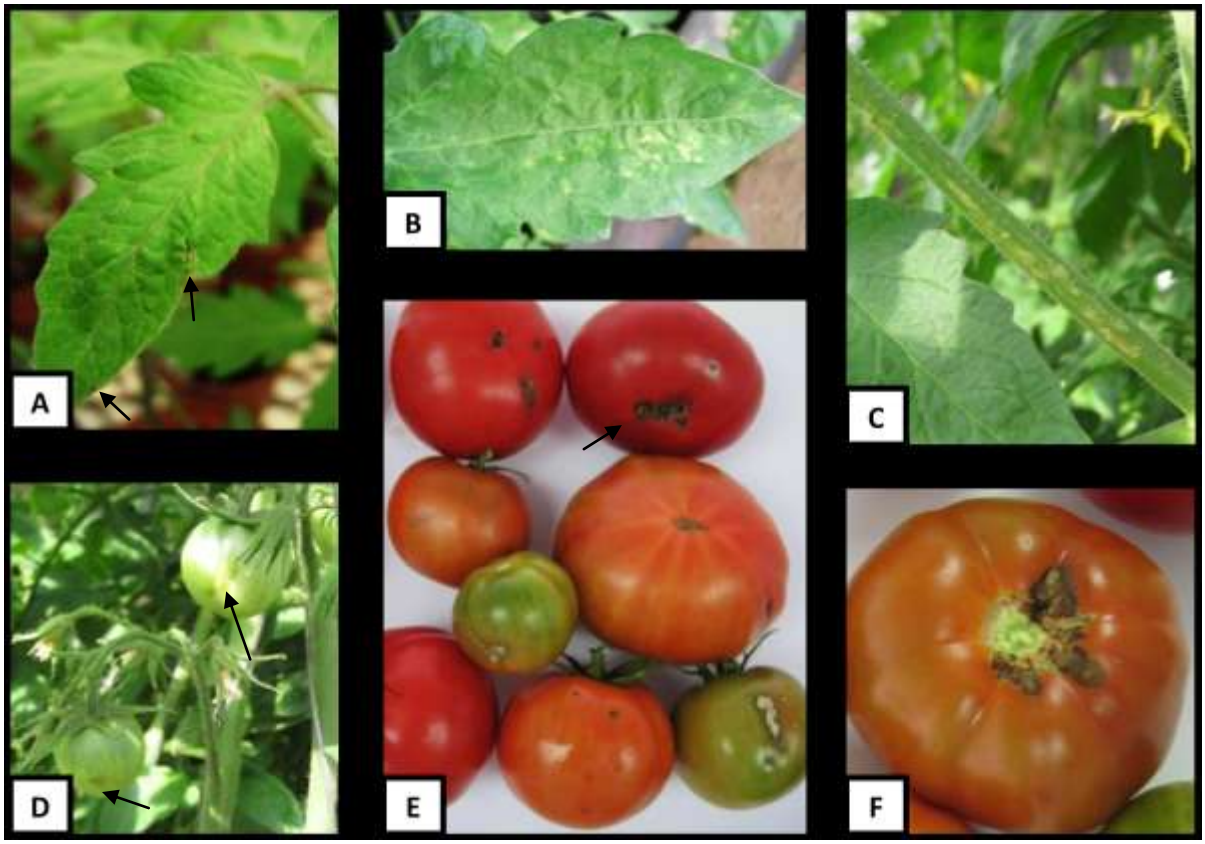

Figure 2.4. 4 Symptoms observed on plants inoculated with a suspect Xcv suspension: black necrotic lesions on leaf margins (arrows) and interveinal regions (A), dried, tan-brown necrotic lesions (B), water-soaked lesions on stems (C), water-soaked lesions developing on immature fruit (arrows) (D), harvested fruit demonstrating varied lesion development and necrotic furrows (arrow) (E), black depressions visible at the peduncle attachment site of fruit (F).

brown/black necrotic regions that gave affected leaves a scorched appearance. The overall height of infected plants was not affected by inoculations and fruit developed normally after approximately 12 weeks. Symptoms on fruit initially developed as green or brownish, water-soaked lesions or white blisters (Figure 2.4. 4 D), which became necrotic and turned brown or black. Some lesions were raised and surrounded by a whitish halo, though these disappeared during fruit ripening. Larger lesions were sunken and black, tan brown or white with a black, raised margin. Large black depressions developed at the site of peduncle attachment (Figure 2.4. 4 F) and a number of immature fruit also developed black necrotic furrows at the basal growth points of fruit which deepened as fruit matured (Figure 2.4. 4 E).

2.4.1.5. Pseudomonas syringae pv. *tomato*

Symptoms observed on plants inoculated with a suspect Pst culture were similar to those inoculated with Xcv, with lesions developing both on leaf margins and interveinal regions, that were best visible 20 dpi. These were also initially visible only on older leaves but spread to all leaves as the disease progressed. Lesions were smaller, however, ranging from 1 – 3 mm in diameter, and either circular or irregularly shaped. Lesions or specks were necrotic and dark brown or black with yellow chlorotic halos which intensified over time (Figure 2.4. 5 A). On some leaves, chlorotic halos expanded across leaf surfaces, causing chlorosis and yellowing of the entire leaf. Numerous lesions developed per leaf and those that formed in close proximity caused slight leaf distortion or coalesced to form large, black, necrotic regions across leaf surfaces (Figure 2.4. 5 B and C). In drier areas of the humidity chamber, leaf lesions dessicated to form numerous tan brown, necrotic holes similar to those observed on Xcv-inoculated plants. Stems, petioles and fruit peduncles developed black necrotic lesions later, which were oval and elongated compared to leaf lesions (Figure 2.4. 5 D). The overall growth and size of diseased plants was similar to healthy control plants, and fruit developed normally despite floral inoculations with the Pst suspension. Maturing fruit on inoculated plants developed water-soaked lesions however (Figure 2.4. 5 E), each approximately 3 cm in diameter. These lesions dried and became whitish or tan brown, elongated lesions that persisted through the fruit ripening process. Plants with severe infections characteristically developed fruit with numerous necrotic, sunken lesions on surfaces which were generally black and up to 1 mm in diameter (Figure 2.4. 5 F and G). In some cases these spots coalesced, forming large necrotic regions on fruit surfaces.

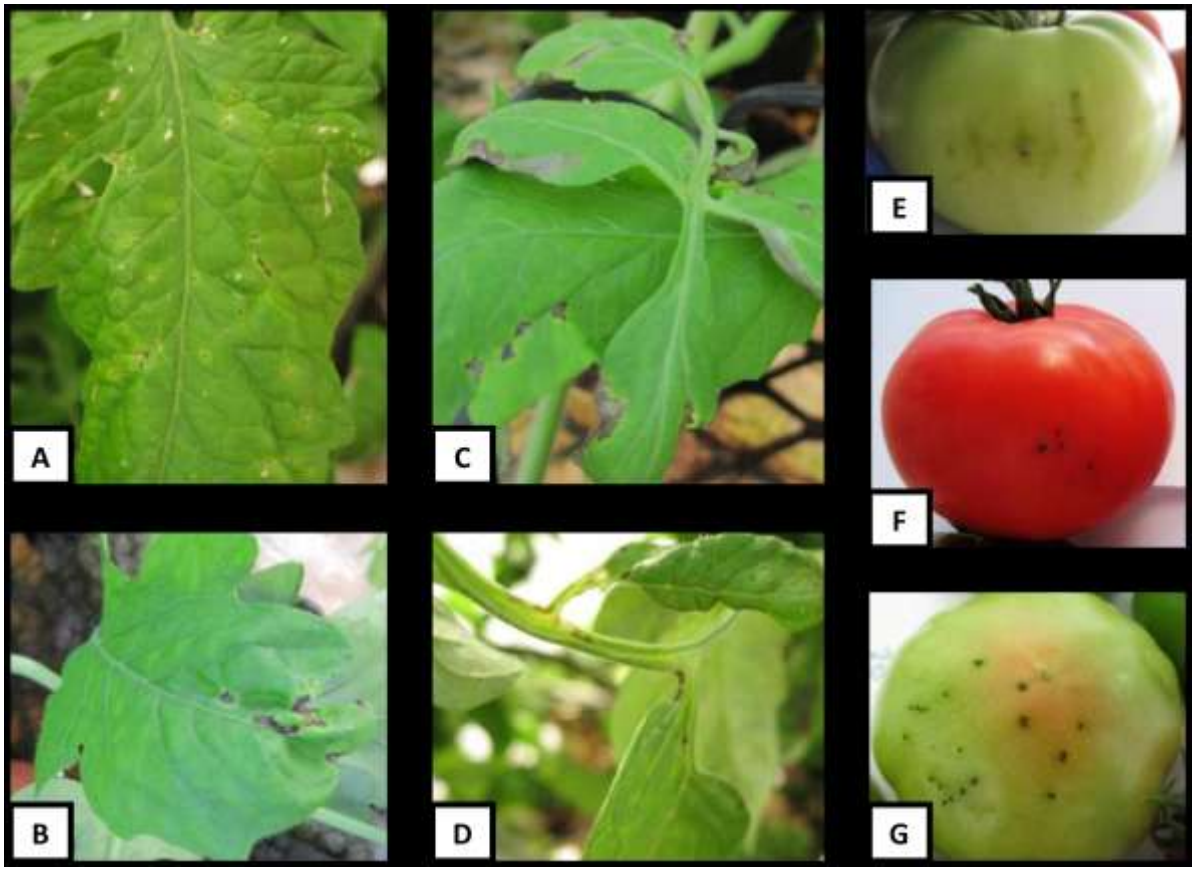

Figure 2.4. 5 Symptoms expressed by plants inoculated with a suspect Pst suspension: numerous small, black necrotic lesions each with a yellow, chlorotic halo on the leaf surface (A), leaf distortion due to lesion development and coalescence (B), expansion of coalesced lesions across leaf surfaces (C), elongated oval lesions visible on petioles and stems (D), water-soaked lesions on immature fruit (E), severely infected immature fruit with numerous small, black, necrotic lesions (F), mature fruit with depressed, black, necrotic lesions (G).

2.4.2. Confirmation of the presence of viral particles in leaf and seed samples

2.4.2.1. Detection of viral particles from symptomatic host samples

Plants and the harvested seed that demonstrated symptoms characteristic of PepMV or ToMV, were tested by DAS-ELISA to confirm the presence of the suspected virus. Samples were tested in separate assays with antibodies complimentary to PepMV or ToMV to validate the presence of a single infection of each respective virus. The hard coat of tomato seed prevented complete homogenization of seed samples compared to the fresh leaf samples, which were easily crushed to release any viral particles present. Nevertheless, homogenates from the partially crushed seed were tested in assays.

Assays of leaf samples suspected to be infected with PepMV, were performed on five sub-samples of the 10 symptomatic plants, i.e. two plants per sub-sample, and a pooled sample of leaves from all of the plants. For reactions with the PepMV-specific antibody, the cut-off limit, determined as

described in section 2.3.4.1, was set at 0.262. Samples containing the original leaf inoculum (suspected to contain PepMV particles) used for pathogenicity trials, had a mean absorbance of 1.352 and produced a strong, yellow colour in test wells. This indicated a high concentration of PepMV particles in these leaf samples. Sub-samples taken from the host plants of pathogenicity trials had absorbance values of over 1.25 with the pooled sample recording an absorbance of 1.387. This confirmed a positive reaction between PepMV-specific antibodies and the test samples. The 'clean' leaf sample, taken from seedlings before inoculation and symptom development, recorded an absorbance of 0.141, indicating a negative reaction with PepMV-specific antibodies. The negative cut-off for ToMV detection with pathogen-specific antibodies for assays with these leaf samples was set at 0.129, determined as described in section 2.3.4.1. The absorbance readings of all five sub-samples and the pooled test sample were below 0.048, indicating a negative reaction with ToMV antibodies in PepMV-positive leaf samples. For detection of PepMV from seed harvested from diseased host plants, six replicate sub-samples of 250 seed, taken from a pooled collection of seed, were tested. In this assay, the cut-off was set at 0.106 according to the absorbance reading of the negative control sample. The absorbance of the six replicates was between 0.229 and 0.667, indicating a low, but positive reaction with PepMV-specific antibodies, compared to the absorbance of the positive control sample of 1.095. These wells correspondingly demonstrated a pale yellow colour upon visual inspection of the test plates in contrast to the strong yellow colour of positive control wells. Reactions of these seed samples with the ToMV antibody were based on a cut-off of 0.056. All replicate samples recorded absorbance values of less than 0.048, therefore indicating the absence of ToMV but the presence of PepMV in the seed samples.

To confirm the presence of ToMV in putatively infected host plants, the four sub-samples from symptomatic plants and a pooled sample were subjected to DAS-ELISA testing. For designation of a positive reaction with ToMV antibodies, absorbance values had to be above the cut-off for this assay, determined as described in section 2.3.4.1, which was set at 0.081 according to the absorbance of the negative control samples. All four samples and the pooled sample had an absorbance reading greater than 0.196. The absorbance of the pooled sample was also in close proximity to that of the positive control sample for ToMV, at 0.260 and 0.267, respectively. Accordingly, a pale yellow colour was observed from the positive control samples and all test sample wells. Clean and buffer-inoculated leaf samples (i.e., negative control leaf samples) recorded absorbance readings of 0.045 and 0.047 respectively, after assays with ToMV-specific antibodies, indicating negative reactions with the antibodies. Reactions of test and control leaf samples with the PepMV antibodies, was based on a cut-off value of 0.038 to distinguish positive or negative results.

Test leaf samples and the pooled sample recorded absorbance values less than or equal to 0.49, indicating a negative reaction with the PepMV-specific antibody in all ToMV-positive samples. For seed assays, six replicate samples of 250 seed were assayed against ToMV- and PepMV-specific antibodies. Positive detection of ToMV from seed was based on a cut-off of 0.060, and all replicates, as well as the positive ToMV control samples, recorded an absorbance value within a range of 0.732 – 0.786. Respective wells for these samples demonstrated a pale to strong yellow colour upon visual inspection. Assays with the PepMV-specific antibody, on the other hand, resulted in absorbance values of less than 0.055 for all replicate samples. The cut-off of 0.095 for this assay indicated that all ToMV-positive seed samples produced a negative reaction with PepMV-specific antibodies, which was validated by the absence of colour in the wells.

2.4.2.2. Confirmation of the viability of detected viral particles

The viability of PepMV or ToMV viral particles detected from tomato leaf and seed samples, was determined by bioassays on host tobacco plants of *N. benthimiana* and *N. tabacum* cv. Xanthi, respectively. Leaf and seed samples that tested positive for the presence of PepMV by DAS-ELISA were analysed for the ability to induce symptoms on inoculated *N. benthamiana* plants. Similar symptoms were observed from trials inoculated with all leaf (Figure 2.4. 7 A – C) and seed homogenates (Figure 2.4. 7 E – F) by 14 dpi, however, the symptoms were less severe on plants inoculated with DAS-ELISA-positive seed homogenates than leaf homogenates. Leaves of the inoculated plants, other than the two leaves inoculated per plant, demonstrated mild to severe, dark/light green mosaic and bubbling. These symptoms were the most severe on the youngest leaves of inoculated plants. Leaves of inoculated plants also demonstrated varying degrees of distortion and curling (Figure 2.4. 7 B and C). Overall, leaf expansion was reduced, diverging from the club-shape of mature leaves of control tobacco plants (Figure 2.4. 7 D). Stunting of apical and, especially, lateral growth was observed, with an overall size reduction of inoculated plants. Control samples, that were DAS-ELISA negative, and buffer-inoculated tobacco plants, did not display any symptoms and developed into mature, flowering plants (Figure 2.4. 7 D). The systemic spread of viral particles within host plants was confirmed by DAS-ELISA of leaf samples from tobacco plants initially inoculated with leaf or seed homogenates, respectively. In both assays, replicates were divided into five sub-samples and one pooled sample from all inoculated tobacco plants. Assays were performed with PepMV-specific antibodies only, and were based on a cut-off of 0.074 and 0.321 for samples from leaf and seed bioassays, respectively. All test samples and pooled samples from both assays recorded absorbance values greater than these cut-offs, indicating positive detection of PepMV viral particles in tobacco hosts.

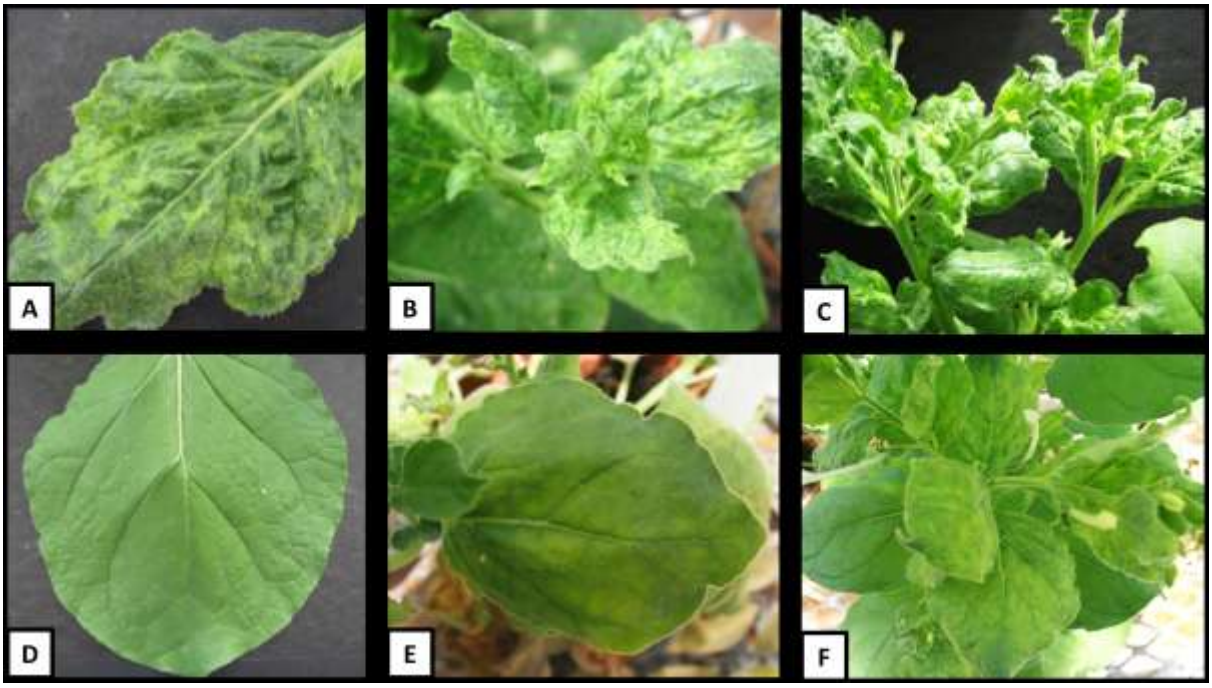

Figure 2.4. 7 Symptom development on tobacco hosts inoculated with PepMV DAS-ELISA-positive leaf or seed homogenates. Symptoms of bubbling, mosaic, distortion and stunting observed from plants inoculated with positive leaf samples (A – C). Healthy leaf of a control tobacco plant with normal club-shape expansion (D). Plants inoculated with positive seed homogenates demonstrating various degrees of leaf mosaic, bubbling and distortion (E and F).

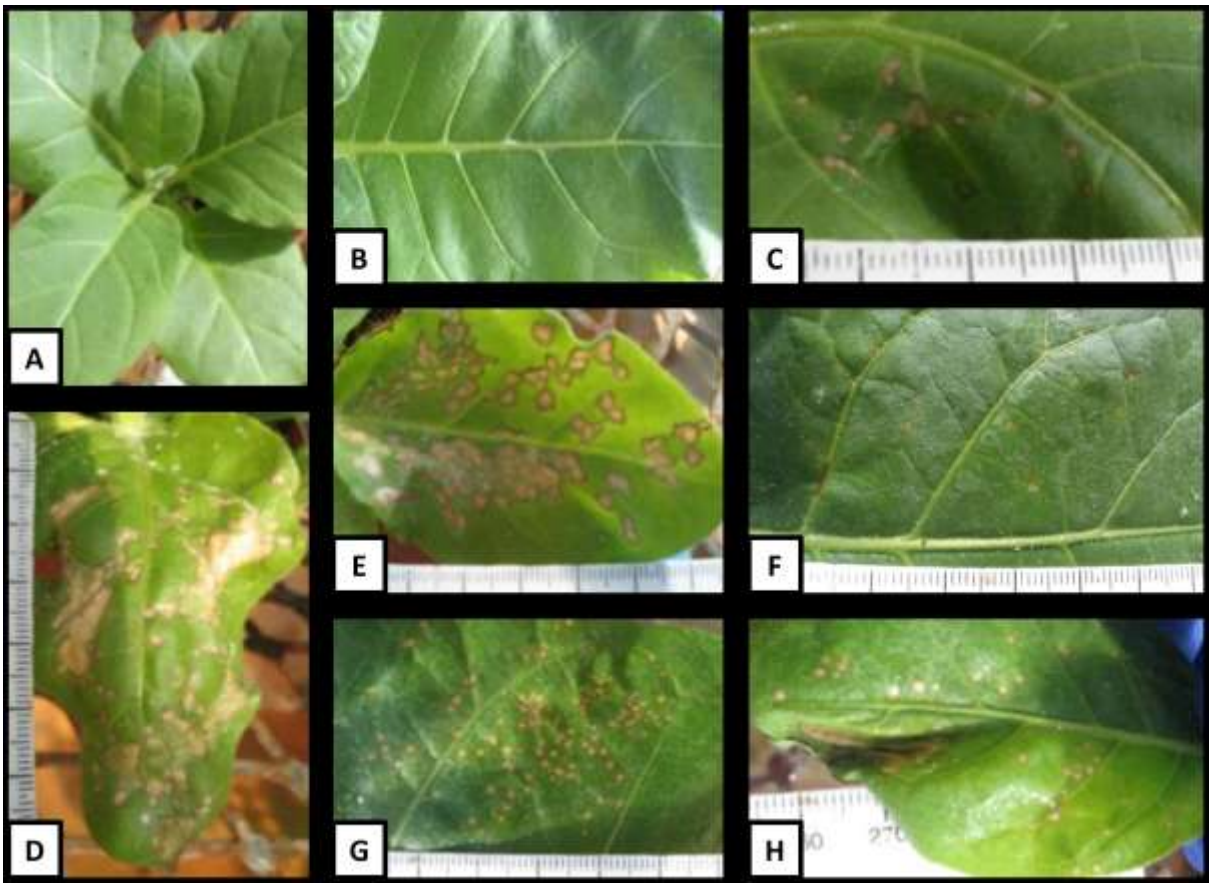

Figure 2.4. 6 Local lesion development on tobacco plants as an indicator of viable ToMV particles. Buffer inoculated control plant demonstrating normal growth and absence of lesion development (A). Fully expanded mature leaf of a healthy, buffer-inoculated plant with no lesion development (B). Sunken, black, necrotic lesions on tobacco leaves inoculated with DAS-ELISA-positive leaf homogenates (C). Lesion coalescence and expansion on leaves inoculated with ToMV positive leaf homogenates (D and E). Varying intensities of lesion development on leaves inoculated with DAS-ELISA positive seed homogenates (F – H).

Confirmation of the viability of ToMV particles in DAS-ELISA-positive leaf and seed samples, was indicated by local lesion development on inoculated leaves of *N. tabacum* cv. Xanthi. Lesions were detected on leaves from both trials after three days and a final evaluation of symptoms was performed after seven days. Tobacco leaves inoculated with buffer in both bioassays developed and expanded as normal with no indication of lesion development (Figure 2.4. 6 A and B). Leaves inoculated with DAS-ELISA confirmed, ToMV-positive tomato leaf homogenates, initially displayed green, water-soaked lesions approximately 1 – 4 mm in diameter which developed into necrotic sunken lesions after seven days (Figure 2.4. 6 C). Numerous necrotic lesions, that were tan brown with black halos, developed on inoculated leaves. All assayed samples caused the development of one to 75 lesions on inoculated leaves, and the lesions expanded along with developing leaves. Lesions in close proximity coalesced to form bigger tan areas, and with leaf expansion, caused necrosis of entire regions of inoculated leaves (Figure 2.4. 6 D and E). Tobacco leaves inoculated with DAS-ELISA positive seed homogenates developed similar symptoms of necrotic, tan brown, localised lesions, each surrounded by a black halo (Figure 2.4. 6 F – H). Between 10 and 70 lesions were observed on inoculated leaves and, in some cases, the lesions coalesced and expanded to form a large, necrotic region across the leaf surface.

2.4.3. Confirmation of the presence of bacteria in diseased leaf and seed samples

2.4.3.1. Identification of bacteria from leaf samples by selective plating

After three to five days of incubation, growth was observed on all plates inoculated with suspect Cmm and Pst-infected plant samples. No bacterial growth was observed on plates inoculated with suspect Xcv-infected plant samples and, therefore, a second isolation was performed from plants demonstrating leaf spot symptoms. The second isolation and inoculation resulted in growth of colonies on the selective media after the same incubation period. Isolations from infected plant samples did not always yield pure cultures of colonies and, in at least 50% of isolations, mixed cultures of colonies with different morphological characteristics were observed. The incidences where mixed cultures were recovered were not accurately recorded or diagnosed in this study, but these results would provide an indication of the microflora present in host plants and whether organisms are pathogens, opportunistic pathogens or beneficial organisms. All assays resulted in the recovery of bacterial colonies that were comparable to the morphology of reference cultures of the respective pathogens suspected to be responsible for the symptoms induced on plants (Table 2. 3).

Suspect Cmm colonies recovered from stem samples of plants symptomatic for bacterial canker, were small, circular and yellow (Table 2. 3), with a mucoid appearance on all media tested except TZC, on which suspect colonies were flat and a deep scarlet colour. The absence of growth on MT medium is a characteristic of Cmm (Goszczynska and Serfontein, 1998); however, growth of white-domed colonies, each surrounded with a yellow zone, was observed. Thus, the bacteria isolated from stem samples could not have been Cmm. Re-isolations from the stems of infected plants demonstrating canker symptoms were performed in an attempt to recover Cmm. Resulting colonies from the second isolation exhibited distinctive Cmm morphology on the tested media, with little or no growth on KBC medium and the absence of growth on MT agar.

Table 2. 3 Growth and morphology of the bacterial colonies isolated from plant samples suspected to be infected with Cmm, Xcv or Pst.

	TGA	TZC	KB	KBC	MT	NA
Cmm	Confluent growth of small, yellow, mucoid colonies	Dark red, flat colonies	Small, yellow, mucoid colonies	Very little growth, colonies are small and yellow	No growth	Small, yellow, mucoid colonies
Xcv	Confluent growth of small, yellow, mucoid colonies	Red colonies	Small, yellow, mucoid colonies	Small, yellow, mucoid colonies	Yellow colonies surrounded by a small opaque zone and a bigger clear zone	Small, yellow, mucoid colonies
Pst	Semi-transparent, off-white colonies	Red colonies with a white halo	Semi-transparent, off-white colonies, fluorescent under UV light	Semi-transparent, off-white colonies	Semi-transparent, off-white colonies	Semi-transparent, off-white colonies

The second isolation from infected plants displaying symptoms of bacterial spot resulted in the growth of single and mixed colonies on selective and non-selective media. The majority of colonies were similar in morphology to Cmm reference cultures, specifically small, mucoid and yellow colonies. However, on MT agar the cultures developed as yellow colonies, each surrounded by two zones, which discounted the isolated bacterium as Cmm. The two zones observed were of different

sizes with the larger generally clear and the smaller opaque due to the deposition of crystals characteristic of growth of Xcv cultures (Table 2. 3) (Goszczynska and Serfontein, 1998).

Bacteria isolated from plants demonstrating symptoms of bacterial speck exhibited a uniform colony morphology of semi-transparent, off-white growth on most of the selective and non-selective media (Table 2. 3). On TZC medium, however, bacterial growth recovered from sample macerate developed as red colonies, each with a white halo, which is typical of Pst (Goszczynska et al., 2000).

Only colonies from each of the isolations that demonstrated growth comparable to the expected pathogens were selected for further analysis. Pure cultures of the colonies were obtained by streaking a loopful of the colony onto non-selective TGA or NA media. After incubation, growth of the pure cultures was examined and the colonies were streaked onto the five selective media again to validate uniformity in culture morphology, and to ensure morphology was comparable to that of the expected pathogens. All of the colonies chosen developed growth as described previously (Table 2. 3) on TGA medium and on the selective media and, therefore, had to be characterized by other detection techniques.

2.4.3.2. Identification of isolated bacteria by physiological, biochemical and pathogenicity properties

Presumptive bacterial cultures of Cmm, Pst and Xcv isolated on selective media were subjected to a set of physiological and biochemical tests for further confirmation of identification by selective plating. Presumptive positive classification was assigned to cultures that demonstrated similar reactions in these tests to that of reference cultures of each of the suspected pathogens (Table 2. 4). Gram positive coccoid bacterial cells capable of solubilising KOH were identified from presumptive Cmm cultures. These cultures were unable to solubilise Kovac's solution and produced flat, yellow colonies on sucrose-supplemented differential medium indicating the absence of levan production (Figure 2.4. 8 A). Sucrose was, however, the only carbohydrate-supplemented medium that could be metabolised by suspect Cmm cultures.

Bacteria tentatively identified as Pst or Xcv on selective media were identified as Gram negative, rod-shaped bacteria incapable of solubilising KOH. Presumptive Xcv cultures produced mucoid yellow cultures on sucrose-supplemented medium and, thus, were negative for levan production. Although a purple colour was not produced immediately upon application of Kovac's solution to

Table 2. 4 Characterization of bacteria isolated from diseased plant samples by physiological, biochemical and pathogenicity tests.

Identification Test	Culture	Cmm	Xcv	Pst
Gram stain	**Control**	+	-	-
	Test	-	-	-
Shape	**Control**	Rod/Coccoid[1]	Rod	Rod
	Test	Coccoid	Rod	Rod
KOH solubility	**Control**	+	-	-
	Test	+	-	-
Oxidase (Kovac's reagent)	**Control**	-	Mildly + or -[2]	-
	Test	-	Mildly +	-
Levan	**Control**	-	-	+
	Test	-	-	+
Carbohydrate utilisation				
Erythitol	**Control**	-	-	-
	Test	-	-	-
Mannitol	**Control**	-	-	+
	Test	-	-	+
Sucrose	**Control**	+	+	+
	Test	+	+	+
Sorbitol	**Control**	-	-	+
	Test	-	-	+
Inositol	**Control**	-	-	+
	Test	-	-	+
THR		+	+	+

Control results refer to the behaviour of previously confirmed positive cultures of the bacterial pathogens in the various tests. Observed results refer to the behaviour of presumptive, isolated cultures.
'+' represents a positive test reaction; '-' represents a negative test reaction.
[1] Cmm is pleomorphic implying bacteria differ in shape according to growth conditions. When isolated from plants, Cmm is usually rod-shaped (Jones et al. 1997).
[2] Either no colour or a light purple colour was observed after approximately 30 s.

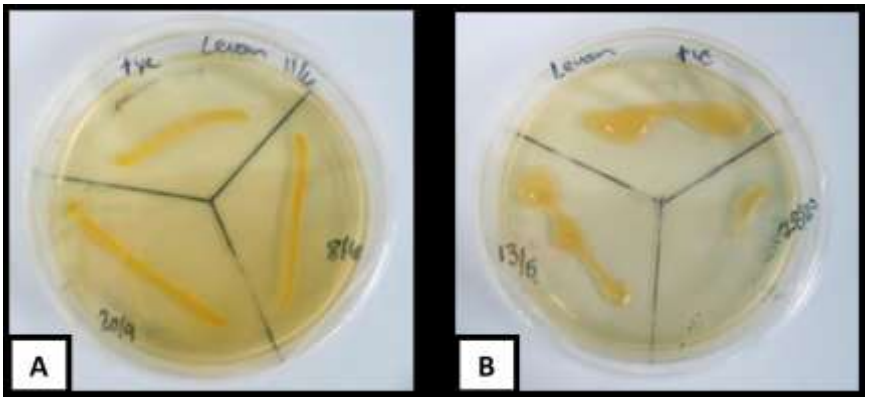

Figure 2.4. 8 Analysis of levan production from sucrose-supplemented medium by isolated bacteria. Non-mucoid, yellow colonies of Cmm-suspect cultures (lower left and right sectors) in combination with a positive Cmm control culture (top sector) indicating the absence of levan production (A). Mucoid, creamy colonies of Pst suspected bacteria (lower left and right sectors) in combination with a positive Pst control culure (top sector) indicating levan production (B).

suspect Xcv colonies, a light purple colour developed after 30 seconds which indicated a mild positive reaction and, thus, partial oxidative metabolism.

Cultures suspected to be Pst were not able to metabolise Kovac's solution but demonstrated domed, mucoid growth of cream-white colonies on sucrose-supplemented medium. This indicated levan production which is a characteristic of all plant-pathogenic Pseudomonads (Goszczynska et al., 2000) (Figure 2.4. 8 B). Although both Xcv and Pst suspect cultures demonstrated abundant growth on sucrose-supplemented medium, no growth was recovered on erythitol-supplemented medium, and thus, neither bacterial culture was able to utilise erythitol as a carbon source. Only presumptive Pst cultures exhibited growth on media supplemented with the other tested carbohydrates: mannitol, inositol and sorbitol; indicating the ability to metabolise these.

Presumptive cultures that conformed to the expected results of control cultures in physiological and biochemical tests were subjected to pathogenicity tests on tobacco seedlings (Table 2. 4). Bacterial cultures were classified as pathogenic if they were able to induce a hypersensitive response on inoculated hosts. This was observed by the production of localised lesions on inoculated leaf surfaces. Two of the presumptive cultures were non-pathogenic and were discounted as causal agents. Non-pathogenic cultures and water-inoculated control samples did not produce lesions or any observable changes on leaf surfaces, though in some cases damage was observed that was attributed to excessive manual pressure applied during inoculations (Figure 2.4. 9 A). At least one of each of the tested Cmm, Xcv and Pst presumptive isolates was pathogenic on tobacco (Table 2. 4).

These cultures produced water-soaked, light green regions after two days on inoculated areas of tobacco plants, which dried out, becoming necrotic (Figure 2.4. 9 B – D).

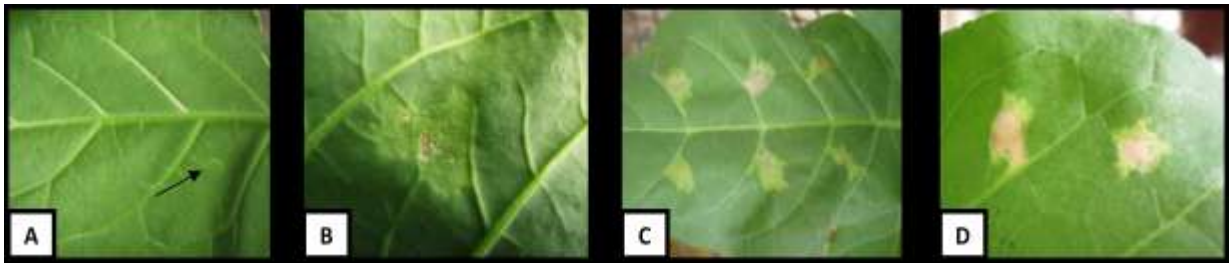

Figure 2.4. 9 Pathogenicity test of isolated bacteria by the tobacco hypersensitive response (THR) assay. Water-inoculated control plants and non-pathogenic cultures did not produce lesions on inoculated leaf surfaces, though inoculation damage was visible (arrow) (A). Production of light-green, water-soaked regions and dried out, necrotic lesions at sites inoculated with pathogenic Cmm (B), Pst (C) and Xcv (D) cultures.

2.4.3.3. Characterization of bacterial isolates by Biolog testing

One bacterial isolate for each pathogen, Cmm, Xcv or Pst, that was tentatively classified positive by the above conventional methods, was characterized by the Biolog microplate system (Biolog Inc., USA). Positive or moderately positive utilisation of a particular carbon source was designated to cultures for test wells that produced a deep to light purple colour, which indicated reduction of the tetrazolium redox dye to violet formazan. For Cmm, a total of 28 out of the 95 carbon sources on the GP2 microplate were metabolised and these included mainly sugar compounds. The isolate was identified by the system as *Leifsonia aquatica* which demonstrated a mean similarity index (SI) of 0.59 with the metabolic profile generated for the test culture. The correct classification of *Clavibacter michiganensis* subsp. *michiganensis* was, however, included in the top 10 organisms with the closest utilisation profiles to the test isolate (Table 2. 5). Another three organisms in this list were subspecies of the *C. michiganensis* species complex, suggesting a high degree of conservation of carbon source utilisation patterns among these close relatives. The isolate classified as Xcv prior to this assay, was capable of utilising 34 out of the 95 carbon sources on the GN2 microplate, 13 of which were the same as those in the metabolic profile of the Cmm isolate. This isolate had the highest mean SI of 0.25 with *X. campestris* pv. *begonia* B. Seven other pathovars of this species were included in the top 10 organisms with the closest matching utilisation profiles to the test isolate (Table 2. 5). Although a metabolic profile for Xcv has been entered into the Biolog database for Gram-negative bacteria (Biolog Inc., 2009), this pathovar was not included in the top 10 closest profile matches (Table 2. 5). The test isolate classified as Pst by other detection methods, utilised 27 of the 95 carbon sources on the GN2 microplate and eight of these were unique to Pst compared to the other two tested isolates. Unique carbon sources metabolised by this isolate included glycogen,

Tween 40, Tween 80, pyruvic acid methyl ester, cis-aconitic acid, citric acid, propionic acid and bromosuccinic acid. Of the top 10 organisms with the most similar utilisation profiles, seven were in the *Pseudomonas* genus (Table 2. 5), none of which are known to infect tomato. Classification by the Biolog system identified the isolate as *P. syringae* pv. *zizaniae* with a mean SI of 0.46 with the test isolate's metabolic profile. Interestingly 7 carbon sources were metabolised by all three isolates and included: dextrin, D-fructose, α-D-glucose, D-psicose, sucrose, L-alanyl-glycine, L-asparagine and L-glutamic acid. Utilisation of sucrose by each of the isolates was consistent with observations in carbohydrate utilisation assays (2.4.3.2 above).

Table 2. 5 The top 10 organisms with the closest matches to the carbon utilisation profile generated for each test bacterial isolate by the Biolog system.[1]

Top Matches	Suspect Isolate		
	Cmm	**Xcv**	**Pst**
1	*Leifsonia aquatica*	*X. campestris* pv. *begonia* B	*P. syringae* pv. *zizaniae*
2	*C. michiganensis* subsp. *nebraskensis*	*X. campestris* pv. *poinsettiicola*	*Janthinobacterium lividum*
3	***C. michiganensis* subsp. *michiganensis***	*X. campestris* pv. *malvacearum*	*P. syringae* pv. *apii*
4	*Cellulomonas flavigena*	*X. campestris* pv. *raphani*	*P. syringae* pv. *sesame*
5	*C. michiganensis* subsp. *tessellarius*	*X. campestris* pv. *juglandis*	*P. syringae* pv. *aptata*
6	*C. michiganensis* subsp. *insidious*	*X. campestris* pv. *dieffenbachiae*	*P. mephitica*
7	*Rathayibacter tritici*	*X. campestris* pv. *begonia* A	*P. chlororaphis*
8	*Anthrobacter ilicis*	*Sphingomonas paucimobilis* A	*Vibrio furnissii*
9	*Curtobacterium albidum*	*Stenotrophomonas maltophilia*	*P. syringae* pv. *persicae*
10	*Sangiobacter inulinus*	*X. campestris* pv. *phaseoli*	*X. campestris* pv. *malvacearum*

[1] Organisms listed form part of the Biolog Microbial Identification Database **(Biolog Inc., 2009)** for which utilisation patterns have been thoroughly tested and validated by independent laboratories.

2.4.3.4. Identification of bacteria isolated from seed samples on selective media

Seed from the fruit of plants demonstrating symptoms of bacterial canker, bacterial spot or bacterial speck were extracted and subjected to assays on appropriate selective media to isolate any viable bacteria that may have been transmitted from diseased parent plants to seed. Seed assays were repeated until conclusive data could be obtained for the presence or absence of each pathogen on appropriate media. Initial isolations from the seed extracts and dilutions thereof on two types of semi-selective media, led to the recovery of both morphologically similar and different bacterial colonies compared to reference cultures on the same media (Table 2. 6). For colonies with both similar and different morphology to that of control cultures, the average number of cfu/mℓ recovered was determined by calculating the average number of colonies multiplied by the appropriate dilution factor and divided by the volume of the homogenate that was streaked onto each selective medium. To analyse the specificity of chosen selective media to recover the target bacterium versus other non-target bacteria, the proportion of colonies similar or different to control colonies was compared to the total number of all colonies recovered from seed extracts on each selective medium (Table 2. 6).

Table 2. 6 The recovery of bacteria from seed samples, each approximately 8 g, on selective media. Results represent an average of the repeat assays and the dilutions that were tested.

Pathogen	Selective Medium	Suspected Pathogen		Other bacteria	
		Average cfu/ml	Specificity of recovery %	Average cfu/ml	Non-specific recovery %
Cmm	D_2ANX	133	43.5	173	56.5
	SCM	580	98.3	10	1.7
Xcv	mTBM	138	0.1	1.08×10^5	99.9
	mKM-1	7.54×10^3	33.4	1.50×10^4	66.6
Pst	KBC	1.00×10^4	99.7	25	0.3
	KBZ	1.64×10^4	2.7	6.00×10^5	97.3

Selective isolation of Cmm from seed was performed on D_2ANX and SCM media. Mixed colonies developed on D_2ANX media inoculated with dilutions of $10^0 – 10^{-2}$ of seed homogenate. Of these, one colony type was characterized by yellow, mucoid, convex colonies which were circular and comparable in morphology to that of positive control colonies. An average of 300 cfu/mℓ was recovered from an assay of 8 g of infected seed (Table 2. 6). Approximately half of these recovered colonies demonstrated similar growth to of known Cmm cultures on D_2ANX medium (Figure 2.4. 10 A). Control Cmm colonies were irregularly shaped, grey and mucoid on SCM medium (Figure 2.4. 10 B). Inoculations with dilutions of seed homogenate on SCM medium resulted in the growth of colonies varying in colour from light and dark grey to brown and black. An average of 580 Cfu/mℓ of suspect Cmm colonies was recovered from 8 g of seed with only a small percentage of colonies differing in morphology to that of positive control colonies (Table 2. 6). The total recovery of bacterial colonies, and suspect Cmm colonies, was 30 and 40% greater on SCM than D_2ANX, respectively. For further confirmation of the identity of recovered bacteria, a representative number of colonies, morphologically similar to Cmm control colonies, were selected from SCM and D_2ANX media and streak-inoculated onto differential YDC medium. In one of the repeat assays, only seven of the 15 selected colonies developed yellow, mucoid colonies similar to positive Cmm control cultures on YDC medium. Of these, five colonies were transferred to another differential medium, CDA, which further excluded one of the suspected colonies as Cmm as this culture did not demonstrate the expected milky yellow/white growth. Due to slight morphological variation observed between suspect colonies and control colonies during this assay, two further characterization tests were performed: Gram staining and an assay on the five general selective media for bacterial isolation from leaf and stem samples. Although the suspect colonies were Gram positive and coccoid, like Cmm control cultures, diverse growth on the five selective media resulted in the conclusion that only two of the selected Cmm presumptive cultures could be confidently classified as Cmm. This contradicts the results observed from selective media isolations.

To selectively isolate Xcv from the seed of plants demonstrating symptoms of bacterial spot, samples were assayed on three semi-selective media: CKTM, mTBM and mKM-1. Variable results were obtained from seed assays and, thus, the assays were repeated five times before a reliable conclusion could be formed. Bacterial growth and recovery on CKTM medium differed significantly among repeat assays with only large white or pale yellow colonies developing from dilutions of the seed homogenate. In contrast, the positive control colonies of Xcv developed circular, mucoid yellow colonies surrounded by a white crystalline halo on CKTM, similar to descriptions of Sijam et al. (1991). The recovery of Xcv from seed, thus, could not be accurately determined for CKTM and was

omitted from calculations. On TBM selective medium, colonies with diverse morphological characteristics were recovered. Only 0.1% of resulting colonies demonstrated similar characteristics to that of positive control Xcv colonies (Table 2. 6) with yellow, mucoid colonies encircled by a white crystalline matrix (Figure 2.4. 10 C). Other contaminating bacteria were characterized by red, pale yellow, dull grey, milky or dull white colony morphologies. The recovery of suspect colonies was 10-fold greater on mKM-1 than TBM media. At least a third of the total colonies recovered on mKM-1 were morphologically similar to colonies of positive Xcv cultures (Table 2. 6). Presumptive Xcv colonies were identified as mucoid, yellow and circular colonies on mKM-1 (Figure 2.4. 10 D) without the development of a surrounding crystalline matrix as observed for colonies on TBM medium. In one of the repeat assays on TBM and mKM-1 media, 22 colonies suspected to be Xcv were isolated onto YDC and CDA differential media for further analysis. On YDC medium, 18/22 suspect isolates developed mucoid, yellow colonies surrounded by a transparent halo and were considered morphologically similar to positive Xcv control colonies. The characteristic clearing of the milky CDA medium by Xcv cultures was, however, absent from the colonies of suspected Xcv cultures. Seven cultures demonstrating the closest morphology to that of control cultures were subjected to further validation tests by Gram stains. All tested cultures were identified as Gram negative, rod-shaped bacteria as expected of Xcv; however, one of the cultures was identified as Gram positive, rod-shaped bacterium and was, therefore, eliminated as a potential Xcv isolate. Inoculation of the

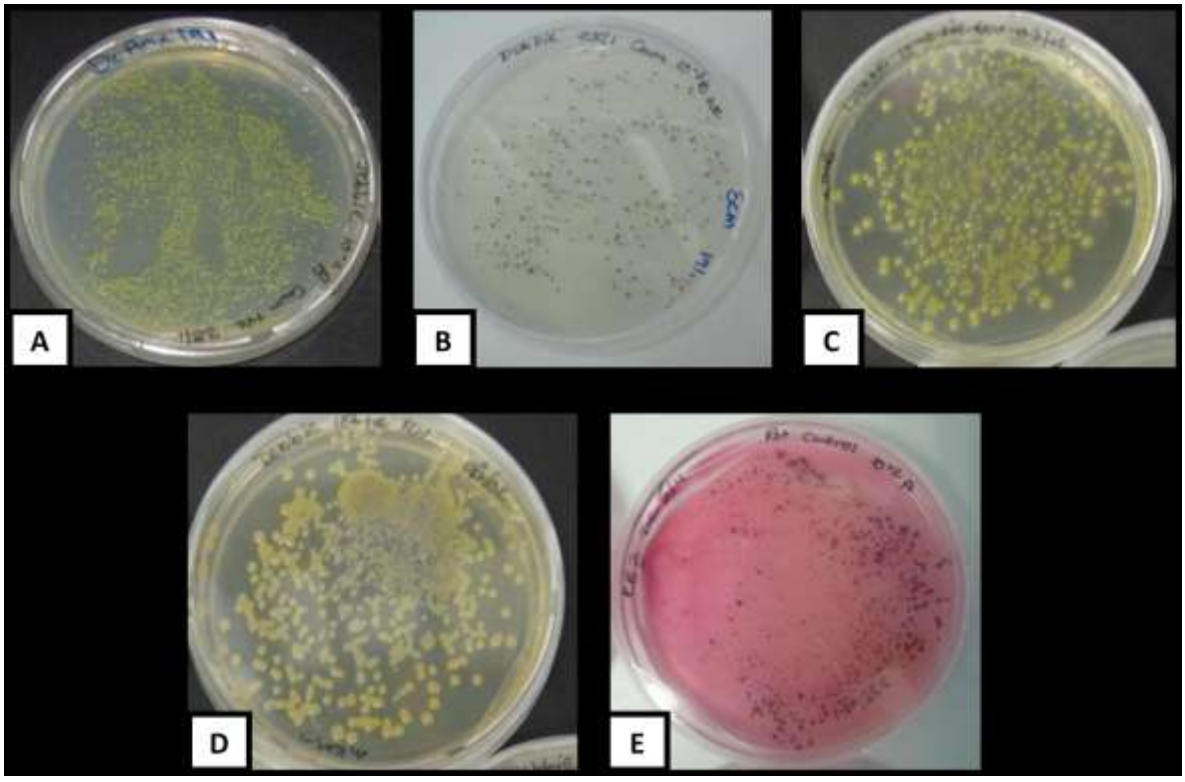

Figure 2.4. 10 The morphology of control cultures on the selective media. Positive Cmm cultures on D_2ANX (A) and SCM (B). Positive Xcv colonies on mTBM (C) and mKM-1 (D). Positive Pst cultures on KBZ (E).

remaining suspect cultures on the five general selective media for bacterial isolation also eliminated another suspected isolate due to variation in morphology compared to colonies of control Xcv cultures. From this assay, the remaining five isolates from infected seed were, therefore, tentatively classified as Xcv, again illustrating the discrepancy between the results from selective media isolations and further characterization tests.

Seed obtained from diseased plants demonstrating symptoms of bacterial speck were assayed for the presence of Pst on KBC and KBZ selective media. Compared to Xcv and Cmm seed assays, between 10- and 100-fold more colonies were recovered from seed homogenate of suspected Pst-infested seed (Table 2. 6). The size of recovered colonies was, however, less than 2 mm in diameter on both media compared to the 1 – 5 mm colonies recovered from Xcv and Cmm assays on selective media. The high yield of colonies on KBC and KBZ media, especially from undiluted seed homogenate, were in many cases too numerous to count, which made accurate quantification challenging by calculating cfu/mℓ. In these instances, a total of 300 colonies was included in calculations and, therefore, results presented in Table 2. 6 indicate the minimum number of colonies that were recovered from seed assays. On KBC medium positive control Pst cultures developed as small, semi-translucent, circular colonies with a whitish tinge and serrated margins. Only 0.3% of colonies recovered from dilutions of seed homogenate did not demonstrate similar growth characteristics to that of positive control colonies on KBC medium (Table 2. 6). High recovery of both suspect and non-target bacteria was obtained on KBZ medium, although contaminants occurred mainly in the more diluted samples. Suspect bacteria demonstrated confluent growth of flat, burgundy colonies, each between 1 and 2 mm in diameter, and were similar to colonies of control Pst cultures (Figure 2.4. 10 E). A total of 17 colonies, that were morphologically similar to positive control Pst colonies, were isolated from KBZ and KBC media onto KB semi-selective medium for further analysis. Positive Pst cultures developed as flat, semi-translucent, whitish colonies on KB medium and were circular with a serrated margin. These were also fluorescent and appeared bluish-green under UV light. Only 8 of the 17 cultures developed colonies with growth similar to that of positive Pst controls and results were sufficiently conclusive, based on the specificity of distinguishing morphological characteristics, to presume these were Pst cultures.

The difference in recovery of both suspect and morphologically different cultures from seed homogenization versus seed wash assays for Xcv and Pst infected seed was analysed by calculating the average number of cfu/mℓ for each assay per gram of seed tested for each selective medium (Table 2. 7). The percentage recovery of all bacterial cultures from seed washes was also determined

by comparing the proportion of bacteria recovered, both similar and different to colonies of the respective positive control cultures, versus the total number of bacteria isolated by both extraction methods. The recovery of bacteria from assays of suspect Xcv-infected seed was 10-fold greater from homogenized seed compared to seed wash assays on both selective media. Although a higher percentage of bacteria were recovered from mTBM medium, these largely constituted non-target bacteria, as indicated by the average cfu/mℓ for target bacteria, which was similar for both media (Table 2. 7). Plating dilutions of presumptive Xcv seed wash suspension on NA medium resulted in the development of three types of colonies: fibrous, whitish-brown colonies, bright, pink colonies and yellow, circular colonies. Of the 12 cultures chosen from mTBM, mKM-1 and NA for further analysis, only 5 developed as yellow mucoid colonies surrounded by a cleared zone of agar on CDA medium. Variable results, however, were obtained from the inoculation of these 5 suspect cultures on NA and YDC media. Only one of these demonstrated consistently positive results on both media and, therefore, could be classified as Xcv. Poor growth of bacteria from Pst seed wash assays was observed on KBZ medium, even after an extended incubation time and, therefore, comparative results could not be determined using this medium. The recovery of suspect bacteria was at least 10-fold greater than that of non-target bacteria in both seed homogenization and wash assays on KBC medium (Table 2. 7). Colonies that developed from homogenized seed however, constituted greater

Table 2. 7 Comparison between the recovery of bacteria infecting the surfaces of seeds by seed homogenization and by seed wash assays.

Pathogen	Selective Medium	Suspected Pathogen		Other Bacteria		% recovery of all bacteria by the seed wash assay
		cfu. mℓ$^{-1}$/g homogenization	cfu. mℓ$^{-1}$/g wash	cfu. mℓ$^{-1}$/g homogenization	cfu.mℓ$^{-1}$/g wash	
Xcv	mTBM	17.2	0.5	1.36 x 10^4	5.73 x 10^3	29.6
	mKM-1	942.8	0.5	1.88 x 10^4	1.5	0.01
Pst	KBC	1.25 x 10^3	76.6	3.1	2	5.9
	KBZ	2.05 x 10^3	ND[1]	7.50 x 10^4	ND[1]	ND[1]

[1] Results from the seed wash assay on KBZ medium were not determined (ND) as colonies demonstrated insufficient growth to allow for accurate quantification.

than 90% of the total recovered bacteria on KBC by both methods. Inoculations of Pst seed wash suspension on NA medium resulted in the development of six morphologically different types of colonies, of which one was characterized by semi-translucent, whitish colonies similar to Pst control colonies. Of the recovered colonies, 18 cultures suspected to be Pst were inoculated onto KB medium for further characterization. Upon evaluation, seven were morphologically similar to colonies of the positive control Pst cultures and were, therefore, tentatively classified as Pst.

2.4.4. Confirming the pathogenicity of isolates on tomato plants

2.4.4.1. Pepino mosaic virus

Leaf samples that were proven to be infected with PepMV by DAS-ELISA and bioassays on tobacco plants were analysed for the ability to induce symptoms on inoculated tomato seedlings. Mild symptom development was noticed 14 dpi, but by 21 dpi, symptoms had become more severe. Older leaves developed a mild dark/light green mosaic which spread to younger leaves over time (Figure 2.4. 11 A). Mild interveinal leaf bubbling developed on a few leaves initially but quickly spread and intensified such that over 90% of leaves demonstrated severe bubbling by 28 dpi. Severe infections on host plants were characterized by severe leaf distortion, bubbling and the curling of leaf margins (Figure 2.4. 11 B and C). Leaves that did not display leaf bubbling developed small yellow spots or unilateral curling and stunted growth. Malformed, distorted leaves were observed that had twisted and curled in orientation and demonstrated a raised mid-rib (Figure 2.4. 11 D arrow). Apical and lateral branches became severely distorted and bent (Figure 2.4. 11 D), often

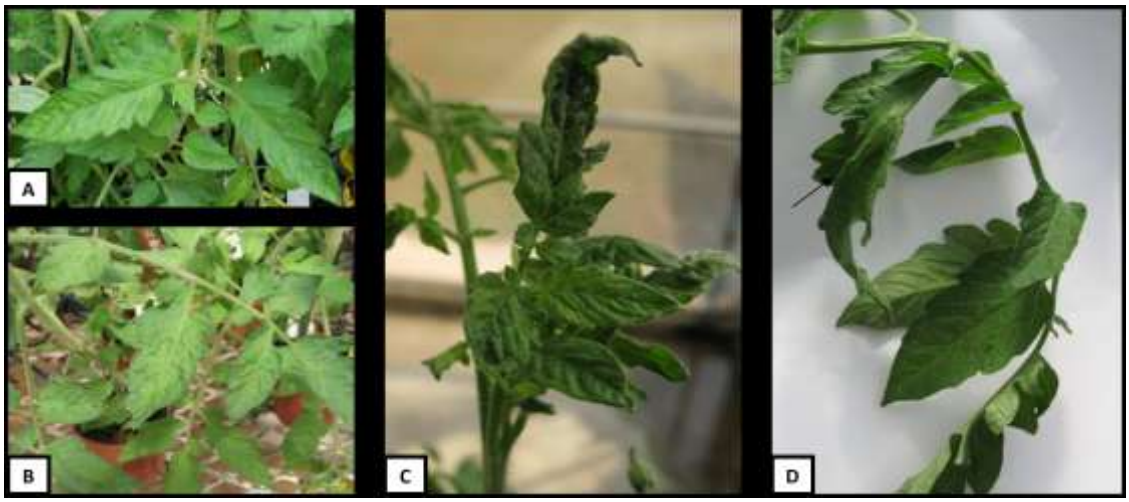

Figure 2.4. 11 The development of symptoms on plants inoculated with suspect PepMV inoculum. Mild dark/light green mosaic on older leaves (A). Curling of leaf margins and severe interveinal bubbling on leaves of inoculated plants (B). Stunting, distortion and bubbling of leaves on apical branches (C). Mature branches of inoculated plants with severe bending and distortion of leaves also demonstrating mid-rib swelling (arrow), severe distortion and twisting (D).

demonstrating asymmetrical growth and stunting. All observed symptoms intensified over time and were similar to those observed in initial trials with suspect PepMV inoculum.

2.4.4.2. Tomato mosaic virus

Healthy tomato plants were inoculated with leaf samples proven to be infected with ToMV by DAS-ELISA and bioassays on tobacco plants. Symptoms that developed on these inoculated plants (Figure 2.4. 12) were characteristic of ToMV infection and resembled those observed in the initial inoculation trial. Apical and lateral shoots were stunted and newly developed leaves were distorted or folded. Leaves of inoculated plants demonstrated a variety of symptoms which included mild leaf bubbling, serrated leaf margins, stunting and upward curling, especially of younger leaves. Older leaves displayed mild to severe yellow/green mosaic and developed chlorotic lesions or mild distortion. Severely infected plants were characterized by malformed leaves that appeared as fern-like or shoe-string like, and leaf surfaces were narrowed or tapered.

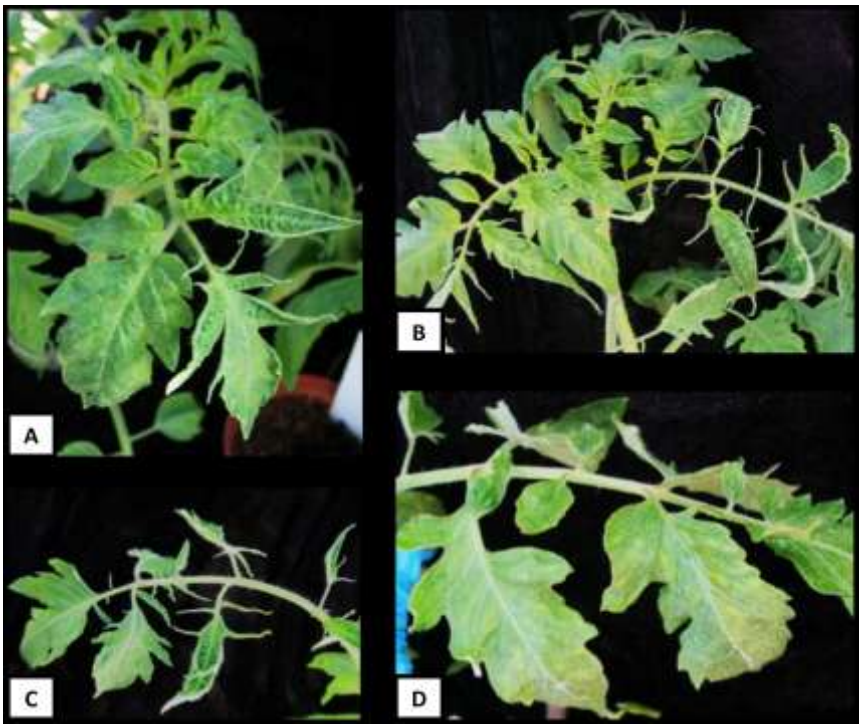

Figure 2.4. 12 Development of symptoms characteristic of ToMV infection on inoculated plants (A –D). Yellow and green mosaic as well as malformed, stunted, distorted leaves were observed.

2.4.4.3. Clavibacter michiganensis subsp. *michiganensis*

A bacterial culture that demonstrated characteristics similar to Cmm in conventional detection and characterization methods, was used to inoculate healthy tomato seedlings, and the inoculated plants

were evaluated for the development of symptoms of bacterial canker. As seen during evaluations of initial trial inoculations, leaves closest to the inoculation site began to droop or demonstrated unilateral curling a few days post inoculation (Figure 2.4. 13 A). With disease progression, loss in turgidity of leaves, petioles and branches of inoculated plants was evident as entire leaves and branches became flaccid or shrivelled up completely (Figure 2.4. 13 B and C). The site of inoculation became brown and necrotic and the most severe wilting symptoms were visible on leaves and branches closest to this region (Figure 2.4. 13 D). Reddish-brown streaks also developed on stems which later split open to form long cankers (Figure 2.4. 13 E). Leaves that were further away from the inoculation site, developed yellow chlorotic lesions that spread across interveinal regions and became necrotic (Figure 2.4. 13 F). Severe infections were characterized by wilting of entire plants, with stems collapsing and leaves becoming dry and brittle. Symptoms observed were similar to those that developed on tomato plants initially inoculated with confirmed cultures of Cmm.

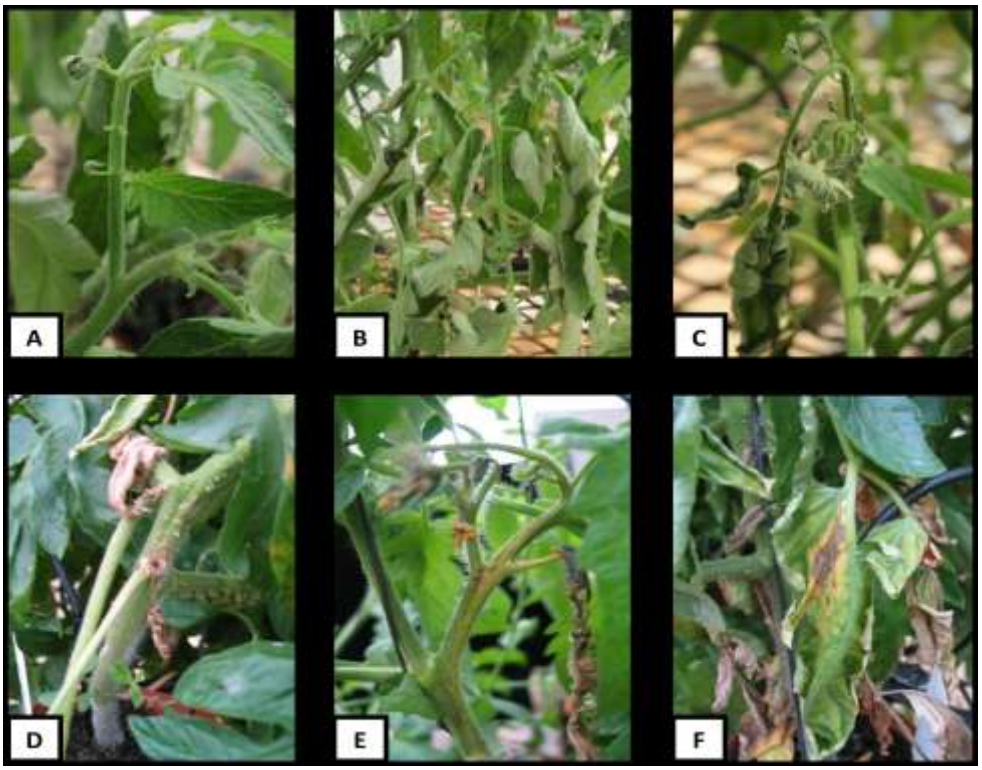

Figure 2.4. 13 Inoculated plants demonstrating symptoms of bacterial canker: unilateral wilting of leaves (A), loss in turgidity of leaves and stem (B and C), formation of adventitious root nodes on stems and necrosis at the site of inoculation (D), canker development on stems (E), chlorotic and necrotic interveinal regions of leaves (F).

2.4.4.4. Xanthomonas campestris pv. *vesicatoria*

Seedlings inoculated with a bacterial culture presumptively identified as Xcv by conventional detection methods, were evaluated for symptom development characteristic of bacterial spot. Initial

symptoms included the development of black and brown regions along leaf margins of older leaves as early as 7 dpi, and interveinal areas around wound sites which expanded into circular lesions (Figure 2.4. 14 A). Numerous small, black spots, developed on older and younger leaves and were best visible on adaxial surfaces (Figure 2.4. 14 B). Spots expanded to form black, necrotic lesions, each approximately 3 mm in diameter on leaf surfaces (Figure 2.4. 14 C), especially in conditions of high humidity. Over time spots developed on all leaves but were more concentrated on older leaves. Spots that developed in close proximity coalesced to form large lesions that expanded to give a scorched appearance. These lesions often resulted in the distortion of leaves or, in dry conditions, caused the formation of brownish, dried out lesions (Figure 2.4. 14 D). Stem necrosis, or the formation of black lesions on stems, was also visible on apical branches of at least 50% of the inoculated plants. Symptoms observed in this second round of inoculations were the same as those observed initially on plants inoculated with known cultures of Xcv.

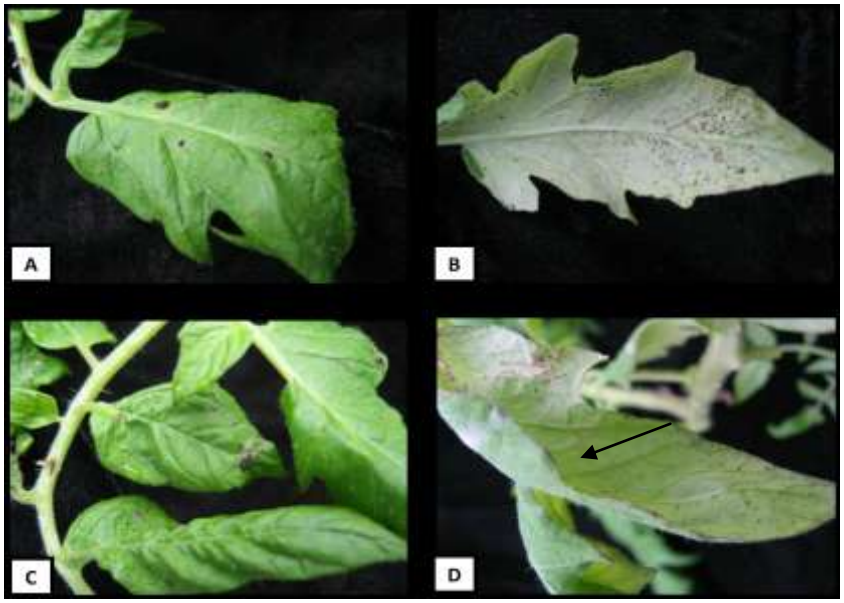

Figure 2.4. 14 The development of symptoms of bacterial spot on plants inoculated with a presumptive Xcv culture. Wounds formed from inoculation with a hypodermic needle were each surrounded by a black halo/lesion (A). Numerous circular black lesions on ventral (A) and dorsal (B) surfaces of leaves under conditions of high humidity. Coalescence and drying out of lesions to form large, brown necrotic lesions on leaf surfaces (arrow) (D).

2.4.4.5. Pseudomonas syringae pv. *tomato*

An isolated bacterial culture that was characterized as Pst by conventional methods, was inoculated onto susceptible tomato plants and analysed for the development of symptoms characteristic of bacterial speck. From 7 dpi, circular, yellow regions had developed on the surfaces of older leaves of the inoculated plants (Figure 2.4. 15 A). These regions developed into black necrotic lesions, each

approximately 1 mm in diameter, surrounded by a chlorotic halo. Lesions increased in size and number on affected leaves and spread to younger leaves (Figure 2.4. 15 B and C). Those that developed in close proximity coalesced to form irregular shaped, necrotic lesions that covered entire interveinal regions. Approximately 4 weeks post inoculation, elongated or circular, necrotic lesions developed on stems and petioles of inoculated plants (Figure 2.4. 15 D). Young, immature fruit also developed water-soaked or brown lesions. Symptoms were similar to those observed on inoculated plants in initial inoculation trials using Pst reference cultures.

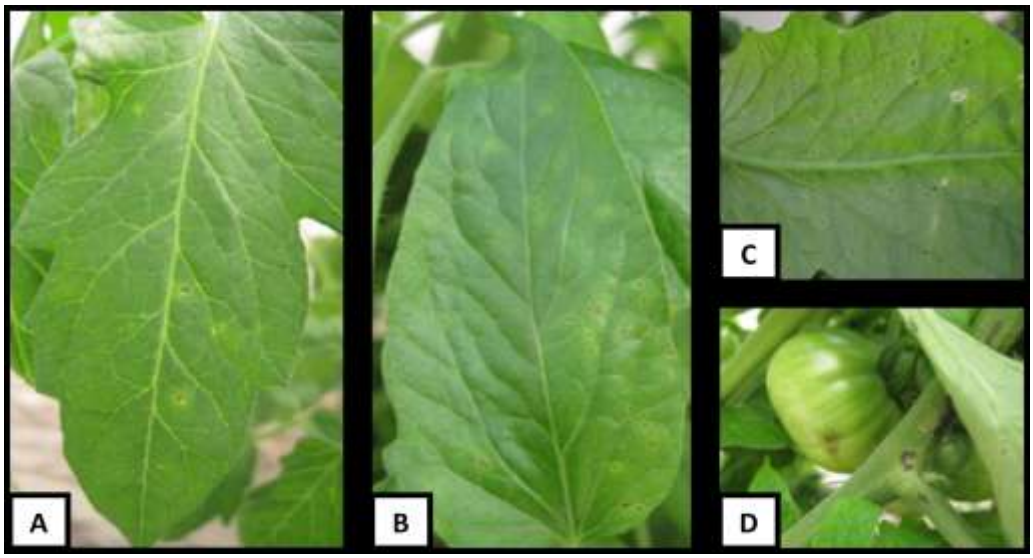

Figure 2.4. 15 Symptom development on plants inoculated with a presumptive Pst culture. Early appearance of specks as yellow, chlorotic lesions, some with a black centre (A). Numerous black lesions with yellow halos visible across both abaxial (B) and adaxial (C) leaf surfaces. Black necrotic lesions developed as streaks or circular lesions on stems, and water-soaked or brown lesions were visible on immature fruit (D).

2.4.5. The development of a mixed viral infection

Seedlings inoculated with either suspect PepMV or confirmed ToMV-inoculum developed symptoms as described in 2.4.1.1 and 2.4.1.2, and single infections by the respective viruses was validated at 25 dpi by DAS-ELISA on leaf samples from each trial. In these assays, leaf samples from plants originally inoculated with suspect PepMV inoculum initially only demonstrated a positive reaction with PepMV-specific antibodies and not ToMV antibodies, whereas those from plants inoculated with ToMV only reacted with ToMV-specific antibodies and not those specific for PepMV. Plants in these two trials however, were kept in close proximity in the greenhouse, approximately 3m apart, for the duration of the study and, although symptoms typical of ToMV and PepMV were still visible on inoculated plants, symptoms characteristic of both viruses had developed on both sets of inoculated plants approximately 16 weeks after inoculation (Figure 2.4. 16). Both ToMV and PepMV-inoculated plants demonstrated severe leaf bubbling and curling of leaf margins. Leaves were also malformed,

with narrowed surfaces and/or a folded phenotype. Irregular, yellow or black, necrotic lesions developed on leaf surfaces and expanded over time (Figure 2.4. 16 A and B). Apical and lateral branches were stunted or demonstrated mild to severe distortion. Mature fruit were reduced in size compared to fruit on control plants, and displayed symptoms of marbling, flaming as well as delayed and uneven ripening (Figure 2.4. 16 E and F). Observed symptoms suggested that a mixed infection had occurred in ToMV and PepMV-inoculated plants.

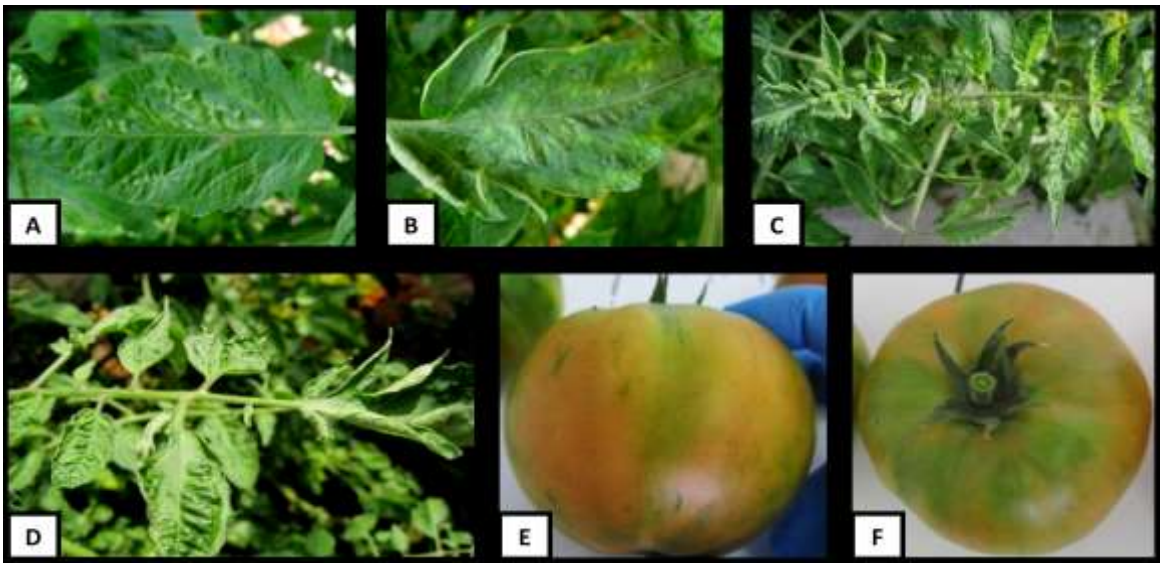

Figure 2.4. 16 Symptoms of a suspected mixed infection in plants originally inoculated with PepMV and ToMV inoculum individually. Development of black, necrotic lesions on leaf surfaces of plants initially inoculated with PepMV (A) and ToMV (B). Stunted apical branches demonstrating narrowed leaf surfaces and malformed leaves folded in an upwards orientation (C). Severe leaf bubbling and curling of leaf margins (D). Harvested fruit demonstrating symptoms of flaming and delayed, uneven ripening, characteristic of both PepMV and ToMV infections (E and F).

Confirmation of the suspected mixed infection was performed by DAS-ELISA on leaf and seed samples from both trials, tested as mentioned previously, using both ToMV and PepMV-specific antibodies. The cut-off for the pathogen-specific antibodies was set at 0.053 and 0.054 for PepMV and ToMV, respectively, based on absorbance of the negative control samples included in both assays. All sub-samples and pooled leaf samples from both trials recorded absorbance values greater than 0.630 in both assays with respective antibodies, indicating positive reactions with PepMV- and ToMV-specific antibodies. In similar assays with seed harvested from both trials, ELISA thresholds were set at 0.234 and 0.334 for PepMV and ToMV, respectively, all seed samples had an absorbance of greater than 2.0 in assays with each antibody. This was in agreement with the strong, yellow colour that developed in all test wells of immunoplates. Positive reactions with PepMV- and ToMV-specific antibodies were, therefore, detected in assayed leaf and seed samples from both trials. Leaf samples that were positive for both PepMV and ToMV particles by DAS-ELISA were inoculated onto two sets of healthy, susceptible tomato host plants and were evaluated for symptom development

after 14 dpi. No significant difference was observed between symptoms that developed on inoculated plants in each trial. On both sets of plants, leaves developed mild bubbling and mosaic symptoms and some had split to form fern-like symptoms usually attributed to infections by ToMV. Apical branches and leaves were distorted and twisted downwards, as seen previously in PepMV pathogenicity trials. By 21 dpi, symptoms had become more severe for both sets of inoculated plants and infections appeared to be more destructive than in the single-infection trials (2.4.1). Two types of lesions or spots were visible on the surfaces of leaves, either irregularly-shaped, chlorotic lesions (Figure 2.4. 17 A) or large, necrotic lesions that ranged from light brown to black (Figure 2.4. 17 B) With disease progression, yellow lesions became more numerous while brown/black lesions expanded to form large necrotic regions on leaf surfaces. Characteristic symptoms of both viruses were visible and included severe bubbling, curling of leaf margins, tapering of leaf surfaces and leaf splitting (Figure 2.4. 17 C and D). Other leaves developed curled margins or were twisted and distorted (Figure 2.4. 17 E). A light/dark green leaf mosaic developed on inoculated plants (Figure 2.4. 17 C) and was especially visible on the older leaves of all inoculated plants. Severe bending and distortion of branches (Figure 2.4. 17 F) in apical regions was also observed. Results proved that a mixed viral infection had developed in plants originally inoculated with ToMV or PepMV. To obtain results mentioned in 2.4.1.1 and 2.4.1.2, repeat inoculations were performed using original inoculum (**Error! Reference source not found.**) and trials were kept separate in the greenhouse to avoid the development of another mixed infection.

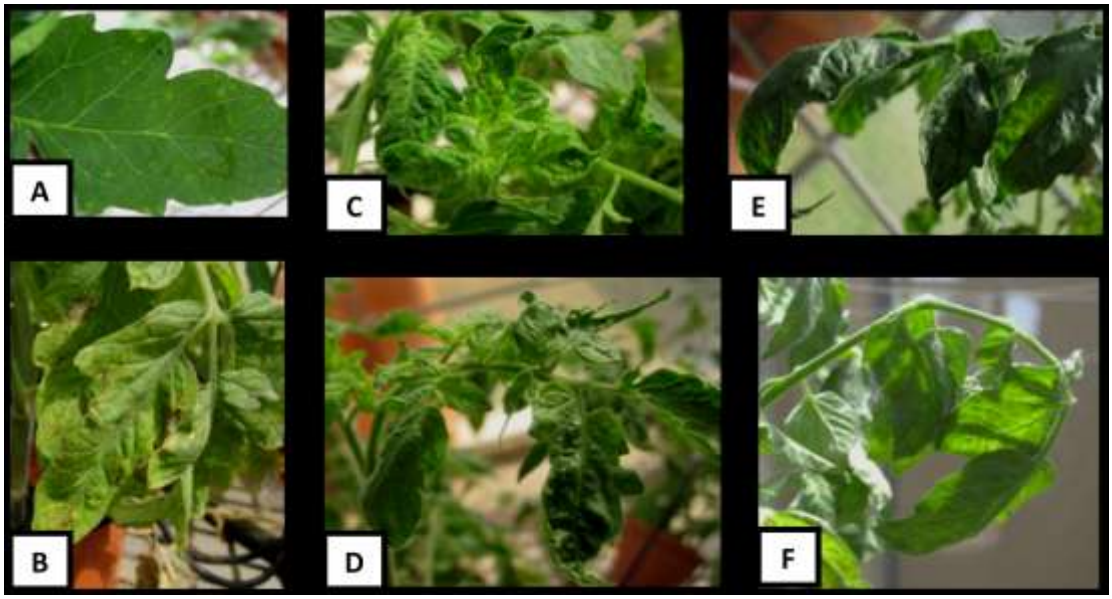

Figure 2.4. 17 Development of symptoms on plants inoculated with leaf samples from a putative mixed infection of ToMV and PepMV. With disease progression yellow, chlorotic lesions (A) as well as large, black necrotic lesions (B) developed on leaf surfaces. Severe bubbling and mild light/dark green mosaic on leaves (C). Malformed leaves demonstrating tapering and fern-like phenotypes with distortion, stunting and severe bubbling (D). Distortion and bending of older leaves of inoculated plants (E) and branches (F).

2.5. Discussion

The detection and identification of seed-borne pathogens on seed before trade is imperative for preventing the spread and reducing the impact of these pathogens on yield and marketability of tomato fruit. It is therefore important that both diseased plants and their harvested seed are accurately and rapidly diagnosed (Lievens et al., 2003; Njambere et al., 2011). In order to confirm the presence of a particular pathogen in host samples, the EPPO have proposed that a minimum of two independent tests, based on different characteristics of an organism, be employed (EPPO/CABI, 2005). In this study, some of the most common detection techniques were selected and evaluated for the capacity to reliably and accurately detect five significant seed-borne pathogens of tomato from plant and seed samples. In order to obtain naturally infected samples to evaluate these detection tests, susceptible tomato seedlings were inoculated with pure reference cultures of ToMV, Cmm, Xcv and Pst from the SVR culture collection. An inoculum source suspected to contain viable PepMV particles was also included in this investigation in an attempt to identify the infectious agent.

Symptom development is one of the first indications of a potential infection, but as a detection method, this method is only suitable for host plants before fruit are harvested as seed seldom display visible symptoms (Kritzman, 1991). Identification of a pathogen based on symptom development is, however, dependent on descriptions from literature (Jones et al., 1997; Hanssen et al., 2009) or prior experience and training. In this study, all plants demonstrated symptoms characteristic of the respective pathogens by 2 – 8 weeks after inoculation. Plants inoculated with suspect PepMV inoculum developed symptoms that have been previously described to be associated with PepMV infection (Fakhro et al., 2011; Hanssen et al., 2009; ISF, 2009a). Fruit that developed from these inoculated plants also demonstrated characteristic symptoms which are responsible for the economic impact of PepMV in the tomato industry (Hanssen et al., 2009, 2010b; Ling, 2008). Although the observed symptoms were similar to those of PepMV infections documented in literature, similar symptoms can be caused by other viral pathogens of tomato (Jones et al., 1997; Hamilton et al., 1981; Van der Vlugt et al., 2002). Thus, characterization based on symptom development alone can be unreliable.

Plants inoculated with selected pathogens in this study did not demonstrate all characteristic symptoms associated with the respective pathogens and displayed a range of symptoms at different times during the trials. The appearance of symptoms is dependent on a variety of factors besides the interaction between the host and pathogen. Changes in environmental conditions, different cultivars

or strains, age of infected plants as well as the various stages in the pathogen infection cycle affect symptom display (Hamilton et al., 1981; Rajeshwari et al., 1998; Van der Vlugt et al., 2002). Tendril formation and fern-like malformations of leaves of plants infected with ToMV for example, were seldom visible before 21 dpi, although the incidence and severity of these symptoms increased over time. Varying symptom display at different stages of infection may, thus, facilitate misdiagnosis of the causal agent. The characteristic 'bird's eye' lesions which are known to develop on fruit of plants infected with Cmm (Gartemann 2003; Jones et al. 1997; Medina-Mora et al. 2001) was not observed in this study. These symptoms are generally associated with late infections of Cmm on floral structures or immature fruit compared to systemic infections established in young seedlings, as done in these trials. Late in the infection cycle of Cmm in inoculated plants, symptoms were observed that were contradictory to those previously reported for bacterial canker. These were attributed to secondary infections by opportunistic pathogens or spores (Fatmi and Schaad 1988; Kaneshiro et al. 2006; Milijašević et al. 2007) that invaded the immune-compromised plants. Identification of Cmm by symptom development is thus difficult during late stages of infection, as the true disease may be masked by symptoms of secondary infections.

Symptom development also varied among leaves and among plants inoculated with the same pathogen. Spots that developed on plants inoculated with Xcv ranged in appearance, and similar variances were observed on plants inoculated with Pst. Both of these pathogens favour moist humid conditions for propagation and infection (Bashan and Okon, 1986; EPPO/CABI, 1995; Getz et al., 1983; Jones et al., 1997; Park et al., 2009) and, therefore, most likely produce characteristic symptoms under these optimal conditions (Abdalla, 2000; EPPO/CABI, 1995). Another possible explanation for the varied appearance of lesions is that new lesions may develop on the same leaf and that the different ages of leaf lesions may affect the comparative appearance of old and new lesions. Plants originally inoculated with Xcv that were located on the edges of the humid chambers and in direct sunlight, typically developed lesions that dried out to form shot-holes in leaf surfaces (Cox et al., 1956; Obradovic et al., 2008), while inoculated plants in more humid areas developed characteristic black circular lesions (Cuppels and Ainsworth, 1995; Cuppels and Elmhirst, 1999; Sahin, 2001; Uppalapati et al., 2008). Spot development observed at the attachment site of the penducles on Xcv-inoculated plants was linked to penducle necrosis and premature fruit drop, which would decrease yields from infected plants.

As a detection technique, symptom observation enabled confirmation of the presence of an infection in this study, especially compared to non-inoculated control plants, and facilitated

preliminary diagnosis of the causal agent. Symptoms that developed in these trials, however, were often variable and dependent on a range of factors. Scouting for potential symptoms in large fields of tomato plants could be impractical if staff are not well-trained and, infected plants may remain unnoticed until the disease has spread to numerous neighbouring plants. Characterization of pathogens based on symptoms alone may, thus, be unreliable and inaccurate, and further techniques to validate observations would be beneficial (Sevİk and Kose-tohumcu, 2011).

Viruses, as obligate pathogens, are not culturable outside of host plants and, thus, detection of viral particles from symptomatic hosts is not possible by media-based detection methods (Lievens et al., 2003). Standard detection protocols for viral detection employ serological techniques, and in particular, DAS-ELISAs, with pathogen-specific antibodies to detect complimentary pathogenic proteins from host samples (Hadas et al., 2004; ISF, 2009a, 2009b). In this study, the production of a yellow colour as a result of enzymatic substrate metabolism in the ELISAs enabled easy visual confirmation of a positive reaction between test samples and selected antibodies, therefore, indicating positive detection of the target virus (Clark and Adams, 1977; Van Regenmortel and Burckard, 1980; Voller et al., 1976). More accurate of detection of viral particles, however, was dependent spectrophotometric analysis which generated precise absorbance readings that could be compared to negative, non-infected control samples or positive, infected samples. Viral detection by this method can be highly accurate and reliable (Alfaro-Fernandez et al., 2009; Hadas et al., 2004; Sevİk and Kose-tohumcu, 2011). Commercialised systems used for detection of a certain viral species, like the one employed in this study, demonstrate high specificity to the target pathogen. Besides the highly specific nature of complimentary binding between diagnostic antibodies and target proteins, antibodies are also extensively validated against numerous strains, related viruses and other pathogens of tomato prior to commercialisation.

A mixed infection of PepMV and ToMV was detected during pathogenicity trials of inoculated plants. Plant samples with a single infection of each virus were needed to analyse the efficiency and specificity of detection by the selected DAS-ELISA systems. Of equal importance was the need to validate the absence of viral particles in seedlings before inoculation to avoid false positive results. Trials were, thus, repeated independently, and the presence of a single infection in diseased hosts was confirmed by testing all samples with both PepMV- and ToMV-specific antibodies. Negative reactions obtained in DAS-ELISA tests with pathogen-specific antibodies confirmed that seedlings did not contain detectable levels of ToMV or PepMV before inoculation and, that antibodies did not react with any host proteins.

Seedlings that were inoculated with plant samples containing an unknown pathogen developed symptoms characteristic of PepMV, suggesting the presence of this virus in the original inoculum. This assumption was confirmed using DAS-ELISA with PepMV-specific antibodies according to the diagnostic process outlined by Van der Vlugt et al. (2002). The strong positive reaction of the original inoculum source with these antibodies validated the presence and pathogenicity of PepMV in these leaf samples. Assayed samples of leaves from pathogenicity trials also produced strong positive DAS-ELISA results, further confirming the detection of this pathogen. Detection of PepMV from seed harvested from symptomatic fruit was, however, more challenging than assays of infected leaves, as the hard seed coats reduced homogenization capacity and, thus, release of viral particles. Accordingly, ELISA absorbance readings for the seed replicate samples were significantly lower than those from leaf assays. Despite this, results confirmed the detection of PepMV particles in seed homogenates. This also proved that viral particles had been transferred from symptomatic hosts to the seed for disease transmission (Córdoba-Sellés et al., 2007; Hanssen et al., 2010b; Ling, 2008). Besides poor homogenization, low absorbance values were attributed to low levels of pathogen particles on host seed and the presence of fragments of homogenized seed in assays, which may have acted as inhibitors for antibody:target binding (Hadas et al., 2004; Sevİk and Kose-tohumcu, 2011). Negative reactions of all leaf and seed samples with ToMV-specific antibodies, confirmed the absence of ToMV particles in samples and proved that no significant cross-reactivity of ToMV-specific antibodies with detected PepMV particles had occurred.

The presence of ToMV in plants that developed symptoms characteristic of this virus was validated by the positive results obtained for all leaf samples assayed by DAS-ELISA with ToMV-specific antibodies. Absorbance readings of the tested samples were comparable to the manufacturer's positive ToMV control samples included in this assay, indicating the strong pathogenicity of the inoculum source. Sub-samples of seed assayed with ToMV-specific antibodies also produced significantly positive reactions for ToMV detection. Unlike PepMV detection assays, absorbance readings from seed were greater than those from leaf samples, despite poor homogenization of the seed. This suggested that viral load on seed affected the detection of a pathogen more than sample homogenization (Sevİk and Kose-tohumcu, 2011). The absence of significant reactions between PepMV-specific antibodies and ToMV-positive leaf and seed samples verified the absence of PepMV and validated the absence of cross-reaction of PepMV antibodies with ToMV.

Detection of target viral pathogens by DAS-ELISA in this study represented a highly specific, simple, reliable and rapid technique with results obtainable within 72 hours for both plant and seed samples

(Alfaro-Fernandez et al., 2009; Clark and Adams, 1977; Hadas et al., 2004; Rajeshwari et al., 1998; Sevİk and Kose-tohumcu, 2011; Vinayarani et al., 2011). Assays were highly robust and capable of detecting target pathogens from all leaf and seed samples, even with poor seed homogenization. Duplicate testing of each sample in adjacent wells and comparison to results of positive and negative control samples included in the assay reduced the possibility of technical errors. The layout of test samples, especially control samples, was extremely important, however, as contamination between adjacent wells could lead to misdiagnosis. Results from DAS-ELISA demonstrated better sensitivity compared to other conventional methods with a minimum of one infected seed detectable in a sample of 250 seeds, as established in previous studies (Gutierrez-Aguirre et al., 2009; Hadas et al., 2004; ISF, 2009b, 2009a). Standard detection protocols have been adjusted to comply with these detection limits to ensure that the lowest possible concentration of the target virus is detectable. Quantification of pathogen infection can be achieved by using relative ELISA absorbance readings, which indicate the degree of substrate utilisation and is considered directly proportional to concentrations of antibody:target complexes (Clark and Adams, 1977). In this study, however, results were highly variable among repeat assays and though results were positive for ToMV detection from leaf samples, some samples recorded poor absorbance values, despite host plants displaying severe symptoms (Kuflu and Cuppels, 1997). Assays are also susceptible to numerous technical and environmental factors such as incubation temperature, buffer washes, buffer composition, sample homogenization, and shelf-life of diagnostic antibodies. Viability of pathogenic particles detected cannot be confirmed by DAS-ELISA because non-viable, fragmented proteins are still detectable by the pathogen-specific antibodies. Detection of viable pathogens is increasingly important in seed-health testing for risk assessment of seed-lots for trade, especially for seed that has been treated to eliminate viral particles. Tests based on DAS-ELISA alone are, thus, often insufficient for certification (Hadas et al., 2004).

In this study, bioassays were performed on solanaceous hosts of *Nicotiana*, as these plants are susceptible to both PepMV and ToMV and produce characteristic responses upon inoculation with viable, pathogenic, viral particles (Hadas et al., 2004; Hamilton et al., 1981; Sijam et al., 1991). The absence of a suitable local lesion indicator host for PepMV necessitated the selection of a systemic host for the bioassay. To confirm the viability of detected PepMV from DAS-ELISA-positive leaf and seed samples, two of the oldest leaves of *Nicotiana benthimiana* were inoculated with seed or leaf homogenates, and the developing leaves of these plants were monitored for characteristic symptom development to confirm the systemic spread of pathogenic PepMV particles (ISF, 2009a; Ling, 2008; Pagán et al., 2006). Milder symptoms induced on hosts inoculated with seed homogenates

correlated with poor absorbance readings in the DAS-ELISA, thus indicating that a low concentration of viral particles was present in these samples. Nevertheless, the development of symptoms on inoculated hosts in both assays confirmed the presence of viable, infectious particles in tested host leaf and seed samples. Further confirmation of this systemic infection had to be proven by a second DAS-ELISA of tobacco leaf samples from both assays with PepMV-specific antibodies, according to the ISF (2009b). This second DAS-ELISA is recommended in standard protocols to eliminate false positive results that may occur as a result of variable environmental effects on host plants. Samples of young, symptomatic leaves from inoculated indicator hosts were DAS-ELISA-positive, thus verifying the development of a systemic infection of PepMV on tobacco host plants and, therefore, the viability of detected particles. The viability of ToMV particles detected in bioassays with both DAS-ELISA-positive leaf and seed homogenates was confirmed by the production of characteristic necrotic lesions on inoculated host plants (Hadas et al., 2004; ISF, 2009c). In this study, the number of lesions induced by leaf homogenates was more than those from seed, which reflected the relative concentration of viral particles within samples. As numerous other factors influence lesion development, however, this assumption could be inaccurate. An additional DAS-ELISA was not necessary to prove the viability of detected ToMV particles as characteristic lesion production is considered sufficient (Hadas et al., 2004).

Coupling DAS-ELISA and bioassay testing for detection of infectious viral particles was sensitive, reliable and accurate for characterization of ToMV and PepMV from infected leaf and seed samples. Including bioassays and, in the case of PepMV, a second DAS-ELISA, lengthened the diagnostic process from 72 hours to 2 - 3 weeks, which is too long for routine screening and seed health testing. If target viruses are not detected in initial DAS-ELISA screening, however, further analysis is not required as results from these assays are considered accurate for target detection (Hadas et al., 2004; ISF, 2009a, 2009b). Simultaneous detection of multiple target pathogens from samples is more complicated using DAS-ELISA or bioassays. Assays should thus be repeated separately, making screening and diagnosis of asymptomatic samples lengthy and laborious (Engel et al., 2010; Hadidi et al., 2004; Vinayarani et al., 2011). The limited number of suitable alternative methods however, has supported continued use of this method in standard protocols to detect significant viral pathogens of tomato.

The causal agents of symptoms characteristic of bacterial diseases were recovered by isolation of symptomatic leaf or stem samples onto five selective and differential media. Media were selected based on their compositions, which have been shown previously to discriminate between

phytobacteria infecting tomato plants (Goszczynska and Serfontein, 1998; Goszczynska et al., 2000; King et al., 1954; Kelman, 1954; Mohan and Schaad, 1987). Media contained components to restrict growth of contaminating organisms, like the antibiotics cycloheximide, to inhibit growth of fungi, and cephalexin, to inhibit *Erwinia herbicola* (McGuire et al., 1986; Sijam et al., 1991). The inclusion of vancomycin in Milk-Tween agar specifically inhibits the growth of Gram positive bacteria and, thus, cultures of Cmm, which facilitated informative discrimination of recovered cultures in this study (Goszczynska and Serfontein 1998; Goszczynska et al. 2000). Initial isolations from plants demonstrating wilt symptoms led to the unexpected recovery of bacterial colonies on MT agar, yet in a second isolation from the same diseased host, sparse growth was observed on MT agar. These results indicated that Cmm that initial isolations had not recovered the target pathogen adequately though the agent was present in symptomatic hosts. The composition of MT agar also facilitated discrimination of Xanthomonads from other bacteria, as recovered cultures produced a characteristic halo of two separate zones on the milky agar (Goszczynska and Serfontein, 1998). The larger zone was a round, clear zone indicating hydrolysis of casein, and the smaller was an opaque zone with crystalline deposits, representing Tween 80 hydrolysis. This specific morphology was observed in this study and easily distinguished Xcv from other contaminants or saprophytes also recovered from necrotic spots of diseased hosts. Components of KB medium supported the selective fluorescence of certain Pseudomonad species (Goszczynska et al., 2000; King et al., 1954), such as the targeted pathogen Pst, which enabled discrimination from other morphologically similar, non-fluorescent bacteria.

Although a single medium may be informative for a particular characteristic of the target bacterium, some bacteria, especially Cmm, can exhibit diverse variations in colony morphology, which necessitates the use of a combination of media to confirm the presence within a sample (Milijašević et al., 2007). Morphological growth of a range of bacteria, including the pathogens of interest in this study, have been well characterized in previous studies using the chosen media (Abdalla, 2000; Cuppels and Elmhirst, 1999; Goszczynska and Serfontein, 1998; Goszczynska et al., 2000; Kaneshiro et al., 2006; Kelman, 1954; King et al., 1954). A combination of these media, therefore, was able to confirm whether the expected pathogens, Cmm, Xcv and Pst, could be reliably recovered from diseased host plants. In order to confirm that a recovered colony was the targeted causal agent, a pure culture this bacterium was required for selective media assays to compare the growth of the isolate to positive control cultures on all media (Fessehaie et al., 2003). Isolation of a single culture with consistent morphological growth characteristics, therefore, required a minimum of two weeks.

Despite the recovery of the target bacterial pathogens, isolations on selective media supported the recovery of other bacterial cultures from leaf and seed samples with both similar and different morphological growth to the target pathogens. Causal agents of bacterial canker and spot, Cmm and Xcv, respectively, characteristically produce yellow, mucoid colonies that are small and circular, on a range of selective media (Fatmi and Schaad, 1988; Goszczynska et al., 2000; Milijašević et al., 2007; Shenge et al., 2007). Despite phenotypic similarity, even Cmm and Xcv have unrelated genetic and physiological characteristics and induce different responses from tomato plants. This emphasises the unreliability of pathogen identification based on colony morphology alone. Symptomatic plants may host numerous saprophytes and opportunistic or secondary pathogens besides the initial causal agent (Fatmi and Schaad, 1988; Goszczynska and Serfontein, 1998; Sijam et al., 1991), especially if host defence responses are suppressed by primary pathogen infection. Samples from mature diseased plants in this study, initially inoculated with Cmm, resulted in high recoveries of bacteria other than those morphologically similar to controls. This complicated isolation of the target bacterium as Cmm are characteristically slow-growing bacteria, only developing visible colonies after seven to 14 days of incubation compared to the more rapid growth of other non-target bacteria (De Leon et al., 2006; Fatmi and Schaad, 1988; Harris-Baldwin and Gudmestad, 1996).

Assays on selective media did not always give reliable results and had to be repeated for Cmm and Xcv to isolate the pathogen originally inoculated on host plants. Cultures were recovered from host samples that displayed similar morphological growth to that of control cultures, but did not behave similarly in other characterization tests, such as pathogenicity tests, confirming that these cultures were false positive non-virulent strains or unrelated saprophytes. These particular isolates were difficult to distinguish from target bacteria on media. Similar observations have been reported where contaminating, non-target organisms demonstrate minimal morphological variation from target pathogens (Goszczynska and Serfontein, 1998; Louws et al., 1998; Milijašević et al., 2007; Shenge et al., 2007; Sijam et al., 1991), which contributes to misdiagnosis. Correct concentration of media components is crucial for the development of characteristic colony morphology, and this error may have been responsible for the comparable colony morphology observed between targets and contaminating organisms (Fatmi and Schaad, 1988; McGuire et al., 1986; Sijam et al., 1991). The presence of Cmm, Xcv or Pst in host plant samples was successfully detected by isolation onto selective media in this study, but required either repeat isolations from host samples or extensive testing of all recovered colonies that were morphologically similar to control cultures.

Detection of bacteria from diseased plants by isolation on selective media has been shown to be a technically simple method to recover and quantify viable bacteria (Chitarra et al., 2000; Du Toit et al., 2005; Fatmi and Schaad, 1988; Kritzman, 1991). Initial isolations provided a means to propagate pure cultures of test isolates that could be used in confirmation or characterization tests. In this study however, isolations were inconsistent and unreliable for bacterial diagnosis from host samples, which was also the case in some other studies (De Leon et al., 2008; Kuflu and Cuppels, 1997; Shenge et al., 2007). This was mainly due to contaminating microflora that obscured the detection of target bacteria. Suspect isolates could, therefore, only be classified as presumptive positives. Media components are relatively inexpensive (Fatmi and Schaad, 1988; Kritzman, 1991) which make this detection technique cost effective for analysis of large samples. However, the repetitions necessary to ensure results are accurate may diminish this advantage. Processing numerous samples, especially for large fields of tomato plants, is also laborious and time-consuming (Chitarra et al., 2000; Harris-Baldwin and Gudmestad, 1996; Kuflu and Cuppels, 1997). These assays were performed using samples from diseased plants inoculated with characterized pathogens. Diagnosis of field samples using selective media would probably be more challenging, particularly if targeted pathogens are present at low concentrations (De Leon et al., 2008). Therefore, selective media assays should rather be used for preliminary identification instead of final diagnoses (Goszczynska and Serfontein, 1998).

Characterization tests of bacteria, based on physiological and biochemical traits, are used to supplement media assays for more accurate diagnosis of recovered cultures (Abdalla, 2000; Du Toit et al., 2005; Klingler et al., 1992; Obradovic et al., 2004; Shenge et al., 2007; Sijam et al., 1991). Because classification is based on characteristics like metabolic profiles, structural features and physiological effects on hosts, such additional characterization can be more reliable than colony morphology. Diagnostic manuals like Bergey's Manual (Bergey et al., 2005), facilitate identification and characterization of test cultures, by relating reactions of key tests to a range of previously characterized organisms. In this study, presumptive positive colonies from selective media were further characterized by a set of physiological and biochemical tests. Tests were selected by the capacity to distinguish between the expected pathogens and morphologically similar non-target bacteria. Reactions of Cmm, Xcv and Pst in these tests have been well characterized and validated (Bergey et al., 2005; Goszczynska et al., 2000), therefore, conclusions were substantiated by a strong record of expected results.

At least one tested isolate behaved as expected for each Cmm, Xcv and Pst in the selected tests. Characterization tests revealed falsely identified positive isolates from media assays. Collation of results from a number of tests also improved the accuracy and reliability of results. The Gram stain assay was an essential test to characterize the shape and cell wall structure of bacteria as suspect cultures that diverged from that of control cultures could be reliably eliminated as false positive isolates. This is also the limiting test for classification when using Bergey's Manual for diagnosis (Bergey et al., 2005). Although KOH solubility can be used to substitute the Gram stain (Goszczynska et al., 2000), because it is faster, simpler and requires fewer reagents than Gram staining, results can be difficult to interpret without control cultures and limited technical experience with this method.

Carbohydrate utilisation was highly informative for characterization of the test isolates as not all bacteria are capable of metabolising all sources of carbon to the same extent. In this study, five carbohydrates were selected to analyse the metabolic capacity of suspect cultures. Incorporation of the indicator, bromothymol blue, in the media also confirmed whether acid was produced from the metabolic reactions with the respective carbohydrates, which is often a unique trait of certain bacterial species (Bergey et al., 2005; Goszczynska et al., 2000). Reactions of test isolates could be compared to control isolates and results from previous studies with Cmm, Xcv and Pst, to facilitate distinction of potential target cultures from other cultures. Production of levan from sucrose-supplemented media was also a valuable discriminatory test as Cmm, Xcv and Pst are all known to utilise sucrose, but only Pst develops mucoid colonies on this medium, which indicates levan production (Abdalla, 2000; Goszczynska et al., 2000; Zaccardelli et al., 2005).

The most critical physiological test was the THR pathogenicity test (Abdalla, 2000; Goszczynska et al., 2000; Kritzman, 1991; Kaneshiro et al., 2006; Obradovic et al., 2004). Isolates that did not produce a positive reaction for this assay were non-pathogenic and could have induced symptoms on diseased tomato plants. This assay confirmed the presence of a pathogen from both plant samples and asymptomatic seeds. One presumptive culture of Cmm was excluded by this test alone, despite behaving similarly to the control isolate in all other tests, as no response was induced from the indicator host upon inoculation. Non-virulent and hypovirulent strains of Cmm have been reported previously (Alvarez et al., 2005; Gartemann, 2003; Kaneshiro et al., 2006), though this isolate may have been just a morphologically similar saprophyte. Opportunistic pathogens also would be clearly distinguishable from true pathogens as these only induce a mild reaction from hosts. At least one presumptive culture of Cmm, Xcv and Pst induced water-soaked or necrotic lesions in this study and, thus, positive detection of each pathogen from diseased host plants was confirmed (Abdalla, 2000).

In order to obtain reliable results, pathogenicity tests must include a suitable indicator host but symptom development can be affected by other factors such as varying environmental conditions (Boonham et al. 2007; Hamilton et al. 1981; Lee et al. 2003). Assays also require a number of seedlings for tests and greenhouses, to maintain uniform growth conditions, which can restrict assays to well equipped facilities.

Physiological and biochemical characterization tests are simple, results are easy to interpret and classification of bacteria is possible beyond genus, unlike other detection methods (Harris-Baldwin and Gudmestad, 1996; Goszczynska et al., 2000). Components for these tests are relatively inexpensive and common in most plant pathology facilities due to their well-validated, characterization capacity. To generate results, however, may still require an additional four – seven days (Harris-Baldwin and Gudmestad, 1996; Sijam et al., 1991). For reliable and accurate classification, a combination of these tests is required which can become an arduous task for testing a large number of samples (Harris-Baldwin and Gudmestad, 1996). Results may be unclear, especially if the target pathogen is unknown and dependence on previous studies for identification is risky if strains of the target pathogen can behave differently. Characterization tests are the most informative where reference cultures are tested in conjunction with test isolates. These can account for variable reactions resulting from incorrect media compositions, erratic culturing conditions and changing environmental conditions. Reference bacterial cultures may lose viability and pathogenicity in storage, however, and must be properly maintained to serve as effective control cultures.

The characterization potential of the Biolog System was examined on three bacterial cultures, isolated from diseased host plants, that demonstrated the most conserved characteristics compared to those of known Cmm, Xcv and Pst cultures. The current Biolog database contains profiles for over 2000 microorganisms including human, animal and plant pathogens which have been thoroughly tested and validated (Biolog Inc., 2009; Harris-Baldwin and Gudmestad, 1996; Kaneshiro et al., 2006; Klingler et al., 1992; Shenge et al., 2007; Truu et al., 1999). Several studies, however, have claimed that the Biolog system is unreliable for precise classification of some phytopathogenic bacteria, particularly beyond the genus level (Harris-Baldwin and Gudmestad, 1996; Kaneshiro et al., 2006; Klingler et al., 1992; Shenge et al., 2007). In this study, none of the three isolates was correctly identified as Cmm, Xcv or Pst, even though metabolic profiles for these pathogens are in the Biolog database (Biolog Inc., 2009). The only isolate with the true classification listed in the top ten closest matches was Cmm. Misidentification of this and the other isolates could be explained by a few false negative utilisation results (Harris-Baldwin and Gudmestad, 1996; Shenge et al., 2007). Variable

utilisation patterns may have resulted from inefficient metabolism by the culture or weak redox reactions with the tetrazolium dye, causing inaccurate absorbance values.

The Biolog system provided valuable insight into similarities of related and unrelated organisms because matches were based on statistical similarities and are not biased by taxonomic classification. Besides Cmm, three other subspecies of *C. michiganensis* were included in the list of the ten closest matches, suggesting that carbon utilisation patterns are highly conserved within this species. Previous studies characterizing *Clavibacter* isolates by the Biolog system (Kaneshiro et al., 2006; Louws et al., 1998) support this assumption. This trend was also observed with results of presumptive Xcv and Pst isolates. Although no tomato-infecting Xanthomonads or Pseudomonads were included in respective lists of the 10 closest matches, more than half of the 10 bacteria belonged to the same genus as the test isolate. The Biolog system, thus, demonstrated high capacity for genus- and, in some cases, species-specific characterization, but results were not always reliable for accurate diagnosis (Harris-Baldwin and Gudmestad, 1996; Kaneshiro et al., 2006; Shenge et al., 2007). Strains with genetic variances could lead to misidentifications, as slightly different utilisation profiles may be considered statistically different. Abdalla (2000) and Bouzar et al. (1999) reported correct identification of Xcv isolates to the pathovar level using Biolog. However, incorrect identification of 35 Xcv strains occurred for Shenge et al. (2007) and the Xcv isolate tested in this study. They proposed that inconsistent results may be due to the complicated taxonomy of Xcv, and that the reference profile may be based on a strain with different metabolic characteristics to the test isolates. The Biolog system may, therefore, be unsuitable for characterization of Xcv strains.

The Biolog system is technically simple as most of the process is automated (Harris-Baldwin and Gudmestad, 1996; Klingler et al., 1992; Shenge et al., 2007; Truu et al., 1999), and results are obtainable within 24 hours. The system automatically generates statistically calculated results and matches from absorbance values, eliminating the need for manual interpretation. Pathogens cannot be characterized directly from plant samples, however, but must be isolated into pure culture for analysis (Harris-Baldwin and Gudmestad, 1996). The Biolog microstation and plates are expensive and routine diagnosis by this method is, thus, not practical for many labs, but tests can be valuable for the preliminary diagnosis of an unknown disease. Results may not always be reliable and may vary among studies as carbohydrate utilisation patterns can be affected by a range of factors including the age of diagnostic plates and nutritional variations between closely related pathovars or strains (Harris-Baldwin and Gudmestad, 1996; Shenge et al., 2007). Results using Biolog analysis are consistent and informative for genus classification, but species characterization can be variable.

The most common method of detection of bacterial pathogens from tomato seed is based on a two phase media system, as suggested in standard protocols of the ISF and EPPO (EPPO/CABI, 2005; ISF, 2008a, 2009c; Milijašević et al., 2007). In this study, dilutions of seed homogenates, extracted from fruit harvested from diseased tomato plants, were plates onto selected media to recover Cmm, Xcv or Pst (Asma, 2005; Chun, 1982; Fatmi and Schaad, 1988; Kim et al., 1982; King et al., 1954; McGuire et al., 1986; Mohan and Schaad, 1987; Schaad, 1988). The first phase employed two semi-selective media supplemented with components suited to the recovery of a single target pathogen – Cmm, Xcv or Pst. The second phase utilised a non-selective differential medium to promote differential colony morphology for discrimination isolated colonies. The two phase system reveals isolated cultures that were misidentified in the first selective assay by using a non-selective second assay, thereby reducing the risk of misdiagnosis. Of the colonies recovered in the first phase from seed putatively infected with Cmm, 15 were selected that were morphologically similar to known Cmm control cultures. These were inoculated onto the second phase YDC media and only seven of these continued to demonstrate comparable morphology to control cultures. Similar discrimination was also possible in assays of seed with suspected Xcv or Pst infection. Surprisingly, all 18 isolates which developed similarly to reference cultures of Xcv on YDC medium, were not comparable in morphology to control Xcv cultures on another differential medium, CDA. This system was, thus, insufficient for characterization of Xcv in this study, and further characterization tests, such as Gram staining, were necessary. Even with these tests, only five of the 18 isolates could be tentatively classified as Xcv.

Although the two-phase system could distinguish among a number of contaminant bacteria from target pathogens, it was not entirely discriminatory and isolates could only be presumptively classified as Cmm, Xcv or Pst (Abdalla, 2000; Boonham et al., 2007; De Leon et al., 2008; Kritzman, 1991; Milijašević et al., 2007). Depending on media tailored for the recovery of a single pathogen also reduced the capacity of this system to detect multiple pathogens simultaneously (Goszczynska and Serfontein 1998). Seed-lots would thus have to undergo multiple assays to confirm the absence of all significant pathogens. Media assays ensure that bacterial cultures isolated from seed are viable (Chitarra et al., 2000; Fatmi and Schaad, 1988), providing an accurate indication of the health risk of each seed-lot. Including a general growth medium instead of a selective medium in this study, however, gave an indication of all viable microflora present on the tested seed, therefore indicating the overall seed health. The absence of restrictive components in general growth media also facilitated natural, characteristic colony morphology of isolated bacteria so that distinguishing the target bacteria from other bacteria was easier than on semi-selective media. The presence of

saprophytes, however, may result in an underestimation of concentrations of targeted pathogens on seed (Sijam et al., 1991). Assays on general media alone may, thus, be insufficient for diagnoses.

The semi-selective media chosen for recovery of Cmm, Xcv and Pst in this study, yielded highly variable results. Characteristic colonies of Cmm were obtained from seed homogenate on D_2ANX medium, however numerous saprophytes as well as hypovirulent and non-virulent strains of this bacterium also develop similar morphologies (Chun, 1982; Hadas et al., 2005; Kaneshiro et al., 2006). Accordingly, some of the presumptive positive colonies selected from this medium, did not behave in line with Cmm control cultures on other media and other characterization tests. Assays on SCM medium resulted in fewer false positives than D_2ANX media, because visual distinction between target and other bacteria was easier. This was due to incorporation of potassium tellurite in SCM media, which altered the morphology of Cmm colonies to a greyish-black compared to the black colonies of other bacteria (De Leon et al., 2006; Fatmi and Schaad, 1988). Nevertheless, false positive cultures isolated from SCM were still revealed in later characterization tests. Assays had to be repeated five times for the isolation of Xcv from putatively infected seed before acceptable results were obtained. In some cases, no growth was observed on culture media and in other cases, no colonies with morphology comparable to controls were recovered. This may have been due to an imbalance in components of the selective media (Fatmi and Schaad, 1988), poor media preparation, or a low concentrations of bacteria on the surfaces of tomato seed (Milijašević et al., 2007). CKTM semi-selective medium, which was developed by Sijam et al. (1991) for specific recovery of Xcv from tomato seed, and has been incorporated into standard detection protocols for Xcv (Abdalla, 2000; Cuppels et al., 2006; ISF, 2009c). In this study however, recovered colonies demonstrated a completely different morphology to that expected for Xcv. Colonies were even morphologically different to those of reference Xcv cultures, assayed in conjunction with seed homogenates, that were used as the initial inoculum source for hosts. Low levels of the target pathogen on seed or inter-strain variation are potential explanations of this. This medium was, therefore, excluded from further analysis and considered unsuitable for detection of the Xcv strain used in this study. Both mTBM and mKM-1, on the other hand, were capable of isolating bacterial colonies cultures that were morphologically similar to the control colonies and these were confirmed as presumptive positives by later tests. This confirmed that Xcv was present in the assayed seed. Although both media recovered more non-target bacteria than target bacteria, the development of a crystalline deposit, around Xcv colonies on mKM-1 medium, aided discrimination from non-target bacteria. The recovery of target bacteria from seed suspected to be infected with Pst was much higher on KBZ and KBC than on semi-selective media for Xcv and Cmm. Assays resulted in very low non-target recovery

and, thus, the media were considered to be highly selective. Further characterization tests were unnecessary as the similarities between growth of control colonies and test colonies were consistent enough to consider the presumptive classification accurate. Recovered colonies from semi-selective media were very small, however, which made quantification difficult.

The method of pathogen extraction from seed may also affect the reliability of detection and diagnosis (Milijašević et al., 2007). Homogenization of seed is used in most studies to extract pathogens but a number of studies have used agitated buffer washes to release pathogens for recovery on selective media (De Leon et al., 2008; Du Toit et al., 2005; Hadas et al., 2005; Leite et al., 1995; Milijašević et al., 2007). Though homogenization ensures good recovery of all bacteria present on and within seed, this process is destructive. Testing of expensive and valuable hybrid seed for trade means that at least 10 000 seed must be sacrificed per pathogen test. Wash assays, on the other hand, leave seed intact, and though few studies have analysed the germination potential of washed seed (Du Toit, pers. com., 2011[1]), seed could be theoretically re-used if the target pathogen is absent from seed. In this study, the recovery of Xcv and Pst from tomato seed was evaluated using homogenization and wash preparations. Recovery of colonies morphologically similar to that of control colonies was greater than 100-fold higher in homogenization assays than wash assays, which was similar to observations in other studies (Hadas et al., 2005; Milijašević et al., 2007). The large difference in recovery is attributed to the recovery of only surface-inhabiting bacteria in wash assays versus entire microflora populations that can be recovered more readily from seed in homogenization assays. Nevertheless, seed wash assays could still be useful for detection provided the media used for recovery promotes sensitive detection of target bacteria. Accurate quantification of levels of pathogen infection was not possible using seed wash assays in this study, and the risk of detecting only surface bacteria may not be sufficiently reliable for test seed for some pathogens.

Analysis of seed homogenates on selective media provided an indication of viable bacteria on seed and quantification of pathogen concentration by plating dilutions is possible (Meng et al., 2004; Milijašević et al., 2007). In this study, the detection threshold or sensitivity of the media was not analysed, but rather the specificity of media to recover target bacteria, which were successful for the detection of Cmm, Xcv and Pst. The media, however, yielded numerous false positive and negative results which obscured detection of the target bacteria (Abdalla, 2000; De Leon et al., 2008; Hadas et al., 2005; Kritzman, 1991; Milijašević et al., 2007). Seed analysed in this study were obtained directly from symptomatic host plants proven to be infected with the target pathogens, yet

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detection of these pathogens from seed was difficult. Detection of natural pathogens from seed-lots for trade would be even more limited and potentially unreliable (De Leon et al., 2008; Kuflu and Cuppels, 1997). Improving the sensitivity and specificity of media for the recovery of target bacteria from seed, and employing seed wash assays over homogenization assays, would make this technique more attractive. Combining the use of a semi-selective medium with a general growth medium in the two-phase system could also be more informative than current systems to detect the target pathogen as well as other potential threats. This technique is laborious and time-consuming for testing large numbers of samples, with results only attainable after 2 – 3 weeks (Chitarra et al., 2000; Harris-Baldwin and Gudmestad, 1996; Kuflu and Cuppels, 1997; Lievens et al., 2003). Combining these tests with other methods, like nucleic-acid based procedures or serological applications (De Leon et al., 2006, 2008; Du Toit et al., 2005; Kaneshiro et al., 2006; Milijašević et al., 2007), may improve the reliability and accuracy of results.

A traditional means to confirm the isolation of the causal agent of observed symptoms is by satisfying Koch's postulates. This may take 8 weeks or longer, which could delay trade of seed-lots and diagnosis of infectious diseases. In this study, it was necessary to prove that isolated organisms could induce the same symptoms on inoculated tomato plants as observed in the initial trails and were, therefore, pathogenic on tomato (Abdalla, 2000; Harris-Baldwin and Gudmestad, 1996; Kaneshiro et al., 2006; Kritzman, 1991; Sijam et al., 1991). The last step of Koch's postulates was not considered necessary to establish this in this study and was, thus omitted, as results from diagnostic tests and all of the symptoms induced on tomato in the second round of infectivity trials were clear indicators of the presence of a single, identifiable causal agent in samples. Isolates of the five pathogens, PepMV, ToMV, Cmm, Xcv and Pst that were confirmed by conventional diagnostic methods, all induced symptoms on inoculated tomato hosts in the second round of infectivity trials that were similar to those seen in the first trial, with the exception of trials where a mixed infection of PepMV and ToMV developed. In some cases, for example with ToMV and Xcv, the second trial resulted in more severe symptoms which could be attributed to varying environmental conditions favouring pathogen spread in the repeat trials. Plants inoculated with Xcv in the second pathogenicity trial demonstrated more uniform production of black lesions compared to the initial trials. This was associated with more suitable climatic conditions as humidity was more finely regulated in the second trial, and is known to affect Xcv infections (EPPO/CABI, 1995; Jones et al., 2005; Thieme et al., 2005). The diagnostic process used in this study was able to facilitate identification of the unknown causal agent in infected leaf samples as PepMV by recognising characteristic symptoms. Some symptoms characteristic of PepMV (Hanssen et al., 2009) that were

not recorded in the initial trial were observed in the second inoculation trial and these included severe twisting and bending of leaves. Variations in symptom development were likely due to seasonal effects on host development and pathogen spread. The length of time required to confirm Koch's postulates is a major drawback for its use in pathogen detection and diagnosis, and current protocols do not focus on proving each stage of the postulates (Adams et al., 2009). Pathogenicity tests also require adequate plant material and controlled environmental conditions (Hamilton et al., 1981; Harris-Baldwin and Gudmestad, 1996). Nevertheless, Koch's postulates are still valuable for determining the identity of new or unexpected pathogens from diseased host samples.

The diagnostic process used in this study was tested further by the unexpected development of a mixed infection of PepMV and ToMV on some plants. Plants were originally inoculated with only PepMV or ToMV, but both sets of inoculated plants developed symptoms characteristic of both viruses, which provided an indication of a potential mixed infection. This observation was insufficient for diagnosis and, thus, leaf and seed samples from these plants were tested by DAS-ELISA using separate assays with ToMV and PepMV-specific antibodies. Positive reactions with both antibodies detected PepMV and ToMV coat proteins in leaf and seed samples, thereby confirming that a mixed infection had developed (Hadas et al., 2004; Hamilton et al., 1981; Van der Vlugt et al., 2002). The two-fold higher ELISA absorbance values recorded in assays with seed compared to leaf samples, also suggested that a mild mixed infection had become established over time. A pathogenicity test was conducted on susceptible tomato seedlings to ensure that both pathogens were viable in the leaf samples. This was confirmed by the development of characteristic symptoms of infection by both viruses (Hollings, 1977; Hanssen et al., 2009; ISF, 2009b, 2009a; Jones et al., 1997). Neither test gave an accurate reflection of the relative proportions of the respective viruses in the diseased plants, but there was a visible increase in severity of symptom development compared to the initial trials. Both viruses have previously been described as highly infectious, virulent and stable outside of plant hosts and, can thus be spread by contact, on infected tools and even by wind (Chitra et al., 1999; Córdoba-Sellés et al., 2007; Davino et al., 2008; Ganoo and Saumtally, 1998; Gutierrez-Aguirre et al., 2009; Hanssen and Thomma, 2010; Hanssen et al., 2010b; Ling, 2008). The close proximity of the two initial trials in this study provided an easy mechanism for cross-contamination between diseased plants. Natural co-infection of PepMV and ToMV in tomato has yet to be reported, but the easy spread of these two pathogens in this study highlights the importance of testing seed for all known pathogens.

Conventional pathogen detection techniques used in this study were relatively inexpensive and simple but may take from four days to a few weeks to confirm the presence of absence of a target pathogen from host samples (Chitarra et al., 2000; Harris-Baldwin and Gudmestad, 1996; Meng et al., 2004). Assays on plants were informative for potential causal agents but symptom display was variable and the assays required greenhouse facilities for the trials (Hamilton et al., 1981; Kritzman, 1991; Ling et al., 2007; Meng et al., 2004; Sijam et al., 1991). Selective media and other characterization tests utilise positive reference cultures to eliminate variable and inconsistent results. Media assays gave a good indication of the viable contaminating microflora present in leaf and seed samples but assays had to be repeated in many cases to obtain reliable results. The presence of morphologically similar saprophytes or related, non-virulent bacteria complicated classification, so other characterization tests had to be employed for accurate diagnosis (Abdalla, 2000; Harris-Baldwin and Gudmestad, 1996; Sijam et al., 1991). Evaluating large numbers of samples using these tests is time-consuming, laborious and expensive, as expensive seed must be sacrificed for assays. Although identification using the Biolog system was rapid and provided statistically significant matches even if the causal agent was unknown, identification by Biolog was not accurate in this study and was considered unsuitable for routine testing. Utilising ELISA tests on viral infected samples was robust, rapid and accurate (Hadas et al., 2004; Hamilton et al., 1981; Sevİk and Kose-tohumcu, 2011) and gave a clear and reliable indication of the presence or absence of target viruses from seed and leaf samples. Detecting one pathogen from seed and leaves was simple and results were available within 72 hours. Detection of more than one pathogen per assay, as required for most seed health testing, would have to be performed by separate assays for each target virus (Koenig, 1981; Van Regenmortel and Burckard, 1980). Serological methods are also typically unreliable for bacterial disease diagnosis (De Leon et al., 2008; Kaneshiro et al., 2006; Kuflu and Cuppels, 1997), as exposed proteins are too conserved between genera to allow for accurate characterization. The viability of pathogens cannot be established by using DAS-ELISA and, thus, results had to be supplemented by bioassays to evaluate the threat of detected pathogens on traded seed.

Conventional and serological detection methods form the basis of current standard protocols for the detection of pathogens from tomato seed, but in this study these methods were less effective and reliable than desired for accurate identification of the target pathogens. More specific and sensitive detection based on molecular methods may improve these protocols (Cuppels et al., 2006; Meng et al., 2004; Njambere et al., 2011; Schaad and Frederick, 2002) by decreasing the risk of misdiagnosis and increasing the number of pathogens detectable in a single assay.

Chapter 3 Validation and Optimisation of PCR-based Pathogen Detection

3.1. Abstract

Molecular-based detection strategies for seed-borne pathogens of tomato typically demonstrate improved specificity, sensitivity and reliability compared to serological and conventional detection techniques. Some of the major drawbacks preventing the incorporation of PCR into standard detection procedures were identified, and amplification reactions were optimised in this study. Previously validated primer pairs for the detection of five significant viral and bacterial seed-borne pathogens of tomato were employed to evaluate their diagnostic capacity on pure cultures, as well as leaf and seed samples from diseased plants. The use of column-based extraction procedures reduced the time, labour and technical requirements for isolating RNA or DNA from tomato leaf and seed samples. Seed extracts, however, demonstrated low DNA or RNA yields and poor DNA or RNA purity. All targets were successfully detected using pathogen-specific primers from all infected leaf and seed samples but demonstrated inconsistent results and variable banding intensity. Viral diagnostic tests evaluated in this study were capable of detecting target pathogens from both leaf and seed samples in individual and mixed infections and will be combined into a multiplex detection test in future research. All diagnostic PCR tests evaluated facilitated detection of each of the five phytopathogens from leaf and seed samples but results were variable between samples in some of the tests. Detection based on conventional PCR protocols is still limited by the presence of PCR inhibitors and low-yielding extraction procedures which must be optimised before more molecular methods can be incorporated into routine diagnostic assays or standard detection protocols.

3.2. Introduction

The global spread of seed-borne pathogens of tomato has been drastically augmented by the increase in international seed trade to meet production and consumption demands. As seed are largely asymptomatic (Kritzman, 1991; Nome et al., n.d.; Zhao et al., 2007), pathogens are moved between countries remaining undetected until infections emerge in tomato production areas, often leading to epidemics and severe economic losses. Rapid detection of pathogens from diseased host plants is essential for limiting the harvest of infected fruit and seed prior to trade. Many tests have been developed to identify causal agents from various symptomatic hosts and, therefore, facilitate implementation of curative strategies. These tests are generally impractical for screening large fields

of cultivated tomato and symptomless plants can transmit pathogens to seed (Du Toit et al., 2005; Kritzman, 1991; Njambere et al., 2011). To ensure the trade of 'pathogen-free' seed, methods to detect important seed-borne pathogens are generally applied directly to samples of seed-lots. Standard protocols for seed health testing mainly employ conventional and serological methods to identify significant bacterial and viral pathogens of tomato (Hadas et al. 2004; ISF 2008, 2009a, 2009b, 2009c). Many of these techniques, however, demonstrate limited sensitivity and specificity for detection and identification of target pathogens from seed. Poor discrimination from closely related organisms and saprophytes, that may also inhabit hosts, can contribute to the unreliable detection capacity of these methods (De Leon et al., 2008; Gutierrez-Aguirre et al., 2009; Kaneshiro et al., 2006; Kuflu and Cuppels, 1997; Lee et al., 2003; Lievens and Thomma, 2005; Njambere et al., 2011; Van Regenmortel and Burckard, 1980; Vinayarani et al., 2011). Results are often inconsistent, difficult to interpret and tests require a few weeks or months to complete. Tests are also limited to certain pathogens, for example, only culturable, non-obligate pathogens may be detected by selective plating (Lievens et al., 2003; Maes, 1993), and pathogens without cell walls and surface proteins, like phytoplasmas and viroids, cannot be detected by serological methods (Boscia and Myrta, 1998; Hadidi et al., 2004).

Nucleic-acid based strategies, and in particular polymerase chain reaction (PCR)-based methods, offer solutions to some of these drawbacks, as well as numerous added advantages for phytopathogen detection. Genomic DNA or RNA of the pathogen of interest is directly targeted for detection and, therefore, methods are suited to detection of all types of pathogens (Mumford et al., 2006; Njambere et al., 2011; Ward et al., 2004). Directly targeting molecular features of organisms can be more specific, consistent and informative than morphological or physiological traits (Bach et al., 2003; Kaneshiro et al., 2006; Schaad and Frederick, 2002), facilitating simultaneous detection and identification of pathogens from host samples. Primers or probes used in these strategies are designed against genetic sequences of the target organism so that this pathogen is detected by complimentary binding, even in the presence of high concentrations of saprophytes and other non-target sequences in samples (Leite et al., 1994; Obradovic et al., 2004; Widmer et al., 1998). The capacity of PCR-based detection is thus more sensitive than many other methods, with various reports indicating between 10 and 1000 times improvement from conventional and serological strategies (Cuppels et al., 2006; Hartung et al., 1996; Leite et al., 1995; McManus and Jones, 1995). This is critical for detection of seed-borne pathogens, as even low titres of infection may be sufficient to cause diseases and destructive epidemics (Córdoba-Sellés et al., 2007; EPPO/CABI, 1999; Hanssen et al., 2010b; Ling, 2008; Milijašević et al., 2007). Successful detection and amplification of

target sequences of the pathogen results in a collection of sequence fragments of the same size that can be separated and visualised by techniques like electrophoresis and southern blotting (Fanelli et al., 2007; Lievens and Thomma, 2005; Park et al., 2009; Schaad and Frederick, 2002).

In phytodiagnostics, PCR has been used in numerous research studies to successfully detect diverse pathogens from various agronomically important plants. Palacio-Bielsa et al. (2009) compiled an in-depth catalogue of all the diagnostic primers that have been developed for phytopathogen detection to date, providing pathologists with a reference of options and target genes for diagnosing unknown diseases or targeted pathogens from host samples. The success of pathogen detection using PCR-based methods is dependent on meticulous primer design which can be manipulated to target different taxonomic levels, e.g., genus, species, sub-species, strain or race, according to the level of classification or discrimination required (Fatmi and Schaad, 2002; Lievens and Thomma, 2005; Ling et al., 2007). This is achieved by designing primers to be complimentary to particular targeted regions in a pathogen genome that demonstrate sufficient conservation or divergence between related and non-target organisms. These regions are identified by aligning sequences of relevant organisms and/or by previous epidemiological studies. Genes that are more conserved between groups of organisms are suited to broad-spectrum detection and pathogen discovery (Jacobi et al., 1998; Kaneshiro et al., 2006; Maes et al., 1996; Nicolaisen and Bertaccini, 2007; Van der Vlugt and Berendsen, 2002; Widmer et al., 1998). More divergent regions, for example, those that play a role in pathogenicity (Cuppels et al., 2006; Leite et al., 1994; Obradovic et al., 2004; Zaccardelli et al., 2005), are better suited to species- or subspecies-specific detection. Synthetic primers must be tested and validated experimentally against target and related organisms as well as host DNA/RNA, to ensure that developed primers bind only to the targeted sequence of the pathogen of interest and produce amplicons of the expected length (Alfaro-Fernandez et al., 2009; Mansilla et al., 2003; McManus and Jones, 1995). Only if primers demonstrate consistent detection of the target pathogen or group of pathogens with these conditions, can they be considered for routine phytodiagnostic assays or standard detection protocols (Asma, 2005; EPPO/CABI, 2005).

Numerous diagnostic primers and amplification reactions have been developed and validated for the detection of five of the most significant seed-borne pathogens of tomato: *Pepino mosaic virus* (PepMV) (Hanssen et al., 2008, 2009; Ling et al., 2008; Mumford and Metcalfe, 2001; Van der Vlugt et al., 2002), *Tomato mosaic virus* (ToMV) (Da Silva et al., 2008; Jacobi et al., 1998; Letschert et al., 2002), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) (Bach et al., 2003; Louws et al., 1998; Ozdemir, 2009; Pastrik and Rainey, 1999), *Xanthomonas campestris* pv. *vesicatoria* (Xcv) (Cuppels et

al., 2006; Leite et al., 1994; Maes, 1993; Obradovic et al., 2004; Ozdemir, 2009; Park et al., 2009) and *Pseudomonas syringae* pv. *tomato* (Pst) (Cuppels et al., 2006; Fanelli et al., 2007; Ozdemir, 2009; Zaccardelli et al., 2005). Besides facilitating rapid, sensitive and specific detection of these pathogens from infected tomato leaf and seed samples, PCR-based diagnostic tests have proven to be valuable for validating presumptive bacterial cultures isolated during selective plating assays of seed. By using the bio-PCR technique and pathogen-specific primers (EPPO/CABI, 2005; Maes, 1993; Schaad et al., 1995), colonies recovered from seed assays that are morphologically similar to control cultures, may be confidently identified and characterized, thereby distinguishing isolates of true pathogens from false-positive organisms. Assays based on bio-PCR, thus, also provide an indication of the viability of recovered pathogens from non-symptomatic seed (Schaad et al., 1995). Detection and characterization of viral pathogens is generally also improved by using PCR compared to routine serological techniques. Amplification of RNA viruses, however, does necessitate an initial reverse transcription (RT)-PCR step. This converts viral RNA to single-stranded complimentary DNA (cDNA), which is more stable than RNA. To produce a detectable fragment, cDNA is then converted to double-stranded DNA (dsDNA) using pathogen-specific primers.

Although significant seed-borne pathogens of tomato have been detected successfully using PCR, few detection tests based on molecular strategies have been incorporated into standard detection protocols for seed trade (Fanelli et al., 2007; Schaad and Frederick, 2002; Sevİk and Kose-tohumcu, 2011). A major limiting factor of PCR applications is the laborious and technically challenging process of nucleic-acid extraction (Boonham et al., 2008; Dovas et al., 2004; Mumford et al., 2006; Sholberg et al., 2005). The introduction of column-based purification procedures has drastically improved the time and labour involved to obtain high quality recovery of DNA and RNA from diverse samples, including plant leaves and seed (Deyong et al., 2005; Lievens and Thomma, 2005). Mastermixes of PCR reaction components, and the development of robust or high-fidelity polymerase enzymes to facilitate amplification, have also eliminated many technical variables associated with amplification reactions to improve the consistency of results. High-throughput testing of numerous samples by PCR has also become more practical by the development of 96- and 384-well PCR plates and complementary thermocycler PCR machines (Lievens and Thomma, 2005). Where most novel techniques, like next generation sequencing and array technology, are limited by expensive equipment requirements, PCR has become broadly adopted in industry for routine testing and most facilities now have equipment to support PCR testing. This suggests that PCR-based methods have a sure future in standard pathogen detection protocols.

The limited incorporation of PCR-based methods into global standard pathogen detection protocols, and in particular, protocols for the detection of the selected pathogens, was investigated in this study. The detection capacity of conventional PCR for five important seed-borne pathogens of tomato was analysed using primers and amplification systems that have shown high discriminatory potential for pathogen detection and identification in previous studies (Bach et al., 2003; Letschert et al., 2002; Mumford and Metcalfe, 2001; Obradovic et al., 2004; Zaccardelli et al., 2005). Samples of infected seed and leaves for each pathogen, that were used to validate these diagnostic PCR tests, were taken from pooled samples previously classified as positive for the presence of each pathogen. This was to ensure that the results from both studies were comparable. Tests were also used to confirm the identification of presumptive bacterial cultures recovered from media assays of putatively infected seed. The development of successful, robust and repeatable diagnostic protocols using conventional PCR would contribute not only to standard detection protocols which are accepted and performed in laboratories world-wide, but could also serve as a foundation for the development of other applications of PCR that may demonstrate improved capacity for detection.

3.3. Materials and methods

3.3.1. Nucleic acid extraction from leaf and seed samples

Pathogen nucleic-acid was extracted from leaf and seed samples from symptomatic tomato host plants. The presence of PepMV, ToMV, Cmm, Xcv and Pst in the respective samples, was previously validated by conventional and serological methods (Chapter 2, 2.4.). Extractions were also performed on leaf samples from plants previously confirmed to have a mixed infection of PepMV and ToMV. These plants were initially inoculated with only PepMV or ToMV inoculum but developed a mixed infection of both viruses. Samples had been validated for the presence of both particles by DAS-ELISA using the respective pathogen-specific antibodies (Chapter 2, 2.4.5). Prior to extraction procedures, all leaf samples were stored at -70°C and seed samples at 4°C. All extractions were performed in conjunction with healthy, uninfected leaf and seed control samples obtained and tested during trials as mentioned in Chapter 2.

Total RNA extraction, from control and viral-infected leaf and seed samples, was performed using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Approximately 85 – 100 mg of leaf tissue, and 35 – 50 mg of seed, was used as starting material for extractions. Samples were obtained from a pooled collection of symptomatic leaves or from a

collection of seeds harvested from symptomatic fruit. These were homogenized in liquid nitrogen using sterilised mortars and pestles in 2 mℓ sterile plastic tubes. Minor modifications to the manufacturer's protocol included heating RNAse-free H_2O for approximately 30 minutes at 65°C prior to use in the elution step, to better dissolve extracted RNA. For elution of RNA, 50 µℓ of this preheated H_2O was applied directly to the membrane of the RNeasy spin column and incubated at room temperature for 5 minutes to dissolve RNA bound to the column. The RNA suspension was eluted at 10 000 rpm (revolutions per minute) for one minute using a MiniSpin Plus centrifuge (Eppendorf, Germany). The concentration and purity of RNA samples was analysed by a ND-1000 spectrophotometer (Nanodrop technologies, USA). Samples were stored at -70°C until used.

Total DNA extractions were performed on 85 – 100 mg of bacterial-infected and control leaf samples, and 45 – 50 mg of seed samples by the DNeasy Plant Mini Kit (Qiagen, Germany). Samples were homogenized using sterile mortar and pestles and liquid nitrogen. Extractions were also performed directly on seed homogenates prepared during seed extraction assays on selective media (Chapter 2, 2.4.3.4.). Briefly, homogenates were prepared by soaking 4 – 8 g of seed, suspected to be infected with Cmm, Xcv or Pst, in 0.05M Phosphate-Tween buffer overnight, and subsequently homogenized using the SilentCrusher M (Heildolph, Germany). Approximately 100 mg of the crushed seed and 200 µℓ of the liquid homogenate were used for extraction procedures. Variations from the manufacturer's protocol for DNA extraction included using larger volumes of extraction buffers AP1 and AP2 of 600 µℓ and 260 µℓ, respectively, to improve the overall yield of DNA. Elution buffer AE, was also preheated to 37°C, and 50 µℓ of this buffer was applied to the column membrane to dissolve bound DNA. Suspensions were incubated at room temperature for five minutes and then eluted at 8 000 rpm for one minute. A second volume of elution buffer was then applied and eluted through the column in a similar manner. The 100 µℓ eluates were incubated for an hour at 37°C to facilitate further dissolution of DNA. The concentration and purity of DNA extracts was analysed using a spectrophotometer and high quality DNA samples were stored at -20°C until use.

3.3.2. Amplification of pathogen nucleic acid

3.3.2.1. Amplification of viral RNA

Extracted RNA from viral-infected and control leaf and seed samples, was converted to ds-DNA by a two-step RT-PCR procedure. First strand synthesis was performed using the ImProm-II Reverse Transcriptase system (Promega, USA) in a total reaction volume of 20.6 µℓ. Approximately 1 µg of total RNA was incubated at 70°C for 10 minutes with 1 U/µℓ RNasin - RNase Inhibitor (Promega,

USA) and 0.1 µM Oligo dT_{18} primers (Fermentas, EU) for suspect PepMV-infected samples, or 0.1 µM random nonamer (pdN9) primers (Inqaba Biotech, South Africa) for suspect ToMV-infected samples. The use of Oligo dT_{18} primers restricted primer-target binding to RNA fragments with a poly-adenaline tail (poly(A) tail) of repeat adrenaline bases whereas nonamer primers were each a non-specific string of nucleotides and, thus, bound to any RNA fragments present in reaction mixes. Random hexamer primers (Fermentas, EU) were incorporated into RT-PCR reactions with RNA extracts from leaf samples with a mixed viral infection. Primer-template mixtures were incubated on ice for one minute before adding the remainder of reaction components: 1X Improm-II Reaction buffer, 3 mM $MgC\ell_2$, 0.5 mM dNTPs (Fermentas, EU) and 1 µℓ of Improm-II Reverse Transcriptase Enzyme. The RT-PCR cycling conditions included one cycle of 25°C for 10 minutes, 42°C for 60 minutes and a 10 minute enzyme deactivation step at 70°C. All RT-PCR reactions were performed in conjunction with a non-template control sample, a negative control sample (using RNA from healthy leaf or seed samples) and an amplification control sample (no polymerase added). Single-stranded cDNA products were stored at -20°C.

Amplification of viral nucleic acid and conversion of single-stranded cDNA to dsDNA was primed using pathogen-specific primers targeting PepMV or ToMV respectively (Table 3. 1). The two sets of primers were designed to bind specificially to flanking regions of the coat protein (CP) genes of the respective viral genomes for amplification (Hanssen et al., 2008; Letschert et al., 2002; Mumford and Metcalfe, 2001). Second-strand synthesis reaction mixes were composed of 5 µℓ of the appropriate cDNA template from RT-PCR reactions, 0.4µM of the forward and reverse primers for the targeted virus (Table 3. 1), 10 mM dNTPs (Fermentas, EU), 10X Dream Taq Buffer with added $MgC\ell_2$ and 1.25U of Dream Taq Polymerase (Fermentas, EU), to a total reaction volume of 50 µℓ. Thermocycling conditions included an initial denaturation step at 95°C for five minutes; 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for one minute; and a final extension step at 72°C for 10 minutes. No template, non-amplification (no polymerase) and negative controls were included in reactions.

Rapid amplification of ToMV and PepMV cDNA from leaf samples with a mixed infection was performed using the KAPA2G Fast PCR system (KAPA, USA). Each sample was subjected to two separate amplification reactions, one with PepMV-specific primers - Ker-1 and PepCP-R, and the other with ToMV-specific primers - ToMV CP-F and TobUni-1 (Table 3. 1). Both reactions contained 5µℓ of the cDNA template, 0.5µM of each primer of the target-specific pair and 1X KAPA2G Fast HotStart ReadyMix (KAPA, USA) to a total reaction volume of 25µℓ. Cycling conditions were the

same for both reactions and included an initial three minute step at 95°C followed by 30 cycles of 15 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C, with a final five minute extension step at 72°C. Non-template and negative control samples were incorporated into these reactions.

Table 3. 1 A list of pathogen-specific primer pairs selected to target each of the pathogens of interest from nucleic acid extracts in this study.

Target[1]	Primer[2]	Sequence (5' – 3')	Target Gene	Amplicon length (nt)	T_a[3]	Reference
PepMV	Ker1 PepCP-R	CACCAATAAATTTAGTTTTAGC CTCTGATTAAGTTTCGAGTG	CP	986	58°C	Hanssen et al. (2009) Mumford and Metcalfe (2001)
ToMV	ToMV CP-F Tob-Uni1	CGGAAGGCCTAAACCAAAAAG ATTTAAGTGGAGGGAAAAACACT	CP	686	58°C	Letschert et al. (2002)
Cmm	FP Cm RP Cm	TGTCGAGGGCATGTTGCACG GGAGACAGAATTGACCAATGAT	ITS	223	60°C	Bach et al. (2003)
Xcv	RST 65 RST 69	GTCGTCGTTACGGCAAGGTGGTCG TCGCCCAGCGTCATCAGGCCATC	HrpB	420	63°C	Obradovic et al. (2004)
Pst	MM5F MM5R	GAACGAGCTGAAGGAAGACA CAGCCTGGGTTAGTCTGGTTA	HrpZ	532	57.5°C	Zaccardelli et al. (2005)

Amplified ds-DNA products were analysed by size-dependent electrophoresis on 1% agarose gels (Bioline, USA) in 1X TAE Buffer (40mM Tris-Acetate and 1mM EDTA) stained with 0.1 mg/mℓ Ethidium Bromide (EtBr) (Sigma Life Sciences, USA). Approximately 5 µℓ of PCR product was combined with 1.7X Orange Loading dye (Fermentas, EU) and loaded in conjunction with 0.5 µg of O'GeneRuler 1 kb Plus DNA ladder (Fermentas, EU; Appendix A) into individual wells of the gel.

[1] The target gene refers to the gene or portion of a gene from a pathogen genome that will be targeted for amplification during PCR reactions.
[2] Primers are listed in the order of binding that is, the forward primer followed by the reverse primer.
[3] Annealing temperature (T_a) refers to the optimal conditions for primer binding to regions flanking the target amplicon as determined during amplification reactions in this study.
All primers were synthesised by Inqaba Biotech and stored as 100µM stock solutions at -20°C until use.

Products were separated on the gel for 50 minutes at 70V using a PowerPac Basic (Bio-Rad, Singapore) and visualised by exposure to UV light with the BioDoc-IT System (UVP Inc, USA). Samples were considered positive for the presence of PepMV nucleic-acid if a fragment of 986 bp was detected whereas those positive for ToMV were detected by the presence of a 686 bp fragment.

3.3.2.2. Bio-PCR of bacterial cultures isolated from leaf and seed samples

Bacterial cultures were previously recovered on nutrient agar from leaf and stem samples of tomato plants demonstrating symptoms of bacterial canker, spot and speck, respectively (Chapter 2, 2.4.1.). Isolated colonies that demonstrated the closest morphology to colonies of known cultures of Cmm, Xcv or Pst, were subjected to bio-PCR tests for further characterization. From each suspect colony, a loopful of inoculum was rinsed in 500 µℓ of sterile, nuclease-free H_2O, and vortexed vigorously using a Vortex-2 Genie (Scientific Industries Inc., USA) to ensure even dissemination of bacterial cells throughout the suspension. From this stock suspension, 2 µℓ was added to a final volume of 5 µℓ with H_2O and used directly in PCR reactions as a template for primer binding. The remaining reaction mix contained 0.4 µM of forward and reverse primers for each target pathogen (Table 3. 1), 10 mM dNTP mix (Fermentas, EU), 10X DreamTaq Buffer with added $MgC\ell_2$ and 1.25U of DreamTaq Polymerase (Fermentas, EU) to a total volume of 50 µℓ. Reactions were performed in conjunction with template and amplification control samples. Cycling conditions varied according to the target pathogen and its diagnostic primer pair. Pathogen-specific primers for Cmm (Table 3. 1), targeted the intergenic transcribed spacer (ITS) sequence of the ribosomal DNA (rDNA) gene (Bach et al., 2003), amplifying a 223 bp fragment under cycling conditions of 95°C for three minutes; 30 cycles of 95°C for 30 seconds, 60°C for one minute and 72°C for one minute; followed by a 10 minute extension step at 72°C (Pitout, unpub.). A gene in the hypersensitive response/pathogenicity (Hrp) complex, an important pathogenicity factor of Xcv, was targeted for primer binding to produce a fragment of 420 bp from the Xcv genome (Obradovic et al., 2004). This fragment was amplified under the following cycling conditions: 95°C for two minutes; 35 cycles of 95°C for 30 seconds, 63°C for 45 seconds and 72°C for 90 seconds; 72°C for five minutes (Pitout, unpub.). Characterization of Pst also depended on pathogen-specific primers binding to an important pathogenicity factor in the Hrp gene complex (Zaccardelli et al., 2005), to amplify a 532 bp target fragment. Thermocycling conditions to generate this fragment included an initial five minute denaturation step at 95°C, followed by 30 cycles of 95°C for 30 seconds, 57.5°C for 30 seconds and 72°C for one minute; and a final 10 minute extension step at 72°C.

Colonies recovered during seed detection assays were also characterized by bio-PCR using the same bacterial-specific primers (Table 3. 1). Homogenates of seed suspected to be infected with Cmm, Xcv or Pst, were inoculated onto selective or differential media that were specially formulated to favour the recovery of each of the targeted bacteria (Chapter 2, 2.4.3.4). Cultures recovered from these assays that demonstrated similar morphology to colonies of known control cultures, were selected for PCR tests. Cell lysates were extracted from these cultures for use in amplification reactions, prepared as outlined in Pelludat et al. (2009). Briefly, a loopful of a suspect colony was suspended in 300 µℓ of sterile, nuclease-free H_2O, vigorously vortexed, and boiled for 30 minutes at 95°C. After centrifugation at 12 000 rpm for five minutes, a 1:10 dilution of the supernatant was performed with nuclease-free H_2O. Bacterial DNA was then amplified by the KAPA2G Robust HotStart System (KAPA, USA), using 1X KAPA2G Robust HotStart ReadyMix, 0.5 µM of forward and reverse primers for the targeted pathogen (Table 3. 1) and 2 µℓ of the template suspension to a total reaction volume of 25µℓ. Cycling conditions for amplification using this system are represented in Table 3. 2, and employed the relevant annealing temperatures selected for the Dream Taq amplification system (Table 3. 1). Reactions included positive control as well as template control samples.

Table 3. 2 Thermocycling conditions for fragment amplification from target pathogens using the KAPA2G Robust HotStart System (KAPA, USA) and pathogen-specific primers.

	Cmm	Xcv	Pst
Denaturation	95°C – 3 min	95°C – 3 min	95°C – 5 min
Amplification - 40 cycles	95°C – 15s 60°C – 30s 72°C – 30s	95°C – 15s 60°C – 15s 72°C – 30s	95°C – 15s 57.5°C – 15s 72°C – 30s
Extension	72°C – 5 min	72°C – 5 min	72°C – 10 min

3.3.2.3. Amplification of bacterial DNA

Extracted DNA from leaf and seed samples from plants symptomatic for Cmm, Xcv or Pst, were subjected to PCR tests with pathogen-specific primers to confirm the presence of these causal agents in host samples. To target Cmm, 5 µℓ of the appropriate leaf or seed DNA extract along with 0.4 µM forward and reverse primers targeting Cmm (Table 3. 1), 10 mM dNTPs (Fermentas, Eu), 10X DreamTaq Buffer with added $MgCℓ_2$ and 1.25U of Dream Taq Polymerase (Fermentas, EU) were coupled to a final reaction volume of 50 µℓ. Amplification of pathogen-specific fragments of Pst was also performed using the DreamTaq system with Pst-specific primer pairs (Table 3. 1) and 5 µℓ of

DNA extracts from leaf and seeds symptomatic for Pst infection. Thermocycling conditions for both Cmm and Pst PCR tests were consistent with those used in 3.3.2.2 for amplification of cultures isolated from symptomatic leaf samples. Production of the 420 bp target fragment from suspect Xcv-infected leaf and seed DNA extracts was performed using the KAPA2G Robust HotStart System (KAPA, USA) as outlined in 3.3.2.2 for colonies recovered in seed detection assays. In these amplification reactions, 2,5 µℓ of seed or leaf DNA extracts were used as templates with 0.5 µM of forward and reverse primers specific for Xcv (Table 3. 1) and 1X KAPA2G Robust HotStart ReadyMix (KAPA, USA) to a final volume of 25 µℓ. Reaction conditions for amplification were performed as outlined in Table 3. 2. The DNA extracted from seed homogenates of Cmm, Xcv or Pst, was subjected to PCR tests to further confirm the presence of target pathogens within samples. Fragments were amplified from 2 µℓ of DNA extracts using the KAPA2G Robust HotStart System (KAPA, USA), with 1X KAPA2G Robust HotStart ReadyMix and 0.5 µM of forward and reverse pathogen-specific primers (Table 3. 1), to a total reaction volume of 25 µℓ. Cycling conditions for the amplification of target-specific fragments from tested samples were maintained as described in Table 3. 2, for each of the targeted bacteria. All Cmm, Xcv and Pst detection tests on leaf, seed and seed homogenates incorporated template control, positive control and negative control samples, the latter from healthy, non-infected leaf or seed DNA extracts.

Products amplified from DNA extracts or bacterial cultures were evaluated by size-dependent electrophoresis on 1% agarose gels stained with 0.1 mg/mℓ EtBr. Samples were mixed with 1.7X Orange Loading Dye and 7 µℓ of the suspension was added to wells of the gel in conjunction with 0.5µg of O'GeneRuler 1 kb Plus DNA ladder (Appendix A). Fragments were separated at 70V for 50 minutes and band patterns were visualised under UV light. The presence of the target pathogens was considered positive if a fragment of the expected length was detected (Table 3. 1).

3.3.3. Rapid detection of bacteria from leaf samples

Fast extraction and amplification of bacterial DNA from infected leaf samples was achieved using the KAPA Express Extract and 2G Robust HotStart PCR system (KAPA, USA). Leaves from plants demonstrating symptoms of bacterial canker, spot or speck, were sampled and a leaf disc, with a diameter of approximately 5mm, of an area demonstrating characteristic symptoms of the target pathogen, was extracted and transferred to a thin-walled 200 µℓ PCR tube. Leaf discs were soaked in 10X KAPA Express Extract Buffer with 2U of KAPA Express Extract Enzyme, to a total reaction volume of 100 µℓ, and briefly crushed with a sterile pipette tip. For cell lysis and disruption of proteins by the Express Extract enzyme, suspensions were incubated for 15 minutes at 75°C with a subsequent 5

minute enzyme inactivation step at 95°C. Extracted DNA was then recovered from suspensions by centrifugation at high speed for 1 minute. The supernatant was diluted 1:5 in 1X TE Buffer (10 mM Tris and 1 mM EDTA at pH 8) and stored at -20°C until use. Bacterial fragments were amplified in 25 µℓ reaction mixes containing 1 µℓ of DNA extracts, 0.5 µM of forward and reverse primers for the targeted pathogen (Table 3. 1) and 1X KAPA2G Robust HotStart ReadyMix. Cycling conditions for amplification were performed as outlined in Table 3. 2. Amplification reactions for the detection of bacteria from infected host leaf samples were performed in conjunction with non-infected tomato leaf samples and template control samples. Amplified fragments were separated and visualised as described previously.

3.4. Results

3.4.1. Detection of viral RNA from infected samples

Column-based extraction of RNA, using the RNeasy Plant Mini Kit, from infected and healthy leaf samples yielded on average 1,01 µg/µℓ of RNA per 85-100 mg sample of tissue. Extracts demonstrated high purity with both 260/280 and 260/230 absorbance ratios calculated as greater than 2.00 for all samples. Yields from seed extractions were considerably lower, only averaging approximately 50 ng/µℓ. Though the 260/280 ratios of RNA samples extracted from seed was good, 260/230 ratios seldom exceeded 1.00, which indicated that high levels of contaminants and inhibitors were present in seed samples. Despite low purity and concentration, these samples were used in RT-PCR and PCR tests to determine whether pathogen nucleic acid could still be detected. Procedures to extract RNA from leaf and seed samples required approximately 60 minutes and extracts could then be used directly in amplification reactions.

3.4.1.1. Detection of PepMV from infected leaves and seed

Single-stranded cDNA samples, reverse transcribed from leaf and seed RNA extracts, were used in PCR tests with the pathogen-specific primers to confirm the presence of complimentary PepMV nucleic acid. Electrophoretic analysis of leaf samples indicated the presence of amplicons of the expected size of 986 bp (Figure 3. 1). Reactions were repeated five times using three replicates from a pooled collection of symptomatic leaves for each test. A single prominent band of approximately 986 bp was detected in two out of three tested leaf samples from one of these five replicate tests (Figure 3. 1 A). This band was absent in all control samples including a sample from healthy tomato

leaves. Faint auxiliary bands of smaller lengths were visible in test samples but not in the healthy leaf sample, suggesting that weak cross-hybridisations of primers had occurred with other regions of the PepMV genome. In the other four replicate tests, only faint bands of the expected length were detected or were entirely absent from some leaf samples. This indicated that target fragments were either not amplified from samples or amplified inefficiently. No banding patterns were detected from the third replicate of leaf samples in one test (Figure 3. 1A – Lane 7). A similar result was obtained from PCR tests on single-stranded cDNA extracts from seed putatively infected with PepMV. A single prominent band approximately 986 bp was detected from all three replicate seed samples in this test (Figure 3. 1 B). One of the bands, however, demonstrated a lower intensity compared to the other two replicates. Auxiliary bands of a smaller size were also more prominent in this replicate, suggesting lower target-specific amplification in that reaction. Although these auxiliary bands were not visible from control samples in this particular test (Figure 3. 1 B), similar bands were detected in the negative control samples of the other four reactions performed on seed extracts, indicating potential cross-hybridisation of the primers within the tomato plant genome.

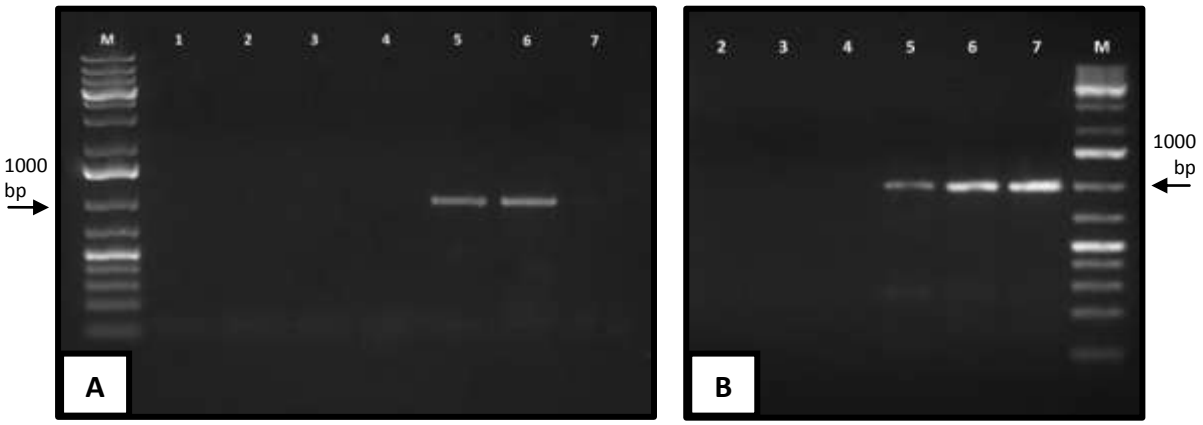

Figure 3. 1 Electrophoretic analysis of pathogen-specific PCR tests on leaf (A) and seed (B) extracts for the detection of PepMV. Positive results were indicated by the presence of a target fragment of roughly 986 bp. Lane M – 5 µg O'GeneRuler 1 kb Plus DNA Ladder (Fermentas EU); lane 1 - non-template negative control (not shown for seed assay); lanes 2 and 3 – Taq and RT- polymerase controls, respectively; lane 4 – negative controls from healthy tomato leaf (A) and seed (B) samples. Lanes 5 – 7 - three replicate samples from symptomatic tomato leaves (A) or infected seed (B).

3.4.1.2. Detection of ToMV from infected leaves and seed

Analysis of resulting amplicons from PCR tests to amplify the target ToMV fragment revealed the presence of a single band of products in both leaf and seed samples (Figure 3. 2). Intense bands of approximately 686 bp were detected from all test samples, indicating a high concentration of the target fragment and, thus, high levels of amplification from template cDNA fragments. Non-specific fragments or bands of different lengths were absent in test samples indicating that diagnostic

primers demonstrated low cross-reactivity within the ToMV genome. Bands representing target or non-specific amplicons were also absent in all control samples, including a sample from non-infected tomato leaves (Figure 3. 2 – Lane 4). Amplification from infected samples was repeatable in replicate tests, producing strong bands for all tested leaf and seed samples suspected to be infected with ToMV.

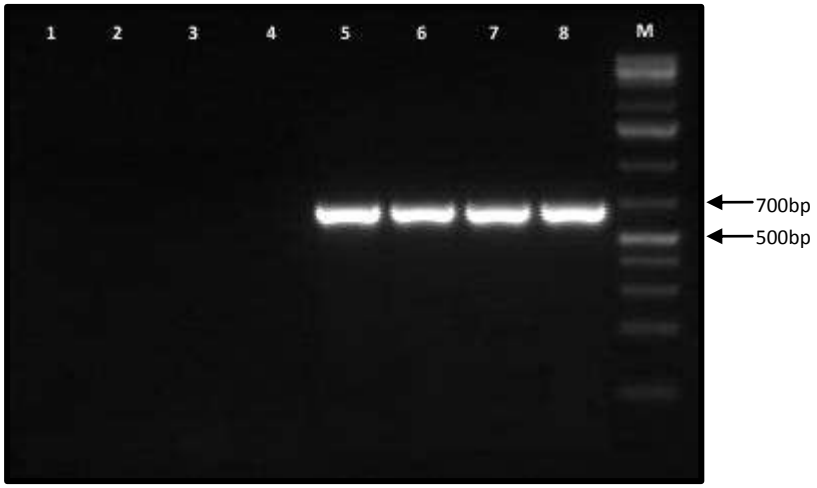

Figure 3. 2 Amplified fragments from PCR tests using pathogen-specific primers for ToMV detection from leaf and seed samples. Positive detection was indicated by the presence of a band of roughly 686 bp. Lane 1 – negative template control; lanes 2 and 3 – Taq and RT- polymerase controls, respectively; lane 4 – non-infected tomato leaf sample; lanes 5 and 6 – infected leaf samples; lane 7 and 8 – infected seed samples; lane M- 5µg O'GeneRuler 1kb Plus DNA Ladder (Fermentas, EU).

3.4.1.3. Confirmation of a mixed PepMV and ToMV infection in leaf samples

To detect both ToMV and PepMV from samples with a mixed infection, separate amplification reactions were performed using diagnostic primer pairs to detect either ToMV or PepMV. Amplicons were detected from two replicate samples of extracts from leaves of plants that were originally infected with PepMV (Figure 3. 3 – Lanes 3 and 4) or with ToMV inoculum (Figure 3. 3 – Lanes 5 and 6). A single prominent band of approximately 986 bp was detected from all leaf samples amplified using Ker1 and PepCP-R primers (Figure 3. 3 - Top), indicating the presence of complimentary PepMV nucleic acid in the samples. Non-specific bands of different lengths were absent in these test samples. No amplified fragments were detected from the template control or the non-infected negative control sample from reactions with the PepMV-specific primer pair. Although amplification of the each of the test samples produced a band of products similar in length, some samples demonstrated weaker band intensity under UV light, for example, lane 3 in Figure 3. 3. In amplification reactions incorporating ToMV-specific primers, ToMV CP-F and Tob-Uni1, the target

fragment of 686 bp was also detected in the same leaf samples (Figure 3. 3 - Bottom). Only a single prominent band was detected in all test samples with no indication of cross-hybridisation. The intensity of detected bands indicated a high concentration of amplified products for three out of four samples tested. The fourth sample only demonstrated a faint band of the expected length in electrophoretic analysis (Figure 3. 3 – Bottom, lane 6), even though this sample was a replicate of the sample in lane 5 in this test. No detectable bands were observed from reactions of the amplification control sample and the negative control sample from non-infected leaves. All tested leaf samples, thus, produced target-specific amplicons with both sets of pathogen-specific primers in individual tests, proving the presence of both of these pathogens.

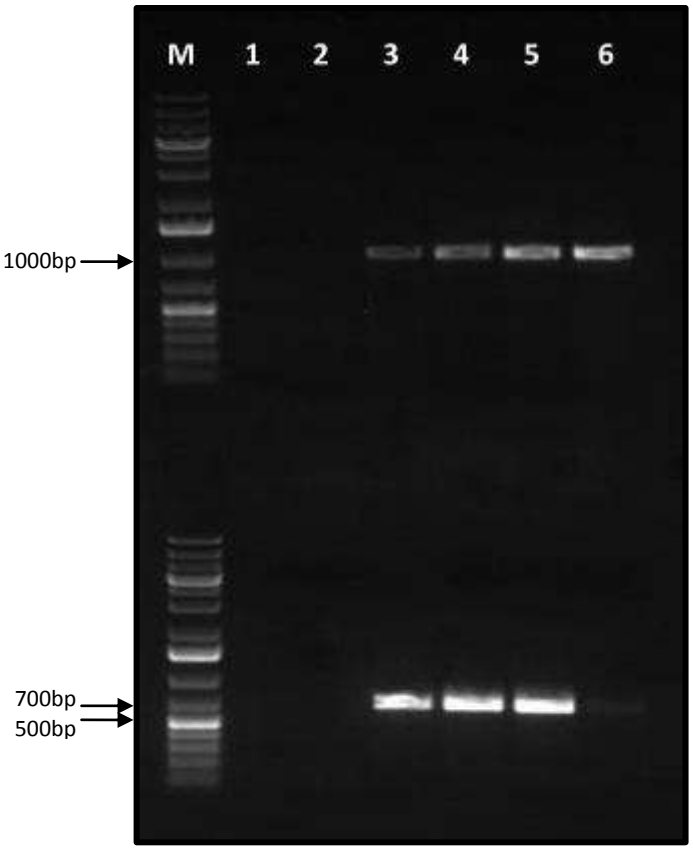

Figure 3. 3 Detection of a mixed viral infection in tomato leaf samples using PepMV-specific primers (top) and ToMV-specific primers (bottom). Production of a fragment approximately 986 bp is indicative for the presence of PepMV and 686 bp, for the presence of ToMV nucleic-acid in samples. Lane M – 5 µg O'GeneRuler 1 kb Plus DNA ladder (Fermentas, EU); lane 1 – non-template negative control; lane 2 – healthy leaf sample; lanes 3 and 4 – samples from tomato plants initially inoculated with PepMV inoculum; lanes 5 and 6 – samples from plants initially inoculated with ToMV inoculum.

3.4.2. Detection of bacterial DNA from infected leaf and seed samples

3.4.2.1. Characterization of bacterial cultures by PCR

To confirm the recovery of Cmm colonies recovered from isolations of stem and leaf samples, primers CmF and CmR were utilised in diagnostic tests for the amplification of a 223 bp target fragment from the Cmm genome. A single, intense band was visualised from the three suspect colonies tested by PCR that corresponded to the expected length of the target fragment (Figure 3.

4). No auxiliary bands were detected from these samples. Non-template and amplification control samples did not produce detectable bands in reactions with Cmm-specific primers, thus confirming the absence of significant primer cross-hybridisation.

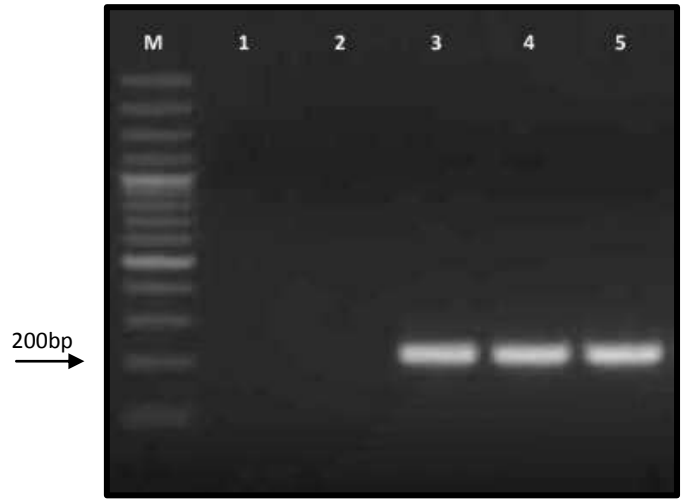

Figure 3. 4 Characterization of suspect Cmm isolates from symptomatic tomato stems. Positive detection of Cmm was indicated by amplification of a pathogen-specific target fragment of approximately 223bp. Lane M – 5 µg O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU); lane 1 – non-template control; lane 2 – polymerase control; lanes 3 – 5 – suspect bacterial isolates.

A strong band of the expected length of 420bp was detected from amplification reactions with Xcv-specific primers for three colonies tested (Figure 3. 5 A). This band was absent in amplification and template control samples. The high intensity of this band indicated a high concentration of target amplicons had been generated by PCR. A second band was also detected in these samples, but this band was faint and smaller than the target fragment, indicating potential cross-hybridisation of diagnostic primers within the Xcv genome. The template control samples from this test (Figure 3. 5 A), and from reactions with the Pst-specific primer pair (Figure 3. 5 B), both produced a faint, low molecular weight band from amplification reactions. These were likely primer dimers formed in the absence of a suitable target template for binding. Successful amplification of a 532 bp target fragment from the Pst genome by PCR using MM5F and MM5R primers facilitated identification of suspect bacterial cultures. An intense, prominent band of approximately 550 bp was detected from all suspect Pst test colonies along with two auxiliary bands, one smaller and one larger than the band of expected length (Figure 3. 5 B). Auxiliary bands were considerably less intense than the prominent central band which was the closest to the expected length of the target amplicon. These three bands were not detected in amplification and non-template control samples. This indicated that suspect cultures could be classified as Pst, but that the primers used in the amplification reactions were

likely cross-hybridising to other regions in the Pst genome, resulting in non-specific amplification of non-targeted regions.

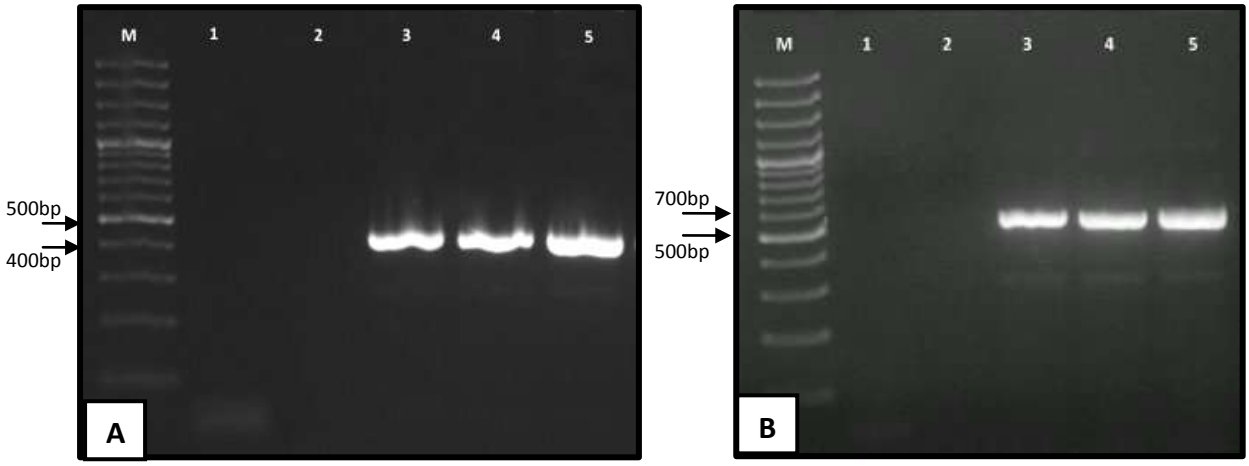

Figure 3. 5 Characterization of respective bacterial isolates using Xcv- (A) and Pst-specific primers (B). A single prominent band of approximately 420 bp identified suspect cultures as Xcv (A). The intense band of roughly 532 bp amplified from suspect cultures confirmed Pst detection. Lane M – 5 µg O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU); lane 1 – amplification control; lane 2 – polymerase control; lanes 3 – 5 – suspect isolates of Xcv (A) or Pst (B).

In bio-PCR assays using seed suspected to be infected with Cmm, Xcv or Pst, colonies were recovered from selective media that demonstrated comparable morphology to known control cultures of each of the targeted pathogens, Cmm, Xcv and Pst. Cell lysates extracted from these suspect colonies were used in amplification reactions to confirm the recovery of each of the bacterial pathogens. Cultures that could be confidently classified as Cmm produced a single band of approximately 223 bp from PCR tests with CmF and CmR primers. In one assay, three isolates recovered on D_2ANX selective medium, and two on SCM selective medium (Chapter 2), were tested in conjunction with known Cmm cultures raised on the respective media. Only one isolate, recovered from SCM medium, produced a detectable band from amplification reactions that was comparable to the band produced by control Cmm cultures (Figure 3. 6). A second culture, isolated from D_2ANX medium, only produced a faint band of a similar length and could not be confidently classified as Cmm. A non-specific band, shorter than the target fragment, was detected from both positive control samples. This band was, however, absent in control samples from other amplification reactions, indicating that mispriming or incomplete target amplification had occurred. In another confirmation test, five isolates that had developed similarly to control Cmm cultures on selective and differential media were all characterized as Cmm-negative by PCR. Assays using these isolates failed to produce a band of the expected length with Cmm-specific primers. Instead, bands of various lengths were detected from amplification reactions and these isolates, therefore, could not be classified as Cmm.

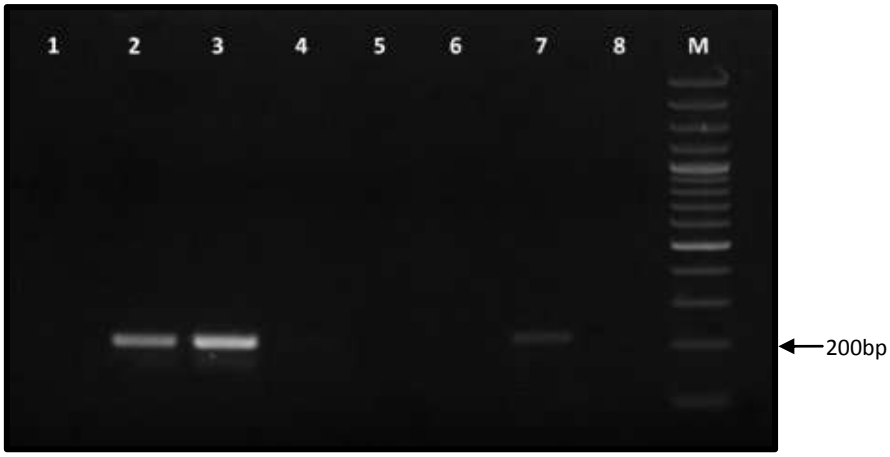

Figure 3. 6 Electrophoretic analysis of bands produced by bio-PCR amplification of cell lysates of suspected Cmm isolates. Characterization was confirmed by the presence of a 223 bp band from amplification assays with pathogen-specific primers. Lane 1 – template control; lane 2 – positive control culture from SCM medium; lane 3 – positive control culture from D_2ANX medium; lanes 4 – 6 – suspect Cmm isolates from D_2ANX medium; lanes 7 and 8 – suspect Cmm isolates from SCM medium; Lane M – 5 µg O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU).

Suspect isolates subjected to bio-PCR tests with the Xcv-specific primer pair were characterized accordingly if detected bands were of the expected length of 420 bp. In one of the repeat tests from seed homogenates, five isolates were selected for PCR testing that had demonstrated the closest morphological similarities to Xcv on selective and differential media. Of these, four produced a single detectable band similar to that produced by the positive Xcv control culture (Figure 3. 7) in pathogen-specific amplification reactions. Bands produced by test isolates were of varying intensities, but all were roughly 400 bp, indicating detection of Xcv. No non-specific fragments were amplified from the positive control or test samples. Despite the uniformity in detection (Figure 3. 7),

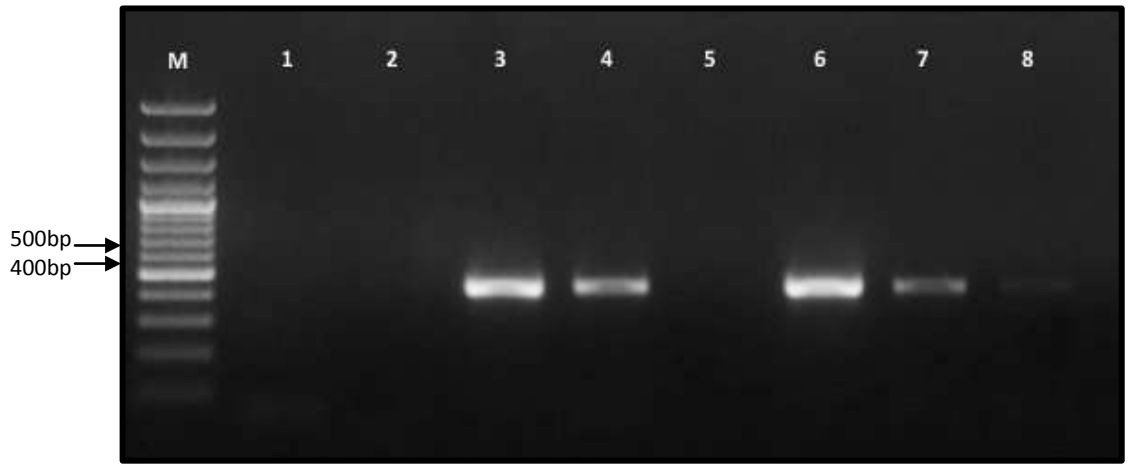

Figure 3. 7 Characterization of suspect Xcv isolates recovered from selective media assays with seed homogenates by bio-PCR. Positive classification was indicated by the production of a fragment of roughly 420 bp. Lane M – 5 µg O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU); lane 1 – template control; lane 2 – negative control (DNA from non-infected tomato seeds); lane 3 – positive Xcv control; lanes 4 – 8 – suspect Xcv isolates.

bands produced from tested isolates in a parallel confirmation test were either absent, or were of different lengths compared to the target fragment produced by reference Xcv cultures, indicating non-specific primer binding.

Characterization of isolates suspected to be Pst, was based on the production of a 532 bp target fragment from amplification reactions with Pst-specific primers. In one of the replicate tests, eight suspect cultures that demonstrated similar morphologies to Pst control cultures on selective media were selected for further confirmation by bio-PCR. A strong prominent band of approximately 550bp was observed from all tested isolates and was comparable to the band detected from the Pst reference culture (Figure 3. 8). No auxiliary bands were detected from any of these samples or from template and negative control samples. Similar to Xcv and Cmm confirmation tests, however, other replicate amplification reactions using other morphologically similar isolates either demonstrated non-specific amplification, or bands were absent. This indicated that other factors may affect recovery and amplification of target pathogens from colonies or seed homogenates.

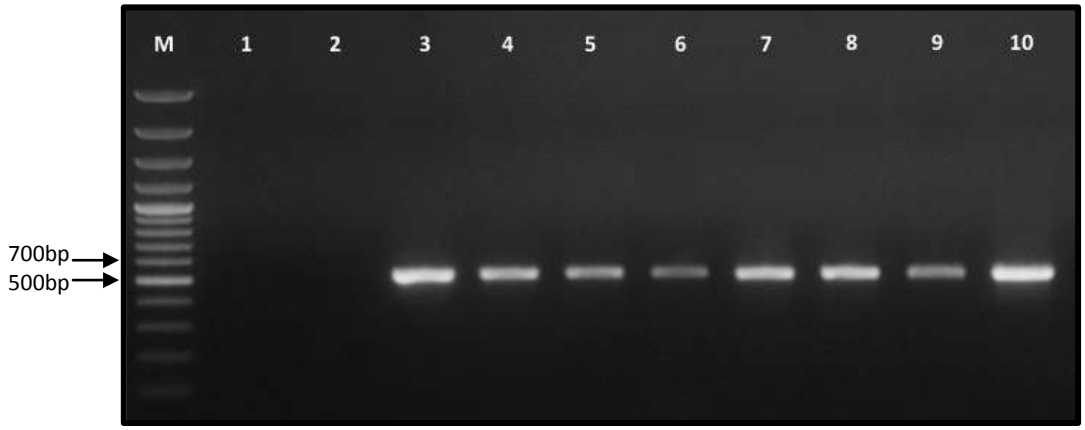

Figure 3. 8 Confirming presumptive classification of suspect isolates as Pst by amplification of a 532 bp target fragment using pathogen-specific primers. Lane M – 5 µg O'GeneRuler 1kb Plus DNA Ladder (Fermentas, EU); lane 1 – template control; lane 2 – negative control (DNA from non-infected seed); lane 3 – positive Pst control; lanes 4 – 10 – suspect bacterial isolates.

3.4.2.2. Detection of bacterial DNA from leaf and seed extracts

The yield of DNA from column-based extraction procedures was considerably lower than RNA column-based extractions from both leaf and seed samples. The average yield of DNA was approximately 150 ng/µℓ from leaf samples of 85 – 100 mg, and only 31 ng/µℓ from seed samples of approximately 50 mg. The quality of DNA extracted, as determined by 260/280 absorbance ratios, was greater than 1.6 and, thus, acceptable for use in PCR tests. The purity of the extracted DNA, however, shown by low 260/230 ratios, indicated the presence of many contaminants. Nevertheless, all samples were tested using pathogen-specific primers in conventional and robust PCR reactions to

detect target pathogens from symptomatic leaf samples or putatively infected seed. Extraction procedures required a preparation time of approximately 60 minutes to obtain purified DNA from leaf and seed samples, excluding additional incubations for improved dissolution of extracted DNA into elution buffers.

As with the classification of suspect bacterial isolates, detection of Cmm from leaf and seed extracts was dependent on the amplification of a 223 bp target fragment from samples tested by diagnostic PCR. Using the DreamTaq amplification system, a single, prominent band with a corresponding length to the target was detected from all three replicates of both seed and leaf extracts in electrophoretic analysis (Figure 3. 9). Extracts from seed, however, demonstrated lower band intensity compared to leaf extracts, indicating a higher concentration of amplified fragments were produced in tests with leaf versus seed samples. All detected bands were comparable to the band produced by the reference culture of Cmm. Secondary, non-specific fragments were absent in both positive control and test samples. Amplification, template and negative control samples did not produce any detectable bands from reactions with Cmm-specific primers. Target fragments were also amplified in repeat reactions with leaf and seed extracts, showing the repeatability of detection and confirming the presence of Cmm in symptomatic leaves and infected seed.

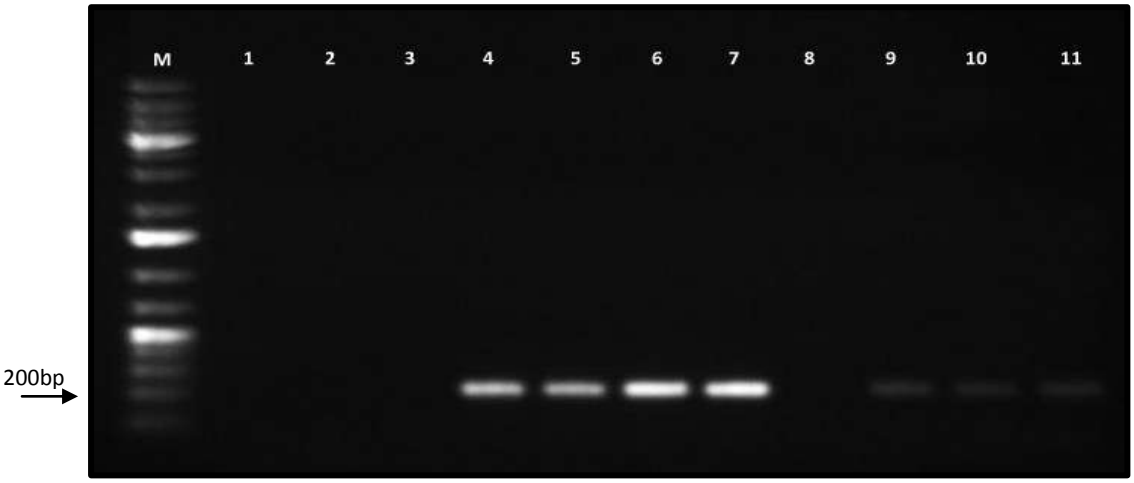

Figure 3. 9 Detection of Cmm from DNA extracts of tomato leaves and seed suspected to be infected with the target pathogen. All tested samples produced a band of approximately 223 bp which was similar to the band produced by a positive control sample of Cmm. Lane M – 5 µg/µℓ O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU); lane 1 – template control; lane 2 – amplification control; lane 3 – negative control sample from healthy tomato leaves; lane 4 – positive control culture of Cmm; lanes 5 – 7 – leaf extracts with putative Cmm infection; lane 8 – negative control sample from non-infected tomato seed; lanes 9 – 11 – seed extracts with putative Cmm infection.

Initial reactions of DNA extracts from leaf and seed for the detection of Xcv using the DreamTaq amplification system yielded poor and inconsistent amplification and thus the KAPA Robust

Detection system was adopted to improve detection. In these reactions, a single prominent band of 420 bp was produced from all leaf and seed samples tested, which was of the expected length and comparable to the band produced from reactions with the reference culture of Xcv (Figure 3. 10). This indicated the presence of Xcv in the two replicate samples of leaf and four replicate samples of seed tested. Varying band intensities were observed from leaf samples, but all samples from seed demonstrated low band intensities indicating a lower accumulation of target amplicons. These positive bands, however, were not present in repeated detection tests of the leaf and seed extracts using the same primers. Although target fragments were absent in amplification, template and negative control samples, the latter from healthy tomato leaves and seed (Figure 3. 10), all samples produced a band of low molecular weight, as did all test samples. These were likely primer dimers and were also observed in repeated detection tests for Xcv from the leaf and seed extracts.

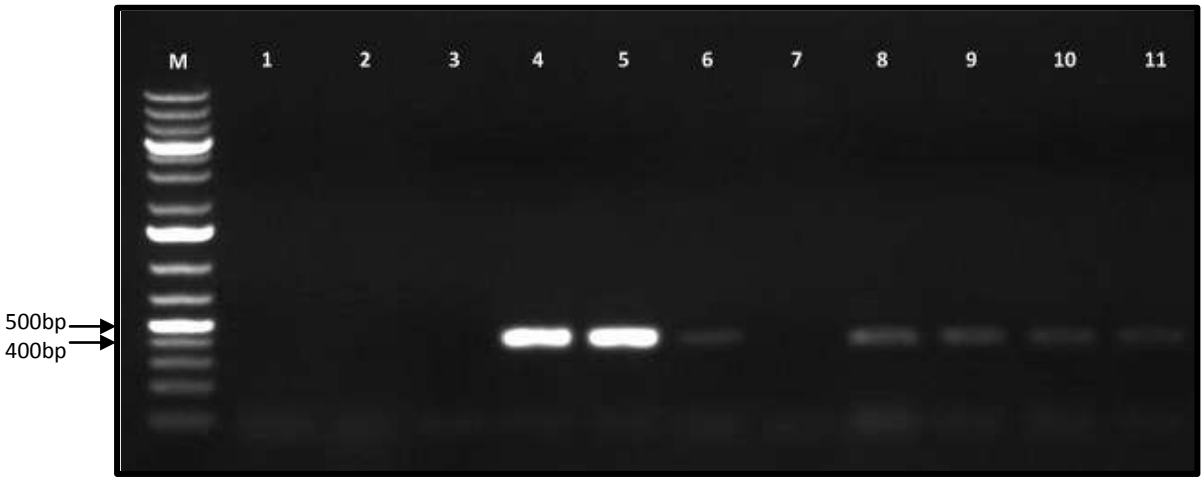

Figure 3. 10 Confirmation of the presence of Xcv in tomato leaf and seed extracts by the production of a 420 bp fragment in PCR tests with Xcv-specific primers. Lane M – 5 µg/µℓ O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU); lane 1 – template control; lane 2 – amplification control; lane 3 – negative control sample from healthy tomato leaves; lane 4 – positive control culture of Xcv; lanes 5 and 6 – samples from putatively infected leaves; lane 7 – negative control sample from non-infected tomato seed; lanes 8 – 11 – test samples from putatively Xcv-infected seed.

Pathogen-specific primers, MM5F and MM5R, demonstrated excellent capacity for the detection of Pst from leaf and seed extracts using the DreamTaq amplification system. Strong, prominent bands of the length expected for the target fragment (532 bp), were detected from three replicates of both seed and leaf extracts (Figure 3. 11). These bands demonstrated good intensity under UV light, indicating high concentrations of amplicons, and were all comparable to the band produced by a reference culture of Pst. No non-specific amplification was detected from test samples, or positive and negative control samples, though faint bands, likely primer dimers, were visible from the seed samples. The results observed were similar to replicate PCR tests on leaf and seed extracts, indicating that Pst was definitely present in symptomatic leaves and infected seed.

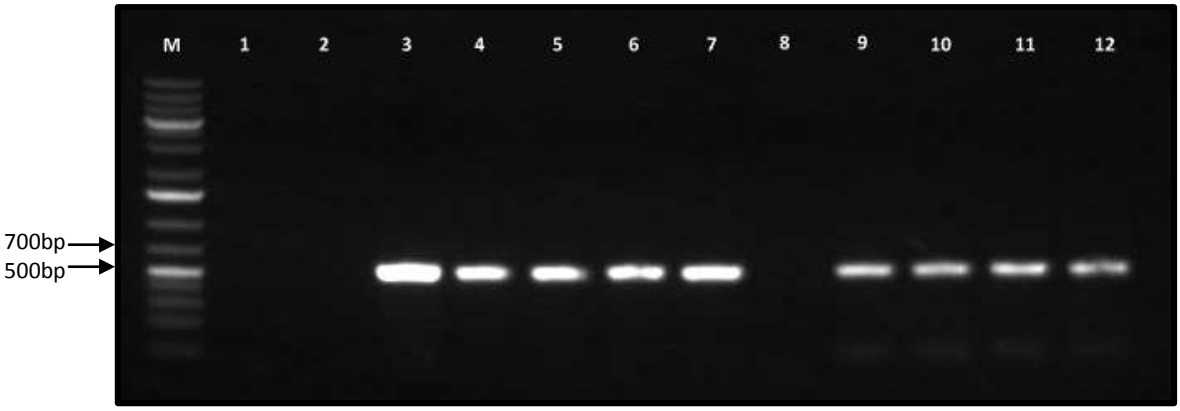

Figure 3. 11 Positive detection of Pst from all leaf and seed extracts by amplification of a 532 bp fragment using pathogen-specific primers. Lane M – 5 µg/µℓ O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU); lane 1 – template control; lane 2 – amplification control; lane 3 – positive control of Pst; lanes 4 – 7 – extracts from symptomatic tomato leaves; lane 8 – negative control from healthy tomato leaves; lanes 9 – 12 – extracts from putatively Pst-infected tomato seed samples.

3.4.2.3. Detection of bacterial DNA from seed homogenates

Direct validation of the presence of bacterial pathogens from seed homogenates differed from other assays as DNA was extracted directly from seeds soaked and homogenized in buffer and used in diagnostic amplification reactions. Extracts from seed with a putative Cmm infection produced a strong band of approximately 200 bp from reactions with CmF and CmR primers (Figure 3. 12). A faint, secondary band of roughly 500 bp was also detected from tested samples which was absent from the reaction with a reference Cmm culture. A similar band, observed from reactions with an extract of DNA from healthy tomato seeds, indicated that the primer pair may have cross-hybridised to a region in the tomato genome. No other non-specific bands were detected from control or test samples. The production of a prominent fragment from the five replicate samples tested, that was similar to that of positive control samples, and close to the expected length of the target fragment, confirmed the detection of Cmm from these seed homogenates (Figure 3. 12).

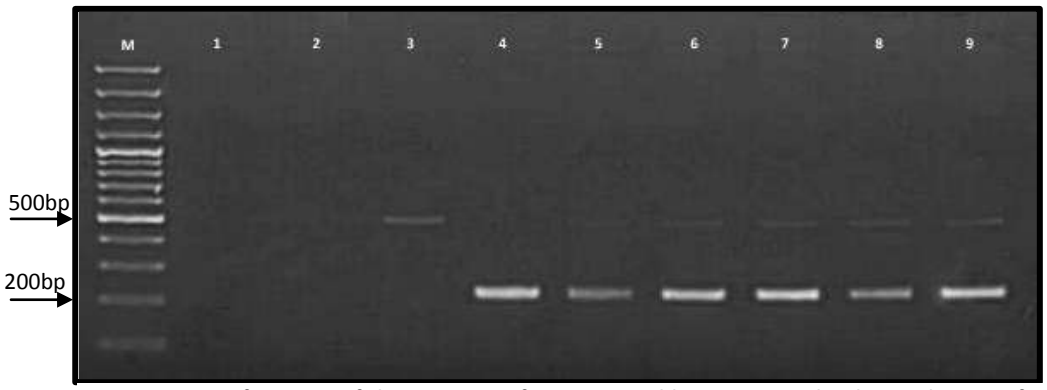

Figure 3. 12 Confirmation of the presence of Cmm in seed homogenates by the production of a band of approximately 223bp from PCR assays with Cmm-specific primers. Lane M – 5 µg/µℓ O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU); lane 1 – template control; lane 2 – amplification control; lane 3 – negative control from healthy tomato seed; lane 4 – positive control sample of Cmm; lanes 5 – 9 – replicate samples from seed homogenates suspected to be infected with Cmm.

In two separate tests to detect Xcv from seed homogenates (Figure 3. 13 A and B), three replicate DNA samples of seed homogenates produced a prominent band which was approximately 400 bp and comparable to the band produced by the reference culture of Xcv. Bands of primer dimers were detected from the three test samples in both replicate assays. Only faint non-specific fragments were detectable from the three test samples, however, more intense non-specific bands were observed in the positive and negative control samples of one of the tests (Figure 3. 13 A). Despite this, consistent detection of the target band from amplification reactions of test samples, confirmed the presence of Xcv in seed homogenates tested.

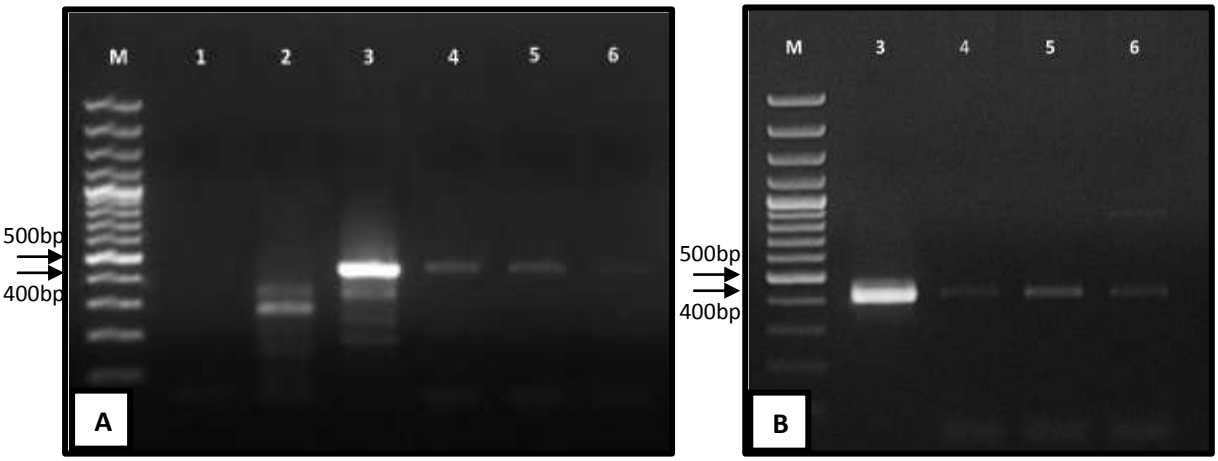

Figure 3. 13 Detection of Xcv from seed homogenates for amplification reactions using Xcv-specific primers. Target-specific bands of approximately 420 bp were detected from positive controls and replicate samples in two separate assays of seed homogenates (A and B). Lane M – 5 µg/µℓ O'GeneRuler 1 kb DNA Ladder (Fermentas, EU); lane 1 – template control; lane 2 – negative control from healthy tomato seed; lane 3 – positive Xcv control culture; lanes 4 – 6 – extracts from seed homogenates suspected to be infected with Xcv.

The presence of Pst cells in seed homogenates was analysed using pathogen-specific primers that targeted a fragment of 532 bp from the Pst genome. Though a band of this length was detected from all three replicate samples tested (Figure 3. 14), in two of these samples, the target band was very faint, indicating a low concentration of the amplicon. Faint, non-specific fragments were also detected from the positive control samples as well as test samples, although these were of different lengths compared to the target fragment. Cross-hybridisation of primers to produce non-specific fragments was also observed in replicate tests with suspect Pst-infected seed homogenates. These fragments, however, were absent from template and negative control samples. Despite the weak band intensity of some tested samples, prominent bands were comparable to the band produced by the reference Pst culture proving the presence of Pst in seed homogenates tested (Figure 3. 14).

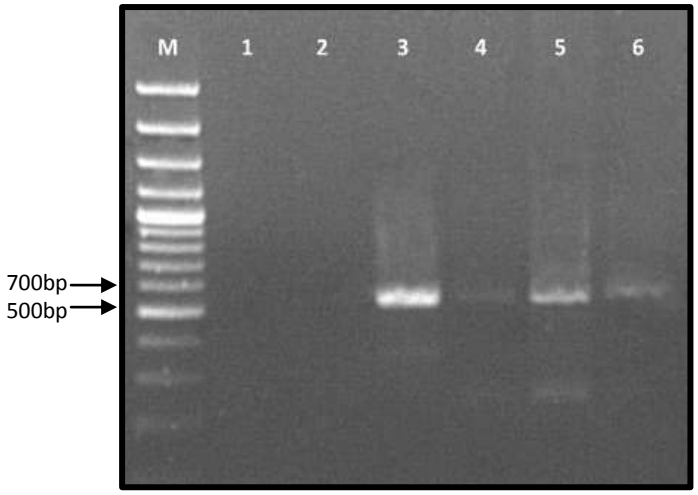

Figure 3. 14 PCR-based detection of Pst from seed homogenates with a suspected infection by amplification of a 532 bp target fragment. Lane M – 5 µg/µℓ O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU); lane 1 – template control; lane 2 – negative control from non-infected tomato seed samples; lane 3 -positive Pst culture; lanes 4 – 6 suspect Pst-infected extracts from seed homogenates.

3.4.3. Rapid detection of bacterial causal agents from symptomatic leaves

A separate extraction and detection system, the KAPA Express Extract system, was evaluated on plants symptomatic for bacterial infections. Confirmation of the presence of target pathogens from leaf samples was indicated by the production of a band similar in length to the target fragment for respective pathogens, as described previously. The best results for pathogen detection using this system was demonstrated by leaf samples suspected to be infected with Pst (Figure 3. 15). Electrophoretic analysis of amplified products indicated a single, intense band of approximately 530bp from both replicate samples, confirming detection of Pst. Non-specific fragments were not detected from test samples and control samples in these reactions. Though faint bands were detected from Cmm and Xcv reactions, results were confusing and inconsistent because these bands

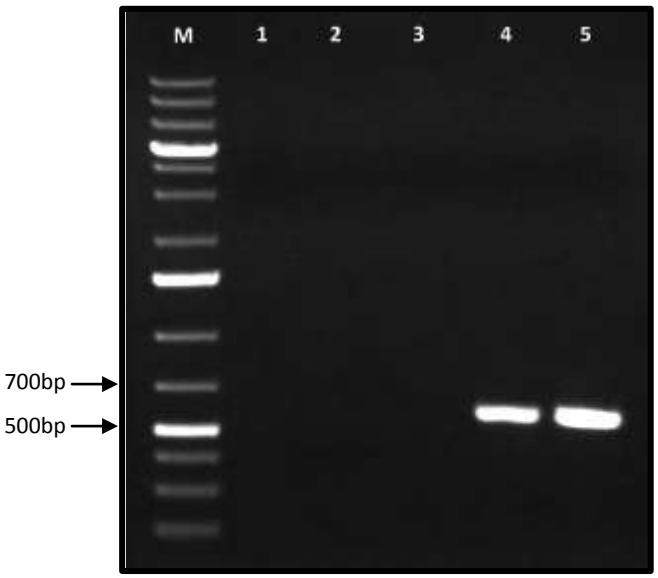

Figure 3. 15 Detection of Pst from symptomatic leaf samples by the production of a 532bp target fragment from PCR tests using Pst-specific primers. Lane M - 5µg/µℓ O'GeneRuler 1kb Plus DNA Ladder (Fermentas, EU); lane 1 – template control; lanes 2 and 3 – negative controls from healthy leaf samples; lanes 4 and 5 – samples from symptomatic tomato leaves.

were slightly shorter than the fragment expected. Results were also not repeatable. Assays were, therefore, considered unreliable for the detection of Cmm and Xcv. This system however, proved to be highly rapid and effective for detection and identification of Pst from tomato leaf samples, with results obtainable within four hours.

3.5. Discussion

Molecular-based strategies for phytopathogen detection demonstrate a significant improvement in time, labour, specificity and sensitivity compared to other available methods, by directly targeting the genetic sequences of the pathogen (Meng et al., 2004; Leite et al., 1995; Park et al., 2009). Despite these stated advantages, adoption of PCR into standard detection procedures can be limited by complex primer design and reaction optimisations required to facilitate highly specific target detection (Mansilla et al., 2003; Sevİk and Kose-tohumcu, 2011). Complicated nucleic acid extraction procedures and limited high-throughput potential of conventional PCR also hamper high-throughput testing of samples. This study sought to evaluate previously developed diagnostic protocols based on conventional PCR, for the detection of significant seed-borne pathogens of tomato, and investigate the limitations preventing these tests from adoption into standard detection protocols. Some recently developed molecular strategies were incorporated into the diagnostic protocols tested which are known to improve the efficiency and sensitivity of extraction and amplification. These included the use of automated column-based extraction procedures, robust readymix amplification systems as well as direct PCR from bacterial cultures or host extracts. Diagnostic assays were evaluated for the capacity to rapidly, repeatedly and explicitly detect target pathogens from pure bacterial cultures and leaf or seed samples.

The development of automated, column-based nucleic-acid extraction procedures have drastically reduced the laborious and technical nature of RNA and DNA extraction from plant samples (Lievens and Thomma, 2005; Lopez et al., 2007). The process employs specialised lysis and binding columns that separate nucleic-acid from cellular components by quick, simple steps with a set of buffers formulated for high recovery of DNA or RNA and the elimination of cellular contaminants. Two commercial kits adopted in this study for RNA extraction (RNeasy-Qiagen, Germany) and DNA extraction (DNeasy – Qiagen, Germany), respectively, have been employed successfully by numerous other studies for extractions from tomato leaves and seeds for pathogen detection (Bach et al., 2003; Da Silva et al., 2008; Ling, 2007, 2008; Lopez et al., 2007; Verhoeven et al., 2003). By extracting the highest possible yield of RNA or DNA from samples (Sholberg et al., 2005), low levels of

pathogen infection may still be detected. This is especially applicable for detecting seed-borne pathogens, which characteristically inhabit tomato seed at low levels (Córdoba-Sellés et al., 2007; Ling, 2008; Milijašević et al., 2007; Nome et al., n.d.).

The yield and purity of RNA and DNA extracted in this study using column-based extraction, as observed from collected absorbance data, was variable among samples and seemed to be dependent on a variety of factors such as sample homogenization, ambient temperature and the nature of host samples – leaf or seed (Bilgin et al., 2009). To facilitate detection of target pathogens from symptomatic leaves, samples had to be extracted from areas on the plant where the pathogen is known to accumulate to establish an infection. Prior knowledge of the target pathogen is thus necessary to prevent misdiagnosis (Schaad and Frederick, 2002). The size of seed samples used for extractions in this study was approximately half the mass of leaf samples. This was to improve the yield of DNA and RNA from seed, as the presence of seed residues is known to block extraction columns and reduce nucleic acid recovery (Bilgin et al., 2009; Lopez et al., 2007). Despite this change, recovery of total RNA or DNA from seed samples was 10 to 100 fold lower than yields from leaves. Low purity of extracts from seed confirmed the presence of contaminants in eluted suspensions of RNA and DNA which could not be precipitated or dissolved by column-based extraction procedures. Average yields of RNA from leaf samples were high, and of good purity. The RNA extraction system was more efficient than the column-based system for DNA extraction as similar sized samples recovered a 10-fold difference in yield. The low purity of leaf and seed extracts was probably from high concentrations of inhibitory components in extracts, like ions, polyphenols, polysaccharides and proteins which are known to occur in leaves and, especially, seeds of plants (Bilgin et al., 2009; Diez and Nuez, 2006; Lopez et al., 2007). Various chemicals can be added during extraction procedures to precipitate or dissolve these components before nucleic acid is eluted (Bilgin et al. 2009; Doyle and Doyle 1987; Momeni et al. 2011; Palacio-Bielsa et al. 2009). Column-based extraction methods however, follow set protocols with pre-formulated buffers and, therefore, optimisation of these procedures is restricted. Crude extraction methods are easy to optimise but are unfavourable due to the lengthy and technical nature of the methods (Dovas et al., 2004; Lopez et al., 2007; Schaad et al., 1995).

Generation of cDNA from RNA templates of viral infected extracts was facilitated by three different primers depending on the targeted virus. For detection of PepMV from leaf and seed samples, Oligo dT_{18} primers were selected, as PepMV is a single-stranded RNA virus of the *Potexvirus* genus which is characterised by a poly(A)-tail at the 3' end of the genome (Aguilar et al., 2002). By targeting this tail,

priming was restricted to templates of the targeted virus and mRNA transcripts in host samples, reducing background amplification. This tail is absent in species of the *Tobamovirus* genus, however (Chapman, 1998; Lartey et al., 1996; Lewandowski, 2000), and thus priming of ToMV RNA was performed using non-specific random nonamer primers. These bind to any genetic sequence available and thus amplified all host and target RNA from samples. Though random nonamers could have been used to amplify sequence fragments of both PepMV and ToMV in leaf extracts with a mixed infection, random hexamer primers were employed. The lower energy required for binding a shorter primer could allow for more efficient amplification and, thus, a higher total amplicon generation in RT-PCR reactions. This assumption was not proven in this study.

Pathogen-specific primers for viral detection and identification were selected based on their ability to detect all known strains of the target pathogen (Dovas et al., 2004; Ling et al., 2007; Van der Vlugt et al., 2002), but discriminate related and non-target organisms or plant material that may have any homology. Primers selected for PepMV and ToMV detection targeted amplification of the entire coat protein gene from each viral genome. The coat protein is crucial for viral infection and transmission and is, therefore, largely conserved between related strains. This region was thus considered suitable for species-specific detection and has been well characterized in previous studies (Hanssen et al., 2008; Letschert et al., 2002; Mumford and Metcalfe, 2001; Vinayarani et al., 2011). This was especially important for PepMV detection, as the taxonomic classification of the virus in samples tested in this study was still unknown. The primers selected for detection of ToMV were originally designed for use in semi-nested PCR assays (Letschert et al., 2002), as the reverse primer was complimentary to species of *Tobamovirus* subgroup 1, while the forward primer was specific for ToMV. This facilitates species-specific distinction in samples with mixed infections of related species (Dovas et al., 2004; Jacobi et al., 1998; Lee et al., 2003; Letschert et al., 2002; Vinayarani et al., 2011). The diagnostic primers for ToMV and PepMV used in this study, were validated extensively by *in silico* analysis before adoption into diagnostic PCR protocols, to ensure primers specifically bound to target fragments from published sequence data with low or no homology to each other, the host genome or other regions within the pathogen genome (Alfaro-Fernandez et al., 2009; Mansilla et al., 2003).

In this study, PepMV was detected from leaf and seed extracts using the selected diagnostic primers in amplification reactions. Minor cross-hybridisations of the primer pair were detected which may have been due to other homologous regions within the PepMV genome or the tomato genome. These were not detrimental to accurate diagnosis however, as target fragments were more

prominent than secondary bands in successful PCR tests. The diagnostic PCR for PepMV demonstrated low repeatability in replicate tests. Target fragments amplified from leaf and seed extracts were faint or absent in about 50% of reactions even though samples were from the same pooled collection of infected leaves or seed. Incorporation of a suitable positive control sample into diagnostic reactions would have validated the amplification and diagnostic capacity of PepMV PCR tests and eliminated the effect of external factors such as user error or non-viable reaction components. Suitable control samples were, however, not accessible as this virus was under quarantine status in South Africa before it was reported in this dissertation (Carmichael et al. 2011; Chapter 4). Assumptions were thus based on negative control and test samples. Low specificity of primer binding was not responsible for variable amplification results as some samples produced strong prominent bands from amplification reactions. The variable intensities of detected bands was instead attributed to varying concentrations of pathogen templates in replicate samples. Incomplete or insufficient generation of cDNA templates from RT-PCR would also contribute to low concentrations of pathogen-specific amplicons. Detection using this method should, therefore, be optimised, for example by optimising cycling conditions or amplification mix components (Lievens and Thomma, 2005; Widmer et al., 1998), to increase the stringency and specificity of detection. Nevertheless, this assay enabled detection and identification of PepMV from some tomato leaf and seed samples.

Diagnostic tests to detect ToMV from leaf and seed samples were consistent and repeatable using the pathogen-specific primers. Reliable production of target-specific bands from all samples tested proved that ToMV particles were present in both leaf and seed samples. Primers were also highly specific for the target pathogen and did not demonstrate any significant cross-hybridisation with control and test samples analysed (Alfaro-Fernandez et al., 2009; Letschert et al., 2002). The high intensity of detected bands from leaf and seed samples reflected a high concentration of the target pathogen in leaf and seed samples. Accurate quantification, however, requires comparative analysis of test samples with standard dilutions of a positive control sample (Meng et al., 2004), which was not performed in this study. Low concentrations of RNA extracted from seed samples did not adversely affect successful amplification or detection of ToMV in this study. This diagnostic test was highly specific and reliable for ToMV detection, demonstrating high potential for use in routine diagnostic protocols.

The development of a mixed infection of ToMV and PepMV in symptomatic tomato leaves was confirmed in this study using pathogen-specific PCR tests for the respective viruses. Previously

proven positive for a mixed infection using pathogen-specific DAS-ELISA tests (Hadas et al. 2004; ISF 2009b, 2009c), diagnostic PCR tests validated these observations. A modified 'fast' amplification system was selected for viral amplification and detection. The system has previously been shown to reduce non-specific priming, thereby increasing the sensitivity of reactions (McManus and Jones, 1995), and facilitating target-specific primer binding. Accordingly, detected bands were uniform and clear with no auxiliary bands amplified from test or control samples. Amplification reactions with leaf extracts using both PepMV- and ToMV-specific primers produced target-specific fragments from all samples proving that both viruses could be detected from a single sample. The intensity of these bands provided an estimation of the concentration of each virus in the sample (Letschert et al., 2002), though quantification would have been more accurate if quantifiable positive control samples were included (Meng et al., 2004). The limited accuracy of visual quantification was exposed by low band production by one of the samples tested using ToMV-specific primers. This sample produced strong bands in reactions with PepMV-specific primers but not with those for ToMV detection. This contradicted the results of a repeat sample tested that produced strong bands with both sets of primers. Varied band intensity in this test was attributed to factors such as the presence of substances that inhibited or reduced the efficiency of amplification (McManus and Jones, 1995; Meng et al., 2004; Widmer et al., 1998).

The specificity of detection of both viruses from leaf samples using the same amplification system supports the development of a multiplex diagnostic system using these primer pairs. Reactions resulted in prominent positive bands with no indication of cross-hybridisation under the same cycling conditions, facilitating specific and accurate diagnosis. Target amplicons were also distinct in length to allow for adequate resolution by electrophoresis if a mixed infection occurs in test samples (Lievens and Thomma, 2005). Multiplex PCR tests are more attractive than conventional PCR as the former reduce the time, labour and cost required to detect more than one pathogen from a single sample (Alfaro-Fernandez et al. 2009; Boonham et al. 2000; Ozdemir 2009). However, multiplex assays require extensive optimisation and complex reaction mixes to detect each pathogen adequately. This multiplex detection system would be extremely valuable for screening seed samples, as tests need not be repeated to prove the absence of PepMV and ToMV, reducing the amount of valuable seed sacrificed for health testing. This method will be developed in future research with the addition of more primers to facilitate strain differentiation for each virus species.

Detection of the three targeted bacterial pathogens from pure cultures, and leaf or seed samples was performed using three selected sets of diagnostic primers that demonstrated different levels of

taxonomic specificity and are applicable for different types of diagnostic tests. The primer pair selected for Cmm detection was designed to target a region of the ITS gene of subspecies of *C. michiganensis* (Bach et al., 2003). As no other subspecies of Cm infects tomato (Bach et al., 2003; Louws et al., 1998), amplification reactions using these primers were specific enough to detect all strains of Cmm in this study, yet are sufficiently conserved to detect any potentially emerging strains or related subspecies infecting tomato samples. Previous tests using these primers have reported successful detection, even in reactions like real-time and multiplex PCR (Bach et al., 2003). The high similarity of symptoms induced by Xcv and Pst infections on tomato support the need for highly discriminatory and specific PCR diagnostic tests for these pathogens from infected plant samples (Cuppels et al., 2006). Primer pairs used in this study targeted different genes of the Hrp cluster which encode pathogenicity determinants for Xcv and Pst (Obradovic et al., 2004; Zaccardelli et al., 2005). Targeting this region for pathogen identification eliminates false positive detection of saprophytes or opportunistic bacteria that may be present in plant samples and do not encode these genes (Obradovic et al., 2004). Diagnostic primers for detection of Xcv were designed to detect all species and/or subspecies causing bacterial spot of tomato (Obradovic et al., 2004). This reduced the risk of misdiagnosing infected samples as the taxonomic classification of the Xcv isolate used in this study was unknown. Primers selected for the detection of Pst, however, were designed to be highly pathovar-specific (Zaccardelli et al., 2005). This primer pair was strongly complimentary to target fragments of the Pst genome but did not bind to similar regions of a close relative, *P. syringae* pv. *syringae* (results not shown), which is also pathogenic on tomato.

Bacterial cultures recovered from leaf and seed samples were characterised using the bio-PCR technique (Maes, 1993; Schaad et al., 1995) and selected pathogen-specific primers. This was quick, simple and did not require complex DNA extraction procedures or hazardous chemicals. In this study, bacterial colonies were added directly to PCR mixes or were boiled briefly prior to amplification reactions, the latter to lyse cells and expose bacterial DNA (Cuppels et al., 2006; Pelludat et al., 2009). Unlike viral detection tests or other methods of conventional PCR, bio-PCR provides an indication of the viability of detected pathogens, as only viable organisms are recovered on growth media inoculated with leaf or seed extracts, and are selected for amplification reactions (Schaad et al., 1995). This is crucial for evaluating the risk of infected seed or plant material for disease transmission, particularly for seed that have been treated to kill any seed-borne pathogens. Culturing also enriches recovered bacteria such that even those present at low concentrations on plant material (EPPO/CABI, 2005; Hadas et al., 2005; Milijašević et al., 2007; Nome et al.; Schaad and Frederick, 2002; Schaad et al., 1995) may still be detected. As diagnostic tests in this study were

performed directly on bacterial cultures, the tests validated the efficacy of selected primers while directly characterising bacteria with comparable colony morphology to that of colonies of reference cultures. The distinction between presumptive positive and negative isolates on growth media is made before colonies were sampled for diagnostic PCR tests. This reduced the presence of competing saprophytes or non-target organisms in the reactions and reducing the risk of misdiagnosing infected samples.

The three selected primer pairs for Cmm, Xcv or Pst detection were capable of detecting each target pathogen from the tested bacterial cultures that were recovered from symptomatic stem and leaf samples. Many saprophytes, opportunistic pathogens and non-virulent related organisms may inhabit host plants and seed, some of which may be morphologically similar to targets on growth media (Goszczynska and Serfontein, 1998), which can complicate accurate detection and diagnosis. Pathogen-specific fragments were detected for at least one replicate isolate presumptively classified as Cmm, Xcv or Pst by previous characterization tests. Primers for Cmm detection proved to be highly specific, generating a single band from positive control and positive test cultures. The production of faint secondary bands detected in reactions of Pst and Xcv- specific primers indicated that cycling conditions were probably not stringent enough for targeted binding (Widmer et al., 1998). As a result, primers could have bound non-specifically to other regions in the pathogen genome. Incorporating positive control samples is, thus, essential in diagnostic PCR to facilitate more reliable diagnoses. Extracting DNA from bacterial cultures may also improve the sensitivity of detection compared to the whole cell templates used for detection in this study (Cuppels et al., 2006; De Leon et al., 2006; Milijašević et al., 2007). The DreamTaq amplification system proved to be suitable for facilitating detection and identification of tested cultures in this study. This system is inexpensive and versatile, though PCR cycling conditions must be optimised to ensure repeatable, specific detection in routine tests.

In this study, bio-PCR assays were performed using selective media to recover suspect bacterial cultures from seed homogenates. Selective media are used in standard diagnostic protocols for detection of bacteria from tomato seed (ISF, 2008a, 2009c), but tests are lengthy and laborious, and classification of bacteria based on colony morphology can be inaccurate and unreliable (Kuflu and Cuppels, 1997; Lievens et al., 2003; McGuire et al., 1986). Targeting nucleic acid of the recovered colonies using bio-PCR was more specific for characterization than assays based only on selective media (Maes, 1993; Schaad et al., 1995). Cell lysates of these bacterial cultures were diluted to reduce the concentration of potential PCR inhibitors derived from bacterial cell components, which

may negatively affect amplification reactions (De Leon et al., 2008; McManus and Jones, 1995). The KAPA Robust amplification system is considered more suitable for sensitive and specific target detection than the DreamTaq system. This 'hot-start' system employed a readymix containing all the required components for reactions (Lopez et al., 2007) at optimised concentrations for amplification. These mixes simplified reaction set-up and eliminated variables associated with reaction components for target amplification. In the readymix, the incorporated polymerase enzyme is maintained in an inactive state until thermal activation during amplification cycling. This prevents random binding of primers at temperatures below optimum annealing temperature for primer/target binding, theoretically reducing the risk of non-specific fragment production (Lopez et al., 2007; McManus and Jones, 1995). Amplification and, thus, disease diagnosis was faster using this system than using the DreamTaq system, as optimised conditions and the robust nature of the incorporated polymerase facilitated rapid primer binding and template extension.

Although at least one bacterial colony isolated from respective seed homogenate samples was confirmed as Cmm, Xcv or Pst using the bio-PCR test, results were largely variable and not always repeatable between replicate tests. This was attributed to the poor discrimination capacity of selective media for recovery of target bacteria, with the result that false-positive isolates were included in diagnostic PCR tests. Detection by bio-PCR is especially affected if targets are present at low concentrations, for example, on assayed seed (De Leon et al., 2006, 2008; Milijašević et al., 2007). Generation of faint target bands or bands with variable intensities in the Cmm and Xcv diagnostic tests demonstrated this, although poor dissemination of bacterial lysates in diluted samples might also explain poor amplification of bacterial templates. The diagnostic assay using media and subsequent bio-PCR tests for Pst detection was the most reliable of the three assays for classification of suspect bacterial cultures in this study, with reactions producing a single amplicon of the expected length in all replicate assays. The variable success of detection using selective media/bio-PCR assays in this study may be influenced by a number of factors other than those mentioned. These include composition of the selective media (Fatmi and Schaad, 1988; McGuire et al., 1986), culturing conditions, cycling conditions (Widmer et al., 1998), visual discrimination among recovered targets and non-target pathogens on media (Maes, 1993), and the presence of growth or amplification inhibitors (Du Toit et al., 2005; Meng et al., 2004).

The production of a detectable band of the expected length from PCR with suspect bacterial cultures was a more reliable means of characterization than diagnosis based on colony morphology (Maes, 1993; Schaad et al., 1995; Schaad and Frederick, 2002). False-positives from selective media assays

were demonstrated by Bio-PCR as amplicons of the target length were absent. Despite the rapid extraction and amplification procedures used to speed up bacterial characterization compared to the standard methods, culturing seed homogenates on selective media prior to PCT tests lengthened the time required to obtain results, making this method less suitable for routine seed-health testing than conventional PCR. A number of studies have supported this observation (De Leon et al., 2006, 2008; McManus and Jones, 1995; Milijašević et al., 2007), that improved detection can be achieved by extracting DNA directly from host plants and eliminating the use of media assays.

Extractions of DNA were thus performed directly from using infected leaf and seed samples and extracts were used in the same pathogen-specific amplification reactions to validate the presence of Cmm, Xcv or Pst. All bacteria were positively detected from DNA extracts, although band intensities were variable, even between replicate samples from the same pooled collections of leaves or seed. Relative band intensities from seed extracts were consistently lower than those from leaf extracts in all diagnostic tests. This may be explained by a low concentration of seed-borne pathogens on host seed (Bach et al., 2003; Hadas et al., 2005; Hausbeck et al., 1999; Sevİk and Kose-tohumcu, 2011; Xu, 2010), and/or the presence of inhibitors from seed. Combined with poor recovery of DNA from seed by extraction procedures in this study, the starting concentration of DNA templates was probably low translating into low banding intensity. Nevertheless, even the faint bands detected from amplification reactions were of the expected length and proved that targeted pathogens were present in seed samples. The Pst diagnostic test again proved to be superior to tests for the other two bacterial pathogens, suggesting that the pathovar-specific Pst primers were adequately sensitive for detection of Pst from various samples. Detection of Cmm using DNA extracts from leaf and seed and the Cmm-specific primers was also specific and repeatable.

Despite using the KAPA amplification system to improve detection of Xcv from leaf and seed DNA extracts, some replicate samples still demonstrated low amplicon production. Nevertheless, target fragments of the expected length were detectable from both leaf and seed extracts. The presence of both strong and faint bands from replicate samples of the same pooled collection of leaves and seed indicated that other contributing factors had affected amplification of Xcv fragments, besides a low starting concentration of pathogen templates. Plant samples may contain various components that are extracted along with nucleic acids during isolation procedures, and may block the activity of polymerase enzymes during amplification reactions (Cuppels et al., 2006; Du Toit et al., 2005; Meng et al., 2004; Ozdemir, 2009). These PCR inhibitors include, for example, metal ions, oxidases, polysaccharides, proteins and polyphenols that are distributed unevenly in plant tissue and may be

up-regulated in the presence of pathogens. The inhibitors are often variable in concentration and are difficult to detect, but may drastically reduce amplification efficiency potentially causing false negative detection (Cuppels et al., 2006; Palacio-Bielsa et al., 2009). Diluting DNA extracts (Du Toit et al., 2005; Meng et al., 2004) and using robust PCR amplification systems usually reduce the effect of these inhibitors, but may still affect the detection of targeted pathogens. In Xcv diagnostic tests, a short fragment of low molecular weight was also detected in all test and control samples. As this band was detected in the amplification control sample, which did not contain DNA polymerase, these bands were suspected to be primer dimers formed by the binding of forward and reverse primers in the absence of sufficient template DNA (Lopez et al., 2007). The formation of primer dimers also indicates that homology exists between forward and reverse primers. This is not ideal for diagnostic tests as primers might bind to each other rather than the target DNA, facilitating false negative results. These bands were faint for samples that exhibited high banding intensities as well as positive control samples, and variable band intensities were, therefore, associated with low template availability in this study rather than significant homology between the diagnostic primers.

Problematic amplification of DNA from some seed samples and the unreliability of media/bio-PCR assays from seed homogenates prompted the evaluation of a third method for the detection of target bacteria from putatively infected seed. In these tests, DNA was extracted, using column-based methods, directly from homogenates of seed soaked in buffer overnight. This method was expected to demonstrate superior detection capacity to media/bio-PCR assays as results were obtainable within 48 hours and extracted DNA served as a better template for specific detection than direct testing of bacterial colonies or cell lysates (De Leon et al., 2006, 2008; McManus and Jones, 1995; Milijašević et al., 2007; Schaad et al., 1995). Incubating larger pooled samples of seed in buffer overnight was also expected to increase the recovery of target pathogens from seed rather than whole seed extractions from samples of only 50mg. Seed contaminants and PCR inhibitors were still expected to be recovered though and thus the KAPA Robust amplification system was selected for diagnostic PCR of DNA extracts. Using this method, all bacteria were positively detected from samples tested using pathogen-specific amplification. The best detection occurred with Cmm, where replicate samples produced a positive band. This validated previous tests where minimal and inconsistent detection of Cmm-specific fragments was obtained from seed samples. Weak cross-hybridisations of the primer with tomato DNA was detected, however. While strong positive bands only confirmed the detection of Xcv and Pst in approximately 60% of samples tested, faint bands were still of the expected length indicating positive detection of the target pathogen. The presence of primer dimers and faint non-specific bands in both Pst and Xcv detection tests, which were also

present in positive and negative control samples, suggested that cycling conditions needed to be optimised to improve the stringency of amplification reactions for detection. A low concentration of bacterial DNA and the presence of PCR inhibitors in extracts may have contributed to poor amplification of targets, forcing the robust polymerase to tolerate mismatches in non-complimentary sequence fragments.

Although diagnosis directly from DNA extracts of leaf and seed, as well as seed homogenates was more accurate and reliable than bio-PCR, the presence of PCR inhibitors from plant samples severely impacted the efficiency of amplification (Ozdemir, 2009; Palacio-Bielsa et al., 2009). Optimising reaction conditions such as annealing temperatures, length of amplification cycles, employing robust enzymes and adding chemicals to reduce the concentration of PCR inhibitor, may eliminate the variability in these reactions (Bilgin et al., 2009; Lopez et al., 2007; Widmer et al., 1998). Once optimised, diagnostic tests based on DNA extracts from seed homogenates would be favoured for bacterial detection from seed as the larger sample size, combined with DNA extraction procedures, could make this technique more sensitive than others investigated in this study.

The rapid detection of the KAPA Express Extract and Robust system makes these kits attractive for seed health testing and diagnostics. Even providing an initial indication of pathogen infection, rapid diagnostic tests could facilitate quicker implementation of curative strategies (Cuppels et al., 2006; Njambere et al., 2011) and assist trade of seed and transplants among countries. Diagnostic tests performed in this study analysed leaf discs from plants with symptoms of Cmm, Xcv or Pst infection. Tests were also performed on seed, but poor detection was achieved. To extract DNA from leaf samples an optimised ready-mix was employed with short incubation periods which halved the sample preparation time compared to column-based and crude extraction procedures. Excellent detection of Pst-specific fragments was obtained from KAPA robust amplification reactions using samples symptomatic for Pst infection. Detection of Cmm and Xcv however, was sometimes inaccurate, as bands were of the incorrect length or too faint. The success of this system is highly dependent on the selection of symptomatic plant material to ensure the pathogen is isolate, which is likely why Pst was more successfully detected than the other two pathogens. Symptoms of Pst are easily visible and localised, making targeted sampling simple (Jones et al., 1997; Uppalapati et al., 2008). The xylem-inhibiting Cmm, however, can be harder to isolate. Including a more intensive homogenization step could facilitate extractions from stem samples which is where highest concentrations of Cmm accumulate during infection (Gartemann, 2003; EPPO/CABI, 2005). Soaking samples in buffer prior to enzyme-mediated extractions would also improve isolation of pathogens

from host tissue. Lowering annealing temperatures for primer/target binding may decrease the stringency of binding, thereby, improving detection of target pathogens (Widmer et al., 1998). Although assays require optimisation before they can be used reliably for detection of these bacterial pathogens, its simplicity and rapid turn-around make the assays well suited to phytopathological applications.

In this study, detection of the five seed-borne pathogens of tomato from leaf and seed samples was partially successful using conventional PCR-based diagnostic assays. Each of the primer pairs tested detected complimentary pathogen DNA or cDNA from plant extracts and facilitated amplification of the target fragment thereby validating the presence of the target pathogen. Diagnosis of bacterial cultures was more reliable and accurate than media based assays. While also limited, PCR-based methods higher sensitivity and specificity for viral and bacterial detection than both conventional and serological detection methods (De Leon et al., 2008; Gutierrez-Aguirre et al., 2009; Kuflu and Cuppels, 1997; Njambere et al., 2011; Schaad and Frederick, 2002) as pathogens could be detected using much lower starting samples of leaf or seed. Results were obtainable within 48 hours and, with some methods, within 24 hours. The modifications incorporated into the methods in this study to make PCR more routine-friendly demonstrated variable success. Automated and rapid extraction systems reduced the time, labour and technical requirements of crude extraction methods (Lievens and Thomma, 2005; Lopez et al., 2007; Palacio-Bielsa et al., 2009) which is valuable for high-throughput testing of numerous samples. The methods, however, could not eliminate PCR inhibitors and contaminants that affected the amplification of target pathogen fragments. High recovery of good quality DNA or RNA is a minimum requirement for adequate pathogen detection (Lopez et al., 2007; Sholberg et al., 2005), however, yields from seed could not be optimised using the column-based detection techniques. While cheaper amplification systems are more suitable for routine diagnostic assays, more robust, and therefore, expensive amplification systems improve the consistency of detection, especially from plant samples in which high concentrations of contaminants may be present.

The variability in the amplification and intensity of bands produced from amplification reactions for all target pathogens except ToMV illustrate the importance of control samples in diagnostic PCR tests (Alfaro-Fernandez et al., 2009; Lievens and Thomma, 2005). These include positive and negative control samples to validate target versus non-specific bands as well as an amplification control sample, containing no polymerase enzymes, to evaluate inter and intra-primer homology or the presence of background signal. Although the targeted pathogens were present in leaf and seed

samples tested, secondary infections, the presence of saprophytes or non-target pathogens, and the presence of homologous regions of the tomato genome increase the risk of false positive and negative results (McManus and Jones, 1995; Lievens and Thomma, 2005; Wilson et al., 2002). Sequence variations or nucleic acid mismatches, between the stains of pathogens used in this study versus those used to validate primers previously, could have also affected amplification (Cuppels et al., 2006; Mansilla et al., 2003). Diagnosis based solely on the visual detection of a band of the expected length by electrophoresis is not completely accurate (Call et al., 2003; Schaad and Frederick, 2002). Additional tests like southern blotting or sequencing would be more more reliable for confirming bands as those of target pathogens. These are, however, labour intensive, technically challenging and too expensive for use in routine diagnostics.

A major drawback of PCR detection is the dependence on knowledge or suspicion of the target pathogen to enable choice of the appropriate diagnostic primers for detection (Schaad and Frederick, 2002). If the target is unknown, for example when testing asymptomatic seed in seed health assays, amplification reactions to validate the absence or presence of all pathogens of concern would have to be performed which could be costly and labour-intensive. The different applications of conventional PCR for pathogen detection investigated in this study were more successful for detection of some target pathogens than others. Formulating multiplex amplification reactions using these diagnostic test to detect all target pathogens would, thus, be a complicated task (Agindotan and Perry, 2007; Lievens and Thomma, 2005; Ozdemir, 2009). The viral diagnostic methods investigated in this study demonstrated high potential for incorporation into a multiplex test and will be optimised in future studies. Proving the viability of pathogens detected from host samples was only possible using bio-PCR (Chitarra et al., 2000; Maes, 1993; Schaad et al., 1995). This is necessary for analysing the health of seed or transplants for trade. In other studies, PCR has been coupled with techniques such as immunomagnetic separation or bioassays (Chitra et al., 1999; De Leon et al., 2006, 2008; EPPO/CABI, 2005), which further complicate the detection assays. Accurate quantification of pathogen infection is also limited when using conventional PCR (Du Toit et al., 2005; Meng et al., 2004) and is better indicated by quantifiable techniques like real-time PCR (Bach et al., 2003; Boonham et al., 2000; Gutierrez-Aguirre et al., 2009; Ling et al., 2007; Ozdemir, 2009). Conventional PCR is typically cheaper than other molecular alternatives, and have become the first standard molecular detection procedure for some seed borne pathogens (Asma, 2005; EPPO/CABI, 2005). More sensitive alternatives with multiplex detection capacity are expected to replace these in the near future when associated costs and technical limitations of the latter become reduced.

Chapter 4 Phylogenetic Characterization of a South African Isolate of *Pepino mosaic virus*

4.1. Abstract

In the past decade, the single stranded RNA virus of the genus *Potexvirus*, *Pepino mosaic virus* (PepMV), has become established as a significant seed-borne pathogen of tomato, inducing severe symptoms on fruit and drastically decreasing market value. Outbreaks of this disease have been reported in Europe, North America, Asia and Australia, but thus far, PepMV has not affected the tomato industry in sub-Saharan Africa. However, tomato plants demonstrating symptoms characteristic of PepMV infection were detected in production areas in Limpopo Province, South Africa, during the winter growing season of 2008. Viral particles were isolated from leaf samples and the causal agent was characterised with PepMV-specific PCR, sequencing of the viral coat protein, and phylogenetic comparisons to reference sequences of PepMV isolates and related species. Particles of PepMV were consistently detected from symptomatic leaf samples by the production of a 986 bp amplicon, which represented the coat protein gene of the target pathogen. The coat protein sequence demonstrated 79 – 99% nucleotide similarity with more than 250 sequence accessions of PepMV in a BLAST analysis. In further phylogenetic analysis, the South African isolate clustered with coat protein sequences of CH2 and US2 PepMV isolates, indicating strong evolutionary relationships with these isolates. Characterization of other regions of the viral genome is required to confirm the identity of this isolate. This study is the first report of PepMV in South Africa. The presence of the virus in this country is most likely due to the importation and cultivation of infected tomato seed, but further research is necessary to establish the source and current distribution of PepMV in this country. Control measures should be implemented without delay to limit the impact that this virus may have on the South African tomato industry.

4.2. Introduction

The emergence of *Pepino mosaic virus* (PepMV) on tomato was first reported in 1999 in the Netherlands (Van der Vlugt et al., 2000, 2002). This virus was originally isolated from infected pepino plants, *Solanum muricatum*, in Peru in 1974 (Jones et al., 1980) and was characterized by mild mosaic and leaf distortion symptoms on these pepino, or pear melon crops, in the Andean region (Lopez et al., 2005). In tomato however, infection was linked to more severe symptom display and economically destructive marbling, flaming and open fruit symptoms on tomato fruit (Hanssen et al.,

2009; Hanssen and Thomma, 2010; Spence et al., 2006; Van der Vlugt et al., 2002). This virus was responsible for successive epidemics in tomato production areas almost simultaneously in Europe and North America (Verhoeven et al., 2003). The disease spread rapidly between countries and caused massive economic losses due the decrease in fruit quality and quantity. The spread of the virus was attributed to its highly virulent, infectious and stable nature, as the virus is transmitted locally by mechanical contact and on contaminated equipment (Davino et al., 2008; Hanssen and Thomma, 2010; Mumford and Metcalfe, 2001; Ling, 2008; Schwarz et al., 2010), and long distances on infected seed (Córdoba-Sellés et al., 2007; Hanssen et al., 2010b; Ling, 2007, 2008). As a result, this destructive virus has been reported in tomato production areas across the world including America (Ling, 2007; Maroon-Lango et al., 2005), Europe (Aguilar et al., 2002; Cotillon et al., 2002; Davino et al., 2008; Efthimiou et al., 2010; Hasiów et al., 2008a; Hasiów-Jaroszewska et al., 2010; Pagán et al., 2006; Mumford and Metcalfe, 2001; Van der Vlugt et al., 2000; Verhoeven et al., 2003), Australia, Asia and the Middle-East (reviewed by Hanssen et al., 2010).

Forming part of the *Potexvirus* genus (Aguilar et al., 2002; Van der Vlugt et al., 2002), PepMV is characterised by a 6410 nt, single-stranded RNA genome, with five open reading frames (ORF) and a 3' poly (A) tail. Putative functions of each of the ORFs of the viral genome have been inferred by well-characterised related viruses and the identification of functional domains within these coding regions (Aguilar et al., 2002). The first ORF encodes a RNA-dependent RNA polymerase (RdRp) for viral replication, ORF 5 encodes the viral coat protein, and a triple gene block (TGB), covering ORF2 – ORF4, is suspected to play a role in cell-to-cell movement (Aguilar et al., 2002; Cotillon et al., 2002; Lopez et al., 2005; Mumford and Metcalfe, 2001). Although the multifunctional nature of viral proteins restricts genetic variability in the genomes, RNA viruses are more prone to recombination events, silent mutations and amino acid changes than pathogens with proof-reading properties (Pagán et al., 2006). These events are thought to be responsible for the shift in host range of PepMV to included tomato as well as the large genome diversity that occurs among isolates of PepMV. In less than 10 years, five different genotypes of PepMV have been isolated from tomato production areas. These include the original Peruvian isolate from pepino (LP), the European isolate (EU) (Aguilar et al., 2002; Cotillon et al., 2002; Lopez et al., 2005; Van der Vlugt et al., 2002), the Chilean isolate (CH2) (Hanssen et al., 2008; Ling, 2007; Ling et al., 2008) and two genotypes isolated from North America, US1/CH1 and US2 (Maroon-Lango et al., 2005; Ling, 2007). The four isolates characterized from infected tomato plants demonstrate a maximum of 95% nucleotide similarity to the original Peruvian isolate (Lopez et al., 2005; Van der Vlugt et al., 2002; Verhoeven et al., 2003) and 79 – 90% similarity among isolates which supported the taxonomic separation of PepMV isolates

into five distinct groups of isolates. Nucleotide identity among these isolates is significantly greater than the closest *Potexvirus* relatives, *Narcissus mosaic virus* (NMV) and *Cymbidium mosaic virus* (CymMV), which demonstrate a maximum sequence identity of 69% to all isolates of PepMV (Cotillon et al., 2002; Mumford and Metcalfe, 2001).

The induction of symptoms by PepMV on tomato hosts is affected by climatic conditions such as temperatures greater than 30°C and high light intensity, which have been associated with mild symptom display (Aguilar et al., 2002; Fakhro et al., 2011; Hanssen et al., 2008, 2009; Pagán et al., 2006; Verhoeven et al., 2003). Isolates also differ in the severity of symptoms induced and the speed that particles accumulate in diseased hosts, which can complicate disease diagnosis. Symptoms range from mild to severe on tomato plants, with the most destructive symptoms reported to be associated with an aggressive isolate of CH2 (Hanssen et al., 2009). Detection and identification of mixed infections with more than one PepMV isolate, as well as the emergence of recombinants of these isolates (Hanssen et al., 2008; Ling et al., 2008; Pagán et al., 2006), have been linked with more severe infections on tomato. This virus has also been reported to co-infect tomato plants with other viruses (Davino et al. 2008), resulting in reduced growth and yields.

The original source of PepMV has not been determined, which affects the efficacy of implemented control methods and preventative approaches. Due to close geographical position to Peru, infected seeds from tomato production areas in Chile are thought to have been the source for US isolates due to the high genomic homology between the US and Chilean isolates (Ling, 2007). Both the US and Chilean isolates, however, diverge from Peruvian and European isolates, the latter pair demonstrating 95% nucleotide identity. This suggests that two independent mutational shifts have occurred (Hanssen and Thomma, 2010; Ling, 2007; Ling et al., 2008). The emergence, spread and co-infection of PepMV isolates in tomato plants emphasises the shortcomings of current detection techniques and control strategies. Even if an isolate has previously been detected and characterized in an area, the risk of introducing other isolates or the emergence of new strains still remains a concern (Gutierrez-Aguirre et al., 2009; Ling, 2007; Pagán et al., 2006). Stringent seed and transplant testing, as well as frequent characterization studies on symptomatic plants, is important for understanding the impact and threat of this virus.

Detection of PepMV from infected tomato seed and leaf samples by various serological and molecular-based techniques has been reported (Alfaro-Fernandez et al., 2009; Gutierrez-Aguirre et al., 2009; ISF, 2009a; Hasiów et al., 2008b; Ling, 2007; Mansilla et al., 2003), but the best method for

sub-species characterization of PepMV isolates is by cloning and sequencing (Van der Vlugt et al., 2002). Using these methods and genus-specific PCR amplification, Van der Vlugt et al. (2002) were able to identify tomato as a host of PepMV for the first time based on sequence homology with the original Peruvian isolate (Jones et al. 1980), and comparison to another tomato-infecting *Potexvirus*, *Potato virus x* (PVX). Sequencing an entire viral genome for diagnostics or phylogenetic studies is costly, time-consuming, and the large data sets generated can be complicated to assemble (Adams et al., 2009; Dovas et al., 2004). Partial genomic sequencing, however, can be sufficient to identify and characterise pathogens detected from plant samples, especially with sequence databases like GenBank (http://www.ncbi.nlm.nih.gov/genbank/) which currently contains more than 200 partial and full genomic sequences of PepMV alone. By sequencing a region known to be informative for the targeted pathogen, an amplified fragment can be used to detect and characterize a pathogen from host samples.

In this study, infected leaf samples obtained from tomato production areas in Limpopo Province, South Africa, were characterized by sequencing a PCR product amplified from RNA extracts of leaf samples. In previous investigations, the causal agent from these samples induced symptoms characteristic of PepMV in susceptible tomato seedlings and demonstrated a positive reaction for PepMV in conventional and serological assays. Therefore, to confirm the presence of PepMV particles from the original source, species-specific primers were employed to amplify the coat protein region of the PepMV genome and the amplified products sequenced. Sequences were subjected to phylogenetic characterization against published sequences of PepMV. Emergence of this destructive virus in South Africa represents a threat to the local tomato industry and may necessitate rapid implementation of control strategies to restrict distribution and spread of PepMV in this country.

4.3. Materials and methods

4.3.1. Viral source

Leaf samples were taken from plants demonstrating mosaic and bubbling symptoms from tomato production areas in the Mooketsi region, Limpopo Province, South Africa, in 2008. Positive detection of viable PepMV particles infecting these samples was demonstrated in a previous chapter of this study (Chapter 2, 2.4.2) by DAS-ELISA, with antibodies specific to the coat protein of PepMV, and by

bioassays on *Nicotiana benthamiana*. Infected leaf samples and leaf samples of healthy *S. lycopersicum* cv. Rooikhaki plants were stored at -70°C until use.

4.3.2. Extraction and amplification of viral RNA

Total RNA was extracted from 85 – 100 mg samples from two healthy and four infected tomato leaf samples using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Samples were homogenised with liquid nitrogen and a pestle in 2 mℓ Eppendorf tubes (Eppendorf, Germany). Modifications to the suggested protocol were performed as previously described (Chapter 3, 3.3.1). Briefly, RNA bound to RNeasy spin columns was dissolved by two replicate washes using 50 µℓ of preheated (65°C), nuclease-free H_2O, incubated for five minutes at room temperature and subsequently eluted at 10 000 rpm for one minute using a MiniSpin Plus centrifuge (Eppendorf, Germany). Analysis of RNA concentration and purity was performed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA) and extracts were stored at -70°C.

Synthesis of single-stranded cDNA was performed using the Improm-II Reverse Transcriptase system (Promega, USA) with 1 µg of total RNA from each replicate sample. For primer-template binding, RNA templates were incubated with 0.1 µM Oligo dT_{18} primers (Fermentas, EU) and 0.5U RNasin - RNase Inhibitor (Promega, USA) for 10 minutes at 70°C, followed by 1 minute incubation on ice. The remaining reaction components were added to a final volume of 20.6 µℓ and included 0.5 mM dNTPs (Fermentas, EU), 1X ImProm-II Reaction buffer, 3 mM $MgCℓ_2$ and 1 µℓ of ImpromII Reverse Transcriptase Enzyme (Promega, USA). Reaction conditions involved a single cycle of primer annealing at 25°C for 10 minutes, cDNA synthesis at 42°C for 60 minutes and a final inactivation step at 70°C for 10 minutes. Besides negative control samples of RNA extracts from healthy leaves, other control samples included a non-template control (no template added to amplification reactions), and an amplification control (no reverse transcriptase added to the reaction mixture). Amplification of viral nucleic acid from cDNA samples was performed by conventional PCR using 5 µℓ of the cDNA template and the DreamTaq Amplification system (Fermentas, EU). Primers selected for PepMV-specific amplification were Ker1 (Hanssen et al., 2009) and PepCP-R (Mumford and Metcalfe 2001), which flanked a 986 nt sequence fragment including the coat protein gene from the PepMV genome. Reaction mixtures contained 0.4 µM of the respective primers, 10 mM dNTPs (Fermentas, EU), 10X DreamTaq Buffer with added $MgCℓ_2$, and 1.25U of DreamTaq Polymerase to a final volume of 50 µℓ. Amplicons were generated under cycling conditions of: denaturation at 95°C for five minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57.5°C for 30 seconds, extension at 72°C for one minute; and a final extension step for 10 minutes at 72°C. In addition to control samples

from the RT-PCR assay, a template control, non-polymerase control (with no DreamTaq polymerase added) and a DNA control (using 5 µℓ of a total nucleic acid extract from infected leaf samples) were included in the assays.

Products from amplification reactions were resolved by electrophoresis on a 2% agarose gel (Bioline, USA) in 1X TAE Buffer (40 mM Tris-Acetate and 1mM EDTA) stained with 0.1 mg/mℓ ethidium bromide (Sigma Life Sciences, USA). Individual wells were each loaded with 5 µℓ of PCR product combined with 1.7X Orange Loading Dye (Fermentas, EU) and separated in conjunction with 5 µg of O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU, Appendix A), for 50 minutes at 70V using a PowerPac Basic (Bio-Rad, Singapore). Amplified fragments were detected under UV light using the BioDoc-IT System (UVP Inc., USA). The presence of a 986 bp fragment from test samples was considered an indication of positive detection of PepMV nucleic acid.

4.3.3. Cloning, transformation and sequencing of suspect PepMV PCR products

Four positive PCR products, detected from extracts of infected leaf samples, were cloned into the pTZ57R/T vector (Figure 4. 1) using the InsTA Cloning Kit (Fermentas, EU). Each ligation reaction of 30 µℓ contained 4 µℓ of the PCR product from the respective replicate sample, 0.165 µg of the uncircularised vector, 30X ligation buffer and 5U T4 DNA Ligase. A positive control ligation reaction was also included in assays, which contained 4 µℓ or 168 µg of a 953 bp control PCR fragment that was provided with the cloning kit. Reaction mixes were incubated overnight at 22°C and stored at -20°C until use in transformation procedures.

Competent cells for transformation were prepared using 100 µℓ of *Escherichia coli* strain DH5α cells which were inoculated into 10 mℓ of sterile Luria-Bertani (LB) broth (1% w/v Bactotryptone, 5% w/v Bacto yeast extract and 10% w/v NaCℓ). The inoculated broth was incubated at 37°C overnight on a SPO-MP15 rotary shaker (Labcom, South Africa) at 150 rpm. Cultures were then split into two 50 mℓ centrifuge tubes, each with 40 mℓ of fresh LB broth, and cultured for a further 90 minutes to ensure that the majority of bacteria had entered the log phase as indicated by an OD_{600} of between 0.3 and 0.5. Cells were concentrated in a 5810R centrifuge (Eppendorf, Germany) for 15 minutes at 6000 rpm at 4°C, and pellets were resuspended in 5 mℓ PIPES buffer (100 mmol/ℓ $CaCl_2$, 10 mmol/ℓ PIPES-HCℓ, 15% glycerol [pH 7.0]). Suspensions were incubated on ice for 20 minutes and then concentrated again at 3500 rpm for 10 minutes at 4°C. Resulting pellets were resuspended in 2 mℓ of PIPES buffer and stored at -70°C for up to six months.

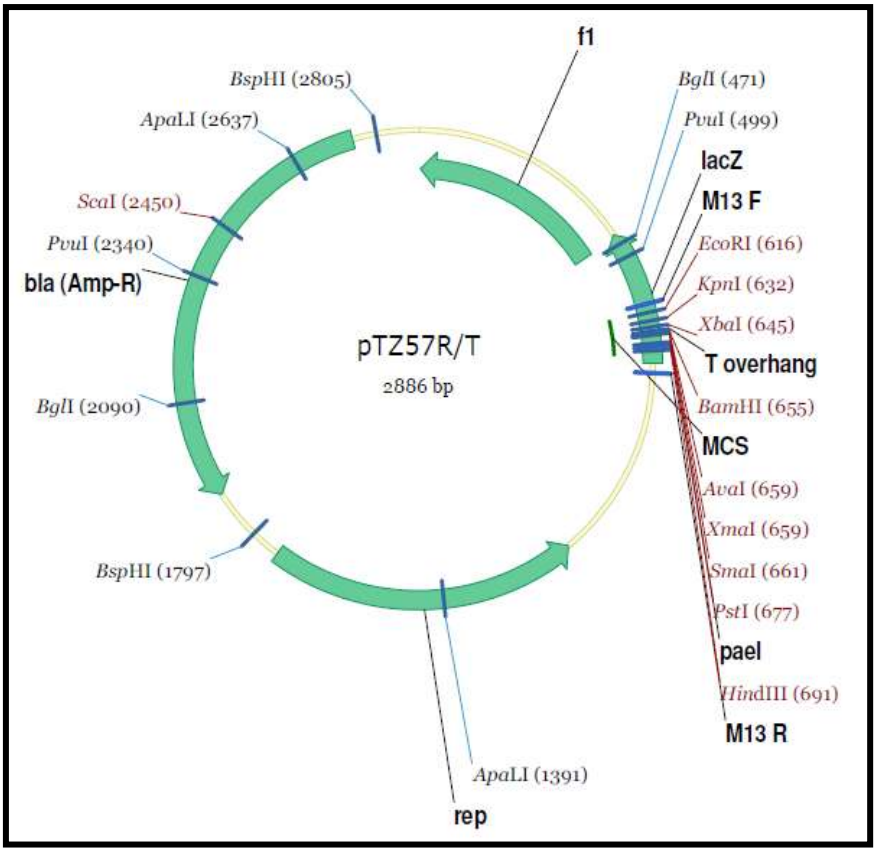

Figure 4. 1 Vector map of the pTZ57R/T plasmid used for TA cloning in this study (Fermentas, EU). The multiple cloning site (MCS) interrupts the lacZ gene in the plasmid, facilitating Blue-White screening for positive transformants of *E.coli* DH5α competent cells. Vector-specific primers M13 forward (F) and reverse (R) flank the MCS, binding to positions 599 and 735 on the vector backbone and amplifying a fragment of approximately 136 bp in the absence of an insert.

Positive control (vector with insert) and PepMV ligation reactions were transformed into 100 µℓ of competent *E. coli* cells, using 15 µℓ of the ligation mix, and incubated on ice for 15 minutes. Mixtures were heat shocked for uptake of vectors into host cells at 42°C for 90 seconds and incubated on ice for a further five minutes. The 100 µℓ suspensions were spread onto 20 mℓ of LB medium (LB broth with 1.5% w/v MicroAgar) supplemented with 100 mg/ℓ ampicillin (Duchefa Biochemie, Netherlands), 4M IPTG solution (Isopropyl-β-thio-galactopyranoside – Roche, USA) and 800 mg/mℓ X-gal solution (5-bromo-4-chloro-indoyl-β-D-galactopyranoside – Fermentas, EU). A negative control sample of 100 µℓ of untransformed competent cells was also spread onto LB/Amp/IPTG/X-gal medium. Inoculated media were incubated for a maximum of 16 hours at 37°C. Inclusion of ampicillin in the growth medium supported the recovery of only transformed cells, as resistance to this antibiotic was conferred to susceptible cells by the vector plasmid. Successful transformed colonies were also detected by blue-white screening, whereby positive transformation of cells with the vector containing the insert, was indicated by the development of white colonies and, therefore, disruption of the *lacZ* gene necessary for X-gal utilisation (reviewed by Horton et al., 2006). The

development of blue colonies indicated transformation of competent cells with vector plasmids that did not contain an insert and, therefore, produced a functional *lacZ* gene product capable of metabolising X-gal to produce an insoluble blue by-product. Cells that were not transformed with the vector should not have developed colonies due to the inclusion of ampicillin in culture media.

Three white colonies from assays with each of the four independent clones, were selected and inoculated into separate centrifuge tubes containing 5 mℓ of LB broth supplemented with 100 mg/ℓ ampicillin. Bacteria were cultured at 37°C overnight on a rotary shaker at 190 rpm to recover pure cultures of the selected clones. Plasmids were extracted from the 12 overnight cultures by diluting 5µℓ of culture in 50 µℓ of nuclease-free H_2O. Dilutions were heated to denature bacterial cells for 20 minutes at 100°C followed by 2 minutes on ice. Cell fragments were separated from the solution by centrifuging samples for 2 minutes at 14 000 rpm using a MiniSpin Plus centrifuge (Eppendorf, Germany). Supernatants containing the vector plasmids were used in PCR tests for amplification of the vector insert.

To confirm the presence of the virus sequence insert from extracted vector plasmids, two sets of PCR assays were performed on each sample. These incorporated the vector-specific primers, M13F (5'-TAAAACGACGGCCAG-3') and M13R (5'-GTCATAGCTGTTTCCTG-3'), and the internal PepMV-specific primer pair, Ker1 and PepCP-R. Both assays employed the DreamTaq amplification system with a total reaction volume of 50 µℓ containing 2 µℓ of plasmid template, 0.4 µM of the relevant forward and reverse primers, 10 mM dNTPs (Fermentas, EU), 10X DreamTaq buffer with $MgCℓ_2$ and 1.25U of DreamTaq polymerase. A non-template control sample, containing no plasmid template, was also included in both assays. Amplification of the PepMV 986 bp fragment was performed under the cycling conditions mentioned in 4.3.2. Amplification of the entire sequence insert using M13 primers was performed under conditions of 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 30 seconds; and an elongation step at 72°C for 5 minutes. Products were resolved as mentioned previously on a 2% agarose gel for 50 minutes at 70V and visualised under UV light. Relative product lengths were indicated by 5 µg of the DNA ladder run in conjunction with PCR products (Appendix A). Plasmid extracts that produced bands of the expected length in both reactions, were selected for sequencing reactions performed by Inqaba Biotech (South Africa), using standard M13 sequencing primers.

4.3.4. Sequence analysis

Sequence data was assembled using CLC Main Workbench v.6.5 (CLC Bio, Denmark). Vector backbone was trimmed from the sequences by identifying the PepMV-specific primer binding regions to generate a single contig of 986 bp for each clone. These contigs were aligned in CLC Main Workbench using default settings of: Gap open cost – 10.0; Gap extension cost – 1.0. Single nucleotide mismatches or sequencing errors, identified by alignments, were corrected and a final consensus sequence for the 986 bp PCR product was determined.

4.3.5. Phylogenetic characterization of the South African PepMV isolate

To characterize the suspected PepMV sequence fragment, BLAST (Basic Local Alignment Search Tool) analysis of public databases (http://blast.ncbi.nlm.nih.gov) was performed using default settings of the standard nucleotide BLAST programme – BLASTN (Altschul et al., 1997). Of the 500 best matches found, the closest relatives to the sequence of interest were identified based on significant scores, high sequence identity and high query coverage. The maximum score for each retrieved match was calculated based on the applied alignment matrix which allocated 2 for every match and -3 for every mismatch, as well as a 5:2 gap creation and extension penalty. Higher scores thus indicated fewer gaps or mismatches with the query sequence, which translated into more significant matches and higher probabilities of evolutionary relatedness.

Representative sequences of the major genotypes of PepMV (Table 4. 1) were obtained from the GenBank Database (http://www.ncbi.nlm.nih.gov/genbank/) for phylogenetic comparison to the query sequence. Sequences of closely related *Potexvirus* species, *Narcissus mosaic virus* (NMV, D13747) and *Cymbidium mosaic virus* (CymMV, NC0011812 and CMU62963), and the *Potexvirus* type species, *Potato virus X* (PVX, AF172259 and NC011620) were included in comparisons. The 986 bp consensus sequence obtained from clones representing the South African (SA) isolate, was trimmed to remove TGB and 3'UTR regions and a putative 714 bp coat protein sequence was extracted, based on pairwise alignment to the closest relative as identified from BLASTN results. Regions encoding the coat protein were also extracted from full genomic sequences of the 19 selected PepMV isolates (Table 4. 1) or *Potexvirus* species for phylogenetic comparisons. These were incorporated into multiple sequence alignments (MSA) with the SA isolate using Clustal X v.2.0.11 (Larkin et al., 2007) with default parameters of: Gap open cost – 15; gap extension cost – 6.66. Phylogenetic trees were inferred from this data by the maximum parsimony (MP) method in MEGA v.4.02 (Tamura et al., 2007) and relationships were validated using 1000 bootstrap replicates.

Table 4. 1 Strains or isolates of *Pepino mosaic virus* used in this study for phylogenetic comparison to the South African isolate.

Strain/Isolate	Origin	Accession No.	Reference
EU	Spain	NC004067	Aguilar et al. (2002)
	Spain	AF484251/NC004067	Aguilar et al. (2002)
	Spain	AJ606360	Lopez et al. (2005)
	Spain	AJ606359	Lopez et al. (2005)
	France	AJ438767	Cotillon et al. (2002)
	Netherlands	FJ940223	Van der Vlugt et al. (2002)
LP	Peru	AJ606361	Lopez et al. (2005)
	Peru	AM109896	Pagán et al. (2006)
Ch1	Chile	DQ000984	Ling (2007)
US1	unknown	FJ940225	Direct submission
	USA	AY509926	Maroon-Lango et al. (2005)
Ch2	Chile	DQ000985	Ling (2007)
US2	unknown	FJ212288	Adams et al. (2009)
	Poland	EF408821	Hasiów et al. (2008)
	USA	AY509927	Maroon-Lango et al. (2005)

4.4. Results

4.4.1. Amplification of a PepMV-specific fragment from infected leaf samples

Total RNA extractions from 85 – 100 mg infected and healthy tomato leaf samples yielded concentrations between 720 and 1700 ng/µℓ. All extracts demonstrated high purity with 260/280 and 260/230 ratios greater than 2.00. Despite high yields, 1 µg of total RNA from these extracts was considered sufficient for first strand cDNA synthesis and subsequent amplification assays.

The likely isolate of PepMV present in the tomato samples was unknown. To prove that the detected isolate belonged to the PepMV species, targeting conserved regions within the PepMV genome was necessary to prime amplification of a target-specific fragment. A strong, prominent band of the

expected length of 986 bp was detected from each the four replicate samples from infected leaves (Figure 4. 2). These bands were absent in extracts from healthy tomato leaves, as well as template and polymerase control samples, indicating the specificity of the amplification reaction for PepMV detection. A non-specific amplicon was detected in one of the replicate samples from infected leaves. This band was not present in other control or test samples. Nevertheless, this sample was still used in subsequent cloning reactions in case results were informative for classification of the pathogen. A low molecular weight band was also detected from reactions with total nucleic acid extracted from infected leaves (Figure 4. 2). This indicated that amplification from this sample did occur, but only short fragments were amplified.

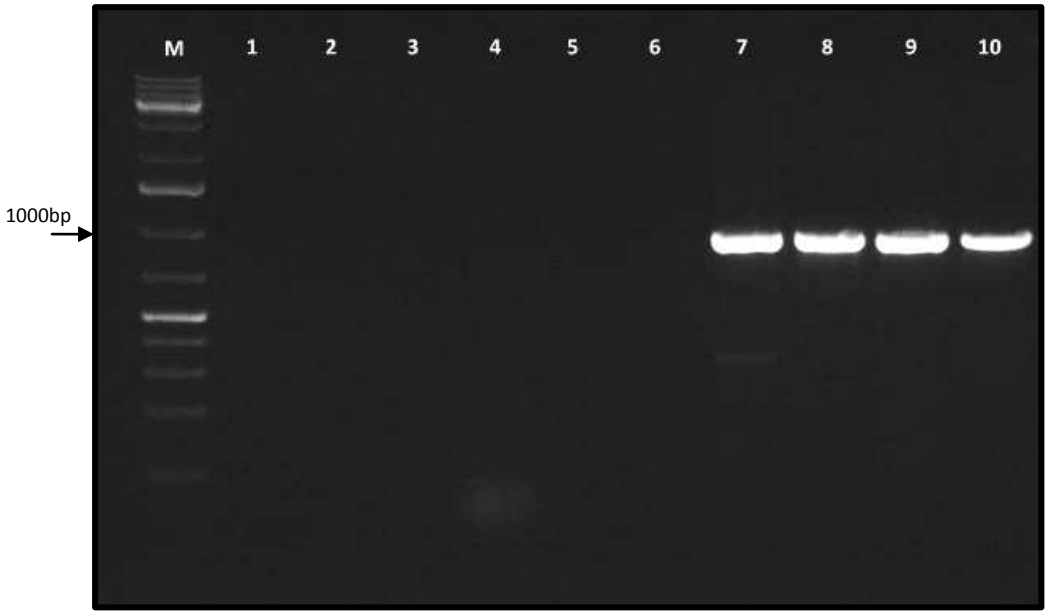

Figure 4. 2 Detection of PepMV from putatively infected tomato leaf samples by amplification of a 986 bp target fragment using pathogen-specific primers. Lane M: 5 µg O'GeneRuler 1 kb Plus DNA ladder (Fermentas, EU; Appendix A); lane 1 – negative template control; lanes 2 and 3 – DreamTaq and reverse transcriptase amplification controls, respectively; lane 4 – sample from total nucleic acid extraction of infected leaves; lane 5 and 6 – negative control sample from healthy tomato leaves; lanes 7 – 10 – replicate samples from a pooled collection of infected leaves.

4.4.2. Cloning of suspect PepMV PCR products

Detection of strong bands of the expected length from PepMV amplification reactions with each of the four replicate leaf samples made these appropriate templates for sequencing. Products from PCR were incorporated into four separate cloning reactions with the pTZ57R/T vector plasmid and transformed into *E. coli* host cells to confirm ligation of the PCR products to vectors. Colonies recovered from the transformation reactions on ampicillin/X-gal/IPTG selective LB medium developed as either light/bright blue or were opaque/clear white. The majority of colonies

recovered on the positive control plate were white, whereas few or no colonies developed on the plates of negative control samples. At least three clear, white colonies developed on each of the plates from the four transformation/cloning reactions, indicating positive ligation of the insert. These were selected for further analysis.

Plasmids extracted from these colonies were assessed by conventional PCR with both PepMV-specific and vector-specific, standard M13, primers. Vectors with the insert were expected to produce the 986 bp band with PepMV-specific primers, as observed previously, but a longer fragment in reactions with M13 primers, as these targeted amplification of the entire MCS region. Amplification reactions demonstrated variable results but no bands were detected in template control samples of both assays, proving the absence of inter-primer homology (Figure 4. 3). No bands were detected from extracts of the first clone with PepMV-specific primers, and only bands of approximately 150 bp were detected from reactions with M13 primers. Unexpected results were

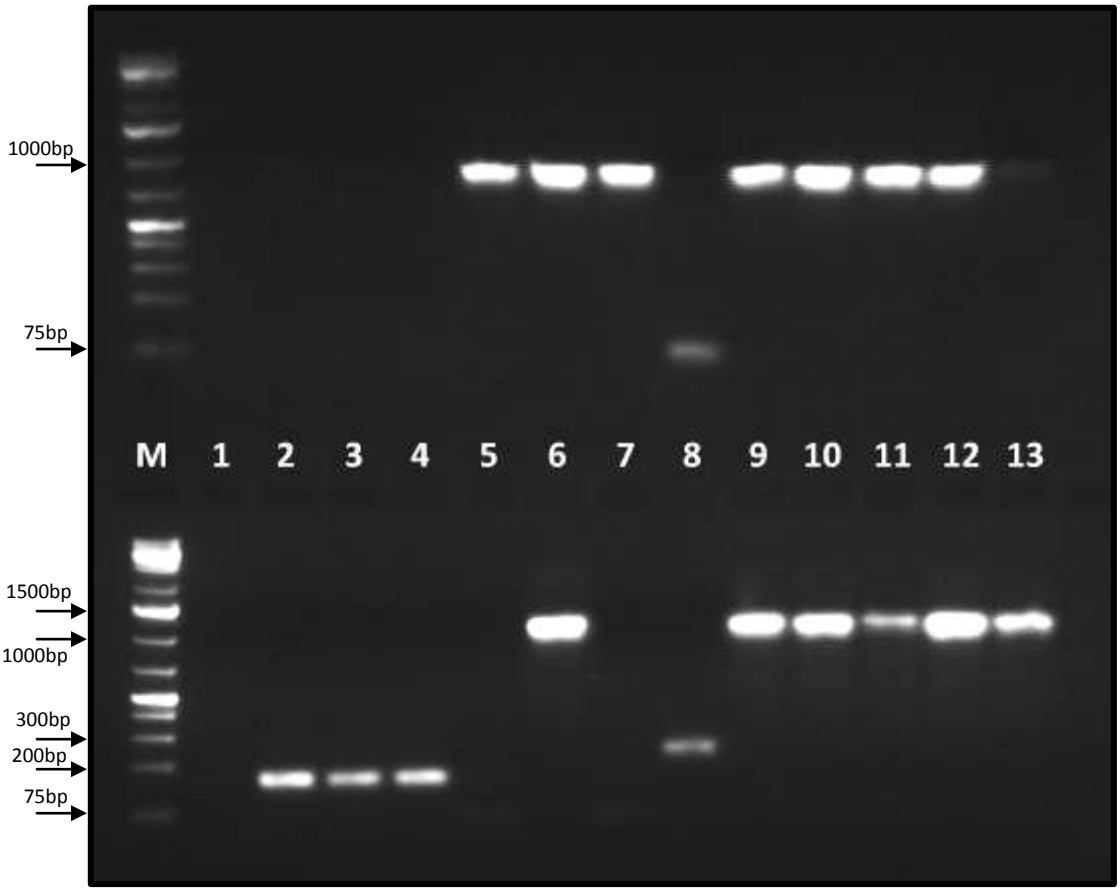

Figure 4. 3 Amplification of the vector insert from cell extracts from selected, successful transformants using PepMV-specific primers (top) and M13 primers (bottom). Positive detection of the target insert was indicated by production of a 986 bp fragment (top) or bands of approximately 1200 bp (bottom) in the respective assays. Lane M: 5 µg O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU; Appendix A); lanes 2 – 4: clone 1; lanes 5 – 7: clone 2; lanes 8 – 10: clone 3; lanes 11 – 13: clone 4.

also obtained from PCR with plasmids of the second clone (Figure 4. 3). Strong, prominent bands of the expected length were detected from all three samples tested with PepMV-specific primers. However, only one of these produced a band of greater than 1000 bp in reactions with M13 primers. This sample had a high concentration of target amplicons as an intense, thick band of the expected length was produced in both amplification reactions. In the remaining two samples from this clone, two faint bands of approximately 150 bp and 50 bp were visible in the M13 assay, which were indicative of potential mispriming events. Strong prominent bands of the expected length were observed in both amplification reactions for two of the three extracts from the third clone (Figure 4. 3), indicating positive detection of the insert from vector plasmids. Amplified fragments from the third sample of that clone, however, were approximately 75 bp and 200 bp from reactions with PepMV- and vector-specific primers, respectively. Samples of the fourth clone demonstrated more uniform results with all three samples producing fragments of the expected length with both sets of primers (Figure 4. 3). From the 12 plasmid extracts of the four clones tested, six samples that produced bands of the expected length from reactions with both primers were selected for sequencing reactions. These bands were of varying intensities indicating variable amplification efficiency.

4.4.3. Phylogenetic characterization of sequence fragments

Sequence data was determined from selected clones in sense and antisense directions of the dsDNA plasmids using forward and reverse standard M13 sequencing primers. These reads were aligned and edited to remove peak ambiguities and flanking sequences, to yield a total fragment length of 1089 bp. Alignments of the six clones revealed only 1 nt sequencing error, proving the reliability of the amplification reactions used in this study. A final consensus sequence was extracted from alignments and trimmed to remove the vector backbone so that a 986 bp sequence, representing the original PCR product, remained after editing.

Comparative analysis of public databases using BLASTN retrieved over 250 matches of the SA isolate to members of the PepMV species. The highest score was with a mild CH2 PepMV isolate (Table 4. 2) from infected tomato plants in Belgium (Hanssen et al., 2009). The query sequence mapped to nucleotides 5264 – 6279 of the genome sequence of this isolate, which included a portion of the TGB3 region and the full coat protein region of the PepMV genome. This isolate and others characterized as PepMV CH2 and US2 isolates, all demonstrated 99% identity with the full length of the query sequence, with 12 nt single nucleotide polymorphisms (SNPs). Members of the US1/CH1, EU and LP isolates of PepMV were more divergent, demonstrating 79 - 80% similarity to the SA

isolate and more than 196 nt mismatches with the SA isolate. Besides PepMV, other *Potexvirus* accessions formed part of the 500 closest matches from the BLASTN analysis. The closest relative was identified as NMV (Table 4. 2) with 66% identity with a portion of the query sequence. All other hits from database searches exhibited less than 40% similarity to the SA isolate, indicating poor or distant evolutionary relatedness.

Pairwise alignment of PepMV to the closest relative, FJ457096 (Table 4. 2), revealed the approximate position of the coat protein region in the sequence fragment. Flanking sequences were, therefore, trimmed to generate a putative 714 nt coat protein sequence, which was incorporated into multiple sequence alignments with representative PepMV and *Potexvirus* isolates. Results of the MSA supported those obtained from BLASTN analysis, demonstrating a sequence identity of greater than 99% with coat protein sequences of the CH2 and US2 PepMV isolates (Table 4. 2). Interestingly, the seven nucleotide mismatches that occurred between the SA isolate and CH2/US2 isolates, were at the same positions as some of the mismatches observed between the South African isolate and all

Table 4. 2 Selected results from BLASTN analysis of the 986 bp sequence from the South African isolate of PepMV.

Accession No.[1]	Species	Isolate	Maximum Score[2]	Query coverage (%)	Maximum Identity (%)
FJ457096	PepMV	Ch2	1728	100	99
AY509927	PepMV	US2	1725	100	99
FJ212288	PepMV	US2	1719	100	99
EF408821	PepMV	US2	1707	100	98
DQ000985	PepMV	Ch2	1698	98	99
FJ940225	PepMV	US1	884	100	80
AY509926	PepMV	US1	883	100	80
DQ000984	PepMV	Ch1	879	100	80
AM109896	PepMV	LP	838	100	79
AJ606631	PepMV	LP	838	100	79
FJ940223	PepMV	EU	836	100	79
AJ606360	PepMV	EU	836	100	79
AJ438767	PepMV	EU	836	100	79
AF484251	PepMV	EU	827	100	79
AJ606359	PepMV	EU	821	100	79
D13747	NMV	n/a	156	65	66
CMU62963	CymMV	n/a	66	34	66
AF172259	PVX	n/a	50	4	84

[1] Accessions chosen represent full genome sequences of the five genotypes of PepMV or other significant species of the *Potexvirus* genus.
[2] Maximum score represents the significance of the match based on the applied alignment matrix.
Query coverage represents the percentage of the 986 bp fragment comparable to the retrieved match and maximum identity, the percentage of nucleotide matches between the two sequences.

other PepMV genotypes. Similar to BLASTN results (Table 4. 2), the other three PepMV genotypes demonstrated a lower similarity with the SA isolate than CH2 and US2 genotypes, as the overall sequence identity of this SA isolate with the coat protein regions of US1/CH1, LP and EU genotypes were 79.7, 77.7 and 77.2, respectively.

Phylogenetic characterization also clustered the SA isolate with Ch2 and US2 sequences in 99% of the Bootstrap replicates (Figure 4. 4), proving the robustness of the topology generated in this study. The 15 representative PepMV sequences clustered into four clades representing the EU, LP, US1/CH1 and CH2/US2 genotypes. Low bootstrap values within branches indicated high sequence identity and close evolutionary relationships among clustered sequences. The clades of PepMV were distinct from PVX sequences, the designated outgroup. The other two *Potexvirus* species, however, demonstrated poor discrimination from other species which may have been indicative of uninformative or inaccurate sequence alignments of these viruses. The coat protein region of the SA isolate was closely related to the US2 isolate AY509927 (Figure 4. 4), obtained from infected tomato plants in the USA (Maroon-Lango et al., 2005). Bootstrap values of less than 50% were considered

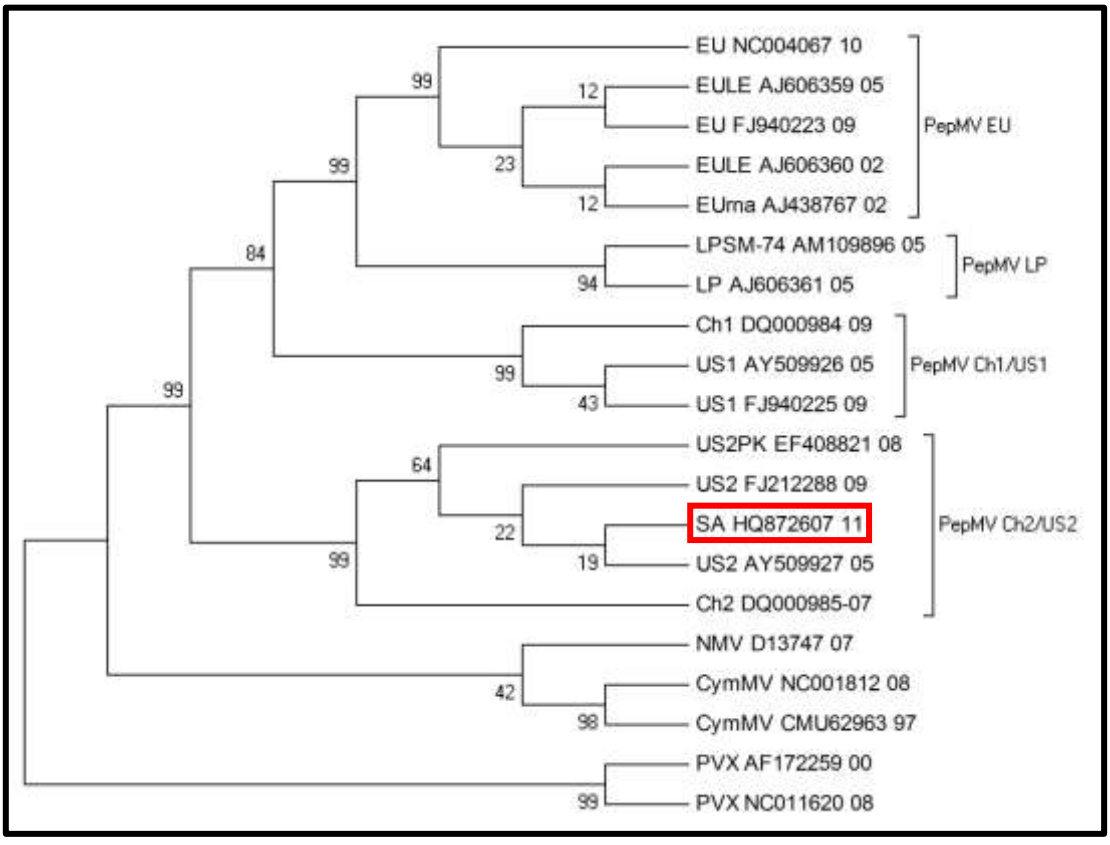

Figure 4. 4 Maximum Parsimony (MP) phylogenetic tree demonstrating evolutionary relationships among the five major genotypes of PepMV and closely related Potexviruses. Relationships were based on the coat protein region for all isolates. The South African isolate (outlined in red) clustered with PepMV Ch2 and US2 isolates indicating high sequence homology for the coat protein region. Labels: US2 (isolate/strain) PK (sample name) EF408821 (Accession No.) 08 (most recent year of modification). Bootstrapping = 1000 replicates.

inadequate for accurate phylogenetic characterization in this study. The more divergent CH2 isolate, DQ000985, on the other hand, clustered out of this clade in 64% of the 1000 bootstrap replicates, which represented reliable sequence divergence from US2 sequences/isolates. There were only four nucleotide mismatches between DQ000985 and AY509927 coat protein sequences in alignment results. The most closely related isolate identified by BLASTN analysis (Table 4. 2), which was omitted from the phylogenetic analysis, is characterized as a PepMV CH2 isolate. This proves that high homology and a potential phylogenetic relationship exists between isolates of CH2 and US2. Results of phylogenetic comparisons and BLASTN analyses confirmed characterization of the SA isolate as part of the PepMV species. This isolate was submitted to GenBank in 2011 as a 714 nt sequence representing the coat protein region of PepMV – Accession number HQ872607.

4.5. Discussion

Since the emergence of PepMV in tomato, this pathogen has spread across tomato production areas in Europe, America and Asia, severely impacting the quality of tomato fruit and resulting in substantial losses for producers and retailers (Hanssen and Thomma, 2010; Ling et al., 2008; Spence et al., 2006). This destructive disease has, until recently, eluded the African tomato industry, most likely due to the high level of contribution of subsistence farming to annual fruit production and, thus, the limited use of imported hybrid seed. The detection of characteristic symptoms of PepMV in commercial tomato production areas of Limpopo Province, South Africa, was, therefore, cause for great concern. Suggested diagnostic protocols demonstrated detection of PepMV particles from leaf samples from these plants, but the high risk of misdiagnosis due to various external factors supported further characterization using more accurate and conclusive techniques. Samples were subjected to amplification and sequencing strategies in this study to facilitate identification and phylogenetic characterization of the causal agent in diseased plants.

The diagnostic method used to amplify targets from leaf samples in this study was successful and reliable as PepMV primers consistently amplified a fragment of the expected length from cDNA templates. The viral coat protein was selected as the targeted gene as it has been well characterized for PepMV (Aguilar et al., 2002; Cotillon et al., 2002; Ling, 2007; Ling et al., 2008; Maroon-Lango et al., 2005; Mumford and Metcalfe, 2001; Pagán et al., 2006), resulting in the availability of an extensive sequence database for comparison. The region demonstrated both conserved and slightly divergent regions to facilitate species and sub-species characterization of the detected PepMV isolate. The coat protein is also located towards the 3’ end of the PepMV genome (Aguilar et al.,

2002; Cotillon et al., 2002) and, thus, more likely to be included in cDNA fragments primed by Oligo dT_{18}, which target the 3' poly (A) tail of RNA extracts for binding. In this study, amplification reactions were successful and efficient for targeted amplification of PepMV nucleic acid from infected leaf and seed samples, and primers did not demonstrate significant cross-hybridisations with the host genome or non-target regions in the pathogen genome. One of the four replicate samples from infected leaves produced a non-specific fragment from amplification reactions which may have resulted from a mispriming event (Maroon-Lango et al., 2005; Mansilla et al., 2003), sequence mutation (Ling, 2007) or fragmentation of cDNA target templates. A sample of total nucleic acid extracts from infected leaves was included in amplification reactions to analyse whether pathogenic RNA could be directly detected from RNA templates in the presence of high concentrations of host DNA and RNA. The target fragment was absent from samples, however, and only a short, low molecular weight band was detected. This was likely due to fragmentation of RNA templates, which are highly unstable, thereby preventing generation of full length amplicons. Direct detection of RNA from plant samples is thus limited, and prior single-strand cDNA synthesis and amplification is required for accurate and sensitive detection of PepMV nucleic acid. Detection of RNA directly from samples may be improved by using a sensitive RdRp-based amplification system, or by employing one-step RT-PCR systems that are commercially available.

Amplifying a fragment similar to the size of a targeted amplicon using pathogen-specific PCR, is not conclusive proof of the presence of the target pathogen in samples (Schaad and Frederick, 2002). Positive PCR products from the four replicate leaf samples in this study were, therefore, ligated into a host vector for sequencing. This further increased the concentration of the target fragment for sequencing and, by using standard M13 forward and reverse primers complimentary to regions on the vector backbone, the process of sequencing was independent of pathogen-specific amplification. The protocol selected for this study required a minimum of 4 days to clone, transform and isolate vectors from successful transformants. This is too lengthy if quick diagnosis and characterization of infected samples is required, but the procedures could be improved by employing rapid cloning kits currently available commercially. These kits are more expensive, however, which reduces their appeal for high-throughput, routine diagnostics.

Selected clones demonstrated positive ligation of the insert by producing bands of the expected length in PCR assays with pathogen- and vector-specific primers. Amplification reactions with both sets of primers proved to be essential for validating successful cloning, as only 50% of tested samples produced target amplicons in both reactions. False positive clones were identified by dual

amplification reactions. These clones either did not contain the vector insert or low copies of non-specific fragments were incorporated into host vectors. Mispriming events (Maroon-Lango et al., 2005) or inefficient amplification reactions may explain these results, though vectors may have also undergone recombination or released inserts during extraction processes which may have facilitated misidentification. Because of the high incidence of false positives generated from cloning and transformation procedures, all six positive clones were sequenced in this study. This reduced the risk of replication and sequencing errors as well as cloning artefacts in the sequence data generated (Mansilla et al., 2003; Louws et al., 1998), ensuring reliability and accuracy of the PepMV characterization results.

The SA isolate was identified as part of the PepMV species based on BLASTN analysis using sequence data generated from clones. High percentage matches with the full length of the 986 bp sequence were obtained with over 250 accessions of PepMV species, proving this method was accurate and reliable (Adams et al., 2009; Schaad and Frederick, 2002). Less significant matches predominantly included other *Potexvirus* species, though these demonstrated lower sequence identity and query coverage. The isolates that were the most similar to the SA PepMV isolate all belonged to the US2 or CH2 genotypes of PepMV. The high overall similarity of the SA isolate with these isolates support its characterization as part of the PepMV US2/CH2 genotype. However, sub-species characterization based only on this small portion of the viral genome could be inaccurate, but a close evolutionary relationship among these isolates can be reliably assumed. Other genotypes of PepMV were more divergent from the SA isolate but still demonstrated a significant evolutionary relationship with the SA PepMV isolate.

The closest related virus to the SA isolate in the *Potexvirus* genus, outside of PepMV, was NMV, which has also been shown in previous studies to be the most homologous to the PepMV species (Hanssen et al., 2008; Mumford and Metcalfe, 2001). Also consistent with previous research (Maroon-Lango et al., 2005; Van der Vlugt et al., 2002; Verhoeven et al., 2003), the type species of the *Potexvirus* genus, PVX, demonstrated poor homology with the isolate and, by extension, members of the PepMV species, only exhibiting identity with less than 50% of the 986 bp query sequence. This supported designation of PVX as an outgroup in the phylogenetic analysis in this study. Such large sequence divergence between species of a genus is characteristic of RNA viruses (Pagán et al., 2006). These demonstrate higher susceptibility to mutations and recombination than other organisms, like bacteria, that demonstrate close evolutionary relationships with species of the same genera. Describing reliable phylogeny between families of RNA viruses is, therefore, extremely

complicated and, in many cases, inaccurate, as these viruses seldom originate from a common ancestor.

As the majority of published sequence data for PepMV represents only portions of the PepMV genome, sub-species characterization and phylogenetic analyses in this study only employed full length sequences to represent the major genotypes of PepMV and related *Potexvirus* species. This was done to ensure that evolutionary relationships were based on true sequence identity for the full length of the target gene. Multiple sequence alignments of the coat protein regions of these selected sequences, and the putative coat protein of the SA isolate, confirmed results of the BLASTN analysis. The SA isolate demonstrated the highest similarity, with few nucleotide mismatches, with coat protein sequences of US2 and CH2 isolates, but more than 150 nucleotide mismatches with LP, EU and US1/CH1 isolates. Though divergent from the SA isolate, the LP isolate demonstrated less SNPs with the SA, CH2, US2 and US1/CH1 isolates compared to the EU isolate. This supports previous hypotheses that Chilean and US isolates emerged in tomato independently of EU isolates (Ling, 2007; Ling et al., 2008; Maroon-Lango et al., 2005).

The seven nucleotide mismatches that were identified between the SA isolate and all other PepMV isolates could be attributed to cloning artefacts or conserved sequencing errors (Louws et al., 1998; Mansilla et al., 2003). However, as the consensus sequence used to establish the identity of the SA isolate was based on data from six clones, such errors are not likely. The conserved positions of these mismatches between the SA isolate and all other PepMV genotypes indicate, rather, that these mismatches are most likely non-random and might demonstrate some kind of functional importance or biological relevance. It would be interesting to determine translated amino acid sequences from the coat protein sequence of this SA isolate to establish whether the SNPs have a significant or silent mutational effect on protein structures. Distinct differences in symptom development and pathogen function, that have been observed for different isolates of PepMV, have been attributed to the small number of nucleotide differences detected in previous comparative studies (Hanssen et al., 2009; Hanssen and Thomma, 2010). Conservation of the nature and position of mismatches for this 714 nt region could indicate that the SA isolate is a divergent form of PepMV, but this could not be established in this study as only one ORF of the viral genome was characterized.

Further proof that the SA isolate was closely related to CH2 and US2 PepMV isolates was indicated by phylogenetic tree construction which clustered these isolates in a single clade. Such trees provide

evidence for evolutionary relationships among isolates and, by including bootstrapping replicates, the topology of relationships was considered highly robust and accurate (Maroon-Lango et al., 2005; Pagán et al., 2006). Bootstrap values of greater than 50% indicated significant clustering or branch topology, with values closer to 100% confirming true evolutionary relationships. Maroon-Lango et al. (2005) even collapsed braches with bootstrap values of less than 50% to eliminate designation of false phylogenetic relationships. In this study, phylogenetic analysis clustered sequences of PepMV into four major clades representing the major genotypes of PepMV: EU, LP, US1/CH1 and US2/CH2. Similar results were observed in other studies comparing the coat protein regions of PepMV isolates (Ling, 2007; Maroon-Lango et al., 2005; Pagán et al., 2006). Distinction of the US1/CH1 sequences from the EU and LP clusters in trees generated in this study, was supported by the overall genome identity of 82% among these isolates (Ling, 2007; Ling et al., 2008; Maroon-Lango et al., 2005). Low bootstrap support of the topology of NMV and CymMV sequences was observed which was similar to results of previous phylogenetic comparisons with isolates of PepMV (Cotillon et al., 2002; Mumford and Metcalfe, 2001). Poor alignments, resulting from the strict alignment matrix applied in the analyses, might explain results of this study. For more informative comparisons of PepMV to its relatives, more representative sequences of other species should be included in the alignments.

Within the US2/CH2 clade, the SA isolate and US2 sequences clustered separately to the representative CH2 sequence. However, BLASTN analysis revealed that the closest related isolate to the SA isolate was a mild CH2 isolate based on the 986 bp query sequence. This closely related isolate was not included in phylogenetic characterization, but Hanssen et al. (2009) reported that the genome of this mild PepMV isolate was 99.4% similar to that of a more aggressive CH2 isolate included in the phylogenetic analysis. The US2 and CH2 isolates have been characterized as distinct genotypes (Hanssen et al., 2008; Ling, 2007; Ling et al., 2008), demonstrating only 90.7% overall nucleotide identity between full genome sequences. The majority of SNPs and sequence variations between CH2 and US2 isolates have been localised to the 5'UTR, replicase and TGB1 regions (Ling, 2007; Ling et al., 2008) and, thus, sub-species discrimination of these isolates is unclear when based on the coat protein alone. Despite low sequence variation that exists in some regions of the viral genomes of US2 and CH2, these two closely related isolates were still capable of inducing differential symptom expression on inoculated plants (Ling, 2007), indicating that the SNPs may exhibit a functional shift. It would be interesting to compare the sequence similarity and variation in symptom expression for mild and aggressive CH2 isolates, the SA isolate and representative US2 isolates, and to establish whether SNPs may be associated with differential symptomology. Nevertheless, the SA isolate demonstrated strong evolutionary relationships with both US2 and CH2 in phylogenetic

analysis in this study. Sub-species classification of the SA isolate will require further sequence analysis of the more informative replicase or TGB1 regions, or of the entire viral genome, in order to determine whether this isolate is more closely related to US2 or CH2 PepMV isolates.

Various mechanisms of transmission have been reported to facilitate the spread of PepMV within and among tomato production areas, which may be responsible for the introduction of the SA isolate of PepMV to South African tomato production areas. The highly infectious nature of this pathogen, supported by the stability of viral particles for up to three months, facilitates the easy mechanical transmission of the virus via infected equipment or by mechanical contact (Hanssen and Thomma, 2010; Ling, 2008). Two vectors have also been reported to support the transmission of PepMV, namely bumblebees (*Bombus impatiens*), which are usually used as pollinators of cultivated tomato (Shipp et al., 2008), and the root fungus, *Olpidium virulentus* (Alfaro-Fernández et al., 2010). These vectors are not likely to account for the long distance spread of PepMV, such as the introduction of PepMV in South Africa which is geographically isolated from regions where the disease has been reported previously. Introduction of PepMV into South Africa is likely to have originated from the import and cultivation of contaminated seeds or seedlings. Seed transmission has been cited as responsible for the introduction of PepMV into North America (Ling, 2007, 2008). Many countries rely on offshore production of hybrid tomato seed for cultivation, which facilitates the dissemination of PepMV through seed trade between countries and continents (Córdoba-Sellés et al., 2007; Hanssen et al., 2010b; Ling, 2008). The limited detection capacity of current PepMV certification protocols, based on testing of sampled seed-lots by DAS-ELISA, may mean that some infected seeds pass undetected, thereby leading to outbreaks and epidemics in destination countries. There is, thus, a need for more sensitive detection protocols and effective curative strategies for PepMV control.

Sequencing as a detection and characterization technique proved to be highly reliable for proving that the unknown South African isolate belonged to the PepMV species in this study. Data generated from cloning and sequencing reactions also facilitated phylogenetic characterization of the SA isolate by revealing closely related isolates within and outside of the PepMV species (Aguilar et al., 2002; Cotillon et al., 2002; Van der Vlugt et al., 2002). Characterization was highly significant for PepMV due to the availability of an extensive comparative database of relevant sequences, including full genome sequences, for this pathogen. This technique was the only tool currently available that was capable of such sensitive comparison among sequences within a subspecies of a pathogen (O'Brien et al., 2011; Studholme et al., 2010). Accurate discrimination among the isolates of PepMV has also

been achieved using RFLP assays (Hanssen et al., 2008), but RFLP assays are usually complex, technical and time-consuming because more than one restriction enzyme must be incorporated for accurate discrimination (Alfaro-Fernandez et al., 2009; Deyong et al., 2005). In some cases (Alfaro-Fernandez et al., 2009; Jones et al., 2004; Widmer et al., 1998), complex patterns are generated from RFLP assays which still require sequencing to clarify results. Molecular characterization based on sequencing used in this study required approximately a week, including molecular tests, data assembly and phylogenetic analysis. This may be impractical for high-throughput routine diagnostics (Dovas et al., 2004) particularly as the results do not indicate viability of the detected pathogens, necessitating the use of parallel viability assays like bioassays (Hadas et al., 2004), which further lengthen the period required for conclusive diagnosis. To isolate a pathogen for sequencing from host plants, prior knowledge of the expected pathogen is necessary to select appropriate pathogen-specific primers (Adams et al., 2009; Mansilla et al., 2003). This reduces the capacity for detection of novel strains or isolates (Mansilla et al., 2003), unless less degenerate primers are selected (Van der Vlugt and Berendsen, 2002; Van der Vlugt et al., 2002). To solve this, entire cDNA libraries of sequences could be generated from total RNA extracts (Adams et al., 2009). Subsequent analysis to remove host sequences and assemble potential pathogenic genomes is, however, currently impractical and time-consuming for use in routine diagnostics. The masses of data generated from sequencing were also unnecessary for basic diagnosis and pathogen identification, suggesting that this technique is only likely to be used where highly specific sub-species characterization is necessary.

The first report of the presence of PepMV in tomato production areas in South Africa was confirmed in this study by characterizing infected tomato leaf samples from the Limpopo Province. The isolate was shown to be closely related to a mild CH2 isolate, and clustered with US2 and CH2 PepMV isolates in phylogenetic comparisons based on the coat protein region. Comparative analysis in this study was restricted to *in silico* testing as reference isolates were not available. Characterization of this isolate by analysing the coat protein region of the PepMV genome was sufficient for species designation. Further sub-species classification will be more informative if more divergent regions like the TGB, or the entire viral genome of the SA isolate is analysed (Aguilar et al., 2002; Ling, 2007; Maroon-Lango et al., 2005; Verhoeven et al., 2003). Identifying the genotype of the SA isolate may facilitate better control of the virus. For example, establishing whether the PepMV isolate is mild or aggressive will determine the degree of sanitation required for an infected production area. That is, whether the infected plants are removed and sterilised or whether the area is placed under quarantine whereby the entire crop is discarded and all soil, equipment and clothing is sterilised.

More research is necessary to establish the exact source of PepMV infection and its current distribution in South Africa. The PepMV species is also known to develop mixed infections with other strains/isolates as well as other viruses of tomato like ToMV (Chapter 2, 2.4.5) and *Tomato chlorosis virus* (Davino et al. 2008), which can have a more severe impact on infected hosts than individual infections. Rapid and stringent control strategies such as: scouting, rouging, seed treatment, sterilisation of production areas and the implementation of strict hygienic measures for workers (GSPP, 2009; EPPO/CABI, 2005; Hanssen and Thomma, 2010), should thus be adopted without delay to prevent introduction of more strains of PepMV into the country, and to restrict further spread in the South African tomato industry.

Chapter 5 Design of a Diagnostic Microarray for Viral and Bacterial Seed-borne Pathogens of Tomato

5.1. Abstract

Microarrays for phytopathogen detection have proven to be highly specific and sensitive, facilitating accurate diagnosis of a range of pathogens, including significant pathogens of tomato. A diagnostic microarray targeting seed-borne pathogens of tomato would be enormously beneficial for seed trade which depends on strict phytosanitary requirements to control the global spread of seed-borne pathogens. Five of the most significant of these tomato pathogens have been identified: *Pepino mosaic virus*, *Tomato mosaic virus, Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *syringae*. A prototype diagnostic microarray for simultaneous detection of these significant viral and bacterial seed-borne pathogens of tomato was developed in this study. The microarray consisted of 152 diagnostic probes that were spotted in 16 replicates on a coated glass substrate. Pathogen-specific probes were designed to target three or four genes of each pathogen, and demonstrated different levels of taxonomic conservation to facilitate both identification and classification of the detected pathogens. The array was validated using pure culture extracts of each of the five pathogens, related and unrelated, non-target pathogens of tomato, and DNA and RNA extracts from healthy tomato leaves. Diagnosis was based on detection of hybridisation signals from probe:target complexes that demonstrated a signal to noise ratio of greater than 2.5. The microarray demonstrated the capacity to detect each of the five pathogens separately and in combination by producing significant hybridisation signals with target templates. Results were, however, highly sensitive to base mismatches, technical variations, external factors and thermodynamic variations that occurred during template preparation and array hybridisation. Optimisation of the diagnostic process, and further validation of the best diagnostic probes identified in this study, are necessary before the array can be adopted as a standard diagnostic tool.

5.2. Introduction

The increasing impact of seed-borne pathogens on the global tomato industry has supported the implementation of strict curative and preventative phytosanitary methods over the past two decades (Deyong et al., 2005; Kritzman, 1991). Accurate detection of the most detrimental of these pathogens, by thorough screening of host plants and seed, has become a prerequisite for

certification of trade to reduce international spread of pathogens (Abdalla, 2000; EPPO/CABI, 2005; GSPP, 2009; Njambere et al., 2011). Current methods, however, lack the specificity and sensitivity required for reliable detection and identification of pathogens for trade certification (Lopez et al., 2007; Mumford et al., 2006; Schaad and Frederick, 2002). Conventional and serological methods are also severely limited by the number of pathogens detectable per assay, making sample screening labour-intensive and time-consuming by requiring weeks or months for diagnoses (Engel et al., 2010; Hadidi et al., 2004; Harris-Baldwin and Gudmestad, 1996; Lievens et al., 2003). Molecular techniques, especially multiplex and real-time PCR assays, can enable faster, culture-independent, specific and sensitive detection capacity for sub-genus as well as sub-species identification of pathogens (Lievens and Thomma, 2005; Njambere et al., 2011; Palacio-Bielsa et al., 2009; Schaad and Frederick, 2002; Wang et al., 2002) and have, therefore, become the focus of many pathogen detection studies.

Tomato plants and seed may host a variety of different classes of pathogens, many of which are capable of infecting plants in mixed infections (Davino et al., 2008; Hanssen et al., 2008; Nome et al.; Ozdemir, 2009; Pelludat et al., 2009; Vinayarani et al., 2011). Screening seed-lots for multiple significant seed-borne pathogens is, thus, an increasingly important requirement for standard protocols. Simultaneous differentiation among multiple pathogens is limited in multiplex assays as well, as incorporating more than six targets per assay can affect the reliability of diagnoses (Call et al., 2003; Engel et al., 2010; Szemes et al., 2005; Wilson et al., 2002). Resolving different target pathogens withthese methods also becomes complicated due to the increasing risk of non-specific primer binding and cross-homology between the multiple primer sets included in each reaction.

Microarrays have become the focus of many phytodiagnostic research efforts due to the increased capacity for specific and parallel detection of 100s and 1000s of targets in a single assay (Boonham et al., 2007; Pelludat et al., 2009; Tiberini et al., 2010; Wilson et al., 2002). The high-throughput capacity of this technique facilitates simultaneous detection and identification of multiple pathogens from a single sample. Microarrays, similar to dot-blot assays, are based on a solid substrate to which thousands of short (18 – 30 nt) or long (50 – 70 nt) oligonucleotide probes may be printed at discrete positions, in uniform spots of homologous probes called features (Boonham et al., 2007; Call et al., 2003; Hadidi et al., 2004). Each oligonucleotide probe is designed to be complimentary to a specifically targeted sequence, which, if present in a sample, will form an irreversible bond with the immobilised probe to form a probe:target complex. Formation of these complexes is confirmed by the incorporation of fluorescent dyes into the samples prior to hybridisation. The fluorescent dyes

are detected by high density scanners which produce a readable image indicating the position of a fluorescing complex. The presence of a target gene or sequence fragment can, therefore, be inferred from the position of the signal detected during scanning (Call et al., 2003). In phytodiagnostic applications, probes are designed to be complimentary to regions of a pathogen genome, enabling specific detection and identification of this pathogen in a sample (Ait Tayeb et al., 2005; Lievens et al., 2005; Njambere et al., 2011; Pelludat et al., 2009). For sensitive detection of targets at a range of concentrations within plant samples, assays are coupled with a preceding amplification step using universal or pathogen-specific primers to augment the pathogen nucleic acid templates (Call et al., 2003; Lievens et al., 2005; Tiberini et al., 2010; Wang et al., 2002). This is essential for screening protocols to detect seed-borne pathogens of tomato as even low levels of infection in seed samples can be sufficient to facilitate seed transmission.

The high-throughput capacity of microarrays supports the incorporation of probe redundancy, or the repetition of the same probe features at various positions within diagnostic arrays (Loy and Bodrossy, 2006; Wang et al., 2002; Wilson et al., 2002), such that an average signal intensity for each probe is evaluated for diagnosis. This improves discrimination of true hybridisation signals from false positive results, which is a major drawback of some conventional diagnostic PCR protocols (Call et al., 2003). In amplification assays, primers may demonstrate poor target detection, especially in complex samples like soil or tomato seed, leading to mispriming events and generation of non-target sequence fragments. This is not a risk with microarray assays, as theoretically, non-specific fragments should have insufficient sequence similarity with diagnostic probes, if probes are designed adequately, and, therefore, should not bind to arrays (Call et al., 2003; Wilson et al., 2002). Sequence variation, base substitutions and horizontal gene transfer are common mechanisms that enable pathogens to evade detection by host plants (Wilson et al., 2002; Yamamoto et al., 2000), but these also negatively impact the consistency and reliability of primer design and binding to target and amplify pathogen nucleic acid fragments. This is usually overcome by introducing multiple primers and probes to target multiple genes per pathogen. Providing further redundancy in diagnostic arrays, the employment of multiple diagnostic targets supports and validates diagnosis to ensure highly accurate and reliable results (Call et al., 2003; Boonham et al., 2003; Lievens et al., 2005; Pelludat et al., 2009; Wang et al., 2002). This is important for viral detection as these pathogens typically demonstrate high sequence variation (Boonham et al., 2007; Pagán et al., 2006; Tiberini et al., 2010; Van der Vlugt and Berendsen, 2002) and, therefore, few conserved regions for reliable detection of related viral pathogens. Different genomic regions demonstrate different levels of taxonomic conservation among related pathogens (Kyselková et al., 2008, 2009). By incorporating

a set of probes for these regions in diagnostic arrays, identification and characterisation of detected pathogens and related species is made possible.

Molecular phytobacterial detection has primarily been based on the 16S ribosomal RNA gene due to high reference sequence availability and high copy numbers within cells, which facilitates sensitive detection (Rajendhran and Gunasekaran, 2011). Limited polymorphic regions, however, limit classification potential of this region to genus level characterization for many bacteria, making it unsuitable for accurate identification of some pathogens (Ait Tayeb et al., 2005; Guasp et al., 2000; Kyselková et al., 2008; Yamamoto et al., 2000). Recent diagnostic strategies have, therefore, targeted other housekeeping genes that evolve faster than the 16S region, but contain both highly conserved regions for reliable primer binding, and polymorphic regions for sub-genus and sub-species differentiation. Some of these targets include: the DNA gyrase subunit B (Parkinson et al., 2007; Richert et al., 2005, 2007; Yamamoto et al., 2000; Zaluga et al., 2011), RNA polymerase beta (rpoB) subunit (Ait Tayeb et al., 2005; Loy and Bodrossy, 2006; Pelludat et al., 2009; Richert et al., 2007; Waleron et al., 2011), sigma factor 70 (rpoD) (Mulet et al. 2009; Parkinson et al. 2011; Yamamoto et al. 2000) and various heat shock proteins like groEL (Pelludat et al. 2009; Rajendhran and Gunasekaran 2011; Waleron et al. 2011). A balance between probe redundancy and gene target selection should be based on a few target genes that demonstrate the greatest diagnostic and characterisation potential for target pathogens. Letowski et al. (2004) have reported that the design of diagnostic probes and the optimisation of hybridisation conditions are the major limiting factors for the development and use of microarrays in diagnostics. Standardisation of the best structure and conditions of diagnostic assays could increase the adoption of this technique in more laboratories and, therefore, in standard diagnostic protocols. Studies have reported improved detection capacity by developing various optimisations of the microarray process or adaptations of probe design (Szemes et al., 2005; Van Doorn et al., 2007). A recent study by Njambere et al. (2011), demonstrated enhanced sensitivity of detection by including diagnostic monomer probes in tandem to form dimer probes (40 – 48 nt) in diagnostic microarrays. In that study, detection of even 0.01 fg of target genomic DNA was possible, as the use of dimer probes led to lower signal variability and, therefore, greater uniformity of results. Such an improvement is ideal for potential certification protocols for which consistency of detection is a priority.

The development of diagnostic microarrays for the detection of viral and bacterial seed-borne pathogens of tomato has been reported previously (Tiberini et al., 2010; Pelludat et al., 2009). Although simultaneous detection of more than one class of pathogen in a single array has also been

described (Wilson et al., 2002), no such array has been developed for concurrent detection of multiple, significant viral and bacterial seed-borne pathogens of tomato. Such a tool would be enormously beneficial to the tomato industry, by enabling the screening of a single host sample for multiple pathogens of different classes or species in a single assay. The aim of the current study was, therefore, to develop a prototype diagnostic microarray for the simultaneous detection and identification of five important seed-borne pathogens of tomato: *Pepino mosaic virus* (PepMV), *Tomato mosaic virus* (ToMV), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Xanthomonas campestris* pv. *vesicatoria* (Xcv) and *Pseudomonas syringae* pv. *tomato* (Pst). Diagnostic probes were developed to target three to four genes of the respective pathogens. These were validated for specificity against pure cultures of each of the targeted pathogens, four related and two unrelated phytopathogens of tomato, and nucleic acid samples extracted from healthy tomato plants. Primers and probes designed in other studies were also included in this array to evaluate the specificity and adaptability to the conditions selected for design and hybridisation in this study. A total of 2560 features were included in the array and analysed for the potential to detect, characterise and differentiate among all targeted pathogens reliably.

5.3. Materials and methods

5.3.1. Viral and bacterial isolates

Pure cultures of relevant bacterial and viral species and pathovars were selected for this study (Table 5. 1). Viral isolates were maintained in infected host leaf samples stored at -70°C. Isolates of PepMV were obtained from leaf samples of inoculated *Solanum lycopersicum* cv. Rooikhaki plants from previous studies (Chapter 2, 2.4.1). Validation and classification of viral particles infecting hosts was proven by DAS-ELISA and PCR testing prior to this study. Pure cultures of selected bacteria were stored at -20°C in milk glycerol solution (0.1% skimmed milk powder and 0.15% glycerol; Goszczynska et al., 2000) and were characterized prior to this study by conventional methods, Biolog testing and pathogen-specific PCR. Cultures designated as Xcv were isolated from inoculated, symptomatic *S. lycopersicum* cv. Rooikhaki plants and were characterised in previous studies (Chapter 2, 2.4.3). Colonies of the respective bacterial isolates (Table 5. 1) were recovered from milk glycerol stock cultures by streaking a loopful of the suspension onto nutrient agar (1.6% w/v nutrient broth and 1.5% w/v bacteriological agar) using a sterile inoculating loop. The inoculated media were incubated for four days at 25°C. Leaf samples from healthy *S. lycopersicum* cv. Rooikhaki plants were used as negative control samples in this study and were stored at -70°C. For each of these isolates

(Table 5. 1), as well as negative control samples, two technical replicates of all of the experimental processes listed below were performed to analyse the robustness of the diagnostic process.

Table 5. 1 Bacterial and viral isolates used in this study.

Isolate[1]	Isolated from/ Host plant	Source
Bacterial		
***Clavibacter michiganensis* subsp. *michiganensis* (Cmm)[2]**	Tomato	SACC - ARC
Enterobacter cloacae	Tomato	SACC – ARC
***Pseudomonas syringae* pv. *tomato* (Pst)[2]**	Tomato	SACC - ARC
Pseudomonas syringae pv. *syringae* (Pss)	Tomato	SACC – ARC
Ralstonia solanacearum	Tomato	SACC – ARC
***Xanthomonas campestris* pv. *vesicatoria* (Xcv)[2]**	Tomato	SVR
Xanthomonas vesicatoria (Xv)	Tomato	SACC – ARC
Viral		
***Pepino mosaic virus* (PepMV)[2]**	Tomato	SVR
Pepper mild mottle virus (PMMoV)	Pepper	ARC
Tobacco mosaic virus (TMV)	Tobacco	ARC
***Tomato mosaic virus* (ToMV)[2]**	Tomato	ARC

5.3.2. Isolation of nucleic acid

5.3.2.1. Extraction of RNA from healthy and infected tomato leaf samples

Total RNA was isolated from leaf samples of viral-infected and healthy tomato plants by the TRI-Reagent method (Simms et al., 1993) with minor modifications. Briefly, samples of 85-100 mg were homogenized using liquid nitrogen and sterile pestles, and incubated in 1 mℓ of chilled (4°C) TRI-Reagent (Sigma Life Sciences, USA) for 5 minutes at room temperature. For isolation of RNA from nucleoprotein complexes, 200 µℓ of chloroform (Merck, Germany) was added to homogenates, which were mixed thoroughly and incubated for 15 minutes at room temperature. Debris was separated from RNA by centrifugation at 13 400 rpm for 10 minutes at 4°C using a MiniSpin Plus centrifuge (Eppendorf, Germany), and the upper aqueous phase was retained for further purification. Precipitation of RNA from this phase was achieved by mixing 500 µℓ of absolute molecular grade isopropanol (Merck, Germany) by inversion of the samples, and centrifuging

[1] Isolates were obtained from symptomatic host plants from the Sakata Vegenetics RSA (SVR) culture collection or the South African Culture Collection (SACC), based at the Agricultural Research Council (ARC) in Gauteng, South Africa.
[2] The five target pathogens for this study are in bold font and others represent related or unrelated pathogens of tomato used for validation of the specificity of detection.

suspensions at 13 400 rpm for a further 10 minutes at 4°C. Precipitates of RNA were washed with 75% molecular grade ethanol (EtOH; Merck, Germany) for 5 minutes at 10 600 rpm at 4°C, and air-dried at room temperature for five minutes. Pellets were resuspended in 100 µℓ of preheated (65°C), RNase-free H_2O with 40U of Ribolock RNase-Inhibitor (Fermentas, EU) per sample. Crude extracts of total RNA were further purified using the RNA Clean-Up Kit (Zymo Research, USA) according to the manufacturer's instructions. Samples with a RNA concentration of greater than 400ng/µℓ were divided between two binding columns to ensure maximum yield of RNA from the clean-up procedures. After the binding and wash steps, RNA bound to spin columns was dissolved by applying 50 µℓ of RNase-free H_2O in two repeat applications of 25 µℓ, directly to the membrane of the column. Suspensions were eluted from columns at 12 000 rpm for 60 seconds and the purity and concentration of extracts was analysed using a ND-1000 spectrophotometer (Nanodrop technologies, USA). High quality RNA extracts were stored at -70°C.

5.3.2.2. Extraction of DNA from bacterial cultures

Colonies recovered on nutrient agar that demonstrated characteristic morphology for the respective bacterial isolates, were selected for extraction of DNA, performed as outlined by Pelludat et al. (2009). Two replicate extractions were performed from separate colonies for each isolate to represent the technical replicates of the assay. For each replicate, a loopful of culture was diluted in 300 µℓ of sterile distilled water (dH_2O) and boiled at 95°C for 30 minutes to denature cellular membranes and proteins. Cell fragments were precipitated from suspensions at 12 000 rpm for 5 minutes. Supernatants of extracts, containing bacterial DNA, were diluted 1:10 with sterile dH_2O and stored at 4°C for short-term storage, or -20°C for long-term storage.

5.3.2.3. Extraction of DNA from healthy leaf samples

Extraction of DNA from leaf samples of healthy tomato plants for use as control samples, was performed using the CTAB method (Doyle and Doyle, 1987), as modified by Momeni et al. (2011). Samples of 85 – 100 mg were homogenized using sterile pestles and liquid nitrogen. Preheated (65°C), 1% CTAB buffer (10 g/ℓ CTAB powder [hexadecyltrimethyl ammonium bromide; Sigma Life Sciences, Denmark], 1.4M NaCℓ, 100 mM Tris and 20 mM EDTA) and 1% β-mercaptoethanol (Merck, Germany) were added to leaf homogenates to a total volume of 750 µℓ. The suspension was mixed by inversion and incubated at 65°C for 20 minutes. The same volume of 24:1 chloroform:isoamylalcohol (Merck, Germany) was added to samples, mixed by inversion for three minutes, and centrifuged at 13 000 rpm for 10 minutes at 4°C to separate DNA from cellular debris.

The upper aqueous phase, containing plant DNA, was isolated from suspensions and a second volume of chloroform:isolamylalcohol was added to the sample to purify extracts further. The aqueous phase was again isolated and DNA was precipitated from this phase by adding two-thirds of the total sample volume of absolute molecular grade isopropanol (Merck, Germany), and centrifuging samples for five minutes at 13 000 rpm at 4°C. Precipitates were washed using 500µℓ of 75% molecular grade EtOH and centrifuged at 13 000 rpm for five minutes. Pellets were air-dried at room temperature for approximately 30 minutes and resuspended in 50 µℓ of pre-warmed (37°C), nuclease-free H_2O with 50 µg of RNase A (Fermentas, EU). Samples were incubated for a further 60 minutes at 37°C, to ensure maximum dissolution of DNA. Two replicate genomic DNA extracts from healthy leaf samples were sheared by nebulisation (Inqaba Biotech, South Africa) to form fragments of less than 1000 bp for more efficient dye-coupling and hybridisation to microarrays. Nebulised samples were stored at -20°C and incorporated directly into labelling reactions.

5.3.3. Design and selection of diagnostic primers and probes

For each of the five pathogens, three or four genes/genomic regions were identified as potential candidates for diagnosis and classification of the respective targets, from previous characterization studies. Viral identification was based on three selected genes which included: regions of the replicase genes, the coat protein, and for PepMV, a region from the more variable triple gene block (TGB) of the *Potexvirus* genome (Hanssen et al., 2009; Jacobi et al., 1998; Lartey et al., 1996; Letschert et al., 2002; Lopez et al., 2005; Mansilla et al., 2003; Maroon-Lango et al., 2005; Mumford and Metcalfe, 2001; Tiberini et al., 2010; Van der Vlugt et al., 2002; Verhoeven et al., 2003). For bacterial characterization, four potential gene targets were selected: the 16S-23S rRNA intergenic transcribed sequence (ITS) region (Bach et al., 2003; Guasp et al., 2000; Pastrik and Rainey, 1999), RNA polymerase β-subunit (rpoB) (Ait Tayeb et al. 2005; Pelludat et al. 2009; Richert et al. 2007; Waleron et al. 2011), DNA gyrase B (gyrB) (Parkinson et al., 2007, 2009; Richert et al., 2005, 2007; Yamamoto et al., 2000; Zaluga et al., 2011), and the chaperonin/heat shock protein, groEL (Pelludat et al., 2009; Waleron et al., 2011).

Full or partial sequences for these genes were obtained from the GenBank database for each pathogen. Sequences of related species and strains within the respective genera of the target pathogens were also included to improve the specificity and discriminatory power of the designed diagnostic probes. Genus-specific, species-specific and isolate- or pathovar-specific multiple sequence alignments were performed using relevant sequence data with the MUSCLE (Multiple Sequence Comparison by Log Expectation) alignment search tool (Edgar, 2004), using default

settings. Alignments were examined and edited in MEGA v.4.02 (Tamura et al., 2007). From these, conserved regions were identified that demonstrated good potential as templates for the design of diagnostic probes and primers. Primers and probes developed in previous studies for selected genes of the targeted pathogens in this study were also included in probe design strategies to analyse their potential for incorporation as diagnostic probes into the microarray.

Probes were designed from selected template regions for all target pathogens using Allele-ID software v.7.7.2 (Premier Biosoft, USA) and a uniform set of parameters to standardise thermodynamic characteristics. Oligonucleotides that were selected from the analyses demonstrated at least 75% agreement with the following selected parameters:

- Length: 18 – 24 nt
- Orientation: complimentary to the sense strand
- Melting temperature (T_m): 50 - 60°C
- GC content: 35 – 70%
- Maximum base repeat: 4 nt
- Hairpin maximum ΔG (Gibb's free energy): -3.0 kcal/mol
- Self-dimer maximum ΔG: -6.0 kcal/mol

Thermodynamic characteristics of hybridisation reactions were set using default settings of the programme during probe design to ensure maximum thermodynamic support for highly specific probe:target binding:

- Nucleic-acid concentration: 0.25 nM
- Monovalent ion concentration: 50 mM
- Free divalent ion concentration: 1.5 mM

The specificity of each of the selected probes was evaluated against the GenBank sequence database by performing standard nucleotide BLAST (BLASTN) analysis with default parameters (Altschul et al., 1997). Highly specific probes were identified as those that demonstrated high alignment scores with sequences of the targeted genus, species or pathovar/isolate for each of the five pathogens. Only probes that did not demonstrate significant sequence homology with the tomato genome or other known pathogens of tomato were retained as potential diagnostic probes. Homology of probes to sequences of other pathogens used in this study was also a discriminatory factor for probe selection.

Probes that demonstrated highly significant, specific and conserved sequence homology with more than one target pathogen, however, were retained as broad spectrum diagnostic probes.

Final *in silico* validation of the specificity of selected probes was performed by pairwise alignment of selected probes and relevant reference sequences of target pathogens and related species or strains using CLC Main Workbench v.6.5 (CLC Bio, Denmark) with default parameters. Probes that demonstrated less than 2 nt mismatches with reference sequences were considered sufficiently specific to detect target pathogens, and were selected for incorporation into the diagnostic microarray. Probes that performed the best in all *in silico* evaluation steps were selected to form dimer probes according to Njambere et al. (2011). Control probes were also included in design strategies and were complimentary to house-keeping genes of the tomato genome or were random control probes, complimentary to organisms unrelated to tomato and any other organisms that might be present in infected tomato samples. Hybridisation control probes (Table 5. 2) to validate efficient slide hybridisation, were also included and were based on the GUS (beta-glucoronidase) and EACMV (*East African cassava mosaic virus*) genes, for which optimised amplification protocols have been developed for routine use by this research group.

For conventional PCR amplification assays, primers were selected or designed to flank diagnostic portions of targeted genes containing the chosen diagnostic probes. To analyse the specificity of these primers for target pathogens, binding regions were validated by BLASTN analysis and pairwise alignments to reference sequences using the CLC Main Workbench. Primer pairs selected for this study are listed in Table 5. 2. To minimise the number of primer sets used in this study, only primers that demonstrated consistent and specific binding from *in silico* analysis to all available sequences of all strains and isolates of the targeted pathogens were considered. Primers that demonstrated broad spectrum specificity for the three targeted bacterial pathogens of this study were favoured for selection. Wobble bases were included in primer sequences, with restraint, to broaden the specificity of attractive primers. Minimal requirements for chosen forward and reverse primer pairs included: maximum difference in T_m of 5°C, amplicon size of 1500 bp or less, minimal intra and inter-primer homology and the absence of significant homology with the tomato genome, other tomato pathogens or other primers used in the study. Where no suitable primers could be identified from previous studies, new primers were designed to target conserved regions identified from sequence alignments. Design was based on the aforementioned criteria for suitable diagnostic primer pairs.

5.3.4. Pathogen-specific amplification from total nucleic-acid extracts

Total RNA extracts from viral-infected and healthy leaf samples were converted to single-stranded cDNA (Figure 5. 1) by reverse-transcription (RT)-PCR using the SuperScript III Reverse Transcriptase System (Invitrogen, USA). Reaction mixes containing 5 µg of RNA, 3 µg of random nonamer primers and 40U of Ribolock RNase-Inhibitor (Fermentas, EU), were incubated at 70°C for 5 minutes for primer annealing. After 10 minutes at room temperature, the remaining reaction components were added, which included 20 mM dNTPs, 40X 1st Strand buffer, 9.6 mM DTT and 400U of reverse transcriptase (Invitrogen, USA) to a total reaction volume of 41.5 µℓ. Samples were incubated at 42°C for four hours, to ensure high yields of cDNA from RNA templates, and stored at -20°C. For improved detection, 10 µg of RNA was used for cDNA synthesis from PepMV extracts, as poor yields were obtained using samples with lower starting concentrations compared to *Tobamovirus* and healthy control extracts.

Samples from pathogen-infected leaves were used directly in PCR assays with pathogen-specific primers, but control samples from healthy leaf extracts were subjected to clean-up procedures for removal of residual RNA and reaction components. To two samples of 41.5 µℓ from healthy leaf extracts, 5 µℓ of sterile 1M NaOH (Merck, South Africa) and 0.5M EDTA (Sigma Aldrich Chemie, Spain) were added and incubated at 65°C for 15 minutes for the hydrolysis of RNA. The pH of samples was subsequently neutralised by adding 7.25 µℓ of 1M HEPES buffer (4-(2-hydroxyethyl)-1-piperazinethansulfonic acid [ph 7.0]; Promega, USA) and RNase-free H_2O to a final volume of 100 µℓ. Purification of cDNA from these samples was performed using the RNeasy Minelute Clean-Up Kit (Qiagen, Germany) according to the suggested protocol. Elution of cDNA from spin columns was performed by two applications of 14 µℓ of RNase-free H_2O and 1 minute incubation at room temperature before centrifugation at full speed for one minute. Purity and concentration of healthy control cDNA samples was analysed using a spectrophotometer and stored at -20°C before incorporation into labelling procedures.

Amplification of multiple gene fragments from viral cDNA and bacterial DNA samples (Figure 5. 1) was performed using the DreamTaq amplification system (Fermentas, EU) and selected pathogen-specific primers (Table 5. 2). Each 50 µℓ reaction mixture contained 5 µℓ of template cDNA or DNA, 0.4 µM of the relevant forward and reverse primers, 10 mM dNTPs (Fermentas, EU), 10X DreamTaq Buffer with added $MgC\ell_2$ and 1.25U of DreamTaq Polymerase. Cycling conditions for amplification were dependent on the length of the target gene fragment as well as the optimum annealing temperature (T_a) of the relevant primer pair (Table 5. 2), established by gradient PCR with reference

Table 5. 2 Primer pairs selected for the amplification of gene fragments, targeted by diagnostic probes, from nucleic acid extracts of viral and bacterial pathogens as well as hybridisation control samples.

Primer	Sequence (5' – 3')[1]	Reference[2]	Target	Gene	Amplicon Length (bp)	T_a [3]
PepMVUTR_F	ACAGCKAAMGCACTTTAC	This Study	PepMV	RdRp	1167	42°C
PepRep_R	AATCATGCACCTCCAGTC	This Study				
PepTGB1F	TTAGTTATAGGWTCACCTAGGC	This Study	PepMV	TGB	630	49°C
PepTGB2R	TGGAAGTAAATGCARGCTGAC	This Study				
PepMVCP_F	TGGAACGGGTTAAGTTTTCCT	This Study	PepMV	CP	820	49°C
CPR_1	AGAAAACCCCACTCTRRTTA	This Study				
ToMVF	CAAYTACAATGGCATACACA	This Study	Tobamovirus	Replicase A[4]	1036	51°C
ToMV10_3R	TTGTARTGCCAYGCGTCTTC	This Study				
ToMV42_2	GCAGGWACWCAATAGCAATTACAG	This Study	Tobamovirus	Replicase B[5]	1518	51°C
ToMVMP_R	CCTTTAACAACTAGAGCCATC	Letschert et al. (2002)				
TobUniF	GTYGTTGATGAGTTCATGGA	Letschert et al. (2002)	Tobamovirus	CP	787	51°C
TobUniR	TTAAGTGGAGGGAAAAACAC	This Study				
16R_Bact	GCTCGTGTCGTGAGATGT	This Study	Eubacteria	ITS	1400	59.6°C
23R_Bact	CCTTTCCCTCACGGTACT	Guasp et al. (2000)				
rpoB1_Bact	RTMRTCAACGGCACCGAG	Waleron et al. (2011)	Eubacteria	rpoB	1220	59.6°C
rpoBR_Bact	CGGRTTGTTCTGGTCCAT	Richert et al. (2007)				
gyrB-2F	ACCGTCGAGTTCGACTACGA	Richert et al. (2005)	Cmm	gyrB	849	55°C
emigyrBR	GTTGAGGATCTTGCCGCGCA	This Study				
Xcv19_gyr	CCCTGCTGCTGACCTTCTT	This Study	Xcv and Pst	gyrB	729	57°C
X.gyrrsp1	CARGGTGYTGAAGATCTGGTC	Parkinson et al. (2007)				
groELF pos	CCTTGATGTTCGAGATCTTCG	Pelludat et al. (2009)	Cmm	groEL	338	62°C
groELR pos	GGCATCGAGAAGGCCGTC	Pelludat et al. (2009)				
GUS Plus F	CAACATCCTCGACGATAGCA	Rossin (2008)	pCAMBIA 1305.1	GUS	181	58°C
GUS Plus R	GGTCACAACCGAGATGTCCT	Rossin (2008)				
EACMV AC1/4 F	TTCTGGCATCGACTTGGAAA	Moralo (2009, unpublished)	EACMV	AC1/4	207	54°C
EACMV AC1/4 R	ATGGGGTGCCTCATCTCCAT	Moralo (2009, unpublished)				

[1] Primer pairs are listed as forward primers followed by antisense-binding reverse primers.
[2] Primers obtained from literature were analysed for the ability to bind to targeted sequences *in silico* and, in some cases, altered slightly to broaden binding specificity.
[3] The optimum annealing temperature (T_a) for each pair was determined using temperature gradient PCRs and used in all subsequent amplification steps.
[4] Region of the ToMV genome covering a portion of the 126 kDa replication protein and an 183 kDa read-through protein that functions in replication of the viral genome.
[5] Region spanning a portion of the 183 kDa read-through protein of the ToMV genome.

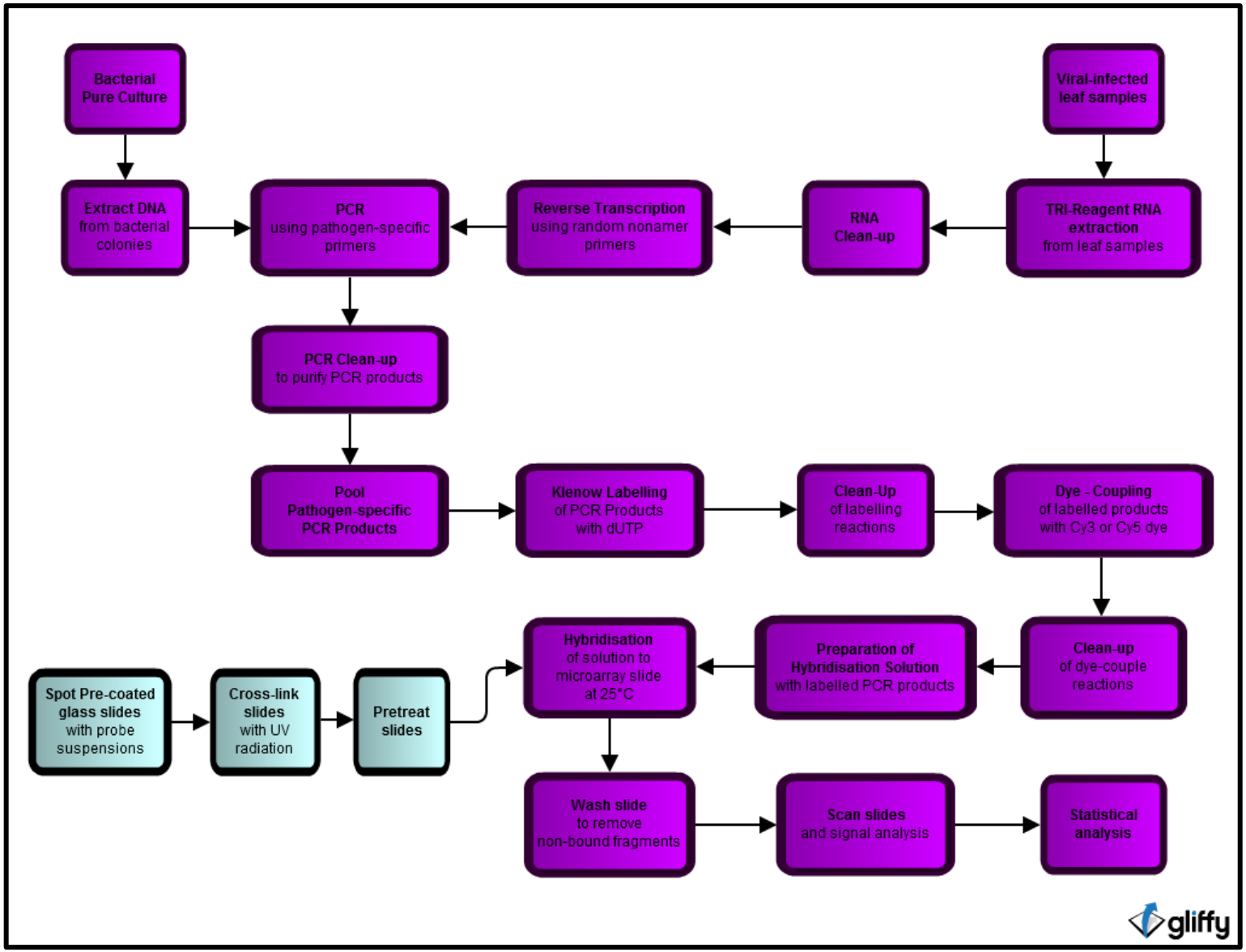

Figure 5. 1 The process of template preparation and hybridisation followed in this study for analysing pure cultures of viral or bacterial isolates in the diagnostic microarray. Two technical replicates from the same source for all tested isolates and healthy control samples were used for comparative evaluation of the robustness of this process.

cultures. Conditions were based on a standard programme of: denaturation for 5 minutes at 95°C; 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at T_a (Table 5. 2) for 30 seconds and elongation at 72°C for 30 seconds/ 500bp of target fragment length (Table 5. 2); final extension at 72°C for 5 minutes/500 bp of target fragment length. Amplification of hybridisation control samples was also performed using the DreamTaq system using the same concentrations of the components, but 1 µℓ of template DNA. The 181 bp fragment of the GUS gene was amplified from pCAMBIA vector plasmid templates (Table 5. 2) under thermocycling conditions of: 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds; and a final elongation step at 72°C for 7 minutes. The EACMV AC1 207 bp gene fragment was amplified directly from EACMV K282 DNA-A plasmids under conditions of 95°C for 3 minutes; 30 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds; and an extension step at 72°C for 5 minutes. Confirmation of the amplification of target amplicons for all reactions was performed by electrophoretic analysis. All PCR products were resolved on a 1% (w/v) agarose gel (Bioline, USA) dissolved in 1X TAE Buffer (4 0mM Tris-acetate and 1 mM EDTA) and stained with 0.1mg/mℓ of ethidium bromide (Sigma Life Sciences, USA). Samples were run in conjunction with 5 µg of O'GeneRuler 1 kb Plus DNA Ladder (Fermentas, EU; Appendix A) to confirm the approximate length of amplified fragments. After 50 minutes at 70V, products were visualised under UV light using the BioDoc-IT System (UVP Inc., USA).

Four successful amplification reactions were prepared per gene target for both replicate samples of all bacterial and viral isolates. Of these selected samples, 45 µℓ was pooled to form a final volume of 180 µℓ per gene target for clean-up procedures (Figure 5. 1). Prior to purification procedures, all pooled samples of viral PCR products were treated with 25 µg of RNase A (Fermentas, EU) and incubated at 37°C for 60 minutes to degrade residual, single-stranded nucleic acid in samples. Pooled PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's suggested protocol, using supplied binding and wash buffers. Briefly, five volumes of PB buffer and 10 µℓ of 3M sodium acetate were added to pooled samples and mixed thoroughly. Suspensions were applied to the spin column and centrifuged at 13 000 rpm for 60 seconds to bind DNA to column membranes. Membranes were each washed with 750 µℓ of buffer PE and centrifuged for a further 60 seconds at 13 000 rpm. The wash step was repeated with an incubation at room temperature for 3 minutes prior to centrifugation, for maximum dissolution of any contaminates still present in spin columns. Columns were dried by an additional centrifugation step of 13 000rpm for 60 seconds. Bound DNA was eluted from spin columns by applying 25 µℓ of nuclease-free H_2O directly to the membrane of spin columns, incubating columns

at room temperature for 1 minute and centrifuging samples at full speed for 60 seconds. This elution step was repeated to yield a total volume of 50 µℓ for each sample. Purity and quantity of samples was analysed with a spectrophotometer and high quality samples were stored at -20°C.

5.3.5. Hybridisation of pathogen-specific gene fragments to printed arrays

5.3.5.1. Labelling of pathogen-specific PCR products

For hybridisation reactions, target DNA was labelled in two steps. The first incorporated a modified nucleotide into the DNA fragments, and the second attached complimentary fluorescent dyes to these modified nucleotides. Amino-allyl deoxyuridine triphosphate (aa-dUTP) nucleotides (Ambion, USA) were incorporated into PCR products (Figure 5. 1) using the Klenow fragment, exo$^-$ labelling system (Fermentas, EU). Purified PCR products of target genes for the pathogen isolates (Table 5. 1) were pooled in ratios based on the target fragment length (Table 5. 3), favouring higher concentrations of longer fragments in reaction mixes. Pooled products were coupled with 250 ng of each hybridisation control sample to form a total sample concentration of 3 µg for labelling reactions. Control DNA and RNA/cDNA samples were also coupled with hybridisation controls to a total concentration of 2 – 3 µg, depending on yields from nebulisation and RT-PCR assays. Pooled

Table 5. 3 Ratio of PCR product incorporation of each of the gene fragments for each pathogen or group of pathogens in labelling procedures.[1]

Pathogen	Gene fragment 1	Gene fragment 2	Gene fragment 3	Gene fragment 4	Gene fragment 5
PepMV	Rep 2	TGB 1	CP 1.5	N/A	N/A
Tobamovirus	RepA 1.5	RepB 2	CP 1	N/A	N/A
Cmm	ITS 3.5	rpoB 2	gyrB 2	groEL 1	N/A
Xanthomonas spp. **and** ***Pseudomonas spp.***	ITS 2	rpoB 1.5	gyrB 1	N/A	N/A
R. solanacearum **and** ***E. cloace***[2]	ITS 1	rpoB 1	gyrB – Cmm 1	gyrB – Xcv 1	groEL 1

[1] Proportions of genes were based on the length of the target fragment with longer fragments added in higher proportions to reduced the effect of size-dependent labelling by the Klenow enzyme.
[2] Unrelated pathogens, Rs and Ec, were amplified using all bacterial-specific primer pairs which resulted in 5 separate PCR products.

samples were incubated with 2.5 µg of random nonamer (pdN9) primers (Fermentas, EU) at 99°C for five minutes, to denature double-stranded DNA products, followed by a further 5 minutes of cooling on ice. The remaining reaction components were added to each sample for a total volume of 50 µℓ, including 2X dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 2 mM dTTP and 8 mM aa-dUTP), 1X Klenow reaction buffer and 5U of Klenow fragment, exo^{-1} (Fermentas, EU). Samples were incubated at 37°C overnight, or a minimum of 16 hours, for maximum dUTP incorporation into gene fragments. After incubation, samples were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the protocol described above (5.3.4). Wash buffers provided with the kit were replaced with a phosphate buffer (1M KPO_4 [pH8.5], 15.25 mℓ nuclease-free H_2O and 84.25 mℓ of 95% EtOH) for removal of contaminants from spin columns. Eluates of 50 µℓ were analysed spectrophotometrically and samples with concentrations greater than 60 ng/µℓ were retained for dye-coupling steps and concentrated for 32 minutes using a RH20-12 SpeedVac concentrator (Savant,USA). Any replicates that yielded concentrations less than 60 ng/µℓ were discarded, as poor levels of amplification were likely associated with poor incorporation of the modified nucleotides. As primers selected for amplification reactions were pathogen-specific, low concentrations of labelled products were expected for replicate samples of non-target pathogens and, therefore, these samples were included in dye-coupling reactions, irrespective of DNA concentration.

Dye-couple reactions (Figure 5. 1) were performed using cyanine fluorescent dyes: Cy3 and/or Cy5, from the Cy Dye Post-Labelling Reactive Dye Pack (GE Healthcare, UK), which were resuspended in molecular grade DMSO (Sigma Life Sciences, EU). Dye coupling reactions incorporated either Cy3 or Cy5 fluorescent dye-molecules, but technical replicates of each isolate were always labelled with the same dye to standardise any possible variations in hybridisation or background signal associated with the use of different fluorescent dyes. Concentrated samples from Klenow reactions were resuspended in 0.2M sodium bicarbonate buffer at pH 9.0 (200mM Na_2CO_3 and 200mM $NaHCO_3$), with 3 µℓ of sterile, nuclease-free H_2O and 2 µℓ of Cy3 or Cy5 dye suspensions. Reactions were incubated in the dark at 22°C for three hours for dye-coupling. Clean-up of reactions (Figure 5. 1) was performed as mentioned previously, using the QIAquick PCR Purification Kit (Qiagen, Germany) and the binding and wash buffers supplied with the kit. Washing steps were altered to reduce the risk of losing coupled dye molecules. The second wash step was only incubated at room temperature for 1 minute before centrifugation and subsequent elution steps, to yield a total eluate of 50 µℓ for each sample. Dye incorporation efficiency and DNA concentration of the labelled sample was analysed using a ND-1000 spectrophotometer. For dual channel hybridisations (i.e., with both Cy3 and Cy5-labelled products), two labelled samples were combined, and samples destined for both

dual channel and single channel (i.e., only Cy3 or Cy5 products) hybridisations were concentrated for 42 and 32 minutes, respectively. Concentrated samples were stored in the dark on ice until incorporation into hybridisation solutions.

5.3.5.2. Preparation and pre-treatment of microarray slides

Arrays were built using glass slides coated with covalently bound, positively charged, gamma amino propyl silane (GAPS) solution (GAPS II Slides; Corning, UK), for improved binding capacity of glass substrates for oligonucleotide probes. Selected diagnostic probes were synthesised on a 0.01 µM scale with cartridge purification (Inqaba Biotech, South Africa). Stock solutions of 40 µM were prepared from concentrates for each probe using sterile, nuclease-free H_2O. A further five dilutions of both hybridisation control probe suspensions were made and, spotting solutions of hybridisation control samples included concentrations of 1.25, 2.5, 5, 10, 20 and the original 40 µM solution. Stock solutions and dilutions of hybridisation control probes were further diluted 1:2 with molecular grade dimethyl sulfoxide (DMSO; Sigma Life Sciences, EU) to a final volume of 10 µℓ for slide spotting. Probe suspensions were printed on glass slides using a Generation 3 Spotter (Molecular Devices, UK) at 20°C and 50% relative humidity (Figure 5. 1). Probes were printed in two adjacent sector repeats with quadruplicate spots replicated twice per sector, resulting in a total of 16 spots per probe per slide and a total of 2560 features per slide. The positions of spotted probes were randomized but adjacent sectors were identical, and positions were compiled into a Gene Array list (GAL) file for subsequent analysis of scan results. Printed slides were dried overnight at 20°C and 50% relative humidity, to ensure uniform spot morphology, and cross-linked with UV (Figure 5. 1) in a 250 mJ X-Linker (UVItec, UK). Slides were stored in a dessicator container in the dark at 22°C.

Prior to hybridisation, slides were pretreated (Figure 5. 1) with a 50 mℓ solution of 3.5X SSC buffer (saline-sodium citrate; Sigma Life Sciences, USA), 0.2% sodium dodecyl sulfate (SDS; Sigma Life Sciences, Switzerland) and 0.1g bovine serum albumin (BSA; Roche, Germany). Slides were incubated in the solution for 20 minutes at 60°C, rinsed briefly in sterile, nuclease-free H_2O and dried using a flat-bed spinner (Labnet, Korea) for four minutes at 1000 rpm. Slides were maintained in the dark at room temperature and a high relative humidity until application of hybridisation solutions.

5.3.5.3. Preparation and hybridisation of labelled target nucleic acid to microarray slides

Labelled DNA samples were incorporated into hybridisation solutions of 55 µℓ (Figure 5. 1) containing 50% formamide (Merck, Germany), 25% nuclease-free H_2O and 25% Amersham

Microarray Hybridisation Buffer (GE Healthcare, UK). DNA was denatured at 95°C for 2 minutes, briefly placed on ice and pipetted in even volumes across the surface of printed microarray slides. To spread the hybridisation solution evenly across slides, a 24mm x 60mm glass coverslip (Marienfeld, Germany) sterilised with 70% EtOH, was gently lowered onto slides. Slides were placed in HybUP hybridisation chambers (Engineering Dept. of Pretoria University, South Africa) and incubated for a minimum of 16 hours at 25°C for hybridisation of targets to complimentary, immobilised probes (Figure 5. 1). Non-bound fragments were removed from slides after incubation, by consecutive 50mℓ buffer washes of increasing stringency (Figure 5. 1). Slides were subjected to an initial wash (1X SSC buffer, 0.2% SDS) for 4 minutes at 25°C, and a further two washes (0.1X SSC buffer, 0.2% SDS) for four minutes at 25°C. These were followed by three washes (0.1X SSC buffer) for 1 minute at room temperature and a final rinse in a 0.01X SSC wash for a few seconds at room temperature. Slides were dried for 4 minutes at 1000 rpm.

5.3.6. Microarray data capture and analysis

Scanning of slides was performed using an Axon, GenePix 4000B Scanner (Molecular Devices, UK) at dual laser wavelengths of 532 nm (Cy3) and 635 nm (Cy5). Laser power was set at 100% for maximum signal detection, and the photomultiplier tube power (PMT) was set at 700 for the 532 nm wavelength (Cy3) scans and 850 for the 635 nm wavelength (Cy5) scans. Positive hybridisation signals from images were indicated by the detection of green (Cy3) or red (Cy5) dots/spots. White spots or features indicated high concentrations of labelled probe:target complexes or saturation of probes (Figure 5. 3). Signal intensity of the microarray spots was analysed and quantified using GenePix Pro software v.6.1 (Molecular Devices, UK). Spots were resized to incorporate maximum intensity readings and spots affected by local technical deformations, or localised blemishes on slides resulting from technical errors during slide processing, were removed from analyses. The position and identity of spots was associated with signal intensity and background intensity readings using the reference GAL file (5.3.5.2). To evaluate the significance of hybridisation signal readings for each probe, the SNR (signal to noise ratio) for each of the 16 spots per probe per slide was selected for statistical analysis. This ratio was generated by the software and based on the equation:

SNR532/635 = (Foreground 532/635 Median – Background 532/635 Median) ÷ Background 532/635 Standard deviation.

This value represented the average true signal of each feature, taking into account the effect of background signal variation. Features with SNR values ≥ 2.5 were considered to have significant

hybridisation signals, with the highest SNR values representing strong, reliable hybridisation of the relevant probe to the target. The proportion (propn), or number of replicate spots or features out of a total of 16 spots that demonstrated SNR values ≥ 2.5, was calculated for each probe per slide. Values closer to 1.00 indicated reliable hybridisation of all replicate probes to complimentary targets within the tested sample.

Statistical analysis of scan results and SNR values from scans of hybridised slides, for all samples and replicates, was performed using R statistical software v.2.13.2 (http://www.r-project.org). For the 16 replicate spots per probe per slide, the mean or average SNR value and the standard deviation of the data set was calculated. Probes that demonstrated a mean SNR of < 2.5 in both replicates of an isolate were excluded from further analysis. The co-efficient of variation (CV), indicating the distribution of the data relative to the mean, was also calculated for each set of data for each probe per slide, based on the equation:

%CV = Standard deviation of data set (16 data points)/Mean of the data set

Probes with CV values of < 20% therefore indicated low variation between SNR readings and good uniformity of hybridisation of the target to replicates of that probe on a particular slide. The normality, or binomial distribution, of SNR values for replicates of each probe was determined using the Shapiro Wilk test using a significance level of 5%. For data sets where the probability (p)-value for this test was > 0.05, i.e., this result was obtained in more than 95% of observations, data was considered normally distributed. A one-tailed student's t-test (for normally distributed data) or a Wilcoxon rank sum test (for data not normally distributed) was performed on SNR values for the 16 replicate features for each probe per slide. These tests were performed to determine whether the mean of this data set was significantly > 2.5 at a 5% level of significance. For data sets with p-values > 0.05, tests concluded that the mean SNR value for that data set was significantly > 2.5. The result of this test indicated whether hybridisation signals from probe:target complexes was reliably significant and could be potential diagnostic probes for the targeted pathogen or isolate. This result was not discriminative for the selection of potential diagnostic probes, but was rather used to indicate superior diagnostic probes that were capable of producing SNR values that were significantly higher than the threshold value of 2.5. For improved accuracy of the result of this test, the p-values for each data set considered were corrected for multiple testing using the false discovery rate (FDR). This ensured that the assumptions based on statistical analyses were valid

despite the presence of various factors during the preparation, hybridisation or analytical process of the assay which may have affected readings.

5.4. Results

5.4.1. Nucleic acid extractions

To ensure that high yields of nucleic acid were obtained from infected (RNA) and control (RNA and DNA) leaf samples of between 85 and 100 mg, crude extraction methods were selected over column-based extraction procedures. Purified crude extracts from PepMV-infected leaf samples yielded RNA concentrations of 1375 – 2856 ng/µℓ with high quality and purity. The quality of the RNA extracted was based on the criteria that the ratio of absorbance values A260/A280 > 1.8, while the purity was based on the criteria that the ratio A260/A230 > 1.8. Extracts from leaf samples infected with ToMV, TMV or PMMoV *Tobamovirus* particles yielded concentrations of 860 – 1206 ng/µℓ, all with satisfactory quality and purity ratios. Two replicate RNA samples representing each of these viral isolates, with the highest RNA concentrations as well as the best quality and purity of RNA, were selected for cDNA synthesis. Negative control samples included both RNA and DNA extracts from healthy tomato leaf samples. Extracted RNA demonstrated adequate quality and purity with concentrations of 344 – 867 ng/µℓ. Extractions of DNA from leaf samples yielded concentrations of 1181 – 2443 ng/µℓ with acceptable quality and purity. The quality of the DNA extracted was based on the criteria that A260/A280 > 1.6, with the purity was based on the criteria that A260/A230 > 1.6. Due to the high amount of DNA lost during nebulisation (30-50% of the DNA), three 50 µℓ samples were pooled and concentrated to represent each technical replicate, yielding final concentrations of 6 – 8 µg of DNA. The nebulised technical replicates of control DNA recorded final concentrations of 2.48 µg (DNA1) and 2.24µg (DNA2) of DNA. These samples were used directly in labelling reactions. Crude bacterial extracts were not quantified prior to DNA amplification reactions as the process of extraction, as well as the dilution of extracts, enabled direct use of these samples in amplification reactions to produce pathogen-specific target DNA fragments.

5.4.2. Selection of pathogen-specific primers and diagnostic probes

Evaluation of conserved regions of pathogen genomes selected from multiple sequence alignments to design probes, revealed hundreds of potential probe sequences for each pathogen, with satisfactory thermodynamic properties for uniform hybridisation. Sequences were filtered so that

only probes between 18 and 24 nt in length, meeting more than 75% of the selected thermodynamic and structural parameters, were subjected to homology investigations using BLASTN and pairwise alignments. High conservation of phytobacterial sequences with related and unrelated bacteria was common for all genes analysed. Chance similarity (e-values of over 0), among the probe query sequences, the tomato genome and sequences of other known non-targeted pathogens was also frequently observed. Query sequences were discarded from further analysis if more than 10 consecutive base pairs were shared with sequences of a non-target organism or with the host genome, as these could lead to false positive diagnoses. A total of 152 probes were selected for incorporation into the diagnostic microarray. These included probes complimentary to selected genomic regions of each of the five targeted pathogens; dimer diagnostic probes; negative control probes for house-keeping genes of the tomato genome; random negative control probes and hybridisation control probes. For a detailed list of all selected probes indicating sequences, references, targeted genes and taxonomic level of specificity, refer to Appendix B.

For specific detection of PepMV, 26 probes were selected that targeted three regions of the viral genome: a 1 kb fragment in the 5' region of the RNA dependent RNA polymerase (RdRp) gene; a region of the triple gene block (TGB) and the entire coat protein coding region. Of these probes, 10 demonstrated high complimentarity during *in silico* analysis to all strains and isolates of the PepMV species, with a further two also demonstrating complimentarity to other *Potexvirus* species in the same genomic regions. For strain/isolate-specific detection, 11 probes were selected that demonstrated high discriminatory potential with CH2 and US2 PepMV isolates, as these were identified as close relatives to the SA PepMV isolate used in this study (Chapter 4, 4.4.3). To confirm previous characterization of this isolate, three additional probes, specific only to other isolates of PepMV, were included in the developed microarray. Two of the selected diagnostic probes that each demonstrated the best specificity during *in silico* analysis, based on high alignment scores and limited SNPs, for members of the PepMV species or US2/CH2 isolates, were selected to form dimer diagnostic probes.

As tomato may be a host to more than one type of *Tobamovirus*, the 27 probes selected for incorporation into the diagnostic microarray included more genus level diagnostic probes than those selected for PepMV detection. These probes targeted three regions in the viral genome, including two portions of the replicase read-through protein, and the entire coat protein coding region. Of these, seven probes demonstrated complimentarity with ToMV and other *Tobamovirus* species and represented potential genus-specific diagnostic probes. To differentiate between infections of TMV

and ToMV, two probes with discriminatory specificity for the TMV genome were also included in the microarray. Remaining probes were complimentary to isolates and strains of ToMV. No sub-species specific probes of ToMV were specially designed for this microarray. Two of the probe sequences with the highest complimentarity, and highest alignment scores, to sequences of ToMV were used to construct dimer probes for incorporation into the microarray.

Probes selected for phytobacterial detection ranged in specificity, with some demonstrating broad spectrum specificity to most bacteria tested and others specific to sub-species of the targeted pathogens. Probes selected for Cmm, Xcv and Pst detection included genus-, species- and pathovar- or subspecies-specific sequence complimentarity. Only regions of the Xcv genome demonstrated limited polymorphisms for sub-genus characterization. A total of 33 diagnostic probes selected for the detection of Cmm were based on regions of four targeted genes: ITS, rpoB, gyrB and groEL. Detection of Xcv and Pst isolates was based on 14 and 17 probes, respectively, which were complimentary to three target genes: ITS, rpoB and gyrB. Although potential diagnostic probes were identified for Pst and Xcv detection using the groEL gene fragment, primers were not available for these regions so they were omitted from the array. Three probes represented potential universal probes, as they demonstrated conserved complimentarity with numerous species of bacteria, including those of interest in this study, as well as the tomato genome. Another two probes, identified from Cmm sequence alignments, were complimentary to numerous Eubacteria and were, therefore, included in the array for broad spectrum detection of bacteria. For each target gene, the most specific probe for bacterial diagnosis was used to form a dimer probe for Cmm, Xcv or Pst. This translated into four probes for each bacterium, including an additional probe from the ribosomal RNA region for Xcv and Pst detection.

An additional nine control probes were incorporated into the diagnostic array, two of which were designed to target alien DNA, i.e., DNA that would not be present in any tomato samples or pure cultures of targeted pathogens, as well as related organisms or non-target pathogens. These random control probes were based on genomic regions of the *Archaea* family and were not complimentary to any relevant organisms during *in silico* analysis. The five negative control probes, complimentary to genomic DNA or mRNA fragments of tomato, were based on common house-keeping genes present in high copy numbers within host plant cells. Some of these specifically targeted RNA transcripts, as these were complimentary to spliced intron borders, while others were complimentary to both RNA and DNA target sequences of the tomato genome. The hybridisation control probes selected acted as positive control probes and targeted specific genomic portions of

two vector plasmids. Templates for these probes were included in assays at the amplification stage, to ensure labelling of templates and incorporation into within hybridisation solutions, to confirm that the solutions, as well as DNA fragments, had successfully hybridised to slides during the assays.
The primers selected for the PCR amplification reactions in this study flanked regions that contained binding sites for the greatest number of the best-rated diagnostic probes. All selected primers demonstrated high specificity, or exact homology, with sequences of the targeted pathogen or groups of pathogens. As mixed infections of ToMV and PepMV have been reported (Chapter 2, 2.4.5), stringent *in silico* analysis was performed using the respective primer sequences to ensure that all pairs selected for amplification of the respective viruses did not demonstrate significant homology. The primer sets selected for amplification of the bacterial ITS and rpoB genes were complimentary to all three targeted bacteria. Amplification of the gyrB gene, however, required the use of two separate sets of primers, one for Cmm detection (designated as primer pair gyrC), and another for Xcv, Pst and closely related bacteria (designated as primer pair gyrX). Amplification of the groEL gene was limited to Cmm isolates in this study and, therefore, primers that targeted only Gram positive bacteria were considered for selection.

5.4.3. PCR amplification and cDNA synthesis

Synthesis of cDNA from RNA extracts was performed using 5 µg of RNA template per reaction. This was sufficient for recovery of total cDNA, which yielded intense bands from subsequent amplification reactions using pathogen-specific primers. Amplification reactions with PepMV-specific primers using these cDNA samples, however, yielded bands of low intensity which were unsuitable for array hybridisation. Starting concentrations of RNA for cDNA synthesis were, therefore, doubled for PepMV templates. This drastically improved the efficiency of PCR and the resulting band intensity. Purification of cDNA to remove residual RNA and reaction components, was initially performed using a clean-up kit with a preceding RNA hydrolysis step for all infected test samples and control cDNA samples. Eluted samples, however, demonstrated inconsistent purity values or 260/230 ratios <1.00. The concentration of purified cDNA recovered from these clean-up procedures was also low in all replicate extractions. This step was, thus, abandoned for infected sample extracts, and residual RNA was instead degraded by incubating samples with RNase. As the cDNA of healthy, negative control samples was used directly in labelling reactions, this degradation step was insufficient to purify samples for array hybridisation. Samples were, thus, still purified using clean-up and hydrolysis procedures. Those with the greatest cDNA concentrations as well as the best quality and purity of cDNA, based on 260/280 and 260/230 ratios >1.8, were pooled and concentrated to

form two samples containing 2.8 µg and 3.0 µg of cDNA, respectively, representing the technical replicate samples for healthy RNA controls in labelling reactions.

Amplification assays using selected primers resulted in the generation of amplicons of the expected length for all targeted genes, except the rpoB gene, of the five pathogens of interest (Figure 5. 2). Fragments of the expected length were also generated in amplification reactions of related pathogen species, but three reactions resulted in the production of non-specific fragments. It was necessary to perform as many as 10 replicate amplification reactions per gene target for each technical replicate of each pathogen isolates, and to validate reactions electrophoretically, as the band intensity of amplicons generated varied among samples and assays. Only four samples demonstrating the strongest bands for each gene target were selected for clean-up procedures. These samples contained high concentrations of target fragments and were, therefore, the best templates for array hybridisation Intense smears of bands were observed in electrophoretic analysis of viral amplicons prior to clean-up procedures. This was associated with residual RNA or reaction components remaining from cDNA synthesis, as these smears were less visible after clean-up procedures were applied to pooled PCR products. Improvement in concentration and quality of amplicons by clean-up procedures was confirmed by electrophoretic analysis (Figure 5. 2), this removed residual DNA/RNA and reaction components from samples, and could then be used to test the diagnostic microarray.

Amplification of target genomic regions from PepMV templates was successful (Figure 5. 2 A) and produced strong bands of amplicons when 10 µg of starting RNA was used in cDNA synthesis reactions. Samples of cDNA synthesised from lower concentrations of RNA only generated visible amplicons from reactions utilising TGB primer pairs. Reactions using these primers were more efficient than reactions with the other two sets of primers, i.e., capable of binding to target templates rapidly and amplifying large copy numbers of fragments, as pooled PCR products produced a dense band after clean-up procedures compared to those representing the RdRp fragment and the coat protein fragment (Figure 5. 2 A). Conditions for amplification of the RdRp gene from templates had to be optimised in this study for consistent amplification, which may be indicative of low levels of this target template within the samples tested. Amplification of the coat protein region from cDNA templates resulted in the weakest band intensity of the three gene targets. No non-specific fragments were detected from any of the amplification reactions, proving that selected primer pairs for PepMV detection were highly specific.

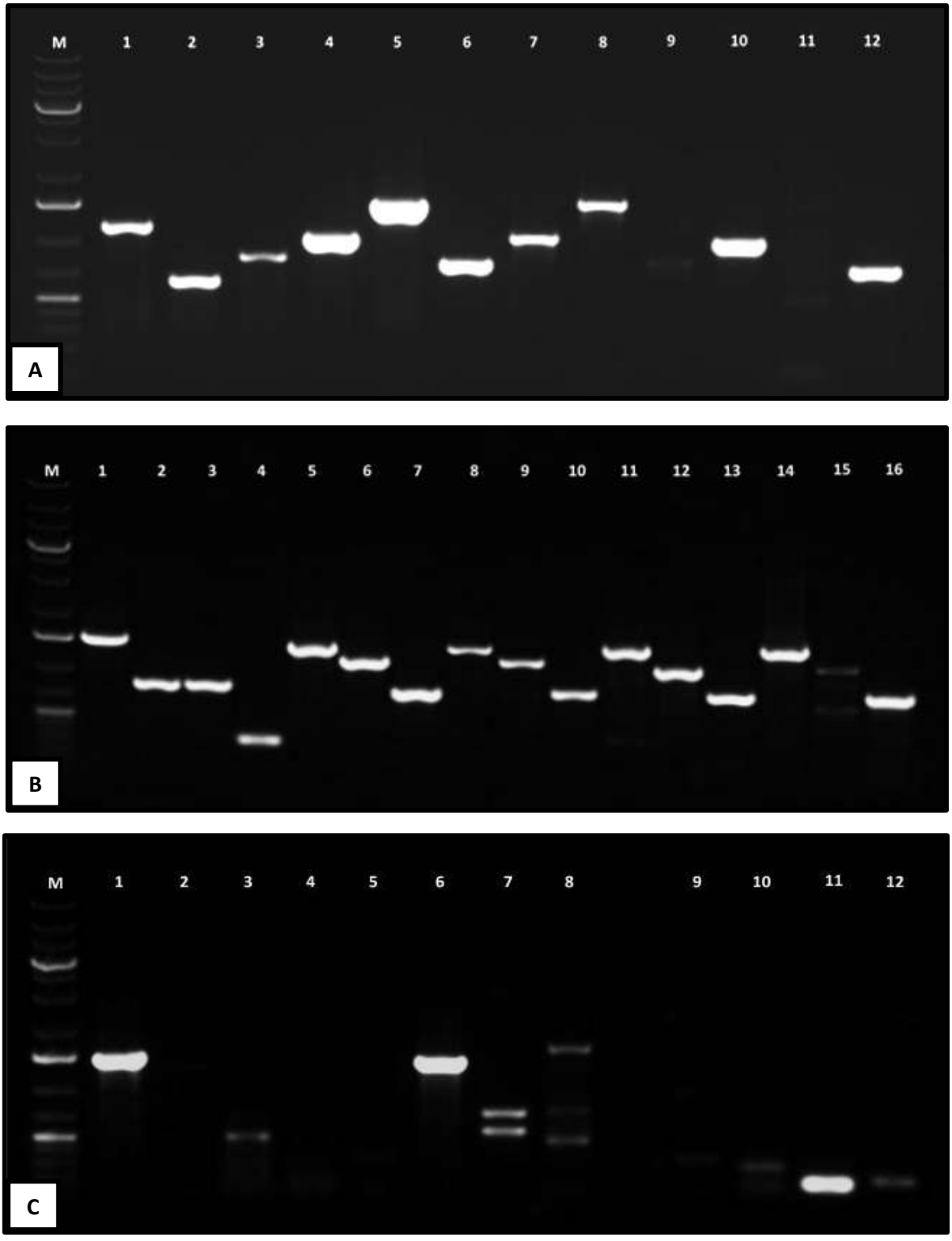

Figure 5. 2 Consolidation of amplification reactions using purified, pooled PCR products that were generated from extracts of tested pathogen isolates by conventional PCR using pathogen-specific primers. Successful reactions demonstrated a strong, intense band of the expected length in electrophoretic analysis. Amplification of viral isolates from cDNA samples (A): Lane 1 – PepMV RdRp; 2 – PepMV TGB; 3 – PepMV CP; 4 – ToMV RepA; 5 – ToMV RepB; 6 – ToMV CP; 7 – TMV Rep A; 8 – TMV – RepB; 9 – TMV CP; 10 – PMMoV RepA; 11 – PMMoV RepB; 12 – PMMoV CP. Amplification of targeted bacterial isolates and related bacteria (B): Lane 1 – Cmm ITS; 2 – Cmm rpoB; 3 – Cmm gyrB; 4 – Cmm groEL; 5 - Xcv ITS; 6 – Xcv rpoB; 7 – Xcv gyrB; 8 – Xv ITS; 9 – Xv rpoB; 10 – Xv gyrB; 11 – Pst ITS; 12 – Pst rpoB; 13 – Pst gyrB; 14 – Pss ITS; 15 – Pss rpoB; 16 – Pss gyrB. Amplification of non-target bacterial pathogens and hybridisation control samples (C): Lane 1 – *R. solanacearum* ITS, 2 - *R. solanacearum* rpoB; 3 - *R. solanacearum* gyrC; 4 - *R. solanacearum* gyrX; 5 - *R. solanacearum* groEL; 6 – *E. cloace* ITS; 7 - *E. cloace* rpoB; 8 - *E. cloace* gyrC; 9 - *E. cloace* gyrX; 10 - *E. cloace* groEL; 11 – GUS; 12 – EACMV. For all agarose gels lane M – 10 µg O'GeneRuler DNA Ladder (Fermentas, EU).

Amplification of target fragments with selected ToMV-specific primers yielded thick, intense bands of the expected length in all assays (Figure 5. 2 A). Assays were repeatable and target-specific, producing the greatest concentration of amplicons with RepB primers. This was in contrast to amplification reactions with related viruses, TMV and PMMoV, which did not produce such intense bands in PCR with all of the relevant primer sets (Figure 5. 2 A). Bands of the expected length were produced in reactions with RepA primers for both TMV and PMMoV. However, faint bands of the expected length were produced from TMV templates using primers targeting the coat protein region. Variable, non-specific amplification of RepB was observed from reactions with PMMoV samples, as two faint bands differing in length from the bands generated with ToMV and TMV samples, were detected by electrophoretic analysis (Figure 5. 2 A).

Amplification of the ITS region from all bacterial cultures was repeatable and efficient using the selected primers (Figure 5. 2 B and C), generating amplicons of the expected size from DNA extracts of targeted bacteria, related bacteria and even non-target bacteria pathogenic on tomato. A small, non-target amplicon was, however, detected from ITS amplification reactions with DNA extracts of Pst. Interestingly, the single set of primers used for amplification of a portion of the rpoB gene, resulted in the production of amplicons of different sizes among groups of related bacteria evaluated. The amplicon generated from *Xanthomonas* templates was the largest while reactions with Cmm resulted in smaller bands (Figure 5. 2 B). Poor specificity of the rpoB primers for Pss amplification was concluded by the detection of fragments of different lengths, including one of the expected length, in electrophoretic analyses. Selected rpoB primers were weakly complimentary to extracts of DNA from *R. solanacearum* cultures and amplicons produced were faintly visible from concentrated, purified PCR product samples (Figure 5. 2 C). Extracts from *E. cloacae* cultures, however, produced two strong, non-specific fragments in reactions with these primers. This confirmed the high specificity of the selected primers to regions in the rpoB gene of the targeted bacteria. Amplification of the gyrB region using appropriate primer sets resulted in repeatable and efficient generation of a single band of the expected length from reactions with bacterial isolates (Figure 5. 2 B). No non-specific fragments were detected in these assays making samples suitable for hybridisation reactions. Amplification reactions using the gyrC and gyrX primer sets with DNA extracts of *R. solanacearum* and *E. cloacae*, however, only resulted in the production of faint, non-specific fragments (Figure 5. 2 C), different from bands generated with Cmm, Xcv or Pst DNA extracts. Primers selected for the amplification of a region of the groEL gene from Cmm extracts facilitated the production of strong bands of the expected length (Figure 5. 2 B). Reactions of these primers with extracts of *R. solanacearum*, however, resulted in faint, non-specific bands, but two,

distinct and intense bands were produced from reactions with extracts of *E. cloacae*. One of these bands was similar in length to the amplicon generated from Cmm templates, indicating potential homology between Cmm and *E. cloacae* for this region.

Amplification of positive control DNA fragments from vector plasmids using primer sets for GUS and EACMV, resulted in successful production of bands of the expected length. Reactions using GUS-specific primers facilitated better amplification of target fragments than those for EACMV. Strong, intense bands of expected length were generated by reactions with the pCAMBIA vector plasmid (Figure 5. 2 C). Reactions with the EACMV vector and relevant primers generated only faint band of the expected length from amplification reactions, as well as a number of non-specific fragments.

5.4.4. Hybridisation of samples to printed microarray slides

5.4.4.1. Labelling of purified gene targets

Labelling reactions for technical replicates of isolates and control samples were prepared by adding fixed ratios of purified PCR products for each of the relevant gene targets and a fixed concentration of hybridisation control products. This standardized labelling conditions for all gene targets and hybridisation controls for each replicate. The concentration of the modified nucleotide, aa-dUTP, in dNTP mixes, proved to be crucial for facilitating optimal labelling of sequence fragments. To favour the incorporation of aa-dUTP over the chemical homolog, dTTP, during polymerization of templates, optimal labelling was obtained using mixes at a 4:1 ratio of aa-dUTP to dTTP. Greater labelling efficiency, concentrations of >60 ng/µℓ after Klenow reactions and clean-up procedures, was also recorded when dUTP was maintained at -70°C with minimal freeze-thaw treatments, compared to older stocks of dUTP or stocks stored at higher temperatures. The phosphate-based wash buffer (section 5.3.5.1) were selected over those supplied with kits for clean-up procedures, as the use of the former resulted in yields < 60 ng/µℓ from Klenow labelling reactions. The recovery of labelled products from Klenow reactions was < 100 ng/µℓ for 88% of technical replicate samples of target pathogen isolates as well as healthy control samples (Table 5. 4). The greatest yields were obtained from samples containing combined amplicons of all five pathogens of interest in this study, proving that numerous different target fragments within reaction mixes did not affect the efficiency of labelling. Non-target isolates, as expected, yielded low levels of labelled fragments.

The efficiency of dye incorporation was calculated for each technical replicate after clean-up procedures (Table 5. 4). This was based on the concentration of template fragments and the

Table 5. 4 The efficiency of indirect labelling of combined, purified PCR products representing technical replicates of all pathogenic isolates or healthy plant control samples.

Pathogen Technical Replicate	Klenow yield ng/µℓ	Dye	Dye Incorporation Efficiency[1]		Hybridised to slides with[2]
			pmol/µℓ	Concentration ng/µℓ	
PepMV1	168.40	Cy3	3.33	155.33	DNA2
PepMV2	102.90	Cy3	1.37	85.59	N/A
ToMV1	128.76	Cy3	2.22	80.32	*R. solanacearum*1
ToMV2	146.85	Cy3	1.17	78.57	*R. solanacearum*2
TMV1	91.92	Cy3	1.50	85.40	*E. cloace* 1
TMV2	80.80	Cy3	2.83	70.50	*E. cloace* 2
PMMoV1	81.90	Cy5	0.83	46.30	Xv2
PMMoV2	109.24	Cy5	0.90	49.20	Pss2
Cmm1	135.28	Cy3	1.01	124.72	Xcv2
Cmm2	113.46	Cy3	0.71	98.13	RNA1
Xcv1	142.07	Cy5	1.09	125.04	Pst2
Xcv2	192.47	Cy5	1.93	159.76	Cmm1
Xv1	184.75	Cy3	3.26	164.09	DNA1
Xv2	101.00	Cy3	0.85	78.48	PMMoV1
Pst1	170.10	Cy3	1.86	142.31	N/A
Pst2	155.56	Cy3	1.80	129.46	Xcv2
Pss1	178.48	Cy3	3.02	157.08	RNA2
Pss2	148.29	Cy3	1.40	78.80	PMMoV2
***R. solanacearum*1**	121.87	Cy5	1.70	69.66	ToMV1
***R. solanacearum*2**	136.13	Cy5	0.74	99.99	ToMV2
***E. cloace* 1**	140.45	Cy5	2.40	133.10	TMV1
***E. cloace* 2**	60.10	Cy5	0.52	46.30	TMV2
RNA1	186.11	Cy5	1.62	145.34	Cmm2
RNA2	132.78	Cy5	0.70	102.90	Pss1
DNA1	218.65	Cy5	3.43	157.44	Xv1
DNA2	194.50	Cy5	4.84	187.8	PepMV1
PCR1[3]	235.00	Cy5	3.69	202.32	N/A
PCR2	223.40	Cy5	3.18	183.52	N/A

For each technical replicate of pathogen isolates and control samples, yields from both labelling reactions were recorded.

Samples were labelled with either Cy3 or Cy5 fluorescent dyes and coupled with samples labelled with the alternate dye for slide hybridisation (i.e., Cy3-labelled fragments hybridised with Cy5-labelled fragments).

[1] The efficiency of dye incorporation indicated the amount of dye incorporated into samples.

[2] Labelled samples were combined so that two samples could be hybridised to a single array. N/A refers to slides that were only hybridised with one labelled sample.

[3] Samples designated as PCR refer to those containing PCR products from all gene targets of the five pathogens of interest in this study.

absorbance of the labelled solution. Higher levels of dye incorporation were usually associated with high yields from Klenow labelling reactions. Five samples, however, that had yields < 100 ng/μℓ from Klenow reactions, had dye incorporation values of less than 1pmol/μℓ. Although samples with dye incorporation values < 1 pmol/μℓ were favoured for hybridisations, samples with lower labelling efficiency were retained to analyse whether this affected the strength of hybridisation signals. Clean-up procedures from dye-coupling reactions, while important to remove residual, unincorporated dye molecules, caused the loss of some of the labelled products. A reduction in the loss of labelled DNA was observed when these clean-up procedures were optimised. Changes included repeating the initial binding step, and reducing the incubation times of wash solutions on clean-up columns from 3 minutes to 1 minute. This improved the recovery of labelled targets so that concentrations of Klenow templates were only reduced by 10-20 ng/μℓ.

5.4.4.2. Microarray hybridisation

A total of 17 spotted microarray slides were processed in the current study under optimised hybridisation conditions of 25°C for 16 hours using 55 μℓ of hybridisation solution. To reduce the number of slides hybridised, as well as associated costs, two samples were combined into each hybridisation solution to be applied to a single slide. As hybridised slides were scanned at dual wavelengths, signals recorded from each sample were considered separate data sets and were not expected to interfere with signals from the other coupled pair. Samples from different classes of pathogens or organisms were paired together (Table 5. 4). For example, replicates of viral isolates were hybridised with bacterial isolates or samples from unrelated pathogens were coupled. Successful hybridisations were obtained for all isolates tested and control samples used in this study (Figure 5. 3). The images produced from hybridised slides by scanners provided a visual indication of hybridisation results. These images, however, also revealed the effect of technical errors from the slide preparation process, on hybridisation and scan results. For about 50% of the slides, scratches or precipitates of dye molecules were distributed across surfaces of slides and interfered with hybridisation signals. On three of the slides, the effects of these blemishes were insufficient to necessitate the repetition of the hybridisation reaction. The hybridisation signals of the affected spots or features were removed from statistical analyses according to suggestions of the Axon Guide to Microarray Analysis (Verdnik, 2004). Calculations dealing with the affected data sets were adjusted to accommodate the new data size of these altered signal sets.

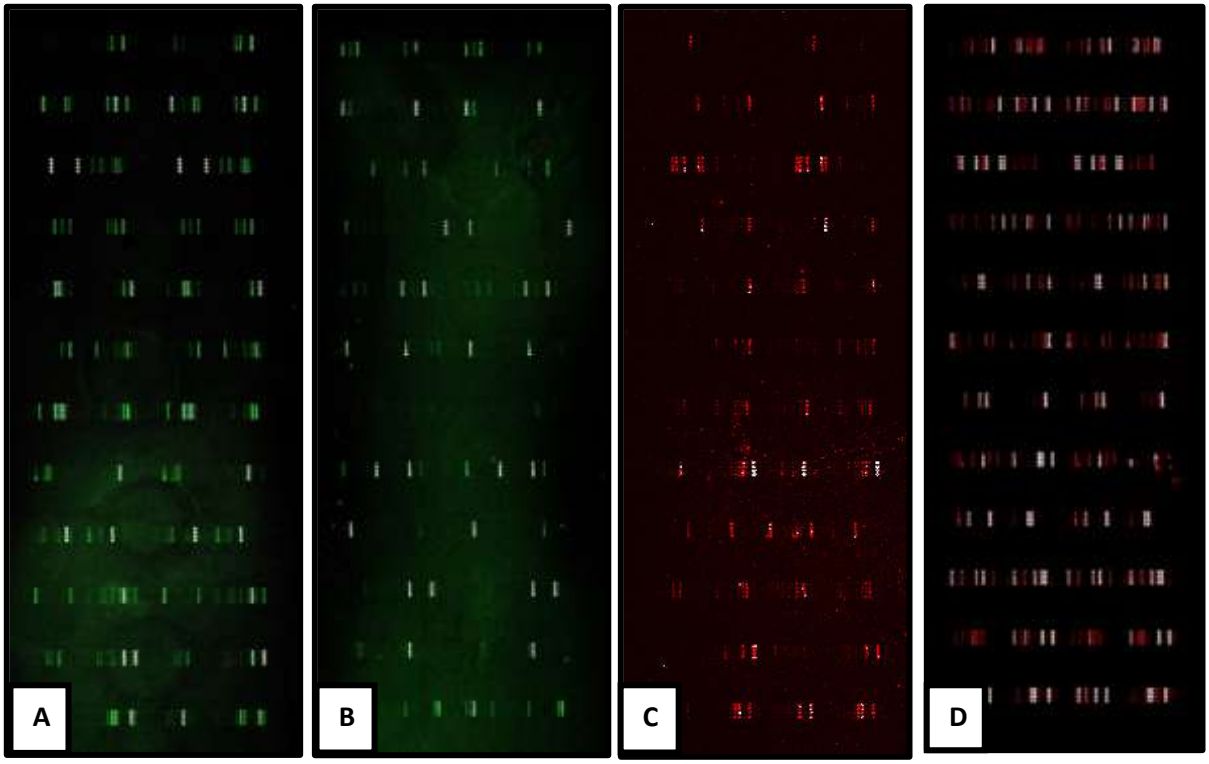

Figure 5. 3 Successful hybridisation reactions on the prototype diagnostic microarray obtained using a GenePix 4000B scanner. Patterns demonstrate target-specific signals from Cy3 labelled PepMV gene products (A), Cy3 labelled Pst gene products (B) and Cy5 labelled, nebulised DNA extracts from healthy tomato leaves (C). Gene products for each of the five target pathogenic isolates were combined, labelled with Cy5 and hybridised onto a single microarray slide (D). Targets labelled with Cy3 fluoresced green when excited with lasers at 532 nm (A and B) while those labelled with Cy5, fluoresced red when scanned with lasers set at wavelengths 635 nm (C and D).

5.4.5. Analysis and interpretation of hybridisation signals

5.4.5.1. Target recognition capacity of designed probes

The probes incorporated into the developed array were analysed for the capacity to detect target sequences, in the presence of other gene fragments by challenging slides with a hybridisation solution containing labelled gene targets from all five pathogens of interest in this study. Overall, SNR values were higher from the second technical PCR replicate than PCR replicate 1, despite a greater concentration and labelling efficiency of PCR replicate 1 prior to hybridisation (Table 5. 4). This affected the consistency of SNR values between replicates for each probe. Of all the probes spotted on arrayed slides, 75 recorded mean SNR values greater than the cut-off of 2.5, in either one or both technical replicate assays, indicating significant hybridisation to labelled targets. For each of these 75 probes, SNR values from each of the two technical replicate samples were used for statistical calculations to determine the statistical significance of SNR results (Table 5. 5). Calculations included determining: the mean or average SNR value from the 16 replicate features of each probe per slide; the standard deviation (SD) of the data set for each probe; the coefficient of

variance (%CV) which indicated the distribution or variance of the SNR values of the 16 features from the mean; the proportion of the 16 features that produced an SNR value ≥ 2.5; and the results of the t-test or Wilcoxon's signed ranked test, indicating whether the mean SNR for each probe was significantly greater than 2.5 at a 0.05% confidence level. Statistical analysis of the 75 probes revealed that 46 probes demonstrated reliable capacity for target detection in spite of signal variation between technical replicates (Table 5. 5). Selection of these probes was based on two criteria for both technical replicates: mean SNR values for the 16 replicate spots significantly > 2.5 at a 0.05% confidence level (a), and a minimum proportion of 0.5 of the replicate spots demonstrating a SNR > 2.5 (b). Probes demonstrating CV values < 40% also indicated superior detection capacity. Probes that did not satisfy these criteria were not considered reliable for detection of a target from samples. Probes designed to detect each of the five pathogens of interest in this study were included in the 46 probes selected (Table 5. 5). The greatest number of these probes were complimentary to regions of the PepMV genome, 15 of the 46 probes. Between seven and nine probes, each specific for ToMV, Cmm, Xcv or Pst, made up the remaining 31 probes. Of the 16 dimer probes that were selected for inclusion in the array, 13 demonstrated significant and reliable detection of targets from both technical replicate hybridisation reactions.

Some results obtained from replicates in these reactions did not conform to those expected. The probe TMV10_4 was designed specifically to target genomic fragments of TMV but templates of this pathogen were not included in hybridisation replicate samples. A mean SNR value of > 2.5 was recorded for this probe in results of PCR replicate 2 (Table 5. 5), but the results were not considered statistically significant and only poor SNR values were obtained in replicate 1 for this probe. Two probes, Cmm2_rpoB2 and Xanth24_rpoB, were classified as borderline probes as both recorded SNR values of > 2.5 for both technical replicate samples, but because proportion values were less than 0.5, probes were not considered specific enough to detect targets in the presence of competing, labelled DNA. Probe 1078-R demonstrated broad specificity for bacterial pathogens during homology searches but in hybridisation results from this assay, SNR values were variable and not significantly >2.5 for both technical replicates. Inconsistent results were also observed between the replicates for 14 other probes in this assay. For these probes, mean SNR, variance and the proportion values were dissimilar between technical replicates and could not be used to form reliable conclusions from data. The PCR products of two hybridisation control samples were included at consistent concentrations of 250 ng in hybridisation solutions of both technical replicate samples. Significant SNR values were, however, only recorded from some spots of the 20 µM and 40 µM GUS probes. Hybridisation signals from even these two standard control samples were not consistent between technical replicates and

Table 5. 5 Probes producing significant signal to noise ratios (SNR) when challenged with samples containing a combination of labelled amplicons from all gene targets of the five pathogens of interest in this study.

Probe	Target	Replicate 1					Replicate 2					Reliable Signal (Y/N)
		Mean	SD	%CV	Propn > 2.5	Mean Sign[1] > 2.5	Mean	SD	%CV	Propn > 2.5	Mean Sign[1] > 2.5	
1078-R	Bacteria	2.36	1.46	61.89	0.44	No	3.70	1.63	44.00	0.75	Yes	X[2]
1114f 16S/IGS	Bacteria	1.15	0.25	21.40	0.00	No	2.76	0.88	31.89	0.56	No	No
CH2-CP-F	PepMV	3.00	1.66	55.43	0.56	No	6.25	3.32	53.14	0.81	Yes	Yes
CH2RdRp_1	PepMV	5.97	3.07	51.36	0.81	Yes	11.29	5.67	50.21	0.94	Yes	Yes
Cm10_rRNA	Cmm	4.71	2.50	53.03	0.75	Yes	14.67	2.85	19.41	1.00	Yes	Yes
Cm13_gyrB2-Di2	Cmm	1.61	0.26	16.29	0.00	No	5.59	1.26	22.60	0.94	Yes	No
Cm13_rpoB	Cmm	6.69	2.09	31.24	1.00	Yes	15.77	4.00	25.36	1.00	Yes	Yes
Cm14_gyrB1	Cmm	5.19	1.86	35.83	1.00	Yes	15.22	2.87	18.83	1.00	Yes	Yes
Cm14_gyrB2	Cmm	1.93	0.66	34.11	0.25	No	4.70	1.19	25.40	1.00	Yes	No
Cm17_rRNA	Cmm	1.23	0.95	77.02	0.25	No	1.24	1.25	101.02	0.25	No	No
Cm22_gyrB	Cmm	6.97	2.84	40.79	0.88	Yes	18.63	6.43	34.53	1.00	Yes	Yes
CmgroEL_5_2	Cmm	2.03	0.49	24.41	0.13	No	8.77	3.14	35.83	1.00	Yes	No
Cmm_groEL18-Di2	Cmm	10.53	3.64	34.53	1.00	Yes	16.01	3.95	24.67	1.00	Yes	Yes
Cmm14_rRNA	Cmm	2.52	1.53	60.56	0.50	No	2.28	1.16	50.71	0.50	No	No
Cmm2_rpoB1	Cmm	6.00	1.51	25.07	1.00	Yes	22.47	5.67	25.24	1.00	Yes	Yes
Cmm2_rpoB2	Cmm	2.50	0.83	33.14	0.31	No	9.56	3.42	35.80	1.00	Yes	X[2]
Cmm2_rpoB2_Di2	Cmm	5.97	3.09	51.76	0.88	Yes	13.57	3.06	22.54	1.00	Yes	Yes
Cmm2_rpoB5	Cmm	0.86	0.29	33.38	0.00	No	3.04	1.01	33.27	0.63	No	No
Cmm2_rpoB6	Cmm	4.24	1.45	34.27	0.94	Yes	18.97	4.24	22.33	1.00	Yes	Yes
Cmmprobe-Di2	Cmm	8.18	4.90	59.92	0.75	Yes	23.94	10.35	43.22	1.00	Yes	Yes
Hyb GUS 20uM	+ve control[3]	2.42	0.95	39.17	0.31	No	3.74	0.97	25.97	0.94	Yes	X[2]

Highlighted probes represent the 46 selected probes demonstrating the most reliable detection of targets from both technical replicate samples.

[1] Refers to whether mean SNR values proved to be significantly greater than 2.5 based on the t-test or Wilcoxon's signed rank test at a 0.05% confidence interval.

[2] Represents borderline results which may be significant but require further tests to prove whether results are statistically significant or not.

[3] The positive (+ve) control is the hybridisation control sample included in hybridisation solutions to prove that labelled PCR products had hybridised to microarray slides.

Probe	Target	Replicate 1					Replicate 2					Reliable Signal (Y/N)
		Mean	SD	%CV	Propn > 2.5	Mean Sign[1] > 2.5	Mean	SD	%CV	Propn > 2.5	Mean Sign[1] > 2.5	
Hyb GUS 40uM	+ve control[3]	1.32	0.32	24.50	0.00	No	3.04	0.70	22.92	0.81	Yes	No
PepMV_15	PepMV	5.44	1.44	26.40	1.00	Yes	17.55	5.01	28.52	1.00	Yes	Yes
PepMV_15_2	PepMV	7.91	1.95	24.63	1.00	Yes	7.75	2.44	31.52	1.00	Yes	Yes
PepMV_16	PepMV	4.23	1.77	41.79	0.81	Yes	13.99	3.75	26.83	1.00	Yes	Yes
PepMV_16_2	PepMV	5.86	4.04	69.05	0.75	Yes	16.40	3.60	21.94	1.00	Yes	Yes
PepMV_16_Di	PepMV	6.80	1.82	26.81	1.00	Yes	15.60	8.95	57.33	1.00	Yes	Yes
PepMV_19	PepMV	4.64	0.89	19.12	1.00	Yes	7.20	2.93	40.63	1.00	Yes	Yes
PepMV_CH2H	PepMV	3.20	1.26	39.21	0.75	No	13.84	3.31	23.92	1.00	Yes	Yes
PepMV_CH2J	PepMV	2.88	1.38	47.75	0.50	No	4.75	1.43	30.13	0.94	Yes	Yes
PepMV_CH2O	PepMV	5.38	3.65	67.93	0.75	Yes	1.54	1.00	64.73	0.19	No	No
PepMVCh210	PepMV	5.60	2.53	45.25	0.75	Yes	14.97	3.68	24.57	1.00	Yes	Yes
PepMVCh215	PepMV	1.51	0.89	59.04	0.19	No	3.12	1.02	32.77	0.75	Yes	No
PepMVCP_1	PepMV	1.31	0.41	31.58	0.06	No	4.95	3.09	62.56	0.75	Yes	No
PepMVTGB1.2	PepMV	1.88	0.77	41.18	0.31	No	4.62	1.17	25.24	1.00	Yes	No
Ps14_rRNA	Pst	1.14	0.84	73.66	0.06	No	4.14	1.96	47.38	0.81	Yes	No
Ps22_gyrB	Pst	5.32	1.78	33.56	1.00	Yes	15.01	3.98	26.53	1.00	Yes	Yes
Ps23_gyrB	Pst	5.37	2.10	39.19	1.00	Yes	15.88	4.05	25.49	1.00	Yes	Yes
Pst1_rpoB_7	Pst	2.93	0.57	19.56	0.75	Yes	11.99	4.13	34.47	1.00	Yes	Yes
Pst1_rpoB_8	Pst	2.06	0.64	30.97	0.25	No	4.83	3.14	64.97	0.94	Yes	No
Pst11_rRNA_Di2	Pst	4.04	2.18	54.05	0.69	Yes	18.38	3.92	21.35	1.00	Yes	Yes
Pst16_gyrB	Pst	1.77	1.13	63.78	0.31	No	6.49	3.23	49.70	0.81	Yes	No
Pst19_gyrB	Pst	1.19	0.31	26.27	0.00	No	2.56	0.75	29.31	0.56	No	No
Pst2_rpoB_1	Pst	4.27	1.15	26.89	0.94	Yes	6.07	1.88	30.95	0.88	Yes	Yes
Pst20gyrB_Di2	Pst	3.95	2.14	54.13	0.75	Yes	3.99	1.87	47.00	0.81	Yes	Yes
Pst3_rpoB_Di2	Pst	4.87	2.61	53.52	0.81	Yes	3.64	1.92	52.84	0.56	No	Yes
TMV10_4	N/A	1.40	1.00	71.22	0.13	No	4.08	3.21	78.71	0.63	No	No
TO/TMV_10_3	ToMV	8.50	2.77	32.58	1.00	Yes	14.87	4.17	28.03	1.00	Yes	Yes

Probe	Target	Replicate 1					Replicate 2					Reliable Signal (Y/N)
		Mean	SD	%CV	Propn > 2.5	Mean Sign[1] > 2.5	Mean	SD	%CV	Propn > 2.5	Mean Sign[1] > 2.5	
TO/TMV_10_5	ToMV	5.23	4.08	78.13	0.56	No	7.30	4.60	63.01	0.81	Yes	Yes
ToMV3 Tib 2	ToMV	5.53	3.51	63.42	0.75	Yes	13.79	4.43	32.11	1.00	Yes	Yes
ToMV4_1	ToMV	0.69	0.25	35.58	0.00	No	2.96	1.36	45.87	0.64	No	No
ToMV4_3	ToMV	3.43	0.82	24.05	0.81	Yes	15.70	2.57	16.34	1.00	Yes	Yes
ToMV46_Di2	ToMV	5.63	2.62	46.59	0.75	Yes	15.83	2.63	16.64	1.00	Yes	Yes
ToMV46	ToMV	5.10	2.49	48.81	0.81	Yes	17.20	3.60	20.96	1.00	Yes	Yes
ToMV53_1	ToMV	2.60	1.54	59.41	0.50	No	1.94	0.50	25.85	0.13	No	No
ToMV53_4	ToMV	4.38	1.42	32.45	1.00	Yes	5.39	1.02	18.88	1.00	Yes	Yes
ToMV70_1	ToMV	1.87	1.18	63.09	0.38	No	8.49	2.39	28.19	1.00	Yes	X^2
ToMV73_2	ToMV	2.85	0.84	29.67	0.75	No	1.03	0.42	40.51	0.00	No	No
ToMV9_3	ToMV	1.72	0.33	18.87	0.00	No	4.20	0.82	19.43	1.00	Yes	No
ToMV9_4	ToMV	5.95	1.51	25.43	1.00	Yes	8.45	1.01	11.98	1.00	Yes	Yes
US2-3	PepMV	3.65	1.03	28.18	0.88	Yes	12.93	3.60	27.85	1.00	Yes	Yes
US2-7	PepMV	3.13	1.41	45.11	0.56	No	6.73	1.57	23.26	1.00	Yes	Yes
US2-7_Di2	PepMV	4.80	1.40	29.14	1.00	Yes	16.82	6.59	39.21	1.00	Yes	Yes
US2-9	PepMV	2.49	0.99	39.81	0.50	No	3.17	1.12	35.30	0.75	Yes	Yes
Xanth17_rpoB_2	Xcv	1.56	0.65	41.50	0.06	No	3.61	0.77	21.37	1.00	Yes	No
Xanth23_rpoB_1	Xcv	1.41	0.85	59.99	0.25	No	2.97	1.31	44.07	0.63	No	No
Xanth24_rpoB	Xcv	2.67	1.04	38.80	0.31	No	5.01	1.76	35.22	1.00	Yes	Yes
Xanth28_rRNA_Di2	Xcv	5.08	2.13	41.85	0.81	Yes	16.61	1.98	11.89	1.00	Yes	Yes
Xcv_rRNA_20_Di2	Xcv	7.01	1.35	19.20	1.00	Yes	22.06	5.16	23.41	1.00	Yes	Yes
Xcv11_rpoB_Di2	Xcv	6.75	2.25	33.29	1.00	Yes	15.86	6.24	39.34	1.00	Yes	Yes
Xcv11_rpoB	Xcv	3.03	0.75	24.74	0.75	Yes	4.24	0.66	15.63	1.00	Yes	Yes
Xcv20_gyrB_1	Xcv	5.82	2.16	37.15	1.00	Yes	11.17	3.81	34.07	1.00	Yes	Yes
Xcv20_gyrB_3	Xcv	4.81	1.37	28.38	1.00	Yes	11.46	3.41	29.79	1.00	Yes	Yes
Xcv22_gyr_Di2	Xcv	5.77	1.82	31.52	1.00	Yes	22.98	5.80	25.24	1.00	Yes	Yes
XgyrPCR2F	Xcv	2.84	1.04	36.52	0.50	No	3.92	1.33	33.91	0.81	Yes	Yes

only produced significant results in PCR replicate 2. Interestingly, a larger proportion of features spotted at 20 µM recorded SNR values of > 2.5, compared to features spotted at 40 µM.

5.4.5.2. Selection of pathogen-specific diagnostic probes

Pepino mosaic virus

A total of 32 probes, designed to target genomic regions of PepMV, were included in the diagnostic microarray (Appendix B). Successful hybridisation reactions were observed from both technical replicates in visual and statistical data analysis of results. Approximately 62% of reactions with PepMV-specific probes recorded SNR values > 2.5 and variance < 50% (Appendix C), indicating significant probe:target interactions. Of the probes demonstrating any SNR values ≥ 2.5 from reactions with PepMV replicate samples, 93% of probes recorded a greater SNR value in PepMV replicate 1 reactions compared to reactions with PepMV replicate 2 (Table 5. 6). This difference was associated with the different efficiencies of dye incorporation that occurred between the replicates of 3.33 pmol/µℓ for PepMV replicate 1 and 1.37 pmol/µℓ for PepMV replicate 2 (Table 5. 4). For example, one of the probes recording the highest SNR values for both replicates in PepMV assays – US2-9 (Table 5. 6), confirmed this observation, as the mean SNR value for the 16 spots of this probe was three times greater in results of PepMV replicate 1 compared to PepMV replicate 2.

A large proportion of probes in the microarray proved to be capable of detecting PepMV fragments from samples by producing significant SNR values from hybridisation reactions with one or both technical replicate PepMV samples (Appendix C). Statistical analysis identified 26 potential diagnostic probes that consistently produced significant SNR results, or mean SNR values > 2.5, with minimal variance, or CV < 50%, from replicate samples (Table 5. 6). Only 18 of these, however, had been designed specifically to target PepMV genomic regions, and the remaining probes had been developed to target ToMV genomic regions. Mean SNR values for these ToMV probes were significantly > 2.5 in both replicate assays and demonstrated variance < 50% (Table 5. 6), indicating that reactions were not random and were target-specific reactions. Other non-target probes that recorded significant SNR values included: Cmm2_rpoB2_Di, Cmmprobe_Di and Eub probes. Interestingly, these probes only produced significant hybridisation signals in reactions with PepMV replicate 1, while the mean SNR values for these probes were less than 1.2 in reactions with PepMV replicate 2. The first PepMV replicate was coupled with a labelled sample of healthy tomato genomic DNA during hybridisation reactions (Table 5. 6), but PepMV replicate 2 was hybridised to the microarray slide alone.

Table 5. 6 Potential diagnostic probes for PepMV that were selected based on statistically significant signals produced from hybridisation reactions with replicate samples containing labelled PepMV amplicons.

Probe	Replicate 1					Replicate 2				
	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5
CH2-CP-F	8.75	3.99	45.58	1.00	yes	3.40	1.71	50.13	0.63	no
CH2RdRp_1[1]	8.43	1.61	19.07	1.00	yes	7.57	2.12	27.99	1.00	yes
PepMV_15	16.92	7.89	46.60	1.00	yes	13.38	6.35	47.44	1.00	yes
PepMV_15_2	7.89	1.53	19.44	1.00	yes	8.49	1.31	15.40	1.00	yes
PepMV_16	8.84	1.82	20.65	1.00	yes	8.52	2.31	27.15	1.00	yes
PepMV_16_2	10.71	2.79	26.07	1.00	yes	8.17	1.10	13.50	1.00	yes
PepMV_16_Di	11.65	5.96	51.15	1.00	yes	14.99	5.65	37.72	1.00	yes
PepMV_19	18.91	8.10	42.84	1.00	yes	5.83	3.65	62.64	0.75	no
PepMV_CH2H	14.56	6.60	45.34	1.00	yes	11.66	4.51	38.65	1.00	yes
PepMV_CH2J	16.57	8.14	49.14	1.00	yes	4.87	2.81	57.79	0.75	no
PepMV_CH2K	9.38	4.50	48.00	1.00	yes	3.07	1.71	55.69	0.50	no
PepMVCh210	9.79	2.28	23.31	1.00	yes	8.93	3.51	39.35	1.00	yes
PepMVCh215	10.82	4.87	45.03	1.00	yes	7.27	3.26	44.88	1.00	yes
PepMVTGB1.2	10.26	4.64	45.25	1.00	yes	5.95	2.11	35.44	1.00	yes
TO/TMV10_5	4.65	2.44	52.50	0.81	yes	4.43	1.93	43.56	0.88	yes
ToMV29_2	6.63	3.08	46.52	0.94	yes	4.96	1.60	32.17	1.00	yes
ToMV3 Tib 2	9.43	2.75	29.13	1.00	yes	12.38	6.99	56.46	1.00	yes
ToMV38	9.79	4.93	50.36	0.94	yes	3.19	1.98	62.05	0.56	no
ToMV4_3	4.87	2.31	47.37	0.81	yes	3.04	2.14	70.52	0.50	no
ToMV46_Di2	10.25	2.16	21.06	1.00	yes	7.30	2.41	32.95	1.00	yes
ToMV53_4	6.01	2.04	34.00	1.00	yes	3.60	1.54	42.80	0.69	yes
ToMV70_1	9.22	3.40	36.92	1.00	yes	6.15	2.34	37.95	1.00	yes
US2-3	14.27	4.16	29.16	1.00	yes	7.62	2.29	29.99	1.00	yes
US2-7	14.93	6.67	44.69	1.00	yes	6.19	2.06	33.22	1.00	yes
US2-7_Di	15.36	4.34	28.23	1.00	yes	14.00	3.06	21.89	1.00	yes
US2-9	22.06	6.59	29.89	1.00	yes	6.90	2.29	33.20	1.00	yes

The overall proportion of probes that demonstrated reliable detection of targets from both technical replicate assays was 0.56, i.e., 18 of the possible 32 probes that were designed to be complimentary to PepMV gene fragments. The best of these probes (highlighted in yellow in Table 5. 6) were characterised by high mean SNR values, that were significantly > 2.5, and proportion values thatwere close to or equal to 1.00 for the 16 features per probe per slide. These 18 probes represented good

[1] Highlighted probes represent those with the best capacity for target detection in both technical replicates. Criteria for selection were SNR values significantly > 2.5, and proportion values of > 0.80 for the 16 features per probe.

diagnostic probes for PepMV and, according to *in silico* homology searches, included five probes conserved within the PepMV species, 10 probes specifically complimentary to sub-species US2 and CH2 PepMV isolates, and three probes of variable isolate-specific conservation. The two dimer probes included for PepMV detection, PepMV16_Di and US2-7, both demonstrated strong hybridisation signals, with mean SNR values > 10 for both technical replicates (Table 5. 6), although SNR values were not drastically higher than that of the monomer counterparts. No significant hybridisation signals were detected for probes: 11 Tiberini, R5R, EU-CP-F and US1_CPR, but these probes were designed specifically to target EU, LP and US1/CH1 isolates of PepMV. The probe PepMVTom11 was expected to bind to a region of the coat protein of all isolates of PepMV, but no hybridisation signals for feature of this probe were over the 2.5 SNR threshold. Three probes designed to target regions in the RdRp or coat protein of PepMV CH2 and US2 isolates, PepMV_CH2I, PepMV_CH2O and PepMVCP_1, demonstrated inconsistent results between replicate tests, only recording statistically significant SNR values from PepMV replicate 1 and poor or borderline values from PepMV replicate 2 assays.

The diagnostic probes selected for PepMV detection specifically targeted PepMV fragments, and did not produce significant hybridisation signals from reactions with other pathogens tested. Results from assays with healthy tomato DNA extracts did, however, produce significant SNR values for 11 of these probes (Appendix C), but only for one of the replicate samples tested. This replicate, DNA2, was the sample replicate coupled with PepMV replicate 1 during hybridisation reactions. Two of the 18 diagnostic probes selected, PepMVCh215 and PepMVTGB1.2, were not part of the PepMV-specific probes that recorded significant and reliable hybridisation reactions in probe specificity assays (Table 5. 5). This may indicate reduced diagnostic capacity of these probes over others selected. The best gene for diagnosis of PepMV was RdRp as 85% of the probes included in the array for this region demonstrated reliable detection, by recording significant SNR values, with the target templates. The TGB region was also associated with a number of high-quality diagnostic probes, but diagnostic probes that targeted the coat protein region best facilitated sub-species characterization of the isolate used in this study.

Tomato mosaic virus and related *Tobamovirus* species

Probes developed for ToMV detection were challenged using PCR products from pure culture extracts of three *Tobamovirus* species: ToMV, TMV and PMMoV. Labelled amplicons from technical replicates of ToMV demonstrated variable labelling efficiency. Replicate 1 demonstrated a higher level of dye incorporation, 2.22 pmol/µℓ, compared to the 1.17 pmol/µℓ of ToMV replicate 2 (Table

5. 4). Accordingly, five probes that demonstrated significant hybridisation signals with ToMV replicate 1, recorded low mean SNR values with ToMV replicate 2 (Appendix C). A similar result was observed for dye incorporation between replicates of TMV (Table 5. 4), however, only two probes were affected by variable hybridisation signals with TMV replicates.

Only four of the 30 probes included in the array for ToMV diagnosis demonstrated reliable detection of target templates of ToMV, with mean SNR values > 2.5 and proportion > 0.50 for both technical replicates (Table 5. 7). This included one of the two dimer probes, ToMV46_Di. This was different from results with samples containing combined amplicons for all pathogens (5.4.5.1 above), where an additional four probes designed for ToMV detection, recorded statistically significant hybridisation signals in the two replicate reactions (Table 5. 5). Two of these additional probes, ToMV 46 and ToMV53_4, produced mean SNR values significantly > 2.5 in pathogen-specific reactions with ToMV replicate 1, but SNR values were only > 2.5 for 37.5% and 12.5% of the 16 features for the two respective probes with ToMV replicate 2.

Table 5. 7 Probes with the best potential for detection and diagnosis of ToMV, identified by the production of significant signals ≥ 2.5 from hybridisation reactions with extracts from reference samples.

Probe	Replicate 1					Replicate 2				
	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5
TO/TMV_10_3	6.01	2.98	49.56	0.75	yes	3.23	1.54	47.67	0.56	no
ToMV4_3	3.81	0.97	25.37	1.00	yes	2.50	0.49	19.48	0.50	no
ToMV46_Di2	6.76	2.65	39.22	1.00	yes	4.69	2.09	44.47	0.75	no
ToMV9_4	4.37	1.12	25.57	1.00	yes	2.93	1.29	44.06	0.56	no

Hybridisation reactions performed using technical replicates of the related viruses, TMV and PMMoV, confirmed which probes could be used as potential genus-specific diagnostic probes (Appendix C). Two probes, To/TMV10_3 and ToMV46_Di, demonstrated high capacity for detection of relatives, as mean SNR values for these probes in reactions with ToMV, TMV and PMMoV labelled samples were significantly > 2.5. The mean SNR values for these probes from reactions with PMMoV replicate samples were > 20.00, indicating high concentrations of probe:target complexes on hybridised slides. Although the labelling efficiency for both PMMoV samples was < 1 pmol/µℓ (Table 5. 4), intense bands of target amplicons were recovered from pathogen-specific amplification reactions with pure culture extracts (Figure 5. 2 A). The probe To/TMV10_5 demonstrated good genus-specific detection in TMV and PMMoV replicate assays, producing significant SNR values in

replicate reactions with TMV and PMMoV templates, but only produced a significant SNR in assays of ToMV replicate 1 (Appendix C).

For differentiation of TMV templates from the targeted ToMV templates, two probes, complimentary to TMV in homology searches, were included on the array. No significant hybridisation signals were detected from assays with technical replicates of ToMV; however, only TMV replicate 1 demonstrated a statistically significant, reliable signal, and only with one probe, TMV10_4. The second TMV replicate only produced low SNR values from hybridisation reactions with this probe. The two replicates of PMMoV demonstrated unexpected, strong hybridisation reactions with TMV10_4, as mean SNR values were 10.27 and 32.14 for replicate 1 and 2, respectively, indicating highly specific and sensitive detection of PMMoV templates by this probe.

Potential diagnostic probes for ToMV only included 13% of those tested, or four out of the total 30 probes, that were designed to be complimentary to the target. These included species-specific and genus-specific diagnostic probes. No probes designed to target other pathogens demonstrated cross-hybridisation with regions of the ToMV genome. Eight probes designed to target ToMV did, however, produce significant signals when hybridised to technical replicate samples of PepMV (Table 5. 6), with a further 11 probes recording significant SNR values. The ToMV46_Di probe also produced highly significant signals in reactions with the Xv replicate 2 (Appendix C). Interestingly, this Xv replicate was coupled with PMMoV replicate 2 in the Cy3 channel during hybridisation reactions (Table 5. 4). Regions from the RepA and RepB genes of ToMV represented the best regions for diagnosis in this study, as 79% of probes that produced any significant signal mapped to the Rep genes, despite the overall low levels of hybridisation signals produced in both replicate tests.

Clavibacter michiganensis subsp. *michiganensis*

A total of 37 probes specific to regions in the four selected gene fragments were designed to target sequences of Cmm and closely related species within the *Clavibacter* genus. Four of these probes demonstrated broad specificity during homology investigations, however, and were included in the array for broad-spectrum detection of phytobacteria. Successful hybridisation signals were detected from technical replicates of Cmm amplicons despite low levels of dye incorporation, 1.01 pmol/µℓ for Cmm replicate 1 and 0.71 pmol/µℓ for Cmm replicate 2 (Table 5. 4). Surprisingly, the lower level of dye incorporation in Cmm replicate 2 did not affect signal intensity recorded with some probes, like Cmm_groEL18_Di, which actually demonstrated a greater mean SNR value with Cmm replicate 2 than Cmm replicate 1 (Table 5. 8).

Table 5. 8 The most reliable diagnostic probes for Cmm detection which produced significant hybridisation signals of ≥ 2.5 in the presence of labelled Cmm target templates during hybridisation reactions.

Probe	Replicate 1					Replicate 2				
	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5
Cm13_rpoB	6.81	3.23	47.51	1.00	yes	6.99	1.13	16.19	1.00	yes
Cmm_groEL18 Di	5.69	2.01	35.29	1.00	yes	9.18	1.91	20.85	1.00	yes
Cmm2_rpoB1	3.60	0.47	12.91	1.00	yes	6.24	1.05	16.90	1.00	yes
Cmm2_rpoB2_Di	5.93	3.19	53.81	1.00	yes	6.37	0.86	13.48	1.00	yes
Cmm2_rpoB6	3.35	0.68	20.22	0.81	yes	4.25	1.05	24.81	1.00	yes
Cmmprobe-Di	4.93	2.41	48.88	0.75	yes	9.84	4.45	45.21	1.00	yes

Probes that demonstrated the best capacity for detection of Cmm templates made 18%, or six of the total 33 probes designed to target Cmm specifically (Table 5. 8). These demonstrated mean SNR values significantly > 2.5 from both replicate reactions with high proportion values. The six selected diagnostic probes included three dimer probes, for which only one of the monomer forms, Cmm2_rpoB2, had significant SNR values for 37.5% and 19% of features in Cmm replicates 1 and 2, respectively. The fourth dimer probe for Cmm detection did not demonstrate significant hybridisation signals with Cmm-specific templates. However, in PCR replicate samples (5.4.5.1), one of the technical replicates had a significant mean SNR value for this probe. In these replicate hybridisation reactions using combined pathogen gene targets, an additional three probes, Cm10_rRNA, Cm14_gyrB1 and Cm22_gyrB, which were designed to be complimentary to Cmm, demonstrated reliable hybridisation signals (Table 5. 5). These may also represent potential diagnostic probes for Cmm detection.

Although probes that were not designed to target Cmm did not demonstrate significant or consistent signal production in hybridisation reactions, a number of features representing Xcv-specific probes produced significant SNR values in one of the two technical replicates. Two dimer probes for Xcv detection, Xcv_rRNA_pv20_Di and Xcv11_rpoB_Di, produced mean SNR values > 2.5, but only in reactions with Cmm replicate 1. This particular replicate was coupled with Cy5-labelled Xcv templates during hybridisation. The threshold of 2.5, prevented these Xcv probes being classified as strong, cross-hybridising probes. Some of the best diagnostic probes for Cmm did demonstrate significant cross-hybridisation reactions with other pathogens evaluated (Appendix C). This was a particularly common observation with the three successful dimer probes of Cmm. The probe Cmm_groEL18_Di demonstrated mean SNR values that were significantly > 2.5 in both technical

replicate assays of *R. solanacearum* and *E. cloacae*. The other two dimer probes only demonstrated significant SNR values with one of the technical replicates tested for *E. cloacae*, Pss, Xcv, PMMoV and PepMV. Variable cross-hybridisations of Cmm-specific probes were also observed with labelled RNA (cDNA) and DNA extracts from healthy tomato leaf samples.

Two of the four probes analysed for broad detection capacity of bacteria demonstrated significant hybridisation signals from some of the replicate samples of the pathogens tested. These were Cm10_rRNA and 1078_R, but signals from these probes were not always consistent for assays with pathogen templates. High mean SNR values were recorded for Cm10_rRNA in both replicate hybridisation reactions for Xv, Pss, *R. solanacearum* and *E. cloacae* samples, but only in one replicate sample of Cmm, Pst and PMMoV (Appendix C). Similarly, reactions with 1078-R only produced significant mean SNR values in both replicates of Pst and *E. cloacae* and with one replicate sample of Xcv, Xv, Pss and *R. solanacearum*. Interestingly, the regions that these two probes targeted differed by only two nucleotides and were complimentary to opposite genomic strands.

The diagnostic probes selected for Cmm detection demonstrated various levels of sub-genus conservation during *in silico* analysis, which should facilitate characterization of the target pathogens detected. All probes that targeted regions within the rpoB amplicon were conserved within *C. michiganensis*, only displaying one nucleotide mismatch, if any, with all subspecies of Cm. The rpoB proved to be the best gene for reliable Cmm detection in this study versus the other three genes for which diagnostic probes were designed, as four of the six probes designed to detect regions within this gene for Cmm detection demonstrated highly significant hybridisation signals with both replicate samples. The probe, Cmm_groEL18, was only homologous to the subspecies Cmm, Cms and Cmi. The potential diagnostic probes that were complimentary to regions within the gyrB gene of Cmm isolates also demonstrated conservation of targets within *C. michiganensis*. The Cmm_probe was homologous specifically to the ITS region of Cmm isolates only, thereby facilitating sub-species identification.

Xanthomonas campestris pv. *vesicatoria*

Development of diagnostic probes was largely limited by the high level of genomic conservation within the *Xanthomonas* genus. Nevertheless, 18 probes, including four dimer probes, were incorporated into the array and were challenged using technical replicates of amplicons of Xcv and the related bacterium, Xv. Successful hybridisations were obtained for Xcv replicate 1, Xcv replicate 2 and Xv replicate 2, however, poor hybridisation signals were detected for Xv replicate 1. This was

attributed to poor technical processing as the dye incorporation efficiency of this replicate was high, at 3.26 pmol/μℓ (Table 5. 4). The second replicate of Xv demonstrated a much lower dye incorporation of 0.85 pmol/μℓ, yet still recorded SNR values > 20 for the best performing probes (Appendix C). The target templates of both replicates assays with Xcv demonstrated average labelling efficiencies, between 1 and 2 pmol/μℓ, which were sufficient for reliable hybridisation signal production (Table 5. 4).

Only three probes, designed to target Xcv, demonstrated statistically significant mean SNR values > 2.5 (Table 5. 9). These probes were all dimer probes for which monomer counterparts demonstrated low mean SNR values, with only Xcv11_rpoB recording significant SNR values for 8% and 19% of features in Xcv replicate 1 and 2, respectively. Despite lower dye incorporation in Xcv replicate 1 compared to Xcv replicate 2, dimer probes were had a greater mean SNR value for the former replicate. Results from pathogen-specific hybridisation reactions were different from reactions using combinations of all pathogen gene targets (5.4.5.1). In PCR replicate samples, an additional four probes demonstrated highly significant and reliable detection of Xcv targets: Xanth28_rRNA_Di, Xcv20_gyrB1, Xcv20_gyrB3 and XgyrPCR2F. Of the 16 features, a proportion of 0.81 for Xcv20_gyrB1 and 0.75 for Xcv20_gyrB3 did, however, demonstrate mean SNR values > 2.5 in the Xcv replicate 2 pathogen-specific hybridisation reaction (Table 5. 9), which may indicate the potential diagnostic capacity of these probes.

The hybridisation pattern of Xv differed from that of Xcv, which may indicate potential capacity of the array for species-specific classification within *Xanthomonas*. Poor results from Xv replicate 1, however, reduced the reliability of any conclusions made from data as mean hybridisation signals from probes in this replicate were only ≥ 2.5 for one probe, Cm10_rNRA. Low signal production was most a result of technical errors which occurred during the processing of slides. Nevertheless, results from Xv replicate 2 indicated that, in addition to Xcv_rRNA20_Di, high mean SNR values were recorded from four other Xcv-specific probes: Xanth28_rRNA_Di, Xanth17_rpoB, Xcv22_gyr_Di and XgyrPCR2F (Appendix C). Interestingly, the dimer Xanth28_rRNA_Di, which did not demonstrate significant hybridisation signals from reactions with replicate samples of Xcv, produced a mean SNR > 20 from reactions with Xv replicate 2. A similar result was observed for the broad diagnostic probe, Cm10_rRNA, and the ToMV-specific dimer probe, ToMV46_Di, with high SNR production from Xv replicate 2 but low SNR values with Xcv replicates.

Table 5. 9 Potential diagnostic probes for Xcv detection, based on results of significant hybridisation signals from hybridisation reactions with labelled amplicons of Xcv extracts.

Probe	Replicate 1					Replicate 2				
	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5
Xcv_rRNA20_Di[1]	16.22	5.99	36.94	1.00	yes	4.06	1.82	44.82	0.88	yes
Xcv11_rpoB_Di	17.60	6.47	36.76	1.00	yes	11.19	7.76	69.39	1.00	yes
Xcv20_gyrB_1	1.74	0.72	41.24	0.19	no	5.10	2.68	52.46	0.81	yes
Xcv20_gyrB_3	1.10	0.39	35.44	0.00	no	3.71	1.63	43.88	0.75	no
Xcv22_gyr_Di	9.08	4.51	49.65	1.00	yes	3.61	0.97	26.82	0.88	yes

For diagnosis of Xcv, only 39% of the 18 probes designed for Xcv detection proved to be promising candidates. The three dimer probes demonstrating the most reliable hybridisation signals with target templates all recorded highly significant cross-hybridisation reactions with other pathogens and control samples evaluated (Appendix C). Two replicate samples, Pst replicate 2 and Cmm replicate 1, which were coupled with Xcv replicates 1 and 2, respectively, produced high SNR values with Xcv-specific probes. The absence of significant signal production in reactions with Pst replicate 1 and Cmm replicate 2, however, confirmed that these results were not consistent. Similarly, probes designed to target Pst and Cmm demonstrated significant signals in Xcv replicate hybridisation reactions when coupled with the respective pathogens.

Despite these cross-hybridisation problems, the dimer and monomer probes that demonstrated potential diagnostic capacity with Xcv technical replicate samples did facilitate taxonomic classification of templates based on prior results of homology analysis. This mainly included genus-specific detection, but probes Xcv22_gyr and Xcv20_gyrB3 demonstrated potential sub-genus characterization, as these sequences only bound to selected species or *X. campestris* pathovars in pairwise alignments. The probes designed to target regions of the rpoB would have facilitated the best sub-genus characterization of detected isolates but, surprisingly, probes for this region demonstrated low SNR values. Exact classification potential of all the probes was still unclear in this study because of inconsistent and unexpected cross-hybridisations observed. Even though the designed probes demonstrated limited diagnostic potential for Xcv detection, probes targeting gyrase B appeared to be the most reliable detection of targets, as three probes designed to bind to this gene produced hybridisation signals > 2.5 from one or both Xcv replicates.

[1] Highlighted probes represent those with the best diagnostic capacity from both technical replicate reactions. Criteria for selection were SNR values significantly > 2.5, and proportion values of > 0.80 for the 16 features per probe.

Pseudomonas syringae pv. *tomato*

For the detection and diagnosis of Pst, 21 probes were incorporated into the microarray and challenged with technical replicate samples of Pst and the closely related pathogen, Pss. Strong visible signals were observed from hybridisation images for reactions with both pathogens though these did not always translate into significant SNR values (Appendix C). The levels of dye incorporation for Pst replicates were similar, approximately 1.80 pmol/μℓ. However, the labelling efficiency of Pss samples was very different, with Pss replicate 1 demonstrating a dye-incorporation two times greater than that of Pss replicate 2 (Table 5. 4). This did not affect the intensity of hybridisation signals for Pss replicates however as Pss replicate 2 consistently demonstrated greater SNR values than Pss replicate 1 for the eight probes that produced SNR values ≥ 2.5 (Appendix C). Despite the comparable labelling efficiency of the Pst technical replicates, signal intensity readings were always greater for Pst replicate 1 (Table 5. 10). This affected the identification of potential diagnostic probes as those that produced statistically significant SNR results for Pst replicate 1 were not significant in Pst replicate 2 reactions. Identification of diagnostic probes for Pst thus also relied on results from reactions using combined templates of all five pathogens for validation (5.4.5.1).

The probes with the best diagnostic potential included six probes that were designed to detect Pst (Table 5. 10), or 29% of the total Pst-specific probes evluated. These included two dimer probes, but hybridisation signals recorded from these dimer probes were similar to the signals of monomer probes. This contrasted with the results of other pathogens, where greater sensitivity was observed for dimers than monomers in relevant hybridisation reactions. Five of the six diagnostic probes selected for Pst reliably produced significant hybridisation signals from replicate reactions of Pss. In addition, Ps14_rRNA, Xcv11_rpoB_Di and Cm10_rRNA demonstrated mean SNR values > 2.5 for both Pss replicate samples. Significant cross-hybridisations of non-Pst/Pss specific probes with Pss- and Pst-labelled samples was common. In reactions with Pst replicate 2, hybridisation signals were greater for Xcv-specific probes than Pst-specific probes, but these were insignificant as poor hybridisation signals were recorded for the same probes with Pst replicate 1 (Appendix C). Similarly, significant cross-hybridisations of Xcv-specific probes were recorded only in reactions with the Pss replicate 2 sample.

The diagnostic probes selected demonstrated mainly species-specific conservation, as confirmed by the production of significant hybridisation signals from both Pst and Pss samples. This was despite homology searches indicating that some of these probes were specific only to Pst isolates. Two probes, Pst2_rpoB1 and Pst_gyrB_Di, did, however, demonstrate good potential as pathovar-specific

diagnostic probes as reactions with Pss replicate samples did not result in any significant signal production. A total of nine Pst-specific probes demonstrated cross-hybridisation with Xcv replicate samples by producing SNR values > 2.5, but these results were only statistically significant for reactions of Xcv replicate 1. This sample was coupled with labelled products of Pst replicate 2 during hybridisation reactions. Other significant cross-hybridisations of Pst-specific probes included two dimer probes, Pst11_rRNA_Di and Pst3_rpoB_Di, which produced SNR values > 2.5 with Xv replicate 2 and *E. cloace* replicate samples, respectively. The unclear diagnostic potential of Pst probes meant that the ideal gene for diagnosis was difficult to determine. However, results did demonstrate the poor diagnostic potential of the rRNA/ITS region.

Table 5. 10 Potential diagnostic probes for Pst based on results from significant hybridisation reactions with pathogen-specific templates as well as replicate samples containing amplicons for all the targeted pathogens in this study.

Pathogen	Probe	Replicate 1					Replicate 2				
		Mean	SD	%CV	Propn >2.5	Mean Sign >2.5	Mean	SD	%CV	Propn >2.5	Mean Sign >2.5
Pst	Ps22_gyrB	5.57	2.40	43.04	0.94	Yes	0.84	0.43	51.06	0.00	No
	Ps23_gyrB	5.89	1.24	21.02	1.00	Yes	0.67	0.39	58.62	0.00	No
	Pst1_rpoB7	7.07	2.27	32.16	1.00	Yes	1.66	0.88	53.04	0.25	No
	Pst11_rRNA_Di	6.08	1.45	23.91	1.00	Yes	3.04	1.61	52.86	0.63	No
	Pst2_rpoB1	8.18	2.16	26.42	1.00	Yes	2.91	1.41	48.31	0.63	No
	Pst20gyrB_Di	3.91	0.83	21.14	1.00	Yes	0.34	0.14	40.50	0.00	No
PCR	Ps22_gyrB	5.32	1.78	33.56	1.00	Yes	15.01	3.98	26.53	1.00	Yes
	Ps23_gyrB	5.37	2.10	39.19	0.75	Yes	15.88	4.05	25.49	1.00	Yes
	Pst1_rpoB7	2.93	0.57	19.56	0.75	Yes	11.99	4.13	34.47	1.00	Yes
	Pst11_rRNA_Di	4.04	2.18	54.05	0.69	Yes	18.38	3.92	21.35	1.00	Yes
	Pst2_rpoB1	4.27	1.15	26.89	0.75	Yes	6.07	1.88	30.95	0.88	Yes
	Pst20gyrB_Di	3.95	2.14	54.13	0.81	Yes	3.99	1.87	47.00	0.81	Yes
	Pst3_rpoB_Di	4.87	2.61	53.52	0.81	Yes	3.64	1.92	52.84	0.56	No

5.4.5.3. Cross-hybridisation of diagnostic probes with non-target tomato pathogens

Although not specifically designed to target *R. solanacearum* or *E. cloacae*, four probes that exhibited broad spectrum bacterial detection in homology analyses were predicted to form significant hybridisation signals in reactions with replicate samples of these non-target bacterial pathogens of tomato: 1078-R, 1114f 16S/IGS, Cm10_rRNA and Eub Pelludat. Besides Eub Pelludat, each of these probes demonstrated significant SNR values with one or both replicates of *R. solanacearum* and *E. cloacae* (Appendix C). The level of signal intensity was expected to be

associated with dye incorporation efficiencies of the replicates but, despite the variable efficiencies that were recorded between replicates of both bacteria (Table 5. 4), SNR values appeared to be unaffected. With the second *E. cloacae* replicate, dye incorporation was estimated at only 0.52pmol/µℓ, yet mean SNR values > 31 and > 41 were recorded for reactions with probes Xanth28_rRNA_Di and Cm10_rRNA, respectively (Appendix C).

Besides general bacterial diagnostic probes, several other pathogen-specific probes demonstrated significant hybridisation signals with *R. solanacearum* or *E. cloacae* (Appendix C). Dimer probes designed for Cmm, Xcv or Pst detection recorded the highest incidence of significant cross-hybridisations with replicate samples of the two non-target bacteria, which included one Cmm dimer, three Xcv dimers and one Pst dimer. Two pathogen-specific monomer probes demonstrated mean SNR values > 2.5 for reactions with *E. cloacae* and both of these targeted regions within the groEL gene. As these bacterial replicates were purposely coupled with viral technical replicate samples and not other bacterial samples, the likelihood that these hybridisation results were random or by chance, was low and results were, therefore, considered significant.

5.4.5.4. Cross-hybridisation of diagnostic probes with the tomato genome

The amount of hybridisation signal generated by non-target, host RNA and DNA was evaluated by challenging the developed array with replicate samples from healthy tomato leaf extracts. The levels of dye incorporation for labelled RNA (cDNA) extracts were much less than labelled DNA extracts in both replicates (Table 5. 4), which translated to poor overall SNR values from hybridisation reactions with RNA versus those from DNA. Only three of the five probes included for detection of tomato mRNA transcripts or genomic DNA (Appendix B), had SNR values > 2.5 for one or both replicates (Appendix D): Eub Pelludat, GAPDH_2 and mRNA E2_1. Two pathogen-specific probes demonstrated mean SNR values significantly > 2.5 for both DNA replicates: Cmmprobe_Di and Xcv_rRNA20_Di. The Xcv-specific dimer probe recorded exceptionally high hybridisation signals with a mean SNR value for DNA replicate 2 of > 40. Only the RNA replicate 1 sample demonstrated significant cross-hybridisation signals with one pathogen-specific probe, Cmmprobe_Di, recording a mean SNR value of 18.62. Interestingly, this replicate was coupled with labelled Cmm replicate 2 templates during hybridisation reactions. Hybridisation control samples only demonstrated significant signal intensity from DNA replicates, despite the addition of the same concentration of control templates in labelling reactions. Though some features of the EACMV probe had SNR values > 2.5 with DNA replicate samples, only the GUS probes spotted at 20 µM and 40 µM demonstrated mean SNR values > 2.5.

Surprisingly, the signal produced from the 20 µM GUS probe was more statistically significant than that of the 40 µM probes and was, therefore the better hybridisation control probe.

The second replicate of DNA recorded high mean SNR values that were likely a result of high levels of dye incorporation of 4.84 pmol/µℓ (Table 5. 4). Mean SNR values were > 10 for 39% of probes from this replicate and 72% of the probes had a proportion ≥ 12/16 features with significant SNR values. Cross-hybridisation reactions were observed with 30 probes designed to target PepMV, ToMV, Cmm, Pst or Xcv. Interestingly, the majority of significant cross-hybridisation reactions were with dimer probes. These included two dimer probes for PepMV, one for ToMV, three for Cmm, two for Pst and four for Xcv. Though the DNA replicate 1 sample did not demonstrate significant hybridisation signals for these same probes, the inconsistency associated with signal detection using healthy plant samples decreased the diagnostic potential of these pathogen-specific probes.

5.5. Discussion

Current detection techniques for tomato plants and seed infected with important viral and bacterial seed-borne pathogens are limited by poor specificity and low multiplex or parallel detection capacity (Engel et al., 2010; Njambere et al., 2011; Pelludat et al., 2009; Schaad and Frederick, 2002). This lengthens the duration for screening host material for all potential threats, increasing the time and labour required for diagnosis as well as the risk of transmitting undetected pathogens. Microarrays can facilitate high-throughput detection by incorporating thousands of sequence-based, pathogen-specific detector probes which target only complimentary sequences for binding (Chou et al., 2006; Hadidi et al., 2004; Letowski et al., 2004). Depending on nucleotide similarity, probe binding does not discriminate between different classes of pathogens and, thus, carefully designed microarrays can be used to detect various pathogens from a single sample (Wilson et al., 2002). By incorporating separate preparation steps to isolate DNA and RNA from a sample, a single array can be used to detect both RNA-based viral and DNA-based bacterial pathogens of tomato. In this study, 152 probes were designed and included in a prototype diagnostic microarray for simultaneous and specific detection and identification of five important viral and bacterial pathogens of tomato. This prototype array was evaluated using a specific diagnostic process and, to gauge the specificity of the array, pure culture extracts of the five pathogens were used to validate the designed probes. All aspects of the diagnostic process, the prototype array and the diagnostic potential of the array are critically assessed below.

Before any new detection technique can be implemented into standard diagnostic protocols, its specificity and capacity for detection have to be validated extensively on the targeted pathogens as well as non-target, related pathogens, unrelated pathogens of tomato, and healthy RNA and DNA extracts of tomato (Call, 2005; Engel et al., 2010; Kritzman, 1991; Mansilla et al., 2003). It was, therefore, first necessary to analyse the reaction of targeted pathogens in the proposed diagnostic process and on the developed microarray. Colonies of pure cultures for the targeted bacteria, and leaf extracts from previously characterized, viral-infected hosts were used for this purpose. This reduced effects that contaminating pathogens, saprophytes and host material (in tests of bacterial cultures) might have on the diagnostic capacity of the developed tool, as these can contribute to misdiagnoses of field samples (Call et al., 2003; Kaneshiro et al., 2006). Previously, PepMV has not been reported to occur in South Africa, but PepMV was detected from tomato samples obtained from the Limpopo Province, as part of this dissertation (Chapter 4; Carmichael et al., 2011). A pure reference culture of this pathogen was, therefore, not available for tests using the developed microarray. Instead, testing was dependent on using infected material from this study. Similarly, the availability of reference cultures of related isolates, strains and species for the pathogens of interest in this study, that were required to validate the diagnostic potential of the microarray, were limited as many of these were prohibited from being cultured in South Africa at the time this study was conducted. Nonetheless, two technical replicates of each isolate to be tested were included in investigations to evaluate the effect of the optimised extraction and detection process on diagnosis, and to validate the uniformity of results obtained in this study.

For extraction of bacterial or viral nucleic acid from samples, crude-extraction methods were selected. These have been used and optimised extensively in previous studies on samples from various sources (Doyle and Doyle, 1987; Lopez et al., 2007; Momeni et al., 2011; Mumford et al., 2006; Porebski et al., 1997) and were amenable to further optimisations to improve yield and purity of isolated nucleic acid. The use of boiling to extract DNA from bacterial colonies was a cheap, quick and simple technique yet is efficient for isolating DNA from cellular components, including PCR inhibitors (Kaneshiro et al., 2006; Pelludat et al., 2009). This technique is ideal for high-throughput testing, especially in laboratories with limited equipment.

Testing RNA and DNA extracts of healthy tomato leaves with the prototype microarray in this study was done to indicate whether the microarray demonstrated any significant cross-hybridisations with the tomato genome, which would result in false positive diagnoses when screening seed or leaf samples (Lievens and Thomma, 2005). Shearing chromosomal DNA of tomato prior to labelling and

hybridisation to the array was necessary to generate shorter fragments for efficient labelling and binding to diagnostic probes (Bodrossy and Sessitsch, 2004; Boonham et al., 2007; Loy and Bodrossy, 2006; Wilson et al., 2002). Sonication to shear DNA was not effective in this study as it resulted in poor labelling efficiency (results not shown). Nebulisation, however, generated short fragments of DNA from total DNA preparations, and demonstrated high labelling efficiency and good signal intensity in hybridisation reactions. The methods selected to prepare total RNA and DNA extracts for hybridisation to the array caused significant losses of overall RNA and DNA concentration. Accurate quantification of background signal produced by unbound host templates on hybridised slides could, therefore, not be determined in this study. Research into more appropriate methods of nucleic acid preparation or ways to optimise preparation methods used in this study will be a necessary extension of this project to enable practical utility of the microarray.

Generation of cDNA fragments from RNA templates using random nonamer primers was successful from all viral-infected and control RNA extracts in this study. The use of random primers facilitated non-specific and, thus, unbiased amplification of all RNA templates (Agindotan and Perry, 2007; Engel et al., 2010; Wang et al., 2002). This would facilitate amplification of both known and unknown viral pathogens, as well as pathogens of mixed infections, from host extracts. The optimal starting RNA concentration for cDNA synthesis from PepMV-infected samples was two times greater than that for samples from *Tobamovirus*-infected leaf extracts. The concentration of PepMV particles in infected tomato samples was likely lower than the level of infection of tobamoviruses in host samples. This was confirmed in subsequent pathogen-specific amplification reactions where only concentrated cDNA samples from PepMV-infected extracts produced detectable bands in electrophoretic analysis compared to PepMV samples with starting concentrations of 5µg. Synthesis of cDNA from *Potexvirus* templates may be improved by incorporating Oligo dT primers (Aguilar et al., 2002) rather than random nonamers, that specifically bind to viral 3' poly(A) tails, but this could reduce the capacity of diagnostic assays to detect unexpected pathogens. Further research should incorporate a range of concentrations of pathogen nucleic acid (Lievens et al., 2005) to optimise the detection capacity of diagnostic protocols for levels of infection likely to be present in tomato field samples in routine diagnostic testing.

Removal of remaining reaction components and residual RNA from cDNA synthesis reactions was challenging. These components may affect downstream processes such as the availability of templates for pathogen-specific amplification. Using conventional cDNA purification kits and RNA hydrolysis drastically decreased the quality and quantity of cDNA yields. Recovered cDNA contained

high levels of salts and performed poorly in subsequent amplification reactions. These results were attributed to poor storage conditions for chemical components, precipitates or remnants of components from synthesis or extraction reactions (Bilgin et al., 2009). This was remedied for viral cDNA products by including a simple RNAse treatment as used by Hanssen et al. (2008), which enabled strong target-specific bands to be generated from amplification reactions. The RNAse treatment was not suitable for healthy tomato control samples however, as samples were used directly in labelling procedures. The absence of a suitable purification procedure for control cDNA samples in this study meant that hybridisation results for these samples did not accurately reflect the binding capacity of host RNA (cDNA) in the diagnostic microarray.

Amplification of pathogen templates prior to array hybridisation facilitates sensitive detection of even low levels of pathogen infection within a host sample (Agindotan and Perry, 2007; Boonham et al., 2003; Kyselková et al., 2009; Lievens et al., 2003; Wang et al., 2011). However, preparation of multiple pathogen-specific targets for microarray detection can become arduous if separate amplification reactions are performed for each pathogen of interest. As the microarray developed in this study contained numerous probes for discrimination among targeted pathogens, selected primers did not need to be pathogen-specific (Bach et al., 2003; Engel et al., 2010; Schaad and Frederick, 2002). Choice of primers in this study, therefore, favoured primer sets that demonstrated broad specificity like those selected for ITS and rpoB amplification, which bound to all bacterial targets during *in silico* homology analysis. This was not possible for amplification of the RNA viruses however, as these pathogens demonstrate limited inter-genus conservation (Boonham et al., 2007; Letschert et al., 2002; Tiberini et al., 2010; Van der Vlugt and Berendsen, 2002) Primers were selected or specially designed to bind to the respective target pathogens under the same thermodynamic conditions instead. This reduced the labour and time required to amplify targeted gene fragments (Schaad and Frederick, 2002) and could be incorporated into multiplex amplification reactions in future studies. Combining multiplex PCR with subsequent microarray discrimination (Call, 2005) could shorten template preparation processes even more and provide improved detection capacity for screening assays. This would, however, introduce more variables for diagnosis as the impact of varying template binding efficiencies and competition between templates could also affect probe binding and hybridisation signals (Wang et al., 2011).

To broaden the specificity of previously developed primers for amplification of target genes, degenerate bases were added to selected primer sequences. These have been associated with lower binding stability and reduced amplification efficiency (Dovas et al., 2004). Only primers requiring one

to three degenerate bases to attain the required specificity were, therefore, considered for reactions in this study. Nevertheless, the reduced efficiency of amplification of Rep and CP gene targets of PepMV compared to the TGB fragment, was attributed to the higher number of degenerate bases included in primer sequences for these targets. The non-specific fragments that were produced in rpoB amplification reactions with *Pseudomonas* cultures, were also attributed to the degenerate bases included in these primers. This likely facilitated cross-hybridisation of rpoB primers with other slightly homologous regions (Mansilla et al., 2003) in the genomes of *P. syringae* pathovars.

Primer pairs selected to bind to gene targets of the five pathogens of interest in this study facilitated amplification of bands of the expected length from all pathogen templates. Some reactions demonstrated variable banding intensity between replicate samples however, which indicated that primer binding and amplification efficiency was variable (Call et al., 2003; Hanssen et al., 2008). Mutations in primer binding regions among strains of a targeted pathogen may have occurred (Mansilla et al., 2003; Wilson et al., 2002), but variable amplification efficiency can be attributed to many other factors besides primer binding. These include poor reactivity of reaction components, uneven distribution of templates in samples, shearing of target templates and high concentrations of PCR inhibitors resulting from the extraction procedures used (Call et al., 2003; Lopez et al., 2007; Meng et al., 2004). Kaneshiro et al. (2006) attributed inconsistent amplification of targets from bacterial extracts to the use of boiling as a DNA extraction method. They claimed that the heat shock from boiling may have prevented uncoiling of all bacterial DNA with the result that not all gene targets were accessible for primer binding. The effect of inefficient template amplification was reduced in this study by only selecting samples that produced the strongest bands analyses.

Selected primer pairs were extensively validated for targeted binding by *in silico* homology analysis prior to use in amplification reactions. However, experimental validation exposed discrepancies for targeted binding in some reactions (Mansilla et al., 2003; Njambere et al., 2011). Primers selected for amplification of *Tobamovirus* gene targets in this study demonstrated conserved homology among related species during *in silico* analysis (Letschert et al., 2002), but faint bands or non-target amplicons were generated from reactions of PMMoV and TMV extracts with RepB and CP primers, respectively. Amplification reactions of ToMV templates in contrast resulted in the generation of intense bands of the expected length from PCR with all three selected primer pairs. Although these results indicated the limited capacity of some of the selected primer sets for genus-specific detection and classification of Tobamoviruses, the results supported pathogen-specific detection of ToMV from infected samples. To maintain a broad spectrum of detection capacity for mixed infections or

emerging *Tobamovirus* species, however (Dovas et al., 2004; Letschert et al., 2002), a combination of a genus-specific primer pair, like RepA, and a pathogen-specific primer pair, such as RepB or CP, could be retained.

Universal primers were selected for amplification of ITS and rpoB gene targets from bacterial extracts of targeted and non-targeted pathogens (Guasp et al., 2000; Richert et al., 2005; Waleron et al., 2011), but intense bands of different lengths were generated from reactions with these primer sets. This was not a result of mispriming or variation in primer binding regions as bands were distinct and similar in replicate samples for each pathogen. The diverse sizes of amplicons generated was better explained by the presence of substitutions, deletions and insertions within primer-binding regions that might have occurred as a result of bacterial evolution (Guasp et al., 2000; Wilson et al., 2002; Yamamoto et al., 2000). Although the 16S and 23S rRNA genes are highly conserved due to their critical cellular functions, the non-coding ITS region is more polymorphic (Guasp et al., 2000), supporting the use of this DNA region for phylogenetic characterization. The rpoB gene is also highly conserved due to the important function of the gene products for cell metabolism (Ait Tayeb et al., 2005), yet this gene has also been shown to contain numerous polymorphic regions, supporting the incorporation of this region into diagnostic protocols in this and other studies (Ait Tayeb et al., 2005; Pelludat et al., 2009; Rajendhran and Gunasekaran, 2011). Varying amplicon size was, therefore, not expected to affect diagnosis but to improve detection capacity of the array, as probes targeting pathogen-specific polymorphisms within the amplicons would prevent false positive hybridisations (Letowski et al., 2004).

The specificity of primer pairs for targeted bacterial detection was validated further in reactions using with two non-target pathogens of tomato, *R. solanacearum* and *E. cloacae*, which proved the capacity of the selected primers for broad spectrum detection. These assays indicated that the gyr primers for Cmm- and Xcv/Pst-specific amplification, which demonstrated highly efficient amplification of target templates, were pathogen-specific and did not produce amplicons of the expected length in reactions with non-target bacterial extracts. Primers for ITS amplification, however, proved to be suitable for 'catch-all' primers to facilitate detection of target and non-target pathogens in host samples using the developed array (Bach et al., 2003; Call et al., 2003). Conserved detection capacity of these primers supports their use in assays to detect other phytobacterial targets. Unexpected homology between Cmm and *E. cloacae* groEL genes was indicated by results in this study. Amplification reactions with selected groEL primers and *E. cloacae* DNA extracts resulted

in the production of an amplicon of the expected length, and labelled fragments also produced significant signal with probes designed for Cmm groEL targets in hybridisation reactions.

Indirect labelling of amplified gene targets was selected over direct labelling in this study as direct incorporation of the large dye molecules during amplification reactions has been shown to reduce amplification efficiency (Boonham et al., 2003; Bystricka et al., 2005; Engel et al., 2010; Hadidi et al., 2004; Szemes et al., 2005). Using separate Klenow labelling and dye-coupling steps increased the length of template preparation steps by 20 hours to facilitate maximum dye-incorporation, but this step can be reduced to six hours with minimal reduction in dye incorporation for rapid diagnosis. Many factors appeared to affect Klenow labelling of targets, which were mainly associated with the modified nucleotide, amino-allyl dUTP. This nucleotide was extremely sensitive to external conditions and performed best when new stocks of this product were used, stored at -70°C and were exposed to minimal freeze/thaw treatments prior to use. The concentration of aa-dUTP also affected the efficiency of labelling and was optimised in this study to a final concentration of 8 mM. The GC content of the template fragment could have also affected the efficiency of labelling and, therefore, dye-coupling. This is because AT rich amplicons would incorporate higher concentrations of aa-dUTP bases than GC rich fragments. Although it could not be proved implicitly in this study, the use of phosphate-based ethanol wash buffers, as opposed to the Tris-Cℓ-based wash buffer included with kits, appeared to improve the recovery of labelled targets during clean-up procedures.

In this study, it was proposed that the length of the target amplicon would affect the efficiency of Klenow labelling and, thus, gene targets were added to reactions at ratios relative to the length of respective amplicons. Shorter templates would, in theory, be labelled more efficiently than longer targets, thereby creating a bias of higher hybridisation signals for probes targeting shorter amplicons. It was hypothesised that adding higher concentrations of longer amplicons in labelling reactions would reduce this bias. This was not proven in this study as the binding capacity of diagnostic probes in the prototype microarray was unknown prior to hybridisation reactions. This can be validated easily however, by utilising small pilot arrays spotted with the most consistent diagnostic probes, and challenging these with a range of differentially labelled samples. The affect of combining numerous templates of different lengths in a single sample was tested in this study. Results showed that these combinations did not affect labelling efficiency as drastically as expected. However, reactions containing amplicons of all gene targets from the five pathogens of interest demonstrated greater levels of dye incorporation compared to pathogen-specific samples, and recorded significant hybridisation signals for probes targeting each of the selected genes. These

reactions contained the same starting concentrations as other pathogen-specific labelling reactions, yet addition of respective gene targets was based on volume, instead of amplicon length and concentration. Target length may, thus, be a contributing factor to labelling efficiency, but was not a limiting factor in this study.

The efficiency of dye-coupling reactions was extremely variable among pathogens, and even between replicates of the same pathogen, which affected signal intensities in hybridisation reactions (Kyselková et al., 2009). The incubation temperature affected dye-incorporation, and targets demonstrated improved dye incorporation when incubated at a stable temperature of approximately 22°C. It was important to use fresh dye solutions completely dissolved in DMSO or those with minimal freeze/thaw treatments that were stored at -20°C. A proportion of labelled products was lost in the clean-up procedures and down-stream processing prior to slide hybridisation. Thus better target detection required optimisation of the sample preparation and clean-up procedures as well as the use of samples with high concentrations of labelled products.

The length of probes selected for incorporation into the developed array were short, between 18 and 24 nt, as shorter probes have been shown to be less tolerant to single base mismatches, facilitating better discrimination between similar sequences than longer probes (Chou et al., 2006; Letowski et al., 2004). This facilitated more specific detection and classification of target pathogens as well as distinction from related pathogens (Boonham et al., 2003; Chou et al., 2006; Kyselková et al., 2008; Lievens and Thomma, 2005). The array enabled simultaneous validation of 152 short diagnostic probes, and probes that demonstrated the most promising diagnostic capacity can easily be incorporated into multiplex or real-time PCR diagnostic assays in future studies. During *in silico* homology analysis, however, shorter probes demonstrated high levels of random or chance similarity with related, non-target tomato pathogens and the genome of tomato. This significantly reduced the number of genomic regions with potential diagnostic capacity that could be used to design probes. Longer probes recorded far fewer incidences of chance similarity and have been shown to demonstrate more intense hybridisation signals (Letowski et al., 2004; Call et al., 2003). These probes are easier to design but are less specific for diagnosis as they are amenable to more mismatches (Chou et al., 2006; Letowski et al., 2004). Previously designed, pathogen-specific probes were included in this study and manipulated to conform to the selected parameters during probe design (Bach et al., 2003; Chou et al., 2006; Letschert et al., 2002; Pelludat et al., 2009; Tiberini et al., 2010). Only a few of these probes, however, were suitable for inclusion into the prototype array, and even fewer produced significant hybridisation signals. This emphasised the need for a standard set

of parameters for probe design in diagnostic microarrays to facilitate faster development of novel detection arrays (Bodrossy and Sessitsch, 2004; Letowski et al., 2004). Those probes that did produce significant signals were designed using a complex algorithm developed by Chou et al. (2006), which reportedly simplifies probe design.

In this study, the design of probes to demonstrate highly specific diagnostic potential was arduous and technical, requiring intensive *in silico* analysis among target, related, unrelated pathogens and host genomic sequence data (Chou et al., 2006; Letowski et al., 2004). Thorough homology analysis, however, reduces laborious and expensive experimental validation reactions. Despite intensive *in silico* specificity analysis, however, selected diagnostic probes did not all react as expected with targeted pathogens in the prototype microarray (Kyselková et al., 2009; Njambere et al., 2011). Other factors may also have contributed to probe:target binding in hybridisation reactions (Kyselková et al., 2009). These include uneven dissemination of labelled amplicons in hybridisation solutions and, therefore, uneven dissemination over the microarray binding regions; local background signal, which reduces SNR readings; local temperature variations creating hybridisation 'hot spots' on the array; and deformations of probe:target complexes caused by technical processing such as scratches on the array surfaces.

During hybridisation of labelled targets to a microarray, the specific incubation temperature used is critical for specific and sensitive binding (Hadidi et al., 2004; Letowski et al., 2004). In this study, the optimal temperature was determined to be 25°C, likely because of the short length of the probes used (Call, 2005; Letowski et al., 2004; Kyselková et al., 2009). Inclusion of formamide into hybridisation solutions lowers the required binding energy between probes and targets (Blake and Delcourt, 1996) so that maintenance of an even temperature distribution during hybridisation is more practical (Zhang et al., 2007). Standard hybridisation temperatures in microarray studies are 42°C (Letowski et al., 2004), but binding at higher temperatures higher than 25°C in this study produced poor signal intensities with only dimer probes demonstrating detectable signals (results not shown). This may be related to the longer length of the probes (Chou et al., 2006; Letowski et al., 2004) or the higher sensitivity associated with dimer probes (Njambere et al., 2011).

Interpretation of signals detected from the scanned, hybridised slides was based on a mean SNR threshold of 2.5 in this study. Implementing this threshold reduced the risk of false positive hybridisations so that remaining results indicated only significant binding results between probes and target templates (Kyselková et al., 2009). This threshold was selected as an intermediary

between the stringent value of 3.0 selected in some studies (Lezar and Barros, 2010; Stewart, 2006) and recommended by software manufacturers, and the more flexible threshold of 2.0 (Szemes et al., 2005) which may reduce the specificity of diagnosis. To be identified as a suitable diagnostic probe, probes had to demonstrate consistent and reliable detection of the target pathogens between replicate samples tested (Kyselková et al., 2009). The variance of signal, while important for probes with borderline signal, was not necessarily an important factor for distinguishing good versus poor probes in this study. Highly specific and sensitive probes caused a saturated signal in reactions which were not easily interpreted by scanners and, therefore, may have resulted in variable SNR values between replicates. The best diagnostic probes were, therefore, identified as those that demonstrated a significant, mean SNR value of > 2.5 in replicate tests, with high proportions of spots per probe demonstrating an SNR of at least 2.5. This simplistic interpretation would reduce the dependence on complicated statistical analyses for screening and diagnostic assays. The confusing effects of various other factors on the diagnostic capacity of probes observed in this study, however, restricted confident classification of good versus poor diagnostic probes, as discussed below.

Hybridisation signals were extremely sensitive to technical processing errors, in some cases requiring that the entire process be restarted, which was costly and time-consuming and limits the adaptability of microarrays for high-throughput sample testing. Dye precipitates resulted during some hybridisation reactions, such as those with ToMV and *R. solanacearum* isolates which impacted signal intensity readings and the diagnostic potential for these pathogens. Precipitates were likely a result of insufficient dissolution or over-mixing of dye-labelled products into hybridisation solutions. Scratched slides were also discovered, and were most likely associated with loss in relative humidity during hybridisation. Low relative humidity would have led to the evaporation of hybridisation solutions, making slides more sensitive to scratching by the coverslips during the washing steps. Scratches severely affected hybridisation results by effectively removing probe:target complexes before the slides could be scanned, resulting in false negative diagnoses. The effect of these errors was clearly demonstrated in results of the Xv replicate 1 where low SNR values prevented accurate diagnosis and probe detection capacity. High redundancy of probes in this and other studies reduced the overall effects of technical errors by basing results on a collection of data from spots of the same probe (Njambere et al., 2011; Wang et al., 2002; Loy and Bodrossy, 2006), but this may not be sufficient if the entire slide is affected. Novel technologies like Agilent Arrays reduce technical errors as automated procedures are largely used for slide preparation (Boonham et al., 2007; Mumford et al., 2006), but these are currently too expensive for most diagnostic testing facilities.

Concentrations of hybridisation control samples added into labelling reactions was kept the same in all hybridisation reactions, yet resulting signals varied and probe:target binding differed from expected results (Kyselková et al., 2009). Probes targeting GUS and EACMV templates were spotted at six different concentrations ranging from 1.25 to 40 µM in attempt to establish the detection limits of the prototype array, as concentrations of both probes and targets were known (Lievens et al., 2005). This was not achievable, however, as only GUS probes spotted in concentrations of 20 and 40 µM produced significant signals from hybridisation reactions. Concentrations of the spike-in hybridisation control samples may have been too low for detection, but significant results were only obtained in 54% of hybridisation reactions. This indicated that other factors, such as technical variations and local hybridisation effects, were affecting hybridisation results (Call, 2005; Kyselková et al., 2009). The control samples were, therefore, not suitable to validate successful hybridisation with this array, and other alternative control samples should be evaluated.

Hybridisation signals appeared to be related to the level of dye incorporation in a sample with higher incorporation values and concentrations translating to more significant and consistent SNR values. Generally, samples with the best hybridisation results demonstrated dye incorporation values > 1.00 pmol/µℓ. Thus, this could be a threshold for microarray validation experiments. Some exceptions were observed, however, for example, in hybridisation reactions with bacterial templates, where samples demonstrating low levels of dye incorporation still produced significant SNR values with complimentary probes. This is promising for diagnostic application where maximum dye incorporation might not exceed the suggested threshold of 1 pmol/µℓ, especially in screening assays of healthy plant samples. It is, however, clear from these results that further optimisation of labelling procedures is necessary to improve the consistency of hybridisation signals.

To increase the number of samples that can be processed simultaneously, samples can be coupled with different fluorescent dyes to enable more than one sample to be screened for multiple pathogens in one hybridisation reaction (Call, 2005; Tiberini et al., 2010). Emission of lasers at different wavelengths by scanners facilitates independent detection of signals from each sample (Hadidi et al., 2004). Thus, if both samples bind to a single probe, relative hybridisation signals should not be affected. In this study, this was tested by combining differentially labelled (Cy3 vs. Cy5) templates from pure cultures of unrelated pathogens in hybridisation reactions. In five of these coupled hybridisation reactions, however, significant hybridisation signals were obtained from probes with templates from the alternate, non-targeted pathogen as well as the targeted pathogen. These results were not duplicated in replicate assays where the same pathogens were hybridised in

different combinations. This proved that cross-hybridisation signals were related to the combination of labelled templates in hybridisation reactions and not due to partial homology with the probes. The best explanation for this unexpected result was that regions in both labelled amplicons, other than those targeted by detector probes, demonstrated sufficient complimentarity to facilitate inter-amplicon binding during hybridisation. The immobilised target fragment would, therefore, act as a detector probe by binding to the non-target fragment. This explanation was supported by the observation that only probes producing significant hybridisation signals in replicate assays produced high cross-hybridisation signals in combined assays. To prove this, further *in silico* comparative analyses should be performed using sequences for the full-length amplicons for target pathogens used in this study. Reducing the size of labelled amplicons, for example, by enzymatic degradation (Wilson et al., 2002), may reduce the risk of cross-hybridisations between samples by disrupting homologous regions. An exception to this reasoning was observed in reactions of Pss and Pst replicate samples, where consistent hybridisation signals were obtained from probes designed to target Xcv templates, suggesting that significant sequence homology was present between these bacterial targets and the detector probes. Hybridisation reactions studies should, therefore, be performed individually for each pathogen so that only the most specific diagnostic probes are retained for dual sample processing.

Although each of the five targeted pathogens were detected successfully by the respective diagnostic probes used in the prototype microarray, many of the probes incorporated demonstrated borderline results that were insufficient to facilitate reliable diagnosis and taxonomic classification of the target pathogens. Hybridisation results of this study were generated from pure culture extracts of targeted pathogens and, therefore, using these borderline probes for diagnosis of infected field samples, which may contain various levels of infection, could be complex with inaccurate results (Call et al., 2003; Letowski et al., 2004). The most informative hybridisation reaction performed in this study contained a combination of all gene amplicons from the five pathogens of interest. This indicated which probes were capable of producing significant hybridisation reactions with targeted templates in the presence of competing templates. The assays also indicated the potential of probes for use in screening assays for multiple targets and the diagnosis of mixed infections. Pathogen-specific assays, or hybridisation reactions where templates for only a single pathogen were incorporated into hybridisation solutions, were, however, still valuable to prove that the probes were specific to the targeted pathogen and did not cross-hybridise to any other significant targets (Kyselková et al., 2009; Letowski et al., 2004; Njambere et al., 2011). Only a third of the designed probes recorded consistently significant hybridisation results in combination assays. Of these, at

least seven were designed to target each of the five pathogens proving, that the array could detect all pathogens simultaneously (Adams et al., 2009). Results could not be used to calculate the relative levels of each pathogen in samples, however, but this has been achieved in other microarray studies (Lievens et al., 2005; Van Doorn et al., 2007). A surprising observation was that some diagnostic probes did not produce significant signals in pathogen-specific assays but recorded highly repeatable and significant hybridisation results when tested in combination with other pathogens. This was especially noticeable for templates of ToMV, Xcv and Pst and may have been a result of poor dye-coupling or loss of templates in preparation procedures used for pathogen-specific hybridisation reactions.

Overall, hybridisation results indicated that dimer probes demonstrated more consistent and sensitive detection than monomer probes for each targeted pathogen (Njambere et al., 2011). In pathogen-specific reactions for the detection of ToMV, Xcv and Pst, for which only a few probes demonstrated significant hybridisation signals, dimer probes still produced significant SNR values compared to the poor values of monomer equivalent probes. Dimer probes also had higher proportions of significant SNR values from replicate spots per slide than other monomer probes. Even though dimer probes were designed based on the best performing monomer probes identified by *in silico* analysis, three dimer probes and their respective monomer equivalents did not produce significant SNR values in any hybridisation reactions. These may have been associated with spontaneous SNPs that occurred among sequences of strains or species used in hybridisation reactions versus those used for probe design (Wilson et al., 2002). Njambere et al. (2011) reported a slight decrease in specificity when using dimer probes for detection compared to monomer diagnostic probes. However, in this study dimer probes demonstrated highly significant and consistent cross-hybridisation results, especially for those that were designed to detect bacterial target pathogens. High SNR values, > 10.00, recorded from hybridisations of labelled fragments from healthy tomato DNA, confirmed that these probes could not be used for accurate diagnosis of pathogens from analysis of infected host samples. This contradicted *in silico* results which indicated that monomer sequences were not sufficiently homologous to the tomato genome which is what prompted their inclusion in the array. The dimer probes should, therefore, be re-evaluated.

Analysis of hybridisation reactions of genomic DNA and mRNA transcripts from healthy tomato leaves with the array exposed cross-hybridisations of pathogen-specific probes with plant nucleic acid, and indicated whether control probes included in this array could be used to quantify background signal. Control probes were based on house-keeping genes to facilitate sensitive

detection of target pathogens in the absence of amplification. Probes that did not demonstrate consistent detection of targets proved that these were unsuitable targets to quantify background signal (Call et al., 2003). Significant cross-hybridisation results of pathogen-specific probes with host nucleic acid were not repeatable in this study, but any potential homology represents a significant risk of misdiagnosis (Engel et al., 2010). The high concentrations of RNA and DNA lost during the processing of samples in this study also emphasised the need for further tests using more replicate samples of host nucleic acid, as further cross-hybridisation reactions may have occurred.

The detection capacity of the developed microarray for each of the five pathogens was based on the proportion of diagnostic probes that demonstrated significant hybridisation signals and was, in descending order: PepMV, Xcv, Pst, Cmm and ToMV. Although probes to facilitate taxonomic classification were incorporated into the array, these probes could not be validated experimentally for taxonomic specificity by the assays due to the unavailability of cultures of key related pathogens. This may have exposed discrepancies in probe design or SNPs among reference sequences and hybridised cultures, which would have explained the poor hybridisation results obtained for some of the designed probes (Call et al., 2003; Kyselková et al., 2009; Lievens et al., 2006). The variability observed between replicates also limited complete evaluation of the diagnostic potential of probes, especially for Xcv- and Pst-specific diagnoses.

For more specific and reliable detection of targeted bacterial pathogens, a number of studies incorporated probes for pathogen-specific genes (Leite et al., 1994), but in this study, house-keeping genes were selected. These occur in higher concentrations within bacterial cells than pathogenicity genes (Schaad and Frederick, 2002) and theoretically, more sequence data is available to support probe design and *in silico* validation. These gene targets also facilitated the development of probes to target different taxonomic levels of pathogens. Taxonomic probe design was, however, difficult for Xcv and Xv, as taxonomic complications between the species causing bacterial spot of tomato meant that databases contained sequences that had been classified using different taxonomic systems (Kyselková et al., 2009; Jones et al., 2004). Some of the probes developed for broad detection of phytobacteria in this study did demonstrate significant hybridisation signals with targeted pathogens as well as the non-targeted bacteria *R. solanacearum* and *E. cloacae*. Such probes are extremely valuable for diagnostic assays as these facilitate detection of contaminating saprophytes, bacteria with critical SNPs, and unknown or emerging pathogens (Adams et al., 2009; Kyselková et al., 2008; Schaad and Frederick, 2002). In this study, the ITS bacterial gene target demonstrated the lowest potential for diagnoses despite highly successful amplification reactions.

Previous studies have attributed similar results to high levels of cross-hybridisation (Lezar and Barros, 2010; Rajendhran and Gunasekaran, 2011), but low hybridisation signals were obtained for the majority of probes targeting this region, irrespective of the targeted pathogen. This may have been a result of the long length of the fragment amplified by selected primers (Kyselková et al., 2009), for which binding to probes may have been limited by steric hindrances.

Hybridisation reactions with labelled templates from PepMV extracts demonstrated consistent cross-hybridisation signals with probes designed to target ToMV, which indicated that a mixed infection may have been present in some of the hybridised samples. Leaf samples used to represent PepMV-infected leaf material in this study were obtained from stock cultures of infected leaf material from previous studies, as reference cultures were not available for this study. A mixed infection of PepMV and ToMV was previously confirmed for one of these pooled collections of tomato leaves (Chapter 2, 2.4.5). The primers and probes included for diagnosis and amplification of PepMV-specific targets in this study, however, were specifically validated for the absence of significant homology with ToMV targets which should have prevented the detection of ToMV templates, even in a mixed infection. Hybridisation results may, thus, be explained by homology of ToMV-specific probes to regions not targeted by detector probes of PepMV. To prove this, purified viral extracts of PepMV should have been used to challenge the developed array. Nevertheless, no significant cross-hybridisation reactions were recorded with PepMV-specific probes in reactions with pure cultures of ToMV extracts. Of the ToMV-specific probes that produced significant reactions with PepMV extracts, many did not record high SNR values in ToMV-specific hybridisation assays. This proves that the ToMV strain detected in a mixed reaction with PepMV differed from that tested in pure culture extracts.

Cultures of closely related pathogens of ToMV, Pst and Xcv included in array validation experiments indicated the level of taxonomic conservation of the pathogen-specific probes developed in this study. The *Tobamovirus* relative of ToMV, TMV, is also known to infect tomato, which makes species-specific discrimination between these pathogens an imperative target of diagnostic protocols (Jacobi et al., 1998; Letschert et al., 2002; Vinayarani et al., 2011). Of the probes included, one did not produce hybridisation signals with ToMV templates, but did produce significant signals with TMV and PMMoV, demonstrating significant potential for incorporation into future diagnostic assays. Similarly, the tomato-infecting Pss is highly homologous to Pst (Jones et al., 1997) and, accordingly, the majority of suitable probes for Pst diagnosis also demonstrated significant hybridisation signals in the presence of Pss templates. An additional two probes, however, were only

specific for Pst detection and demonstrated poor hybridisation signals with Pss replicate tests. Although the taxonomic difference between the two bacterial spot species, Xcv and Xv, used in hybridisation reactions in this study was unknown, both pathogens demonstrated very different hybridisation profiles. For example, a dimer probe and two monomer probes recorded significant hybridisation signals in reactions with Xv templates, but not in reactions with Xcv templates. Although these results may have been affected by technical variations, observations indicated that the developed array may have potential to distinguish between the two pathogens.

In this study, the developed probes demonstrated differential hybridisation patterns to those expected for target pathogens (Kyselková et al., 2009) as only a portion of probes recorded significant hybridisation signals, and many of these recorded cross-hybridisations with templates of other pathogens. This may be attributed to poor specificity of the designed probes (Chou et al., 2006; Letowski et al., 2004), but hybridisation was also affected by other factors (Kyselková et al., 2009; Zhang et al., 2007). The high levels of true and random homology that resulted from the use of short probes in this diagnostic microarray might facilitate misdiagnosis of infected or healthy host samples. However, if extensively validated against a number of strains and species of targeted pathogens, a diagnostic pattern can be developed for each pathogen (Call et al., 2003; Engel et al., 2010). This pattern would enable diagnosis of infections based on probes that demonstrate significant hybridisation signals, whether designed to be complimentary to targets or not. By only including probes that consistently produce signals in the presence of the five targeted pathogens, the high levels of cross-hybridisation observed in this study would not affect accurate diagnosis if a valid pattern of hybridisation is used as a reference.

A prototype diagnostic microarray was developed in this study that produced significant hybridisation reactions with pure cultures of the five targeted pathogens. The large effect of external and local factors during hybridisation, as well as technical errors in preparation processes, however, affected the consistency of hybridisation and limited the reliable diagnosis of that target pathogens (Szemes et al., 2005; Zhang et al., 2007). This study represented a preliminary analysis of the diagnostic capacity of the array by using two technical replicate samples of a single culture for targeted pathogens, accessible related and non-target, unrelated pathogens of tomato. More in depth validation of the array using more replicates, different strains and more related species is required (Call et al., 2003; Call, 2005).

The diagnostic process selected for template preparation in this study supported the concurrent analysis of RNA and DNA templates in a single hybridisation reaction, facilitating the detection of both viral and bacterial pathogens from a single sample (Wilson et al., 2002). Preparation of RNA and DNA templates for hybridisation, however, was labour intensive and technical that required training and approximately four days to final data interpretation (Lievens and Thomma, 2005; Njambere et al., 2011; Sholberg et al., 2005; Van Doorn et al., 2007). This is still shorter than conventional detection methods, however, which require two – three weeks for diagnosis. The length of the diagnostic process used in this study can easily be reduced in future studies by incorporating mastermixes for amplification reactions, high-throughput 96 well plates, automated extraction processes and automated slide processing (Call, 2005; Lievens and Thomma, 2005; Schaad and Frederick, 2002). Some of these, however, increase the cost of sample processing, making assays too expensive for most diagnostic laboratories. Eliminating PCR amplification steps would eliminate any bias introduced for a targeted pathogen from samples containing unexpected mixed infections (Szemes et al., 2005; Van Doorn et al., 2007, 2009). This would impact the sensitivity of pathogen detection, however, potentially causing in false negative results. Although coupling samples during hybridisation reduces the time and cost of screening multiple samples (Tiberini et al., 2010), this also affected hybridisation results in this study. Therefore, samples should be processed individually until dual processing is optimised.

The microarray used in this study did not validate the viability of the detected pathogens (Call, 2005; Lievens et al., 2005). Including viability confirmation assays would drastically increase the length of diagnostic assays. Probe design could be manipulated to target only mRNA transcripts from bacteria and negative RNA strands of positive sense viruses, providing a measure of pathogen viability (Call et al., 2003). Transcripts are known to be transient and unstable, which may reduce the sensitivity of pathogen detection when using these templates for hybridisation. Results of this study were based on pure bacterial cultures and viral-infected leaf samples, but for adoption into standard diagnostic protocols, infected seed samples should also be used to validate the microarray. Tomato seed are known to contain high concentrations of polysaccharides, polyphenols and proteins (Bilgin et al., 2009) which reduce the efficiency of extraction and amplification procedures and could, therefore, affect hybridisation results further (Call et al., 2003; Momeni et al., 2011; Palacio-Bielsa et al., 2009). Tests on these samples will require intensive optimisation and validation experiments.

As a diagnostic tool, microarrays have demonstrated sensitive and specific detection of targets even in the presence of competing pathogen templates (Boonham et al., 2007; Call et al., 2003; Hadidi et

al., 2004; Njambere et al., 2011; Pelludat et al., 2009; Tiberini et al., 2010; Wilson et al., 2002). The diagnostic process used for microarray-based detection is considerably shorter than conventional detection methods and longer than PCR tests, but can enable identification and classification of amplified fragments (Call et al., 2003; Wilson et al., 2002). Probe design, however, is complicated and requires extensive validation by *in silico* tests and experimental assays (Boonham et al., 2007; Call, 2005; Engel et al., 2010). The process of template preparation is easily affected by technical errors and is highly labour intensive. The high number of probes that can be incorporated into a single array (Adams et al., 2009; Boonham et al., 2007; Hadidi et al., 2004; Kumar, 2009) makes this tool one of the best options available for multiplex screening of different classes of important tomato pathogens. Provided that the numerous factors affecting pathogen detection are optimised and refined, microarrays could benefit the protection of global tomato production and trade industries.

Chapter 6 Concluding Remarks

The growing demand for tomato fruit on a global scale has caused increased pressure on tomato production industries (Diez and Nuez, 2006). Global trade of hybrid tomato seed improves fruit yields but production can be affected drastically by seed-borne pathogens that are moved between countries via infected seed (Kritzman, 1991; Pelludat et al., 2009). Phytosanitary regulations have been implemented to prevent the trade of seed lots until they have been validated for the absence of all significant threats using standard diagnostic protocols (Deyong et al., 2005; EPPO/CABI, 2005; GSPP, 2009). Effective detection tools can restrict the spread of destructive pathogens into tomato production areas and facilitate faster implementation of curative strategies (Lievens and Thomma, 2005; Njambere et al., 2011; Pelludat et al., 2009). Current protocols employed for certification, however, detect only a single pathogen per assay (De Leon et al., 2008; Hadidi et al., 2004), requiring numerous tests and large samples of expensive hybrid seed to prove the absence of detrimental pathogens. In this study, methods currently used for certification, as well as alternative techniques, were evaluated for their capacity to detect five significant seed-borne pathogens of tomato, to establish which demonstrated the best potential for standard diagnostic assays (Table 6. 1). A prototype diagnostic microarray tool was also developed that was capable of screening a single sample for the presence of multiple viral and bacterial pathogens simultaneously, although significant technical problems limit this tool to experimental evaluations currently.

Infected seed and leaf samples were used to evaluate selected conventional and serological methods that form the basis of most phytodiagnostic protocols for the five pathogens selected (ISF 2008, 2009a; 2009b; 2009c). These methods are relatively simple, economical and require minimal complex equipment (Fatmi and Schaad, 1988). Viral detection using double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) demonstrated rapid, robust and specific detection of a targeted species from infected leaf and seed samples (Table 6. 1) (Hadas et al., 2004; Sevİk and Kose-tohumcu, 2011). The technique did not indicate the viability of detected pathogens, however, which can result in false positive diagnoses of samples chemically treated to eliminate pathogens. Selective media plating is the standard method for bacterial detection, yet in this study, assays were not always reliable (Kuflu and Cuppels, 1997), demonstrating variable recovery of targeted bacterial colonies from infected host material. Only viable organisms were recovered by this method, but the assays were lengthy (Table 6. 1) and typically laborious (Harris-Baldwin and Gudmestad, 1996; Lievens et al., 2003), requiring supplementary tests to prove presumptive identification. To improve detection capacity of this method, the best performing selective media

should be employed in combination with a general growth medium instead of two selective media, as suggested by standard protocols. This combination should recover all viable organisms from host material, while still analysing the presence of targeted bacteria. Bioassays and pathogenicity tests were essential to prove the viability and pathogenicity of detected organisms in this study. Duration of tests depended on targeted pathogens (Table 6. 1), required greenhouse facilities and discriminative symptoms were highly dependent on suitable environmental conditions (Hadas et al., 2004). Conventional methods demonstrated limited capacity for multiplex detection (Hadidi et al., 2004; Lee et al., 2003) and the specificity as well as sensitivity of these methods, except when using DAS-ELISA, was typically low for accurate diagnosis and identification of detected pathogens.

Table 6. 1 Comparative analysis of the techniques evaluated in this study for diagnosis of the five viral and bacterial seed-borne pathogens of tomato targeted.

Technique	Target Pathogen	Sensitivity	Highest Level of Specificity	Duration (days)	Viability	Taxonomic Classification	Multiplex detection	Multi-class detection
DAS-ELISA	Viral	Intermediate	Species	2 – 3	No	No	No	No
Selective media plating	Bacterial	Low	Genus	4 – 14	Yes	No	No	No
Bioassay/ Pathogenicity Test	Viral Bacterial	Low	Genus - Subspecies	4 – 14	Yes	Potential	No	No
Conventional PCR	Viral Bacterial	Intermediate	Subspecies	1 – 2	No	Potential	Potential	Potential
Bio-PCR	Bacterial	Intermediate	Species - Subspecies	5 – 15	Yes	Potential	No	No
Sequencing	Viral Bacterial	Intermediate – High	Subspecies	7 – 10	No	Yes	Potential	Potential
Diagnostic Microarray	Viral Bacterial	High	Subspecies	3 – 4	No	Yes	Yes	Yes

Nucleic acid based approaches were more suitable for diagnostic protocols as these were more specific and sensitive than most conventional methods for targeted detection of pathogens from infected host samples (Table 6. 1) (Schaad and Frederick, 2002). In this study, conventional PCR was selected for comparative analysis of diagnostic protocols as this method was rapid, relatively inexpensive and can be expanded for high-throughput screening (Lievens and Thomma, 2005). Five targeted viral and bacterial pathogens were detected from pure cultures, and from leaf and seed extracts of tomato using PCR that incorporated previously validated primer pairs. The efficiency of amplification procedures was, however, affected by various factors like the presence of PCR inhibitors co-extracted with nucleic acid from host samples (McManus and Jones, 1995; Meng et al., 2004). Positive reference cultures, therefore, had to be included in diagnostic assays to validate pathogen detection. Nucleic acid extraction procedures limit incorporation of molecular techniques

into standard diagnostic protocols (Dovas et al., 2004) as these are technically challenging and not always conducive to large-scale testing. Column-based extraction procedures incorporated into this study facilitated simple and quicker RNA and DNA extractions (Lievens and Thomma 2005; Lopez et al. 2007; Mumford et al. 2006). Poor quality and low yields from seed samples were recovered using these kits as these were able to eliminate high concentrations of polysaccharides, proteins and polyphenols that occur in seed (Bilgin et al., 2009; Momeni et al., 2011). Viability of detected pathogens was only possible using bio-PCR out of all the molecular methods evaluated (Table 6. 1) (Maes, 1993; Schaad et al., 1995), and this method facilitated relatively reliable discrimination between true and false positive colonies recovered from media assays. Only one targeted pathogen could be detected per amplification reaction using conventional PCR, but the methods could be extended to facilitate multiple pathogen detection from a single sample (Ozdemir, 2009). Design of multiplex PCR is complicated and reactions are currently limited to a maximum of six primer pairs (Boonham et al., 2007; Lievens and Thomma, 2005).

An important seed-borne, viral pathogen of tomato, *Pepino mosaic virus* (PepMV) was reported in South Africa for the first time in this study. This highly virulent and infectious pathogen has caused considerable economic losses in Europe and North America (Hanssen and Thomma, 2010; Ling, 2008). Thus, its detection in South African tomato production areas represents a threat to the local tomato industry. Introduction of PepMV into South Africa is suspected to be linked to the import and cultivation of infected tomato seed but further research is necessary to prove this assumption and to determine the current distribution of the virus in this country. Detection and identification of PepMV was achieved by symptom evaluation, DAS-ELISA, bioassays and pathogen-specific PCR, but for certain identification and characterization, PCR products were sequenced. Although this latter method is more labour-intensive and longer than other molecular techniques (Table 6. 1), sequencing enables highly accurate subspecies characterization as genomic sequences can be compared directly to a comprehensive database of pathogenic sequences (Adams et al., 2009; Schaad and Frederick, 2002). Sequencing pathogen genomes generates large amounts of unnecessary sequence data of regions that are uninformative for the identification and classification of detected pathogens. The recent development of high-throughput sequencing methods has improved the turnover of results and enabled multiplex pathogen detection from host samples (Adams et al., 2009; Studholme et al., 2010). Interpreting sequence data is complicated, however, and must be assembled using reference genomes.

The high binding capacity of microarrays for hundreds of unrelated probes make this tool attractive for multiplex pathogen detection and taxonomic classification (Bodrossy and Sessitsch, 2004; Boonham et al., 2007; Call, 2005; Hadidi et al., 2004). The prototype microarray developed in this study was capable of detecting the five targeted pathogens from individual and combined samples of pure culture extracts (Table 6. 1). Using separate preparation steps, RNA and DNA extracts were labelled and hybridised to a single slide, facilitating simultaneous detection of different classes of pathogens from a single sample (Wilson et al., 2002). The technique was fairly rapid but did not indicate viability of detected pathogens and, therefore, bioassays or pathogenicity tests are needed to supplement microarray testing. The design of capture probes for pathogen templates was technically challenging and arduous, requiring intensive *in silico* and experimental validation (Njambere et al., 2011). Targeted genomic regions also had to demonstrate sufficient polymorphisms to facilitate taxonomic differentiation (Pelludat et al., 2009). High sensitivity of this technique (Table 6. 1) is achieved through combination of microarrays with PCR (Call, 2005; Szemes et al., 2005), which augment pathogen templates so that these are detectable in the presence of high concentrations of host nucleic acid. Amplification steps can become labour-intensive if separate reactions are required to prepare multiple pathogens for array hybridisation and, thus, must be optimised. The developed array was highly susceptible to technical and external factors (Kyselková et al., 2009) which affected the consistency of hybridisation signals between replicate samples of the same pathogen. This also affected the diagnostic capacity of the prototype array. Tested only on pure cultures of pathogens, the developed microarray also requires further validation using complex host and field samples before it can be incorporated into diagnostic assays. As a detection tool, microarrays are technical, labour-intensive and currently too expensive for use in standard diagnostic protocols (Lievens and Thomma, 2005). The high multiplex capacity of microarrays and their easy extendibility to include other significant pathogens, however, make microarrays potentially ideal for reliable, sensitive and specific detection of multiple pathogens from a single sample (Table 6. 1). Intensive optimisation of the prototype array developed in this study and sample preparation processes are necessary before this and other microarrays can be adopted into diagnostic protocols.

Many other diagnostic techniques currently under development demonstrate varying potential for use in pathogen detection assays. Real-time PCR and ligation dependent microarrays, for example, demonstrate improved sensitivity and specificity for pathogen detection (Schaad and Frederick, 2002; Van Doorn et al., 2007; Wang et al., 2011), yet are costly and still impractical for routine diagnostic assays. All of the detection techniques evaluated in this study demonstrate some limits in

potential for the detection of unknown and emerging pathogens as all depend on pathogen-specific primers, probes or antibodies for identification (Adams et al., 2009). Further studies could focus on improving current tools or developing new multiplex detection methods to support the identification of new pathogens infecting tomato. Inefficient detection systems, especially for novel pathogens, may have an adverse effect on the global tomato industry (Njambere et al., 2011), potentially facilitating spread of pathogens and yield losses before control measures can be implemented adequately.

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Appendix A

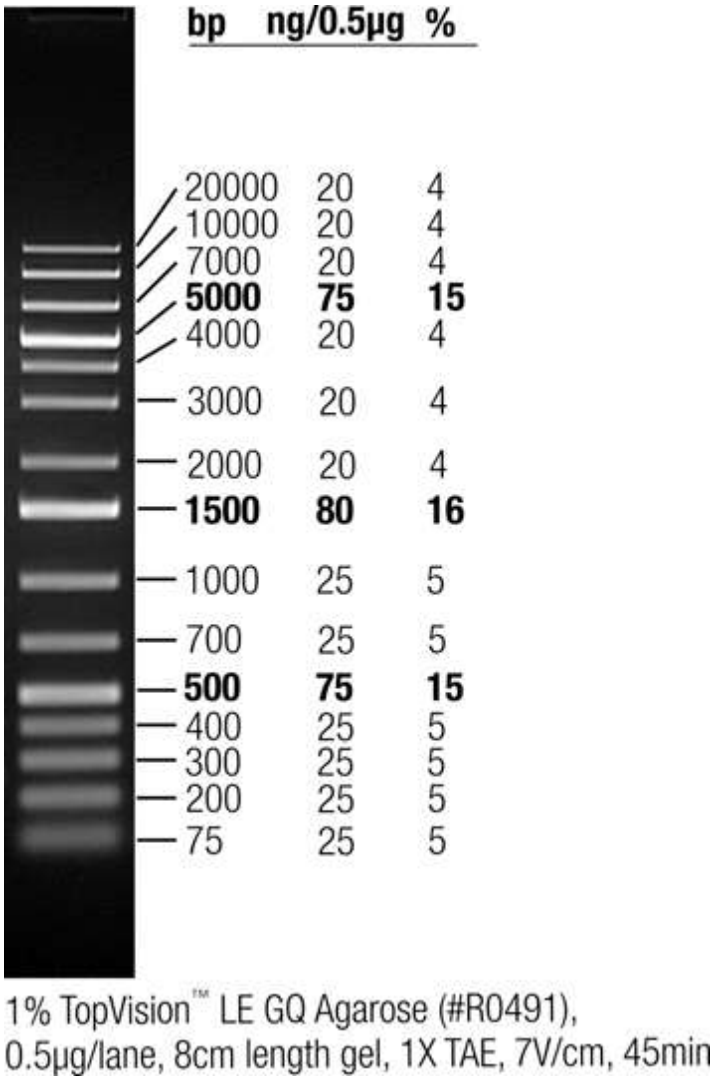

Figure representing the 1 kb O'Generuler DNA ladder used to confirm the length of amplicons generated from conventional PCR tests (Fermentas, EU).

Appendix B

Table representing the diagnostic probes designed and selected for incorporation into the prototype diagnostic microarray.

Target	Accession Name	Gene	Sequence (5' – 3')	Taxonomic level of characterization	Reference
Clavibacter michiganensis* subsp. *michiganensis					
1	Cmm8_rRNA	16S rRNA	TGCGGTGAATACGTTCCC	Eubacteria	
2	1078-R Kaneshiro	16S rRNA	CCAACATCTCACGACACGAG	Eubacteria	Kaneshiro et al. (2006)
3	Cm17_rRNA	16s rRNA	ATCGGTGATTAGGACTAAGTCGT	Genus	
4	Cm22_rRNA	ITS	GGTGTGTCTGGTTTCTTGTCG	Species	
5	Cm23_rRNA1	ITS	GCGAGCATCTTAGATTCACCG	Species	
6	Cm23_rRNA2	ITS	CCGTATATTGAGAACTACACAGTG	Species	
7	Cm24_rRNA	ITS	AGATCATTGGTCAATTCTGTCTCC	Species	
8	Cmm11_rRNA	ITS	GGTGGAACATTGACATTGATGC	Subspecies	
9	Cmm12_rRNA	ITS	TGCTAGTACGCCTCCTTGTG	Subspecies	
10	Cmm14_rRNA	ITS	TGTGTCTGGTTTCTTGTCGGA	Species	
11	Cmm17_rRNA	ITS	GGGAGGCGAAACGATTCAAT	Species	
12	FPCm Bach	ITS	TGTCGAGGGCATGTTGCA	Species	Bach et al. (2003)
13	RPCm Bach	ITS	GGAGACAGAATTGACCAATGAT	Species	Bach et al. (2003)
14	PSA-8 Pastrik	ITS	TTGGTCAATTCTGTCTCCCTTC	Subspecies	Pastrik and Rainey (1999)
15	PSA-4 Pastrik	ITS	TCATTGGTCAATTCTGTCTCCC	Subspecies	Pastrik and Rainey 1999)
16	Cmmprobe	ITS	TTCCGTCGTCCTGTTGTGG	Subspecies	Bach et al. (2003)
17	Cm12_gyrB	gyrB	ACCGCACCGAGAAGTTCC	Species	
18	Cm13_gyrB2	gyrB	AAGACCGAGGTCGTCAACG	Species	
19	Cm14_gyrB1	gyrB	CACGGTCAAGAAGATCAGCC	Species	
20	Cm14_gyrB2	gyrB	TCATCGCCTTCGAGTCCG	Species	

Target	Accession Name	Gene	Sequence (5' – 3')	Taxonomic level of characterization	Reference
21	Cmm9_gyrB1	gyrB	AGAAGGACGAGAACCTCACG	Subspecies	
22	Cmm9_gyrB3	gyrB	GCTCGTCAACCGCTACGC	Subspecies	
23	Cm20_gyrB	gyrB	AGGCGAAGGACATCATCCG	Subspecies	
24	Cm22_gyrB	gyrB	CAAGCTCAAGGACTGCCAGA	Subspecies	
25	CmgroEL_5_2	groEL	GCATCGAGAAGGCCGTCG	Species	
26	Cmm_groEL_18	groEL	CGTACATCCTGATCGTCAACTC	Species	
27	g-C.mm3 Pelludat	groEL	CTCCTCCTTGGTCTCGATCT	Subspecies	Pelludat et al. (2009)
28	Cm13_rpoB	rpoB	CGCTACAAGATCAACCGCAA	Species	
29	Cmm2_rpoB1	rpoB	CTGCTGGACAACTTCTACTTCAA	Species	
30	Cmm2_rpoB2	rpoB	CGAAGATGAACGGCACGC	Species	
31	Cmm2_rpoB5	rpoB	TCATCCAGAACCAGGTGCG	Subspecies	
32	Cmm2_rpoB6	rpoB	GTCGCTACAAGATCAACCGC	Species	
33	Cmm2_rpoB10	rpoB	GACTCGGTGCTGACGGTC	Species	
Pseudomonas syringae* pv. *tomato					
1	Ps11_rRNA1	ITS	GGCTCCACCACTTACTGCT	Genus/Species	
2	Ps14_rRNA	ITS	TTCACAGTATAACCAGATTGCTTG	Species	
3	Pst11_rRNA	ITS	TGGTGAAGTTGGTCAGAGCG	Species	
4	Ps17_rRNA	23S rRNA	CTACTGACTGACCGATAGTGAAC	Genus	
5	Ps11_rRNA2	ITS	CAGGAGGTCAGCGGTTCG	Genus	
6	Ps22_gyrB	gyrB	CCTTCTTCTTCCGTCAGTTGC	Genus	
7	Ps23_gyrB	gyrB	CGTGGCTACATATACATCGCTC	Pathovar	
8	Pst16_gyrB	gyrB	GGTCAAGAAAGGCAAGCAGG	Species	
9	Pst19_gyrB	gyrB	GACTTCTTCGGCAGCAACG	Species	
10	Pst20_gyrB	gyrB	TTCAAACAAGCCCTCGCCT	Pathovar	
11	Pst1_rpoB7	rpoB	TACCGTGGTTCGTGGCTG	Pathovar	
12	Pst1_rpoB8	rpoB	CCTGCGTCCGTGCTTCTG	Pathovar	
13	Pst1_rpoB12	rpoB	CTGGAAGTACCTCTGGACTACG	Species	

Target	Accession Name	Gene	Sequence (5' – 3')	Taxonomic level of characterization	Reference
14	Pst2_rpoB1	rpoB	TGTCTGCTGTAGGTCGTATGAA	Pathovar	
15	Pst2_rpoB3	rpoB	GCAAGGAAGACATCGTAGCG	Pathovar	
16	Pst2_rpoB4	rpoB	CGTGTTCGTTGCGTAGGC	Pathovar	
17	Pst3_rpoB	rpoB	CGGCAGCGGTCAAGGAAT	Species	
Xanthomonas campestris* pv. *vesicatoria					
1	Xanth27_rRNA2	16S rRNA	GGGAGTTTGTTGCACCAGAAG	Genus	
2	Xanth28_rRNA	16S rRNA	CTTAACCTTCGGGAGGGCG	Genus	
3	Xcv3_rRNA_pv11	ITS	GTCGTTGAGGCTAAGGCGG	Genus	
4	Xcv3_rRNA_pv20	ITS	CGTCATCGTCCTGTCGGG	Species	
5	Xcv3_rRNA_pv21	ITS	GATAAGGGTGAGGTCGGTGG	Genus	
6	XgyrPCR2F	gyrB	AAGCAGGGCAAGAGCGAG	Genus	
7	Xcv20_gyrB1	gyrB	AACGCACACCGCTACGAC	Genus	
8	Xcv20_gyrB3	gyrB	GACGAACCGCCGATCACC	Genus/Species	
9	Xcv22_gyrB	gyrB	ATGCCTGGTTGCTGGACG	Genus/Species	
10	Xcv11_rpoB	rpoB	CGAGATCAACGAAGACCAGC	Genus/Species	
11	Xanth17_rpoB	rpoB	TACCTGTCCAACACCTTGCG	Species/Pathovar	
12	Xanth23_rpoB	rpoB	GGCGAGTCGGTGCTGTAC	Pathovar	
13	Xanth24_rpoB	rpoB	TAACGACGAAGAATCCAAGCG	Species/Pathovar	
14	Xcv14_rpoB	rpoB	CTCACCGAAATCCGCAACG	Pathovar	
Tomato mosaic virus					
1	ToMV4_species_1	Rep gp1 and 2	CGTTCACTGCTGTATGCCG	Genus/Species	
2	ToMV4_species_3	Rep	GTTCACTGCTGTATGCCGAAT	Genus/Species	
3	ToMV5_species1	Rep	CGACATAATGCGGCACGAG	Species	
4	ToMV5_species2	Rep	AACGAAGTAGTCTGTCACGATAC	Species	
5	ToMV7_species5	Rep	TGAAATGCCAAACGAAGTAGTCT	Species	
6	ToMV9_species3	Rep	CGATATACCTGCCGACGAGT	Species	
7	ToMV9_species4	Rep	ATACGATATACCTGCCGACGAG	Species	

Target	Accession Name	Gene	Sequence (5’ – 3’)	Taxonomic level of characterization	Reference
8	ToMV16_species1	Rep	ACAAGGCTATGGAAGACGCA	Genus/Species	
9	ToMV46_species	Rep 2	CGAACGGCGGCAGAAATG	Species	
10	ToMV53_species_1	Rep 2	GTAATACCGTCATCATTGCTTCG	Species	
11	ToMV53_species_4	Rep 2	ATACCGTCATCATTGCTTCGTG	Species	
12	ToMV54_species4	Rep 2	GAGCCTTCTGCGGAGATGA	Species	
13	ToMV54_species	Rep 2	AGGAGCCTTCTGCGGAGAT	Species	
14	ToMV58_species_6	Rep 2	GTGATGTTGCTGAGTCGTTGAA	Species	
15	ToMV70_species1	CP	TTACTCAATCACTTCTCCATCGC	Species	
16	ToMV73_species2	CP	CCTCAGAGCACCGTCAGATT	Species	
17	ToMV75_species1	CP	GATCCTCTAATTACTGCGTTGCT	Species	
18	ToMV75_species4	CP	CCTCTAATTACTGCGTTGCTGG	Species	
19	ToMV79_species2	CP	TTGAAAGTATGTCTGGGTTGGTC	Genus/Species	
20	ToMV79_species3	CP	AGTATGTCTGGGTTGGTCTGG	Genus/Species	
21	TMV10_species4	Rep	ATGGAAGACGCATGGCATTAC	Species	
22	TO/TMV_genus_10_3	Rep	GAAGACGCATGGCACTACAA	Genus/Species	
23	TO/TMV_genus_10_5	Rep	ATGGAAGACGCATGGCACTA	Genus/Species	
24	TMVCP-F Letschert	CP	GGTCAGTGCCGAACAAGAA	Species	Letschert et al. (2002)
25	ToMV-3 Jacobi 1	3'UTR	TTCAACAGCAGTTCAGCGAG	Species	Jacobi et al. (1998)
26	ToMV3 Tib 1	CP	TCTTACTCAATCACTTCTCCATCG	Species	Tiberini et al. (2010)
27	ToMV3 Tib 2	CP	CTCAATCACTTCTCCATCGCAAT	Species	Tiberini et al. (2010)
Pepino mosaic virus					
1	PepMV_CH2B	RdRp	CCTAATCAACTGTCCTTATGCG	Species/Strain	
2	PepMV_CH2H	RdRp	CCTGTTGAAGCCATCCACAA	Strain	
3	PepMV_CH2I	RdRp	ACATTAGACTGGCTCAAATACTCT	Strain	
4	PepMV_CH2J	RdRp	CCTTAACTGGACTCCGATACAC	Strain	
5	PepMV_CH2K	RdRp	CCTTTCCTAACATCTTCCATCCC	Strain	
6	PepMV_CH2O	RdRp	GCTCAATCAACGACTATGACAATA	Strain	

Target	Accession Name	Gene	Sequence (5' – 3')	Taxonomic level of characterization	Reference
7	PepMV_15	RdRp	GATCAGGGACCCACAAGTACA	Species	
8	PepMV_15_2	RdRp	AAAGATCAGGGACCCACAAGTA	Species	
9	PepMV_16	RdRp	TGAAGCAGACACCCTTGAGAA	Species	
10	PepMV_16_2	RdRp	TATGAAGCAGACACCCTTGAGA	Species	
11	PepMV_19	TGB1.2	TCCTAGAGCTGATCTTACTGACAC	Species	
12	CH2-CP-F	CP	GGGTTTAGCAGCCAATGAGA	Strain	Gutierrez-Aguirre et al. (2009)
13	11 Tiberini	TGB1	CTGAAGGAAGTGTAATGTGGGAC	Strain	Tiberini et al. (2010)
14	R5R	CP	ACATCAGCATAAGCACGAGC	Genus/Species	Hasiow-jaroszewska et al. (2009)
15	Eur-CP-F	CP	GGAACATACTTCTCGACAGCAA	Strain	Gutierrez-Aguirre et al. (2009)
16	US1-CP-R	CP	CGTGAGAGTGCTGGATTTGAA	Strain	Gutierrez-Aguirre et al. (2009)
20	Potex3		GACTTCACAGCATTTGACCAGT	Genus	Molecular Diagnostics Lab - Sinica (2006)
21	US2-3	TGB1	CACCCAACTACACATCCTTCAG	Strain	Molecular Diagnostics Lab - Sinica (2006)
22	US2-4	RdRp	CCAAGGCTGCTGATAACAATCT	Strain	Molecular Diagnostics Lab - Sinica (2006)
23	US2-7	RdRp	CTCCGATACACCATAACCATTCAA	Strain	Molecular Diagnostics Lab - Sinica (2006)
24	US2-9	TGB1.2	GGTGAACTAAATGCCAGGTCT	Strain	Molecular Diagnostics Lab - Sinica (2006)
25	PepMVTom11	CP	GACTTCTCAAATCCCAATACAGC	Species	Tiberini et al. (2010)
26	PepMVCh210	CP	GCCGACTTCTCAAATCCTAATACA	Species/Strain	Tiberini et al. (2010)
27	PepMVCP_1	CP	GCTCCTTCCCTAAGTGATTTGAA	Strain	
28	PepMVTGB1.2	TGB1	GAACTAAATGCCAGGTCTGACTC	Species/Strain	
29	CH2RdRp_1	RdRp	ATCAGGGACCCACAAGTACAAT	Species/Strain	
Universal Probes					
1	1114f 16S/IGS	16S	GCAACGAGCGCAACCCT	Bacteria; Tomato	
2	Eub Pelludat	16S	TGCTGCCTCCCGTAGGA	Bacteria; Tomato	
3	Cm10_rRNA	16S rRNA	GCTCGTGTCGTGAGATGTTG		
Internal Controls					
1	EFalpha1_1F	Efα1	AGATTGACAGGCGTTCAGGT		Allie, F (unpublished)
2	GAPDH _1	GADPH	GCCTTCAGAGTACCAACTGCT		Allie, F (unpublished)

Target	Accession Name	Gene	Sequence (5’ – 3’)	Taxonomic level of characterization	Reference
3	EFalpha1 _1	Efα1	AGGCTGACTGTGCTGTTCTC		Allie, F (unpublished)
4	mRNA E2_1	Ubiqitin-conjugating enzyme	CTGGACAGCAGACTGACATC		Allie, F (unpublished)
5	GAPDH_2	GADPH	GACCTGCTGTCACCAACAA		Allie, F (unpublished)
Random Control					
1	Archea_1	ArsR	TGAAGAGAACAGAAGGAGGATACT		
2	Archea_3	ArsR	GAGGAAGATATACAGTATTGCGGA		
Dimer Probes					
Cmm					
1	Cmmprobe-Di2	ITS	TTCCGTCGTCCTGTTGTGGTTCCGTCGTCCTGTTGTGG		
2	Cm13_gyrB2-Di2	gyrB	AAGACCGAGGTCGTCAACGAAGACCGAGGTCGTCAACG		
3	Cmm_groEL18-Di2	groEL	CGTACATCCTGATCGTCAACTCCGTACATCCTGATCGTCAACTC		
4	Cmm2_rpoB2_Di2	rpoB	CGAAGATGAACGGCACGCCGAAGATGAACGGCACGC		
Pst					
1	Ps11_rRNA_Di2	ITS	GGCTCCACCACTTACTGCTGGCTCCACCACTTACTGCT		
2	Pst20gyrB_Di2	gyrB	TTCAAACAAGCCCTCGCCTTTCAAACAAGCCCTCGCCT		
3	Pst3_rpoB_Di2	rpoB	CGGCAGCGGTCAAGGAATCGGCAGCGGTCAAGGAAT		
4	Pst11_rRNA_Di2	rRNA	TGGTGAAGTTGGTCAGAGCGTGGTGAAGTTGGTCAGAGCG		
Xcv					
1	Xanth28_rRNA_Di2	16S rRNA	CTTAACCTTCGGGAGGGCGCTTAACCTTCGGGAGGGCG		
2	Xcv22_gyr_Di2	gyrB	ATGCCTGGTTGCTGGACGATGCCTGGTTGCTGGACG		
3	Xcv11_rpoB_di2	rpoB	CGAGATCAACGAAGACCAGCCGAGATCAACGAAGACCAGC		
4	Xcv_rRNA_pv20_Di2	rRNA	CGTCATCGTCCTGTCGGGCGTCATCGTCCTGTCGGG		
PepMV					
1	US2-7	RdRp	CTCCGATACACCATAACCATTCAACTCCGATACACCATAACCATTCAA		
2	PepMV_16	RdRp	TGAAGCAGACACCCTTGAGAATGAAGCAGACACCCTTGAGAA		

Target	Accession Name	Gene	Sequence (5' – 3')	Taxonomic level of characterization	Reference
ToMV					
1	ToMV7_spe5_Di2	Rep	TGAAATGCCAAACGAAGTAGTCTTGAAATGCCAAACGAAGTAGTCT		
2	ToMV46_spe_Di2	Rep 2	CGAACGGCGGCAGAAATGCGAACGGCGGCAGAAATG		

Appendix C

Table of probes that demonstrated significant hybridisation signals (SNR ≥ 2.5) obtained from reactions with one or both replicate samples of the relevant, labelled pathogen templates.[1]

Targeted Pathogen	Probe	Replicate 1						Replicate 2						Reliable signal (Y/N)
		Mean	SD	%CV	Propn >2.5	Data Distribution	Mean Sign >2.5[2]	Mean	SD	%CV	Propn >2.5	Data Distribution	Mean Sign >2.5[2]	
PepMV	CH2-CP-F [3]	8.75	3.99	45.58	1.00	not normal	yes	3.40	1.71	50.13	0.63	normal	no	Yes
	CH2RdRp_1	8.43	1.61	19.07	1.00	normal	yes	7.57	2.12	27.99	1.00	not normal	yes	Yes
	Cmm2_rpoB2_Di2	5.03	3.17	62.88	0.88	not normal	yes	1.18	0.74	63.22	0.06	not normal	no	No
	Cmmprobe-Di2	4.54	2.11	46.49	0.75	not normal	yes	0.72	0.43	59.57	0.00	normal	no	No
	Eub Pelludat	3.01	0.86	28.63	0.81	not normal	yes	1.19	0.73	61.51	0.06	not normal	no	No
	PepMV_15	16.92	7.89	46.60	1.00	normal	yes	13.38	6.35	47.44	1.00	not normal	yes	Yes

[1] Data presented in this table indicates the statistical evidence for the significance of detected hybridisation signals from reactions with labelled replicate samples based on the signal to noise ratio (SNR) and a cut-off of 2.5. Any probes with mean SNR values below this cut-off for one or both replicates were considered indicative of poor or random hybridisation reactions with labelled templates and could therefore not be used for accurate diagnosis of the targeted pathogen.

[2] Refers to whether mean SNR values proved to be significantly greater than 2.5 based on the t-test or Wilcoxon's signed rank test at a 0.05% confidence interval.

[3] Highlighted probes represent those that demonstrated significant, consistent and reliable signal intensity from both replicates tested for a single pathogen, therefore representing potential diagnostic probes for the respective pathogens.

PepMV	PepMV_15_2	7.89	1.53	19.44	1.00	normal	yes	8.49	1.31	15.40	1.00	normal	yes	Yes
	PepMV_16	8.84	1.82	20.65	1.00	not normal	yes	8.52	2.31	27.15	1.00	normal	yes	Yes
	PepMV_16_2	10.71	2.79	26.07	1.00	normal	yes	8.17	1.10	13.50	1.00	normal	yes	Yes
	PepMV_16_Di	11.65	5.96	51.15	1.00	not normal	yes	14.99	5.65	37.72	1.00	normal	yes	yes
	PepMV_19	18.91	8.10	42.84	1.00	normal	yes	5.83	3.65	62.64	0.75	not normal	no	yes
	PepMV_CH2H	14.56	6.60	45.34	1.00	normal	yes	11.66	4.51	38.65	1.00	not normal	yes	yes
	PepMV_CH2I	4.11	1.15	28.03	0.94	normal	yes	0.95	0.21	21.63	0.00	normal	no	no
	PepMV_CH2J	16.57	8.14	49.14	1.00	normal	yes	4.87	2.81	57.79	0.75	not normal	no	yes
	PepMV_CH2K	9.38	4.50	48.00	1.00	normal	yes	3.07	1.71	55.69	0.50	not normal	no	yes
	PepMV_CH2O	7.90	1.88	23.76	1.00	normal	yes	2.42	1.26	51.95	0.50	not normal	no	x[4]
	PepMVCh210	9.79	2.28	23.31	1.00	normal	yes	8.93	3.51	39.35	1.00	not normal	yes	yes
	PepMVCh215	10.82	4.87	45.03	1.00	not normal	yes	7.27	3.26	44.88	1.00	normal	yes	yes
	PepMVCP_1	8.88	4.55	51.21	1.00	not normal	yes	1.13	0.80	70.89	0.06	not normal	no	no
	PepMVTGB1.2	10.26	4.64	45.25	1.00	normal	yes	5.95	2.11	35.44	1.00	normal	yes	yes
	TMV10_SPECIES4	2.76	0.60	21.68	0.69	normal	no	0.96	0.51	53.41	0.00	normal	no	no
	TO/TMV_genus_10_3	3.49	1.22	34.89	0.69	normal	yes	2.11	1.45	68.64	0.25	not normal	no	no
	TO/TMV_genus_10_5	4.65	2.44	52.50	0.81	normal	yes	4.43	1.93	43.56	0.88	normal	yes	yes
	ToMV16_species1	2.98	0.53	17.89	0.81	normal	yes	0.77	0.31	40.49	0.00	normal	no	no
	ToMV29_species_2	6.63	3.08	46.52	0.94	not normal	yes	4.96	1.60	32.17	1.00	normal	yes	yes
	ToMV3 Tib 1	5.68	2.96	52.10	0.81	normal	yes	1.40	0.86	61.57	0.13	not normal	no	no
	ToMV3 Tib 2	9.43	2.75	29.13	1.00	normal	yes	12.38	6.99	56.46	1.00	normal	yes	yes
	ToMV38_species	9.79	4.93	50.36	0.94	normal	yes	3.19	1.98	62.05	0.56	normal	no	yes
	ToMV4_species_3	4.87	2.31	47.37	0.81	normal	yes	3.04	2.14	70.52	0.50	not normal	no	yes
	ToMV46_spe_Di2	10.25	2.16	21.06	1.00	normal	yes	7.30	2.41	32.95	1.00	normal	yes	yes
	ToMV46_species	2.84	1.64	57.83	0.50	normal	no	1.15	0.60	52.25	0.06	not normal	no	no
	ToMV53_species_1	3.95	1.05	26.54	1.00	not normal	yes	1.36	0.36	26.47	0.00	normal	no	no
	ToMV53_species_4	6.01	2.04	34.00	1.00	normal	yes	3.60	1.54	42.80	0.69	normal	yes	yes

[4] The 'x' refers to probes which have borderline statistical results and can, therefore, not clearly be defined as good or poor for diagnosis of the target pathogen. Further tests are required to confirm the diagnostic capacity of these probes.

PepMV	ToMV54_species4	5.50	1.81	33.00	1.00	not normal	yes	1.55	0.76	48.88	0.25	not normal	no	no
	ToMV58_species_6	4.64	2.35	50.64	0.69	normal	yes	1.42	0.96	67.81	0.06	normal	no	no
	TOMV7_SPECIES2	4.00	0.78	19.45	1.00	normal	yes	0.68	0.29	42.19	0.00	not normal	no	no
	ToMV70_species1	9.22	3.40	36.92	1.00	not normal	yes	6.15	2.34	37.95	1.00	normal	yes	yes
	ToMV73_species2	2.52	0.57	22.61	0.50	normal	no	0.84	0.18	21.33	0.00	normal	no	no
	ToMV9_species4	3.04	0.75	24.77	0.81	normal	yes	0.83	0.24	29.24	0.00	normal	no	no
	US2-3	14.27	4.16	29.16	1.00	normal	yes	7.62	2.29	29.99	1.00	normal	yes	yes
	US2-7	14.93	6.67	44.69	1.00	not normal	yes	6.19	2.06	33.22	1.00	normal	yes	yes
	US2-7_Di	15.36	4.34	28.23	1.00	normal	yes	14.00	3.06	21.89	1.00	normal	yes	yes
	US2-9	22.06	6.59	29.89	1.00	not normal	yes	6.90	2.29	33.20	1.00	normal	yes	yes
ToMV	TO/TMV_genus_10_3	6.01	2.98	49.56	0.75	normal	yes	3.23	1.54	47.67	0.56	normal	no	yes
	TO/TMV_genus_10_5	2.85	2.18	76.50	0.50	not normal	no	1.59	0.88	55.48	0.19	not normal	no	no
	ToMV3 Tib 2	4.31	3.47	80.38	0.50	not normal	no	2.19	2.22	101.18	0.38	not normal	no	x
	ToMV4_species_3	3.81	0.97	25.37	1.00	not normal	yes	2.50	0.49	19.48	0.50	normal	no	yes
	ToMV46_spe_Di2	6.76	2.65	39.22	1.00	not normal	yes	4.69	2.09	44.47	0.75	normal	no	yes
	ToMV46_species	4.30	0.89	20.72	1.00	normal	yes	2.21	0.71	31.97	0.38	normal	no	x[4]
	ToMV53_species_4	3.64	1.11	30.43	1.00	not normal	yes	1.87	0.69	36.65	0.13	not normal	no	no
	ToMV9_species4	4.37	1.12	25.57	1.00	normal	yes	2.93	1.29	44.06	0.56	normal	no	yes
TMV	TMV10_SPECIES4	5.28	2.83	53.60	0.75	normal	yes	1.29	1.25	97.10	0.19	not normal	no	no
	TO/TMV_genus_10_3	12.67	5.91	46.69	1.00	not normal	yes	14.42	8.95	62.08	1.00	not normal	yes	yes
	TO/TMV_genus_10_5	14.98	7.44	49.66	1.00	normal	yes	9.85	9.71	98.60	0.75	not normal	no	yes
	ToMV46_spe_Di2	9.70	1.69	17.39	1.00	normal	yes	19.66	5.57	28.34	1.00	normal	yes	yes
	ToMV46_species	10.34	1.84	17.79	1.00	normal	yes	1.50	0.42	28.14	0.00	normal	no	no
PMMoV	Cm10_rRNA	0.53	0.16	29.57	0.00	normal	no	4.85	1.81	37.43	0.94	normal	yes	no
	Cmmprobe-Di2	-0.12	0.06	-50.31	0.00	normal	no	2.55	2.87	112.58	0.25	not normal	no	no
	TMV10_SPECIES4	10.27	8.12	79.03	0.81	not normal	yes	32.14	10.35	32.21	1.00	normal	yes	yes
	TO/TMV_genus_10_3	66.17	39.16	59.17	1.00	not normal	yes	43.49	13.43	30.89	1.00	normal	yes	yes
	TO/TMV_genus_10_5	32.74	19.00	58.04	1.00	not normal	yes	37.54	10.12	26.94	1.00	normal	yes	yes
	ToMV46_spe_Di2	41.55	14.10	33.93	1.00	normal	yes	20.62	5.55	26.90	1.00	normal	yes	yes

PMMoV	ToMV53_species_4	2.96	1.54	51.99	0.63	normal	no	0.42	0.13	29.70	0.00	normal	no	no
Cmm	Cm10_rRNA	1.59	0.54	33.96	0.00	not normal	no	2.80	0.70	25.14	0.63	normal	no	x^4
	Cm13_rpoB	6.81	3.23	47.51	1.00	not normal	yes	6.99	1.13	16.19	1.00	normal	yes	yes
	Cm14_gyrB1	2.64	1.25	47.45	0.50	normal	no	2.24	1.04	46.31	0.31	normal	no	x^4
	Cm22_gyrB	2.22	1.09	49.27	0.31	normal	no	6.66	4.26	64.03	0.88	not normal	yes	x^4
	Cmm_groEL18-Di2	5.69	2.01	35.29	1.00	normal	yes	9.18	1.91	20.85	1.00	normal	yes	yes
	Cmm2_rpoB1	3.60	0.47	12.91	1.00	normal	yes	6.24	1.05	16.90	1.00	normal	yes	yes
	Cmm2_rpoB2_Di2	5.93	3.19	53.81	1.00	not normal	yes	6.37	0.86	13.48	1.00	normal	yes	yes
	Cmm2_rpoB6	3.35	0.68	20.22	0.81	normal	yes	4.25	1.05	24.81	1.00	normal	yes	yes
	Cmmprobe-Di2	4.93	2.41	48.88	0.75	not normal	yes	9.84	4.45	45.21	1.00	not normal	yes	yes
	Xcv_rRNA_pv20_Di2	2.80	1.22	43.69	0.50	not normal	no	0.51	0.22	42.51	0.00	normal	no	no
	Xcv11_rpoB_di2	4.12	2.21	53.64	0.69	not normal	no	0.15	0.15	97.84	0.00	normal	no	no
Xcv	1078-R Kaneshiro	3.46	1.62	46.75	0.75	normal	no	0.55	0.26	47.53	0.00	normal	no	no
	Cmmprobe-Di2	0.19	0.27	142.37	0.00	normal	no	4.22	2.83	67.13	0.75	normal	no	no
	Ps22_gyrB_species	6.06	3.23	53.31	0.75	normal	yes	-0.05	0.22	-410.86	0.00	normal	no	no
	Ps23_gyrB_species	4.96	2.50	50.48	0.75	normal	yes	0.16	0.39	246.37	0.00	normal	no	no
	Pst1_rpoB_pv7	8.33	1.05	12.58	1.00	normal	yes	0.15	0.18	123.77	0.00	normal	no	no
	Pst1_rpoB_pv8	3.05	1.53	50.23	0.50	normal	no	0.15	0.50	332.06	0.00	not normal	no	no
	Pst11_rRNA_Di2	4.54	2.64	58.20	0.63	not normal	no	-0.08	0.41	-537.75	0.00	not normal	no	no
	Pst16_gyrB_pv	2.91	1.76	60.49	0.63	normal	no	0.04	0.35	871.52	0.00	not normal	no	no
	Pst19_gyrB_pv	3.00	0.79	26.17	0.75	normal	no	0.05	0.22	413.28	0.00	normal	no	no
	PSt2_rpoB_pv1	11.28	6.40	56.74	0.94	not normal	yes	-0.31	0.16	-53.28	0.00	normal	no	no
	Pst20gyrB_Di2	2.64	0.69	26.06	0.50	normal	no	0.17	0.31	184.94	0.00	not normal	no	no
	Xcv_rRNA_pv20_Di2	16.22	5.99	36.94	1.00	normal	yes	4.06	1.82	44.82	0.88	not normal	yes	yes
	Xcv11_rpoB_Di	17.60	6.47	36.76	1.00	not normal	yes	11.19	7.76	69.39	1.00	not normal	yes	yes
	Xcv20_gyrB_pv1	1.86	0.52	28.17	0.20	not normal	no	5.10	2.68	52.46	0.81	not normal	yes	no
	Xcv20_gyrB_pv3	1.10	0.39	35.44	0.00	normal	no	3.71	1.63	43.88	0.75	normal	no	no
	Xcv22_gyr_Di	9.08	4.51	49.65	1.00	normal	yes	3.61	0.97	26.82	0.88	not normal	yes	yes
Xv	1078-R	0.13	0.14	105.65	0.00	normal	no	4.13	2.93	70.85	0.50	not normal	no	no

Xv	1114f 16S/IGS	0.27	0.12	48.40	0.00	normal	no	5.88	1.13	19.21	1.00	normal	yes	no
	Cm10_rRNA	2.74	1.42	51.65	0.75	normal	no	22.81	7.36	32.29	1.00	normal	yes	yes
	Pst11_rRNA_Di	0.08	0.28	365.24	0.00	not normal	no	13.89	7.45	53.64	0.88	normal	yes	no
	ToMV46_spe_Di				ND			24.32	7.26	29.87	1.00	normal	yes	yes
	Xanth17_rpoB_2	0.31	0.37	122.62	0.00	not normal	no	6.92	2.47	35.67	1.00	normal	yes	no
	Xanth28_rRNA_Di	0.41	0.44	106.91	0.00	normal	no	22.40	5.14	22.95	1.00	normal	yes	no
	Xcv_rRNA_pv20_Di	0.12	0.26	219.60	0.00	normal	no	5.60	3.14	56.03	0.81	normal	yes	no
	Xcv22_gyr_Di	0.14	0.21	154.44	0.00	not normal	no	6.27	4.52	72.18	0.63	not normal	no	no
	XgyrPCR2F	1.82	0.68	37.54	0.22	Not normal	No	7.23	3.09	42.73	1.00	normal	yes	yes
Pst	1078-R	2.52	1.09	43.35	0.50	Normal	No	2.64	0.95	36.12	0.50	Not normal	no	yes
	Cm10_rRNA	5.35	0.90	16.82	1.00	normal	Yes	0.72	0.51	70.87	0.00	normal	no	no
	Ps14_rRNA	4.15	1.16	27.88	0.94	normal	Yes	0.49	0.35	70.87	0.00	normal	no	no
	Ps22_gyrB	5.57	2.40	43.04	0.94	normal	Yes	0.84	0.43	51.06	0.00	normal	no	no
	Ps23_gyrB	5.89	1.24	21.02	1.00	normal	Yes	0.67	0.39	58.62	0.00	not normal	no	no
	Pst1_rpoB_pv7	7.07	2.27	32.16	1.00	normal	yes	1.66	0.88	53.04	0.25	normal	no	no
	Pst1_rpoB_pv8	4.04	1.16	28.72	0.94	normal	yes	1.01	0.38	37.27	0.00	normal	no	no
	Pst11_rRNA_Di2	6.08	1.45	23.91	1.00	not normal	yes	3.04	1.61	52.86	0.63	normal	no	no
	Pst16_gyrB_pv	5.92	2.21	37.36	0.94	normal	yes	0.69	0.47	68.59	0.00	not normal	no	no
	Pst19_gyrB_pv	3.76	1.11	29.48	0.88	normal	yes	0.48	0.31	65.43	0.00	normal	no	no
	PSt2_rpoB_pv1	8.18	2.16	26.42	1.00	normal	yes	2.91	1.41	48.31	0.63	normal	no	yes
	Pst20gyrB_Di2	3.91	0.83	21.14	1.00	normal	yes	0.34	0.14	40.50	0.00	normal	no	no
	Xcv_rRNA_pv20_Di2	0.26	0.18	71.68	0.00	normal	no	10.77	3.39	31.50	1.00	normal	yes	no
	Xcv11_rpoB_di2	0.11	0.13	121.92	0.00	normal	no	9.98	3.53	35.34	1.00	not normal	yes	no
	Xcv11_rpoB_pv	0.14	0.15	109.37	0.00	normal	no	3.01	1.11	37.05	0.67	normal	no	no
	Xcv20_gyrB_pv1	0.08	0.11	140.38	0.00	normal	no	5.89	1.50	25.46	1.00	normal	yes	no
	Xcv20_gyrB_pv3	0.17	0.12	68.86	0.00	normal	no	4.40	1.08	24.44	1.00	normal	yes	no
	Xcv22_gyr_Di2	0.16	0.11	67.92	0.00	normal	no	11.08	4.48	40.42	1.00	not normal	yes	no

	1078-R Kaneshiro	0.55	0.56	101.53	0.00	normal	no	4.19	1.61	38.46	0.88	normal	yes	no
	Cm10_rRNA	2.79	0.57	20.50	0.63	normal	no	8.32	1.11	13.36	1.00	normal	yes	yes
	Cmmprobe-Di2	0.26	0.38	148.75	0.00	normal	no	2.95	2.62	88.95	0.44	not normal	no	no
	Ps14_rRNA	4.47	1.54	34.32	0.94	normal	yes	8.64	1.63	18.88	1.00	normal	yes	yes
	Ps22_gyrB_species	5.96	4.50	75.43	1.00	not normal	yes	10.64	3.83	36.03	1.00	normal	yes	yes
	Ps23_gyrB_species	5.90	4.72	79.90	0.88	not normal	yes	8.36	1.90	22.71	1.00	normal	yes	yes
	Pst1_rpoB_pv7	1.55	2.83	183.06	0.13	not normal	no	8.49	2.48	29.23	1.00	not normal	yes	no
Pss	Pst1_rpoB_pv8	0.47	0.21	44.97	0.00	normal	no	2.98	1.49	50.15	0.38	not normal	no	no
	Pst16_gyrB_pv	6.78	5.21	76.87	0.69	normal	no	14.49	3.90	26.92	1.00	not normal	yes	yes
	Pst19_gyrB_pv	3.46	1.57	45.30	0.69	normal	no	11.08	2.73	24.67	1.00	normal	yes	yes
	Pst3_rpoB_Di2	0.15	0.14	92.19	0.00	normal	no	4.57	0.95	20.84	1.00	normal	yes	no
	Xanth17_rpoB_2	0.17	0.12	72.80	0.00	normal	no	2.95	0.62	20.94	0.63	normal	no	no
	Xanth24_rpoB	1.20	0.47	39.28	0.00	normal	no	6.04	1.48	24.45	1.00	normal	yes	no
	Xcv11_rpoB_di2	6.51	3.45	52.97	0.81	not normal	yes	9.73	2.49	25.62	1.00	normal	yes	yes
	Xcv11_rpoB_pv	0.63	0.60	95.16	0.00	not normal	no	3.27	0.69	21.00	0.81	normal	yes	no
	Xcv22_gyr_Di2	0.74	0.81	109.49	0.06	not normal	no	5.01	1.86	37.23	0.88	normal	yes	no
	1078-R Kaneshiro	4.44	3.04	68.59	0.75	normal	no	0.90	0.67	74.18	0.00	not normal	no	no
R. solanacearum	Cm10_rRNA	11.94	3.12	26.16	1.00	normal	yes	4.65	2.92	62.80	0.63	not normal	no	yes
	Cmm_groEL18-Di2	21.33	5.79	27.16	1.00	not normal	yes	13.04	4.75	36.40	1.00	normal	yes	yes
	Xcv11_rpoB_di2	0.58	0.21	36.42	0.00	normal	no	9.05	2.54	28.10	1.00	normal	yes	no
	Xcv22_gyr_Di2	3.35	1.09	32.44	0.75	normal	no	-0.05	0.30	-647.80	0.00	not normal	no	no
	1078-R Kaneshiro	11.51	6.88	59.75	1.00	not normal	yes	5.62	4.99	88.86	0.50	not normal	no	yes
	1114f 16S/IGS	2.27	0.37	16.46	0.19	normal	no	4.27	1.03	24.05	0.88	normal	yes	no
	Cm10_rRNA	15.10	2.43	16.07	1.00	normal	yes	41.70	20.22	48.51	1.00	normal	yes	yes
E. cloace	CmgroEL_5_2	6.21	1.21	19.40	1.00	normal	yes	1.03	0.27	26.52	0.00	normal	no	no
	Cmm_groEL_18	4.36	1.15	26.47	0.94	normal	yes	0.07	0.14	193.37	0.00	normal	no	no
	Cmm_groEL18-Di2	33.35	11.12	33.33	1.00	normal	yes	12.77	8.68	68.02	1.00	normal	yes	yes
	Cmmprobe-Di2	2.75	2.25	81.75	0.25	not normal	no	-0.16	0.13	-84.28	0.00	normal	no	no

E. cloace	Pst3_rpoB_Di2	5.59	1.00	17.93	1.00	not normal	yes	0.24	0.19	77.79	0.00	normal	no	no
	Xanth28_rRNA_Di2	0.42	0.41	98.21	0.00	not normal	no	31.32	10.21	32.59	1.00	not normal	yes	no
	Xcv11_rpoB_di2	1.32	0.44	33.06	0.00	normal	no	8.26	4.54	54.93	1.00	not normal	yes	no
	Xcv22_gyr_Di2	8.71	3.17	36.33	1.00	normal	yes	3.20	1.57	49.08	0.63	not normal	no	yes

Appendix D

Table representing significant hybridisation signals obtained from reactions with labelled RNA (cDNA) and DNA extracts from healthy tomato leaves.[5]

Control	Probe	Replicate 1						Replicate 2					
		Mean	SD	%CV	Propn >2.5	Data Distribution	Mean Sign >2.5	Mean	SD	%CV	Propn >2.5	Data Distribution	Mean Sign >2.5
RNA	Cmmprobe-Di2	18.62	9.07	48.70	1.00	not normal	yes	0.18	0.37	201.93	0.00	not normal	no
	Eub Pelludat	4.37	2.68	61.36	0.56	not normal	no	2.35	0.63	26.67	0.25	not normal	no
DNA	CH2-CP-F	0.22	0.26	119.04	0.00	not normal	no	3.41	1.64	48.08	0.69	normal	no
	CH2RdRp_1	0.12	0.24	200.40	0.00	not normal	no	7.15	2.34	32.65	1.00	normal	yes
	Cmm_groEL18-Di2	0.16	0.17	105.51	0.00	normal	no	6.36	5.27	82.91	0.69	not normal	yes
	Cmm14_rRNA	0.03	0.09	279.77	0.00	normal	no	2.73	1.51	55.45	0.50	normal	no
	Cmm17_rRNA	2.13	0.72	33.68	0.38	normal	no	9.31	7.18	77.09	0.94	not normal	yes
	Cmm2_rpoB2	0.33	0.23	70.62	0.00	normal	no	4.91	1.74	35.39	0.94	normal	yes
	Cmm2_rpoB2_Di2	1.14	0.56	49.06	0.00	normal	no	16.08	11.66	72.49	0.94	not normal	yes

[5] Data represented in this table indicates the statistical evidence for the significance of detected hybridisation signals based on the signal to noise ratio (SNR) and a cut-off of 2.5. Any probes with SNR values below this cut-off for one or both replicates were considered indicative of poor or random hybridisation reactions with labelled templates and therefore indicated insignificant cross-hybridisation with the tomato genome.

Control	Probe	Replicate 1						Replicate 2					Mean Sign >2.5
		Mean	SD	%CV	Propn >2.5	Data Distribution	Mean Sign >2.5	Mean	SD	%CV	Propn >2.5	Data Distribution	
DNA	Cmmprobe-Di2	3.35	3.18	94.89	0.50	normal	no	21.18	12.98	61.29	1.00	normal	yes
	Eub Pelludat	0.17	0.25	147.15	0.00	not normal	no	2.55	1.74	68.26	0.50	not normal	no
	FPCm Bach	0.09	0.17	205.68	0.00	normal	no	3.20	0.93	29.08	0.81	normal	yes
	GAPDH_2	0.06	0.14	242.73	0.00	normal	no	2.55	1.73	67.65	0.38	not normal	no
	Hyb EACMV 40uM	0.23	0.21	92.38	0.00	not normal	no	5.62	3.11	55.44	0.81	normal	yes
	Hyb GUS 20uM	3.17	1.94	61.20	0.50	not normal	no	6.94	2.68	38.62	1.00	normal	yes
	Hyb GUS 40uM	2.31	1.05	45.29	0.31	not normal	no	5.99	3.46	57.74	0.81	normal	yes
	mRNA E2_1	0.09	0.09	104.94	0.00	normal	no	3.48	2.39	68.75	0.56	normal	no
	PepMV_15	0.11	0.10	89.62	0.00	normal	no	6.97	4.50	64.63	0.75	normal	yes
	PepMV_15_2	0.31	0.17	54.28	0.00	normal	no	12.10	4.75	39.25	1.00	not normal	yes
	PepMV_16	0.26	0.11	41.53	0.00	normal	no	10.96	4.90	44.71	1.00	normal	yes
	PepMV_16_2	0.31	0.33	104.60	0.00	normal	no	14.36	6.71	46.72	1.00	normal	yes
	PepMV_16_Di	1.05	0.52	49.27	0.00	normal	no	29.46	11.79	40.02	1.00	normal	yes
	PepMV_CH2H	0.12	0.14	116.09	0.00	normal	no	4.25	1.68	39.50	0.81	normal	yes
	PepMVCh210	0.02	0.14	818.76	0.00	normal	no	5.49	2.10	38.15	1.00	normal	yes
	PepMVCh215	0.00	0.07	-2749.33	0.00	normal	no	2.87	0.95	33.24	0.69	normal	no
	Ps17_rRNA	-0.06	0.13	-200.00	0.00	normal	no	2.97	2.88	96.88	0.50	not normal	no
	Pst11_rRNA_Di2	0.23	0.36	152.80	0.00	not normal	no	25.58	18.23	71.28	1.00	not normal	yes
	Pst3_rpoB_Di2	1.35	0.47	34.64	0.00	normal	no	33.59	11.80	35.14	1.00	normal	yes
	ToMV29_species_2	0.03	0.10	369.83	0.00	normal	no	4.48	3.05	68.12	0.81	not normal	yes
	ToMV46_spe_Di2	1.29	0.69	53.26	0.00	normal	no	17.50	5.65	32.29	1.00	normal	yes
	ToMV46_species	0.68	0.37	54.15	0.00	normal	no	2.86	1.77	61.91	0.56	normal	no
	US2-7_Di	0.23	0.29	124.22	0.00	normal	no	16.86	9.24	54.84	1.00	not normal	yes
	Xanth28_rRNA_Di2	-0.14	0.15	-104.76	0.00	normal	no	14.00	5.06	36.13	1.00	normal	yes
	Xcv_rRNA_pv20_Di2	8.12	4.69	57.78	1.00	not normal	yes	42.81	17.94	41.91	1.00	normal	yes
	Xcv11_rpoB_di2	1.69	0.82	48.68	0.19	normal	no	13.04	10.79	82.74	0.88	not normal	yes

DNA	**Xcv20_gyrB_pv1**	0.35	0.16	46.13	0.00	normal	no	4.79	1.89	39.42	0.88	normal	yes
	Xcv20_gyrB_pv3	0.64	0.32	50.46	0.00	normal	no	10.97	3.94	35.96	1.00	not normal	yes
	Xcv22_gyr_Di2	2.35	3.01	127.68	0.31	not normal	no	53.75	22.39	41.65	1.00	normal	yes