# ISOLATION AND MOLECULAR CHARACTERISATION OF TOMATO SPOTTED WILT VIRUS (TSWV) ISOLATES OCCURING IN SOUTH AFRICA

by

**Benice Sivparsad**

**(201297209)**

Submitted in partial fulfilment

of the requirements for the degree of

**MASTER OF SCIENCE**

in the

Discipline of Plant Pathology

School of Biochemistry, Genetics, Microbiology and Plant Pathology

Faculty of Science and Agriculture

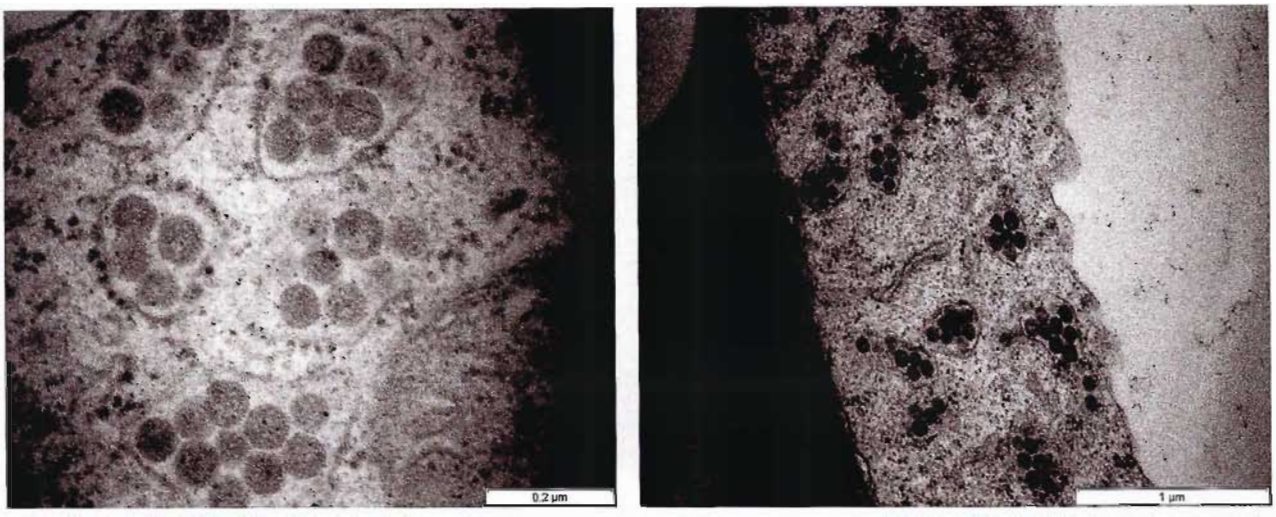
University of KwaZulu-Natal

Pietermaritzburg

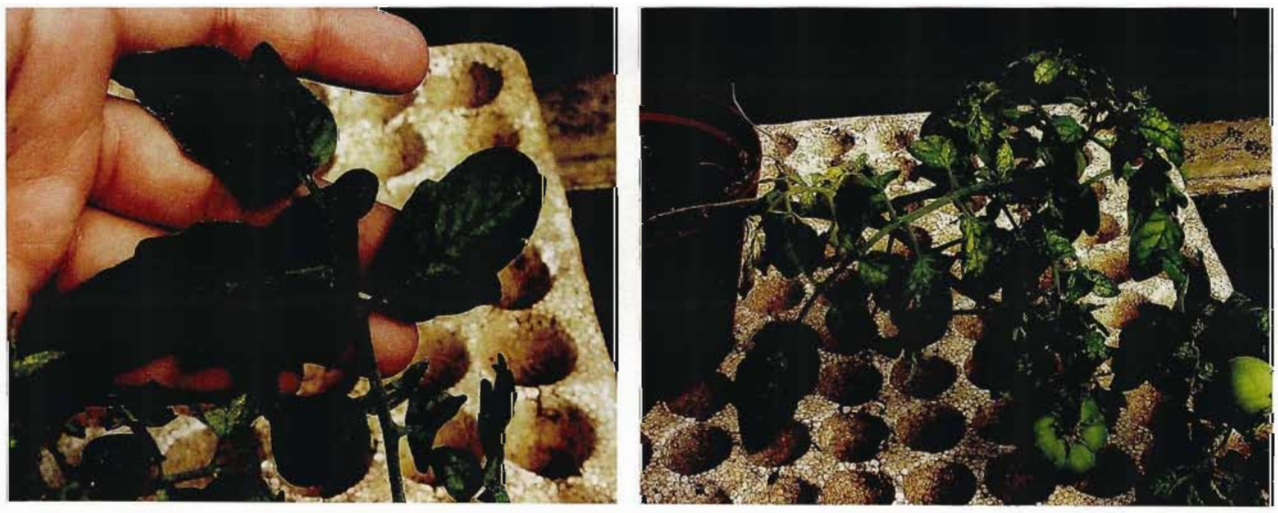
Republic of South Africa

December 2006

# FRONTISPIECE




Transmission electron micrographs of negatively stained ultra-thin leaf sections from *Nicotinia rustica* L. plants infected with the tomato spotted wilt virus (TSWV).



Symptoms induced by tomato spotted wilt virus (TSWV) on tomato (*Lycopersicon esculentum* Mill.).

# ABSTRACT

Tomato spotted wilt virus (TSWV), a Tospovirus, is one of the ten most economically destructive plant viruses worldwide, causing losses exceeding one billion U.S. dollars annually on several crops. In South Africa (SA), TSWV has become an important virus in many economically important crops. The main objective of this research project was to isolate, identify and characterise TSWV isolates occurring in SA.

A review of current literature assembled background information on TSWV molecular biology, epidemiology, transmission, detection and control.

A TSWV isolate infecting pepper (*Capsicum* sp.) occurring in KZN was isolated and partially characterised. The virus was positively identified as TSWV using the enzyme-linked immunosorbent assay (ELISA) and the presence of typical necrotic TSWV symptoms on *Nicotinia rustica* L. Symptomatic leaves were harvested and the virus was partially purified using standard procedures. Under the transmission electron microscope (TEM), typical quasi-spherical and dumbbell-shaped particles of 80-100nm in diameter were observed in negatively stained preparations of both crude and purified virus samples. In negatively stained ultra-thin virus infected leaf sections, an abundance of mature viral particles (100nm) housed in the cisternae of the endoplasmic reticulum (ER) were observed among typical viroplasm inclusions (30nm) and hollow tubules (200-300nm). A viral protein migrating as a 29kDa band, which corresponds to the TSWV nucleocapsid (N) protein, was observed after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Total plant RNA, isolated from *N. rustica* displaying typical symptoms was subjected to reverse-transcription polymerase chain reaction (RT-PCR) using primers specific to the nucleocapsid (N) gene. An expected 760bp product was amplified. The results obtained in this study confirm the presence of TSWV in infected pepper plants from KZN.

The genetic diversity of TSWV isolates occurring in SA was examined. The nucleocapsid (N) gene sequences of six SA TSWV isolates originating from Gauteng, KwaZulu-Natal, North West, Limpopo and Mpumulanga provinces were determined and used in a phylogenetic tree comparison with TSWV isolates occurring in different geographical locations in the world. Nucleotide sequence comparisons of the N gene revealed high levels of similarity between the SA isolates and TSWV isolates from Asia and Europe. SA isolates showed a high degree of sequence similarity (99-100%) which was reflected in their distinct clustering pattern.

The resistance of tomato (*Lycopersicon esculentum* Mill.) plants with natural and transgenic resistance against mechanical inoculation with TSWV isolates occurring in SA was evaluated. The Stevens cultivar which has natural resistance conferred by the Sw-5 gene and the transgenic 13-1 line, which expresses the nucleocapsid (N) protein gene of the TSWV-BL isolate, was used as test cultivars. Plants were assessed for TSWV resistance using a disease severity rating scale and measurements of virion accumulation levels ($A_{405}$nm). There were no significant differences among the reactions produced by the six TSWV isolates on the test plants. Although both plants were susceptible to the SA TSWV isolates by exhibiting similarly high viral accumulation levels, the transgenic tomato line showed milder disease severity compared to the natural resistant cultivar. Results suggest that transgenic resistance is a more effective approach in the control of TSWV in SA.

The information generated in this study will be useful in formulating effective control measures using genetic engineering approaches for this economically important virus. Such approaches will be used as a tool to make strategic decisions in an integrated control programme for TSWV.

## DECLARATION

I, Benice Sivparsad, hereby declare that this research project and all the work described herein was done by the author alone, except where otherwise acknowledged and has not been submitted in any other form to another university. Work of other authors has been duly acknowledged in the text.

Benice Sivparsad
CANDIDATE

Dr A. Gubba
SUPERVISOR

# ACKNOWLEDGEMENTS

I would like to express my sincere thanks and gratitude to the following people, without whom this project would not have been possible:

Dr A. Gubba, my supervisor, for his constructive suggestions, encouragement, faith in my ability and for editing this manuscript.

Kim Govender and the technical staff of the Department of Microbiology and Plant Pathology, for all their assistance.

Vijay Bandu and the staff of the Centre of Electron Microscopy for all their assistance.

Veron Ramsuran for his help with the phylogenetic analysis.

Eyob Kidane for his help with the statistical analysis.

Jacolene Meyer, Research Scientist at the Agricultural Research Council (ARC), for providing the tomato spotted wilt virus isolates used in this study.

Carol Gonsalves, for providing the seed of the transgenic 13-1 line and Stevens cultivar.

The National Research Foundation (NRF) for financial support.

My dear friends: Archana Nunkumar, Dael Visser, Krishna Naicker, Tanya Naidoo, Akshanee Singh and Jiren Reddy for all the encouragement, fun and laughter. Having friends like you made the rough times that much easier.

The Sivparsad and Pillay families, especially my parents, for their sacrifices, love, encouragement and faith in me.

Treslyn Pillay, for all his love, encouragement and support throughout my studies. A special thanks for all his help with the greenhouse work.

Finally, I would like to express my heartfelt gratitude to the Almighty God for seeing me through this study.

## DEDICATION

To my parents for all the love and encouragement throughout my studies.

# CONTENTS

## CHAPTER 2. ISOLATION AND PARTIAL CHARACTERISATION OF A TOMATO SPOTTED WILT VIRUS (TSWV) ISOLATE INFECTING PEPPER (*Capsicum* sp.) IN KWAZULU-NATAL

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Adenine |
| ANOVA | Analysis of variance |
| $A_{260}$ | Absorbance at 260nm |
| $A_{280}$ | Absorbance at 280nm |
| $A_{405}$ | Absorbance at 405nm |
| bp | Base pairs |
| C | Cytosine |
| ch | chloroplast |
| CP | Coat protein |
| DAS | Double antibody sandwich |
| DEPC | Diethylpyrocarbonate |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethyledediaminetetra-acetic acid disodium salt |
| ELISA | Enzyme-linked immunosorbent assay |
| EM | Electron microscope |
| ER | Endoplasmic reticulum |
| G | Guanine |
| G-80 | Geneva 80 |
| h | Hour |
| ICTV | International Committee for the Taxonomy of Viruses |
| IEM | Immunosorbent electron microscopy |
| INSV | Impatiens necrotic spot virus |
| IPM | Integrated pest management |
| LSD | Least significant difference |
| kb | Kilobases |
| kDa | Kilodalton |
| LB | Luria-Bertini |
| m | mitochondria |
| min | minute |

| | |
|---|---|
| ml | milliliter |
| MOPS | 4-Morpholinepropanesulfonic acid |
| mRNA | messenger ribonucleic acid |
| MWM | Molecular weight marker |
| N | Nucleocapsid |
| Nc | Nucleocapsid |
| nca | Nucleocapsid aggregates |
| nt | nucleotide |
| nm | nanometers |
| NPK | Nitrogen phosphate potassium |
| ORF | Open reading frame |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| PDR | Pathogen derived resistance |
| pNpp | p-Nitrophenyl phosphate |
| PTGS | Post transcriptional gene silencing |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| RT-PCR | Reverse transcription polymerase chain reaction |
| s | second |
| SDS | Sodium dodecylsulphate |
| SDS-PAGE | Sodium dodecylsulphate polyacrylamide gel electrophoresis |
| T | Thymine |
| TEM | Transmission electron microscope |
| TMV | Tobacco mosaic virus |
| TSWV | Tomato spotted wilt virus |
| µl | Microliter |
| U.S. | United States |
| vp | Viroplasm |
| X-Gal | 5-bromo-4-chloro-3-indoyl-ß-D-galactoside |

# FOREWARD

All research presented in this thesis was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal (UKZN), Pietermaritzburg, under the sole supervision of Dr Augustine Gubba.

The science of virology has evolved in less than a century to become paramount in studies regarding life itself. Viruses have been exceedingly valuable as ideal models for various important principles, especially in molecular genetics and developmental biology. Within the field of virology, three major groups are recognisable: depending on the model host, the groups are separable into plant, animal and bacterial viruses. Of these three groups, plant virology is the oldest since the discovery of tobacco mosaic virus (TMV) gave birth to the science.

The main objective of our plant virology research team at UKZN is to isolate and identify important viruses infecting major economic crops in South Africa, so that this information can be used in the formulation of effective control measures against these viruses. Considering its vast host range and wide geographical distribution, the tomato spotted wilt virus (TSWV) was identified as a major problem in the cultivation of many important crops in SA.

Therefore the main objective of this research project was to isolate, identify and characterise TSWV isolates occurring in SA. This information could be used as a tool to make strategic decisions in an integrated control programme for TSWV in SA. The scope of this research is presented in five chapters:

Chapter One provides a review of current literature on TSWV molecular biology, epidemiology, transmission, detection and control.

Chapter Two describes the first report of TSWV in KZN. A virus infecting pepper was identified as being TSWV using symptomatology, ELISA, electron microscopy, protein

banding patterns via SDS-PAGE and the amplification of the nucleocapsid (N) gene using the RT-PCR procedure.

Chapter Three covers the investigation into the genetic diversity of TSWV in SA. N gene sequences from SA isolates were obtained and compared to N gene sequences of TSWV isolates from different geographical locations using phylogenetic analysis.

Chapter Four depicts the evaluation of resistance of transgenic tomato expressing the N gene and natural resistant tomato carrying the Sw-5 gene against TSWV isolates occurring in SA.

Chapter Five reviews the experimental results, conclusions and recommendations for future research.

# CHAPTER 1
# LITERATURE REVIEW

## 1.1 INTRODUCTION

Pests and pathogens affect virtually all crops, causing substantial and often devastating losses. Losses due to viruses are of particular importance in developing countries that are highly dependent on agricultural production for food security, employment and export earnings (Thresh, 2003). Actual losses due to plant viruses are difficult to assess, but estimates indicate total economic damage is as high as several billion U.S. dollars per year. Tomato spotted wilt virus (TSWV), the type species of the genus Tospovirus in the family *Bunyaviridae,* is among the most detrimental of all known plant viruses (Rudolph *et al.*, 2003).

The first report on the 'spotted wilt' disease dates back to 1915 when Brittlebank described this disease in tomatoes in Australia (Brittlebank, 1919). Later it was demonstrated that the causal agent of the disease had viral etiology, for which the name tomato spotted wilt virus (TSWV) was coined (Samuel *et al.*, 1972). The virus was shown to be the causal agent of a variety of other diseases, including leaf curl of gram, brown pod of pea, tomato and tobacco carcova, tobacco kromnek, pineapple yellow spot, pineapple side rot, tomato bronze leaf, Kat river disease, makhorka tip chlorosis and vira cabeca (Francki & Hatta, 1981, Mumford *et al.*, 1996).

During the first 50 years since its discovery, TSWV was considered to be unique amongst known plants viruses and remained the sole member of the tomato spotted wilt virus group (Matthews, 1982). In 1991, the placement and unique status of TSWV was significantly challenged. Data on the molecular biology of the virus revealed a taxonomic relationship with the *Bunyaviridae*, a recognised family of animal-infecting viruses. As a result, TSWV was reclassified as the type member of the genus Tospovirus, created to contain the plant-infecting members of the family

*Bunyaviridae*. Since then, a number of other Tospoviruses, distinct from TSWV, have been added to the Tospovirus genus (Francki *et al.*, 1991).

Although TSWV was identified in 1930 as the causal agent of a major viral disease, it was not until the 1980s that studies on the molecular biology of this pathogen intensified. This resurgence of interest coincided with a marked re-emergence of the virus, as a result of increased world trade, and the rapid spread of one of its major vectors, the western flower thrips (*Frankliniella occidentalis*) (Prins & Goldbach, 1998). Today, with an estimated annual crop loss of over one billion dollars for several important crops, TSWV ranks among the top ten most economically destructive plant viruses worldwide (Goldbach & Peters, 1994).

The economic impact of TSWV is huge mainly due to its extremely broad host range and world-wide distribution (Roselló *et al.*, 1996). It possesses one of the largest host ranges of any plant virus, with over 1090 plant species in over 100 families cited (German *et al.*, 1992; Peters, 2003). The economic effect of this virus has been thoroughly documented in Hawaii. Since 1929 when the virus was first described, farmers in Hawaii have been combating this viral disease. TSWV has been devastating to the agricultural industry. At first, losses in lettuce and tomato were tolerated because epidemics occurred only during the summer months; however, the problem increased forcing several growers out of production and others to produce alternative non-susceptible crops. A look at some of Hawaii's production statistics shows that lettuce and tomato production has decreased by over 65% and 35%, respectively since 1984 when TSWV began causing major epidemics statewide (Cho *et al.*, 1998). Similarly, in the USA, TSWV is ranked the most serious pathogen of field crops in several areas (Mumford *et al.*, 1996). These alarming statistics demonstrate the virulent and disastrous capabilities of TSWV on a global scale.

The emergence of TSWV is a significant problem in the cultivation of many important crops in South Africa (SA). By 1939, TSWV was reported to occur in the Western Cape, the Free State and throughout the former Transvaal. Since these initial

reports, TSWV has been found periodically in various crops throughout SA. Today it is prevalent in the Eastern and Western Cape where it can seriously limit crop production. Since the introduction of *Frankliniella occidentalis* into SA in 1988 and its subsequent spread throughout the country, the incidence of TSWV has increased in other provinces (Thompson & van Zijl, 1996). A disease survey has ranked TSWV as the most prevalent viral disease in tomato (*Lycopersicon esculentum* Mill.) in SA (Uys *et al.*, 1996).

The wide host range and corresponding economic importance of TSWV, coupled to its fascinating molecular biology, make it an interesting and significant virus for both the applied and basic scientist. Infection rates of 50-90% lead to major losses in most commercial vegetable crops. Therefore, great strides have been made in elucidating the basic biology of this complex virus, so that such information may contribute to the formulation of effective control measures for this economically destructive plant virus (Mumford *et al.*, 1996; Adkins, 2000). In this chapter, a review of TSWV molecular biology, epidemiology, transmission, detection and control is presented.

## 1.2 VIRUS CHARACTERISATION

### 1.2.1 Taxonomy and Classification

Prior to 1990, TSWV was classified as the single representative of a monotypic plant virus group, the 'tomato spotted wilt virus group'. With the unraveling of its molecular biology, this virus was shown to have some properties in common with members of the *Bunyaviridae*. Therefore, TSWV has recently been classified as the type species of a newly-created genus within the *Bunyaviridae*, for which the genus name Tospovirus has been accepted by the International Committee for the Taxonomy of Viruses (ICTV) (Matthews, 1982). The bunyavirus family (*Bunyaviridae*) is one of the largest known, with well over 300 serologically distinct viruses (Wagner & Hewlett, 1999). This large family of viruses infects both vertebrates and invertebrates. There is only one genus, Tospoviruses that infects plants and its invertebrate vector (Goldbach & Peters, 1996; Hull, 2001).

### 1.2.2 Particle Morphology

Over the past 30 years, extensive electron microscopy studies have been performed on TSWV in infected plant cells. The analyses have shown that the viral particles, like all bunyaviruses, are spherical in shape (80-120nm in diameter). Each viral particle consists of a granular core of nucleocapsids, bounded by a lipid envelope (5nm thick), which is covered with surface projections (Fig. 1.1; van Kammen *et al.*, 1966; Kormelink *et al.*, 1992b; de Haan, 1994; Roselló *et al.*, 1996). The surface projections (spikes) are 5-10nm in length, and consist of two glycoproteins (G1 and G2) (Lararowitz, 2001; Goldbach & Kormelink, 2002).

### 1.2.3 Biochemical Properties

Each plant virus consists of at least a nucleic acid and a protein. The nucleic acid makes up 5 to 40 percent of the virus, with the protein making up the remaining 60 to 95 percent (Agrios, 2005). Virions of tospoviruses comprise 5% nucleic acid, 70% protein, 20% lipid and 5% carbohydrate (Brunt *et al.*, 1996; Adkins, 2000). Elucidation of the genetic properties of the virus has revealed that the tripartite TSWV genome contains five genes, expressing six functional proteins (Table 1.1; Goldbach & Peters, 1994).

#### *1.2.3.1 Viral genome*

TSWV particles contain a tripartite genome consisting of three separately encapsidated segments of linear single-stranded RNA: two ambisense RNA segments, S (small) and M (medium), and one negative-stranded segment, L (large). These RNAs are found in tight association with the 29 kDa nucleocapsid (N) protein forming the pseudo-circular nucleocapsid complexes (Fig. 1.1; van den Hurk *et al.*, 1977; Mohamed, 1981; Verkleij *et al.*, 1982; de Haan *et al.*, 1990, 1991; German *et al.*, 1992; Kormelink *et al.*, 1992a; Wijkamp *et al.*, 1993; Levy *et al.*, 1994).

#### *1.2.3.2 Viral protein*

Genomic RNA possesses an ambisense coding strategy, whereby gene products are expressed from subgenomic mRNAs of opposite sense (Fig. 1.1; Mumford *et al.*, 1996). The L RNA has a single open reading frame (ORF) coding for the RNA dependent RNA polymerase (RdRp), and a specific mRNA for its translation is transcribed from the genomic RNA (de Haan *et al.*, 1991; Law *et al.*, 1991; Mandahar, 1999). M RNA of tospoviruses has the ambisense coding strategy. It encodes a nonstructural (NSm) protein in the viral (v) sense and a protein to serve as the precursor for the G1 and G2 glycoproteins in the viral complementary (vc) sense (Tas

*et al.*, 1977; Chu *et al.*, 2001). The NSm protein facilitates cell-to-cell movement of the non-enveloped ribonucleocapsid structure through structurally modified plasmodesmata (Paape *et al.*, 2006). The G1 and G2 glycoproteins are considered to form spikes on the viral envelope (Kormelink *et al.*, 1992b; Chu & Yeh, 1998). The S RNA also has an ambisense coding strategy. It codes for a nonstructural (NSs) protein in the v sense, and a nucleocapsid (N) protein, which encapsidates the viral RNAs, in the vc sense (Law & Moyer, 1990; Wijkamp *et al.*, 1993; Chu *et al.*, 2001). The NSs protein is found in dense aggregates within the host cytoplasm, often forming fibrous or crystalline structures. Its function remains unclear, although the protein does appear to have a role in pathogenicity and vector transmission (Kormelink *et al.*, 1991; Mumford *et al.*, 1996).

### 1.2.4 Physical Properties

TSWV particles are extremely unstable. The thermal inactivation point is 42°C, the dilution end-point in 1: $10^{-4}$, and the longevity *in vitro* is very short, about 5 hours at room temperature (Smith, 1937; Samuel *et al.*, 1972; Lucas *et al.*, 1985; Sutic *et al.*, 1999). There is one sedimenting component in purified preparations with a sedimentation coefficient of 550 S. Particles have a density of 1.21 g $cm^{-3}$ in sucrose (Brunt *et al.*, 1990).

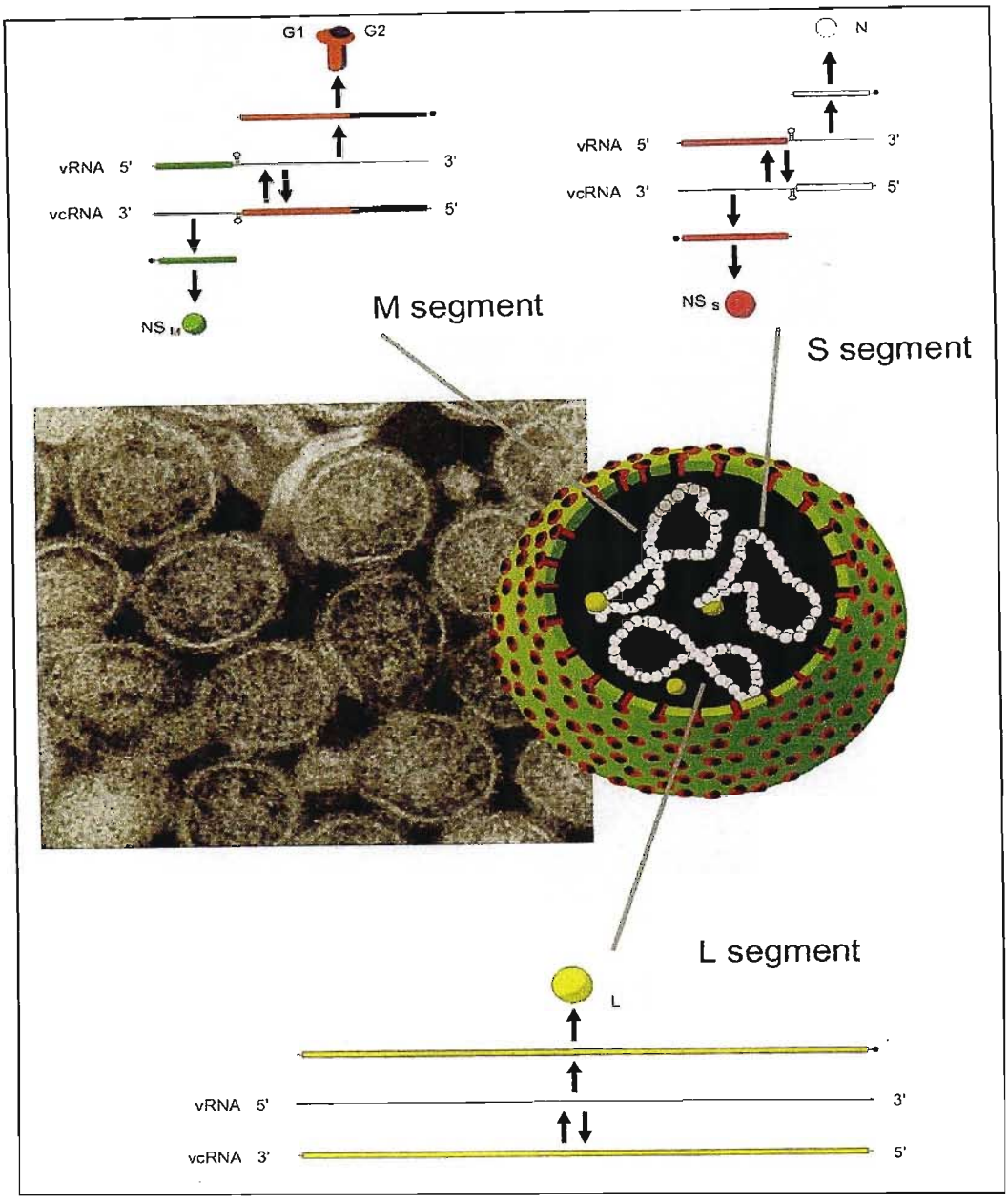



**Figure 1.1** Electron microscopy of tomato spotted wilt virus, its schematic morphology and outline of genome expression. The virus particles show the lipid membrane with the projections consisting of the G1 and G2 proteins. The core of the schematic particle consists of the three viral nucleocapsids. White: nucleocapsid (N), yellow: RNA-dependent RNA (L) polymerase. The particle is enveloped by two glycoproteins (red: G1, blue: G2). NSs (pink) and NSm (green) are the nonstructural proteins. Double arrows indicate replication; where single arrows indicate transcription and translation (Peters, 2003).

**Table 1.1** Summary of main genome characteristics of the tomato spotted wilt virus (TSWV) (Goldbach & Peters, 1996; Wagner & Hewlett, 1999; van Regenmortel, 2000).

| RNA Protein | size(kDa) | Function |
|---|---|---|
| L segment | 8.9 | |
| L | 331 | RdRp |
| M segment | 4.8-4.9 | |
| G1 | 78 | glycoprotein projection |
| G2 | 52-58 | glycoprotein projection |
| NSm | 34 | movement protein |
| S segment | 2.9 | |
| N | 28.8 | nucleocapsid protein |
| NSs | 52.4 | pathogenicity, vector transmission |

## 1.3 VIRUS REPLICATION

After infection, a virus's main objective is its replication (Agrios, 1988). Although major advances in understanding the replication processes of TSWV have been made over the last decade through genetic, molecular and cellular studies, many gaps remain in our knowledge (Bishop, 1990).

### 1.3.1 Replication Strategy

In natural infections, TSWV enters the plant cell during the probing or feeding actions of its insect vector, thrips. Under laboratory conditions, virus infection can be mimicked by mechanical inoculation (Prins & Goldbach, 1998). A schematic representation of the TSWV infection cycle is depicted in Figure 1.2. On entering the plant cell, the virus will fuse with acidic lysosomes, after which the membrane is removed. The infectious nucleocapsids are then released into the cytoplasm. At this stage, the viral RNA will either be transcribed or replicated. This transcription-to-replication switch is thought to be mediated by the concentration of the free N protein in the cytoplasm. At low N concentrations, the replicase will produce mRNAs, resulting, after translation, in an accumulation of the various viral-encoded proteins. As the concentration of N protein increases, the polymerase switches to replicase mode and the viral genomic RNAs are multiplied (Peters, 2003). In some isolates, large amounts of the NSs proteins accumulate to form paracrystalline arrays in the cytoplasm. The purpose of these structures, as well as the function of the protein, has yet to be discovered (Kormelink *et al.*, 1991). The glycoprotein precursor contains a signal that allows translation on the endoplasmic reticulum (ER). After glycosylation and proteolytic cleavage, the G1/G2 glycoproteins are transported to the site of budding. The replicated viral RNAs then associate with N protein to form progeny nucleocapsids to which L protein attaches. The NSm protein enables the spread of the nucleocapsids to neighbouring cells through tubular structures, where the transcription and replication cycle can start again. Alternatively, nucleocapsid structures can form new virus particles by associating with the glycoproteins and

budding through Golgi membranes (Kikkert *et al.*, 1999). Eventually, newly formed particles can be taken up by thrips during feeding (Prins & Goldbach, 1998).

### 1.3.2 Host Response

An infected plant may respond to infection in either one of three ways: immunity, resistance or susceptibility. In the case of susceptible plants, the plant may exhibit characteristic macroscopic symptoms or microscopic cytopathological effects (cellular changes) (Flint *et al.*, 2000; Narayanasamy, 2001).

#### *1.3.2.1 Cytopathology*

One of the most specific changes induced by virus infection of cells is the production of microscopic bodies differing from other cellular changes. These inclusion bodies vary in morphology and composition (Rubio-Huertos, 1972). Cytopathological studies of ultra-thin sections of infected plant cells reveal the presence of numerous virus particles and distinct cytopathological structures (Kitajima, 1965; Milne, 1970; Ie, 1971). Mature particles occur customarily and exclusively in the cisternae of the ER system (Fig. 1.3A). Several to many particles may occur, usually in a single cavity. However a few species are singularly bounded by distinct membranes of the ER. In addition, moderately dense, amorphous masses, which are referred to as viroplasms, are also found in each cell (Ie, 1982; Francki *et al.*, 1985). They occur relatively close to the regions where the virus particles accumulate (Fig. 1.3A). Further studies have shown that viroplasms often contain numerous small complexes of material of higher electron density, often either loosely arranged in apparent chains or strings or grouped in clusters (Fig. 1.3B). These complexes have elliptic, cubic or circular profiles with diameters ranging from 30 to 120nm in size. They are believed to be aggregates of nucleocapsids, which have either not yet enveloped or failed to acquire a membrane during the replication of the virus (Matthews, 1993; Goldbach & Peters, 1996). A second type of inclusion body consists of loose aggregates or bundles of filamentous material consisting of NSs protein (Kormelink *et al.*, 1991; Lawson *et al.*,

1996). The filaments found in TSWV-infected cells are loosely aggregated (Fig. 1.3C). In contrast, the filaments in cells infected with impatiens necrotic spot virus (INSV), a member of the Tospovirus genus, inclusions are ordered in a crystalline structure of alternating rows of threads and points (Fig. 1.3D). Therefore, the arrangement of filaments is a feature by which TSWV can be distinguished from INSV (Urban *et al.*, 1991; Goldbach and Peters, 1996).

### *1.3.2.2 Symptomatology*

The symptoms induced by TSWV vary greatly with the host affected, plant organ affected, and age of plant or organ at the time of infection (Agrios, 2005). Plants may respond locally with necrotic lesions or chlorotic spots (Peters, 2003). Systemic symptoms appear as chlorotic (Fig. 1.4A) or necrotic rings, line patterns (Fig. 1.4B) or purple flecking (Fig. 1.4C) on leaves; bronzing, curling, and wilting of leaves; necrotic streaks on stems (Fig. 1.4D) and necrosis of parts or whole plants. In severely infected plants, fruits show characteristic symptoms: they have a speckled appearance with concentric circles ranging from yellow or brown to green or red depending on the maturity stage of the fruit (Fig. 1.5A). Circular necrotic spots, malformations and necrosis are also observed on infected fruit (Fig. 1.5B; Roselló *et al.*, 1996). The severity of symptoms may vary with age, nutritional status as well as growth governing factors such as temperature and light intensity. The various strains of TSWV are the main cause of symptom variation within a plant species. The incidence of strains responsible for the variation in symptoms may depend on the vector populations prevailing in the different locations and seasons, and the strains in natural vegetation (Peters, 2003).

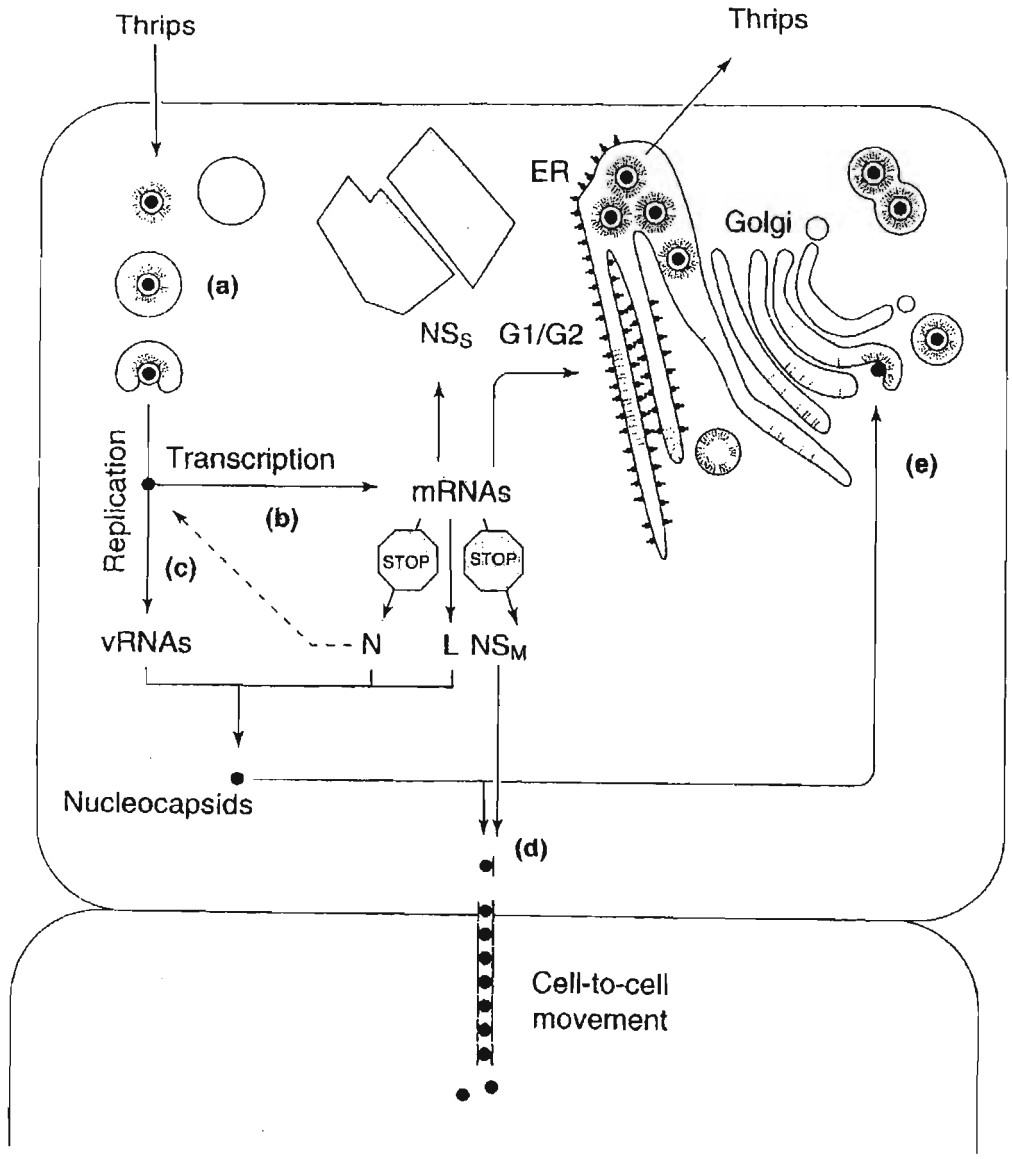



**Figure 1.2** Infection cycle of tomato spotted wilt virus (TSWV) in plant cells. (a) After initial (thrips-mediated) entry of the virus into the cell, the viral nucleocapsids are released. (b) At low N protein concentrations, the polymerase produces viral mRNAs that can be translated into viral proteins. (c) Upon the increase of N protein levels, the polymerase switches its mode to replication, and viral progeny are produced (vRNAs). These can either (d) move to an adjacent cell via NSm-induced tubules or (e) bud through Golgi membranes to form mature virus particles. These particles can then be taken up by thrips and transmitted to other plants (Prins & Goldbach, 1998).

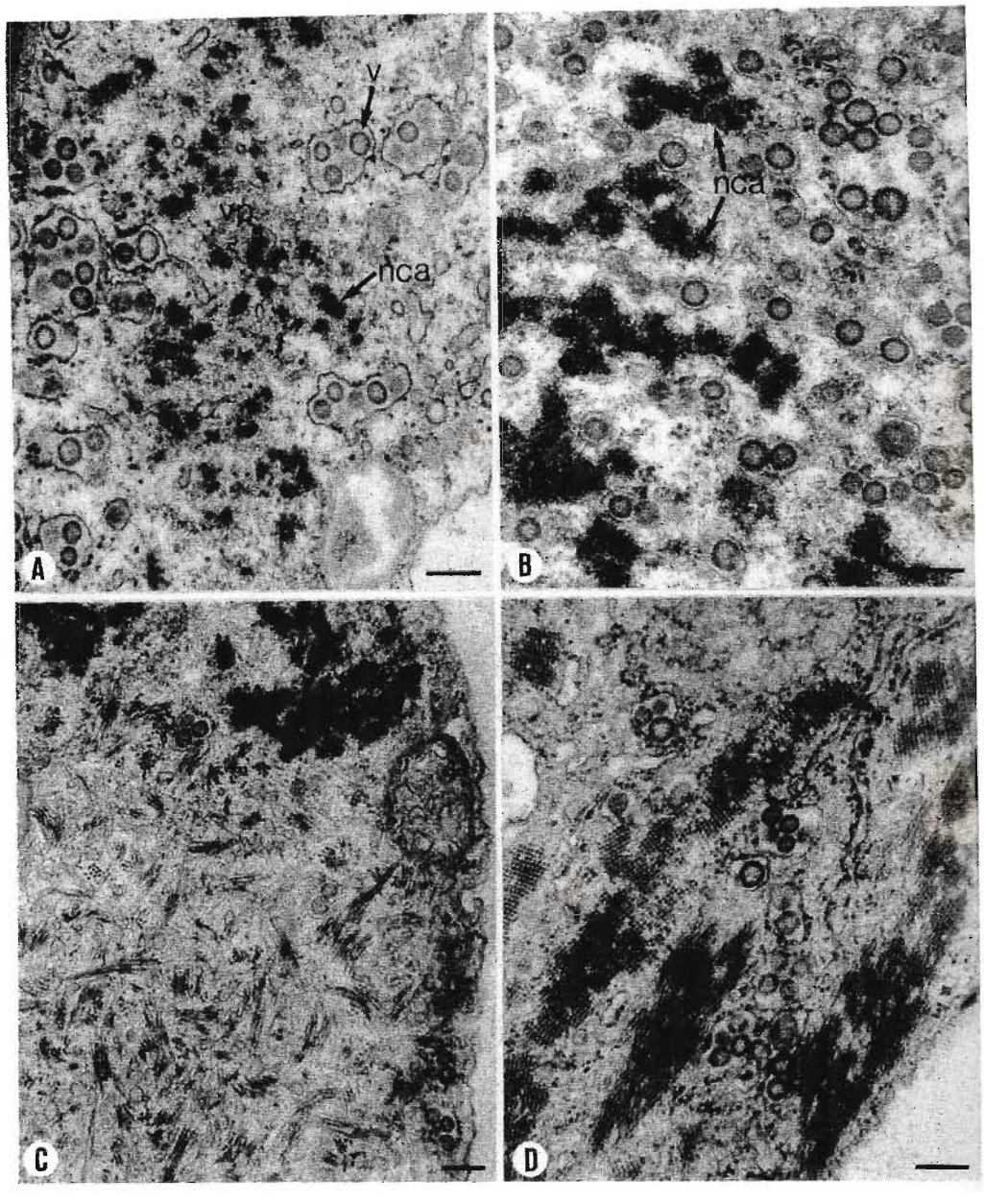



**Figure 1.3** Cytopathology of Tospoviruses. Osium-fixed ultrathin sections of tomato spotted wilt-infected *Nicotinia rustica* L. plants reveal the following cytopathological structures: (A) mature virion particles (v) in the ER system, viroplasm (vp), and nucleocapsid aggregates (nca); (B) nucleocapsid aggregates; (C,D) fibrillar structures in TSWV- and INSV- infected plant cells, respectively. Bars = 200 nm (Goldbach & Peters, 1996).


**Figure 1.4** Foliar symptoms of tomato spotted wilt virus (TSWV). (A) Chlorosis of tomato leaves (Sivparsad, 2006). (B) Chlorotic line pattern on Dahlia leaves (Moyer *et al.*, 1999). (C) Purple flecking of young tomato leaves (Tisserat, 2003). (D) Vascular necrosis on a tobacco plant (Moyer *et al.*, 1999).

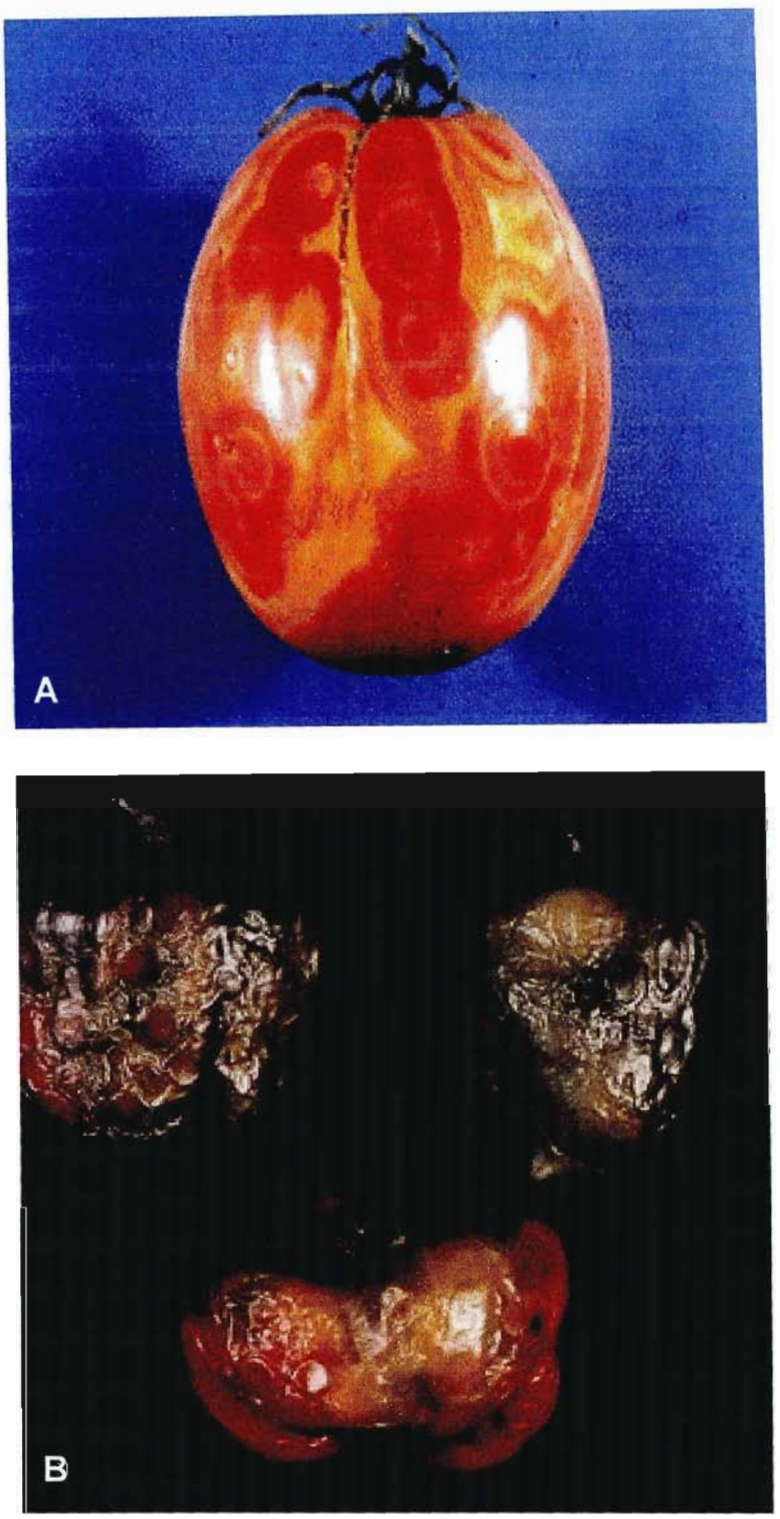



**Figure 1.5** Fruit symptoms of tomato spotted wilt virus (TSWV). (A) Concentric circles on tomato (Jett, 2003). (B) Spotting and distortion on pepper fruit (Hall, 2003).

## 1.4 EPIDEMIOLOGY

When a plant pathogen spreads to affect many individuals within a population over a relatively large area and within a relatively short time, the phenomenon is called an epidemic. The study of epidemics and the factors that influence them is called epidemiology (Agrios, 1988).

### 1.4.1 Transmission

Plant viruses rarely, if ever, come out of the plant spontaneously. For this reason, viruses are not disseminated as such by wind or water, and even when they are carried in plant sap or debris they generally do not cause infection unless they come into contact with the contents of a wounded living cell. Viruses are transmitted from plant to plant in a number of ways such as vegetative propagation; mechanically through sap; and by seed, pollen, insects, mites, nematodes, dodder, and fungi (Agrios, 1988).

#### *1.4.1.1 Vector*

The Tospoviruses are transmitted by minute insect pests called thrips (order, Thysanoptera, family *Thripidae*) (Sakimura 1962, 1963; Amin *et al.*, 1981; German *et al.*, 1992; Wijkamp *et al.*, 1995; Ullman, 1996). Thus far, several thrips species have been reported as vectors of TSWV, of which the western flower thrips, *Frankliniella occidentalis*, is apparently the most important (Table 1.2; Wijkamp *et al.*, 1993; Ullman *et al.*, 1997).

Tospoviruses replicate in their thrips vectors, thus the insects not only spread the virus, but also serve as a virus host. Therefore, Tospoviruses are transmitted in a persistent manner (Harris & Maramorosch, 1980). Although other viruses can be transmitted by thrips species, Tospoviruses are the only ones transmitted in a persistent, circulative manner (Mumford *et al.*, 1996).

The close link between thrips development and TSWV epidemiology is crucial to understanding the TSWV infection cycle (Fig. 1.6). One of the most outstanding features of the thrips-TSWV relationship is the inability of adults to acquire the virus. Only adult thrips that acquire the virus at the first larval stage are able to transmit it (Sakimura, 1963; Lewis, 1973; Ullman *et al.*, 1992, 1997). Initiation of the infection cycle can occur when female adult thrips lay eggs on suitable TSWV-infected leaves (Paliwal, 1975; Wijkamp & Peters, 1993; Ullman *et al.*, 2005). Larvae may acquire the virus within a period of 10 minutes or even less, though the chance that they will become infected increases with the length of the acquisition period (Fig. 1.7A). Acquisition may last as long as the larvae remain on the infected plant surface (Goldbach and Peters, 1996). The recently identified 55kDa protein which occurs in the midgut of the *F. occidentalis* and which binds to TSWV glycoprotein may have a key role in the acquisition process (Bandla *et al.*, 1998; Sin *et al.*, 2005).

In infected thrips, the virus is passaged through moulting, pupation, and emergence to the adult stage. For TSWV, two phases of viral replication have been identified during circulation. The first takes place in the cells of the midgut whilst the second takes place in the salivary glands (Kikkert *et al.*, 1998). Transmission is primarily carried out by adult thrips (Fig. 1.7B,C), although also rarely by larvae (Sakimura, 1963; Goldbach and Peters, 1996). Once a viruliferous thrips enters adulthood, its potential for infecting plants is very high. TSWV can persist within its vectors throughout their lives. This implies a period of between 30 to 40 days on average (Best, 1968). During this period the adult thrips will feed actively, dispersing the virus widely upon feeding on many different plants (Lewis, 1973).

**Table 1.2** Thrips species reported as vectors of tomato spotted wilt virus (TSWV) (German *et al.*, 1992; Goldbach & Peters, 1996; Peters, 2003).

| Latin name | Common name |
|---|---|
| *Frankliniella bispinosa* | Florida flower thrips |
| *Frankliniella fusca* | Tobacco thrips |
| *Frankliniella intonsa* | Eastern flower thrips |
| *Frankliniella occidentalis* | Western flower thrips |
| *Frankliniella schutlzei* | Cotton bud thrips or Common blossom thrips |
| *Thrips setosus* | No common name |
| *Thrips tabaci* | Onion thrips |

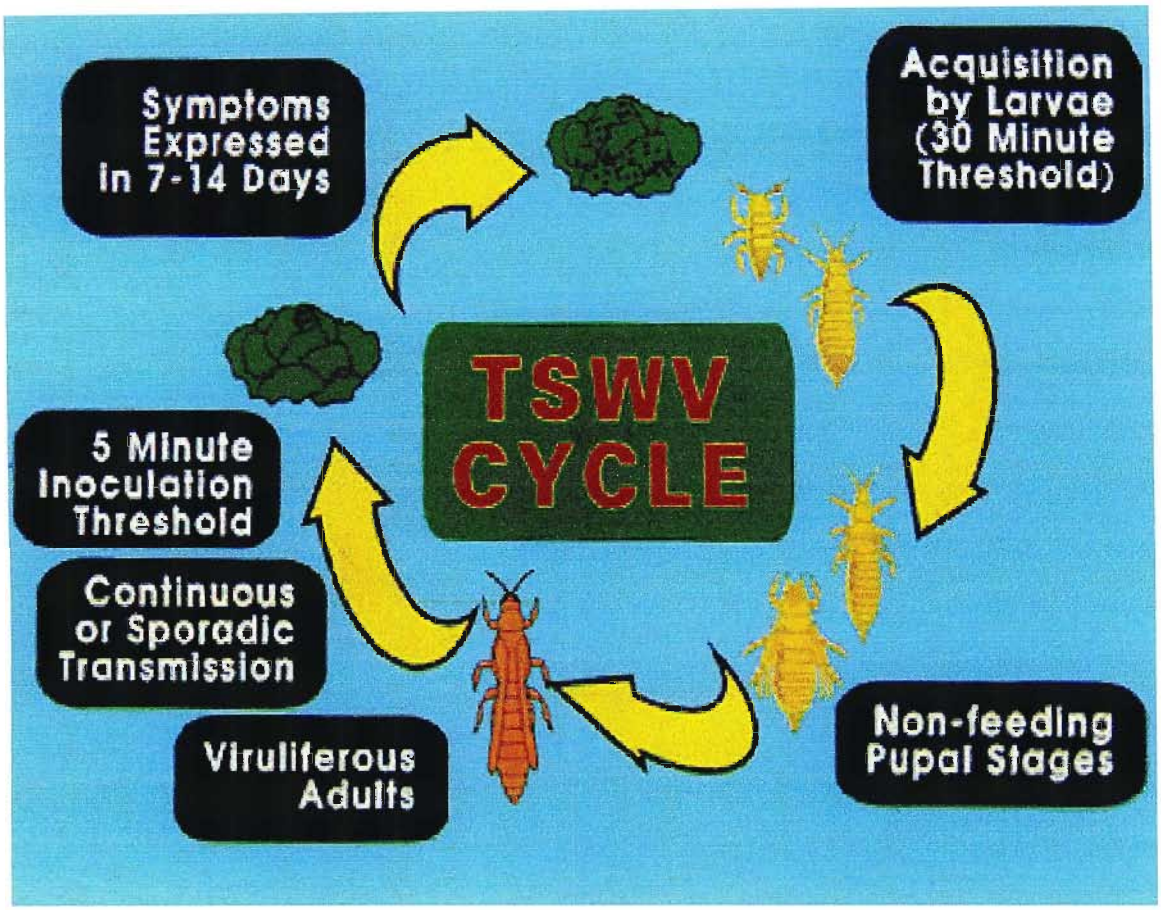



**Figure 1.6** Schematic representation of thrips development within the disease cycle of tomato spotted wilt virus (TSWV) (Moyer *et al.*, 1999).

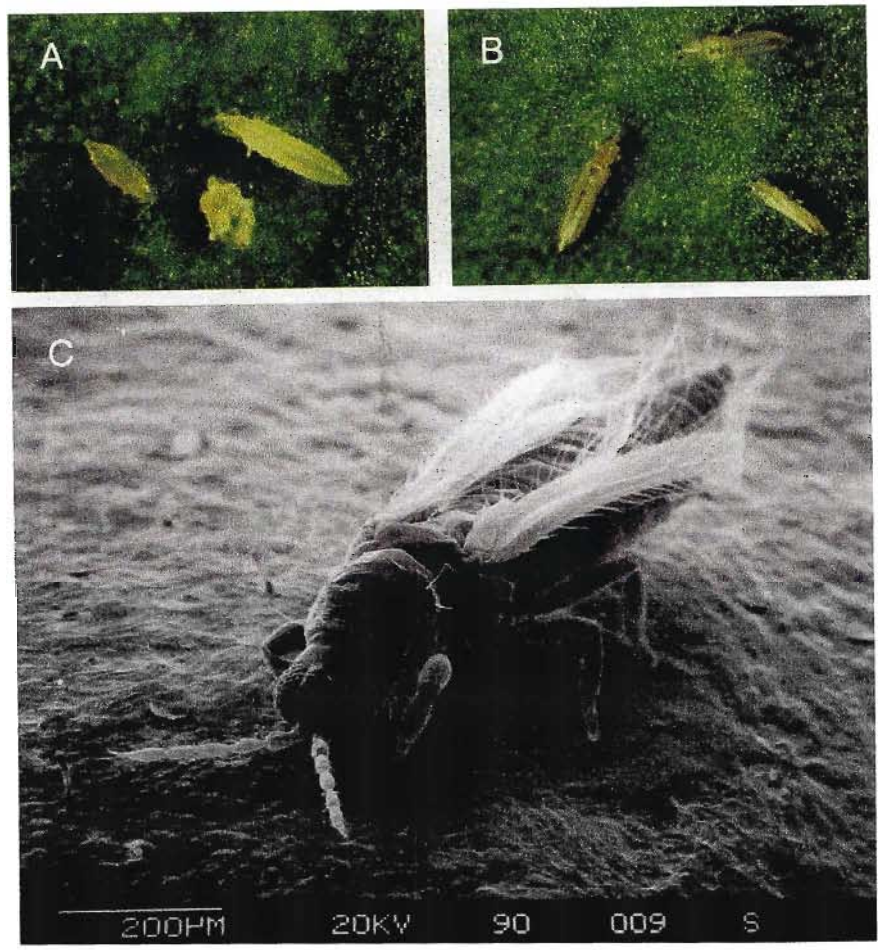



**Figure 1.7** Thrips vectors of tomato spotted wilt virus (TSWV): (A, left to right) First instar larva, first instar larval exuvia (molted exoskeleton), and second instar larva of the western flower thrips, *Frankliniella occidentalis*. (B) Adult female (lower left) and adult male (lower right) stages of *F. occidentalis* compared with adult *F. schultzei*, the common blossom thrips. (C) Scanning electron micrograph of *F. occidentalis* (Cho *et al.*, 1989).

#### *1.4.1.2 Non-vector*

TSWV can be transmitted by mechanical inoculation and grafting. The virus is not transmitted by contact between plants, seeds or pollen (Best, 1968; Brunt *et al.*, 1996).

### 1.4.2 Host Range

TSWV can infect 1090 plant species of the 1200 known to be susceptible to Tospoviruses. These 1090 species belong to more than 100 families of ornamental, vegetable, fruit and other annual and perennial plants (Peters, 2003). Solanaceous, composite and leguminous plants are particularly susceptible (Agrios, 1988). Some of the hosts severely affected by TSWV are tomato, tobacco, peanut, pineapple, papaya, lettuce, dahlia, gloxinia, and *Impatiens* (Agrios, 2005). In addition, up to 25 species are now considered important reservoir weed hosts of the virus (Cho *et al.*, 1986; Hobbs *et al.*, 1996; Mertelik & Mokra, 1999).

### 1.4.3 Geographical Distribution

The economic impact of TSWV is enormous, not only because of its extremely broad host range, but also because of its world wide geographic distribution (Fig. 1.8; de Haan, 1994). First reported in southern Australia, the virus is now widespread in temperate and subtropical regions throughout the world, having been recorded in South Africa, Asia, North and South America, and also in warm climates and glasshouses in Europe (Cho *et al.*, 1989; Sutic *et al.*, 1999). Its widespread distribution seems to be due to the international movement of infected ornamentals, whereas its local abundance and severity depend on the populations of its thrips vectors (Agrios, 2005).

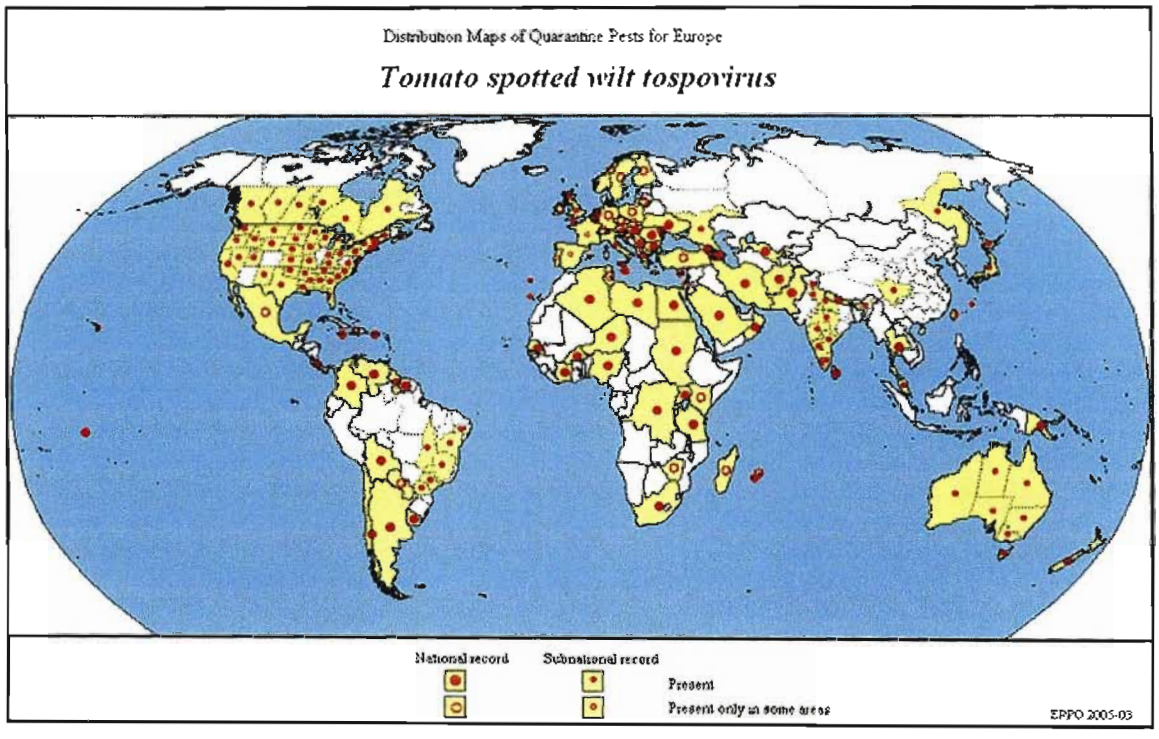



**Figure 1.8** Geographical distribution map of tomato spotted wilt virus (TSWV). (CAB/EPPO, 2005).

## 1.5 DETECTION AND DIAGNOSIS

The reliable detection and diagnosis of TSWV is not straightforward. It is complicated by the numerous aspects of TSWV biology, especially the broad host range and varied symptomatology (Mumford *et al.*, 1996). However, when diagnostic techniques are used together, they can provide a secure means of detection.

### 1.5.1 Serological Tests

Serological techniques used to detect viruses are based on the reaction between viral nucleoprotein or viral protein and its specific antibody (Hsu, 1996). At present the detection and diagnosis of TSWV, and plant viruses in general, are mainly reliant on serologically-based procedures (Mumford *et al.*, 1996). Some of these include: the enzyme-linked immunosorbent assay (ELISA) (Gonsalves & Trujillo, 1986); the agar diffusion test (Feldman & Boninsegna, 1968); and dot blot immunobinding assay (Hsu & Lawson, 1991).

#### *1.5.1.1 ELISA*

A turning point in TSWV detection and diagnosis came with the development of an enzyme-linked immunosorbent assay (ELISA; Gonsalves & Trujillo, 1986; German *et al.*, 1992). The microplate method of ELISA can be applied very effectively to the detection and assay of TSWV. The method has come to be more and more widely used. Many variations of the basic procedure have been described, with the objective of optimising the tests for particular purposes. However, the underlying principle of these variations remains the same (Flint *et al.*, 2000). Detection of the viral antigen or antibody can be accomplished by solid phase methods in which the viral antibody or protein is absorbed to a plastic surface. To detect the viral antigen, a 'capture' antibody, directed against the virus, is linked to a solid support, a plastic dish or bead. The specimen is added to the plastic support, and if viral antigens are present, they will be captured by the bound antibody. The bound viral antigen is detected by using

a second antibody linked to an enzyme. A substrate molecule is then added and is converted by the enzyme to an easily detectable product (Voller *et al.*, 1976; Wang & Gonsalves, 1990; Matthews, 1992). ELISA has also been adapted to allow for the detection of TSWV within individual thrips (Cho *et al.*, 1988; Bandla *et al.*, 1994), demonstrating both the versatility and sensitivity of the technique.

#### *1.5.1.2 Agar gel diffusion test*

Gel diffusion tests involving precipitation reactions between antigen and antibody take place on a semi-solid rather than in a liquid media. There are numerous variations to this technique. The Ouchterlony immunodiffusion method in which both antigen and antibody diffuse toward each other through the gel from separate reservoir wells is, however, the most commonly used technique in Tospovirus comparsions (Hsu, 1996).

#### *1.5.1.3 Dot immunobinding assay*

Also known as the dot blot immunoassay, dot blot immunobinding assay or tissue blot assay, it is similar to ELISA with the exception of a membrane matrix rather than a microtiter plate used as a solid-phase supporting material (Hsu, 1996). The technique is increasingly used for the direct transfer of virus-containing material by pressing the cut surface of plant tissue, squashing single seeds or insects to be tested for infection onto the membrane matrix. The membranes can also be taken into the field, where plant tissues and insects can be blotted or squashed directly onto them (Bos, 1999). The increased sensitivity of the detection limit with dot immunobinding assay on nitrocellulose membranes over the ELISA procedure in microtiter plates is a definite advantage (Hsu, 1996).

An immunoblotting procedure known as the western blot also provides a powerful yet simple method for detecting specific antigens in a complex mixture of proteins. It has

been used effectively to compare and identify proteins of Tospoviruses based on molecular weight and serology. Firstly, Tospovirus proteins are separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred either by diffusion or electrophoretically onto a nitrocellulose membrane. Once on the membrane, the viral proteins are detected and identified using peptide-specific antibodies. This is followed by enzyme-labeled antibodies reacting to the first antibodies (Law & Moyer, 1990).

### 1.5.2 Electron Microscopy

Electron microscopy has become indispensable as a spot-check on infected material and virus preparations, in revealing virus structure, and in showing the distribution and development of viruses in infected tissue and the effects they have on the host (Kado, 1972). Tospoviruses are the only spherical, enveloped plant viruses; thus members of this group are directly identified by transmission electron microscopy (TEM) of leaf-dip preparations or in thin sections of infected plants (Ie, 1964, Kitajima, 1965; Milne, 1970; Ie, 1971; German *et al.*, 1992). Alternatively, these viruses can be identified by the cytoplasmic inclusions associated with infection (Fig. 1.3; Christie & Edwardson, 1986; Roselló *et al.*, 1996). Immunosorbent electron microscopy (IEM) has combined the ease of TEM with the specificity of serology. The technique, which focuses on the specific trapping of viral particles on grids that have been pretreated with antiserum, has increased the sensitivity and specificity of TEM up to 10,000 times. Additional improvement is possible by further labeling of decorated particles with colloidal gold (Immunogold labeling, Bos, 1999).

### 1.5.3 Biological Indexing

Biological indexing is one of the oldest methods employing reactions of plants when infected by viruses. One approach is by visual inspection of the material to be tested for characteristic symptoms. Another is indexing for infective viruses on indicator hosts and their examination for symptoms (Bos, 1999). TSWV is sap-transmissible,

and under carefully controlled conditions can be mechanically inoculated onto indicator plants (Best, 1968). A small number of plant species have been used frequently in the detection of TSWV (Table 1.3). Of these, *Petunia* is one of the most useful due to the rapidity with which the symptoms develop. Preference is also given to *Nicotinia* species as indicator plants for TSWV because of their greater sensitivity under glasshouse conditions (Francki & Hatta, 1981). However, no definite identification can be made as slight differences in symptom expression on indicator plants makes identification almost impossible. Moreover, symptoms produced are often similar to those evoked by viruses of other families (Peters, 2003).

### 1.5.4 Transmission Tests

The transmission of Tospoviruses by thrips is a distinctive characteristic of this genus of virus and can be used to identify them (Best, 1968; Roselló *et al.*, 1996). However, it is not recommended for routine diagnostic work. Vector transmission trails require careful maintenance and management of these fragile insect colonies. Tests require long incubation periods and hence identification cannot be obtained quickly. In addition, it has been observed that TSWV can lose its vector transmissibility after prolonged culture by sap transmission (Best, 1968; Francki & Hatta, 1981).

### 1.5.5 Virus Stability

The stability of a virus in crude sap, assessed by its thermal inactivation point (TIP), longevity in vitro (LIV) and dilution end point (DEP), has been used for diagnosis. It has since been considered an unreliable criterion for identification due to the variability of values reported in literature. In addition, the observed TIPs and LIVs of TSWV overlap with a number of viruses within at least four other plant virus groups (Francki & Hatta, 1981). However, information about the stability of a virus outside the plant cell is still useful for optimal success in mechanical inoculation, selection of plant material for maintenance of virus isolates and their purification. Moreover, tests

that assess virus stability in crude sap are valuable as a basis for further investigations of an unidentified virus (Dijkstra & de Jager, 1998).

### 1.5.6 Hybridisation Techniques

The final and most recent approaches used for the detection of Tospoviruses are those based on molecular biology techniques. Viral nucleic acid hybridisation techniques have been developed and evaluated for their use in the detection of TSWV. Both riboprobes and cDNA probes have been used successfully. These techniques show great promise, and although the sensitivity of these tests is not greater than those recorded for ELISA, probes circumvent the problems associated with serological testing (Rice *et al.*, 1990; Mumford *et al.*, 1996).

#### *1.5.6.1 RT-PCR*

Reverse transcriptase polymerase chain reaction (RT-PCR) is an effective new technique for detecting small amounts of plant viral nucleic acids. This procedure involves the enzymatic synthesis of cDNA from templates of viral RNA with reverse transcriptase, followed by the amplification of the resulting product with oligonucleotides complementary to the viral nucleic acid with the thermostable Taq-polymerase. The reaction can be executed on small amounts of crude RNA from plant sap. Following the amplification, the specific DNA products can be detected by agarose gel electrophoresis (German *et al.*, 1992; Weeks *et al.*, 1996a, 1996b; Giovanna *et al.*, 2003).

**Table 1.3** Some suitable indicator plants for the identification of the tomato spotted wilt virus (TSWV) (Francki & Hatta, 1981; Peters, 2003).

| Plant species | Reactions to infection |
|---|---|
| *Arachis hypogaea* | + |
| *Capsicum annuum* | + |
| *Chenopodium quinoa* | L |
| *Cucumis sativus* | L |
| *Datura stramonium* | + |
| *Emilia sonchifolia* | + |
| *Gomphrena globosa* | L |
| *Impatiens spp* | + |
| *Lycopersicum esculentum* | + |
| *Nicotinia benthamiana* | + |
| *Nicotinia clevelandii* | + |
| *Nicotinia glutinosa* | + |
| *Nicotinia rustica* | + |
| *Nicotinia tabacum* | + |
| *Petunia hybrida* | L |
| *Phaseolus vulgaris* | + |
| *Pisum sativum* | + |
| *Vigna unguiculata* | + |

L = local reactions on the inoculated leaves; + = react locally and systemically

## 1.6 DISEASE MANAGEMENT

The eradication of TSWV in affected regions is difficult due to the wide range of virus reservoirs and the efficiency of the vectors in their transmission. Once the disease has entered the plant, the host-pathogen relationship is so intimate that is practically impossible to break it or to reduce disease development. Therefore, possible control methods are basically preventative. These methods include: (a) controlling its transmission, by controlling vector transmission by means of chemical, physical and biological methods; (b) decreasing the amount of virus (inoculum), by cultural methods and (c), the use of resistant cultivars, when possible (Roselló *et al.*, 1996). However, no single management tool is available that provides adequate control of TSWV. Therefore, an interdisciplinary and multifaceted approach that integrates all applicable measures should be employed (Cho *et al.*, 1989; Culbreath *et al.*, 2003).

### 1.6.1 Vector Control

A practical way of controlling Tospovirus-induced diseases is to reduce its spread. This is mostly achieved by controlling the vector thrips populations (Mumford *et al.*, 1996). This can be accomplished by chemical, physical and biological control.

#### *1.6.1.1 Chemical control*

At present only chemical control of thrips provides significant reductions in population size, resulting in effective management of TSWV diseases (Mumford *et al.*, 1996). Unfortunately, thrips have developed resistance to many available insecticides and move in readily from other crops, so insecticidal sprays often have little or no effect on the spread of the virus (Agrios, 1988; Mumford *et al.*, 1996). In addition, some of these compounds are highly toxic and hence environmentally undesirable. These agents may act in a non-specific manner, which results in elimination of insect predators often introduced as biological control agents (Mumford *et al.*, 1996). Among the chemical products commonly used against thrips are: Acrinathrin,

Avermectin, Dimethoate, Endosulfan, Fenvalerate, Formetanate, Malathion, Chlorpyrifos-methyl, Methiocarb, Naled, Phosfolan and the Piretroids: Deltamethrin and Polybutene (Thripstick) (Hill, 1987; Hussey, 1985). One area of chemical control that does show some promise is the use of horticultural oils, insecticidal soaps and film-forming agents. Products representing all three classes were tested and results show a significant reduction in TSWV transmission. In addition, all products have low-toxicity and are compatible with biological control strategies (Mumford *et al.*, 1996).

#### *1.6.1.2 Physical control*

Among the physical methods of vector control, the use of fine mesh screens on doors and windows is a reliable solution for greenhouse crops. This practice produces a considerable decline in the problem by avoiding early crop contamination by thrips (Roselló *et al.*, 1996). Other physical barriers, such as plastic mulch placed around the base of the plant, have acted to reduce TSWV, presumably by reducing thrips' access to the growing medium, where they produce pupae (Brown & Brown, 1992; Mumford *et al.*, 1996). The use of aluminum-surfaced plastic film mulch reduces the immigration of thrips and the occurrence of TSWV. Thrips are colour sensitive, therefore, they respond negatively to reflective mulches (Greenough *et al.*, 1990). The use of yellow or blue sticky plates for the early detection of thrips in greenhouses is also useful, as it allows for the rapid adoption of control measures (Roselló *et al.*, 1996).

#### *1.6.1.3 Biological control*

Biological control of thrips has been demonstrated using the predatory mite, *Amblyseius cucumeris*, and hemipteran bugs of the genus *Orius* (Higgins, 1992; Cook *et al.*, 1995). Both of these agents are now commercially available. The use of these predators is promising, since they reduce thrips populations, providing effective pest

control in some crops. However, when thrips populations are high, predators are unable to stop virus transmission (Roselló *et al.*, 1996). Nevertheless, the combined use of biological control with other non-chemical methods could prove effective as part of an integrated pest management (IPM) scheme (Mumford *et al.*, 1996).

### 1.6.2 Cultural Control

Cultural practices and varietal selection have proven effective in minimising losses. A series of measures such as prior history of land, changing planting dates, cultivar selection, sanitation, tillage, weed control and plant row spacing have been identified as important factors in the control of TSWV (Brown *et al.*, 1996; Culbreath *et al.*, 2003).

As TSWV can only occur when a crop is invaded by viruliferous thrips, its incidence can be reduced by sowing the crop when the most sensitive stage of its development (usually the seedling) is least likely to be invaded. Physical barriers that limit the movement of thrips have also been shown to reduce TSWV incidence. These include intercropping with fast growing, tall cereals which are non-hosts of TSWV and the vector; and growing crops, especially vegetables and ornamentals, in greenhouses covered with screening or under polyvinyl fabric (Reddy & Wightman, 1988). Spread of TSWV and thrips can also occur via the movement of infected plant material, such as cuttings. This type of spread is most effectively controlled by the use of certified virus-free material (Mumford *et al.*, 1996).

The role weeds play in the incidence and spread of TSWV is significant. In addition, the majority of these are also host to the vectors. Removal of reservoir weed hosts in a wide area around a crop may relieve the infection pressure. Keeping fields bare, including weeding and tilling, may prevent the survival of thrips pupae (Peters, 2003).

### 1.6.3 Resistance

Cultural practices and vector control have only been marginally effective in the management of TSWV (Cho *et al.*, 1989). Host resistance to TSWV is the most promising means of controlling the disease in the long term (de Haan, 1994).

#### *1.6.3.1 Natural resistance*

Over the past 50 years, considerable effort has been spent on developing crops with increased resistance or tolerance to TSWV infections (Goldbach & Peters 1996). However, limited progress has been made in breeding tomato, tobacco and lettuce with increased resistance to TSWV, partly because of the lack of suitable forms of resistance. In most cases, naturally occurring TSWV resistance or tolerance is polygenic, based on complex interactions between virus, vector and plant (Cho *et al.*, 1998). A single TSWV-tolerance gene from *Capsicum chinense* L. has been successfully introduced into pepper. However, the pepper plants are tolerant of TSWV and not other Tospoviruses. Moreover, the tolerance is linked to many of the agronomically undesired traits. Despite numerous attempts in tobacco and lettuce, no clear source of resistance to TSWV could be identified. Genetic resistance to TSWV has been found in *Lycopersicon* spp. which has been introduced into commercial tomato (*Lycopersicon esculenta* Mill.) cultivars (Goldbach & Peters, 1996). Several tomato lines have now been released with this resistance (Peters, 2003). A single dominant gene, tentatively identified as Sw-5, provided broad resistance to TSWV isolates from many geographical areas (Stevens *et al.*, 1992). However, TSWV resistance-breaking strains are now encountered in the field in tomato cultivars carrying the Sw-5 gene (Thompson & van Zijl, 1996).

### *1.6.3.2 Engineered resistance*

In light of the limited utility of natural resistance genes, and the time span required to produce resistant plants using conventional plant breeding, scientists are actively investigating genetically-engineered resistance strategies as an alternative (Mumford *et al.*, 1996). Most of these strategies are based on the concept of 'pathogen-derived resistance' (PDR), which proposes that pathogen resistance genes may be developed from the pathogen's own genetic material (Sanford & Johnson, 1985). Selected genes from a virus, when inserted into the host plant genome, may render that host resistant to the virus (Mumford *et al.*, 1996). This type of resistance is known as RNA-mediated resistance, which operates via a post-transcriptional gene silencing (PTGS) of the virus-derived transgene (Baulcombe, 1996; Prins & Goldbach, 1998). In general, this resistance is effective only against viral strains which have a high degree of sequence similarity to the transgene (Gubba *et al.*, 2002). Several other forms of PDR may be envisaged for TSWV. These include: nucleocapsid-mediated resistance, antisense resistance and defective-interfering (DI)-RNA mediated resistance (Prins & Goldbach, 1998).

Initial studies reported the production of transgenic tobacco, which by expressing the TSWV N gene, show significant levels of resistance to TSWV (Gielen *et al.*, 1991; MacKenzie & Ellis, 1992; Pang *et al.*, 1992). These were the first examples of nucleocapsid-mediated resistance directed at a negative-stranded plant virus. Besides being protected against mechanical inoculation, transgenic tobacco plants are also resistant to inoculation via viruliferous thrips, e.g., *F. occidentalis.* Sequences of other genes were not effective in inducing resistance in transgenic plants (Prins & Goldbach, 1998). This new approach is an important contribution to the control of TSWV in the near future (de Haan *et al.*, 1996).

## 1.7 REFERENCES


**Adkins S. (2000).** Tomato spotted wilt virus-positive steps towards negative success. *Molecular Plant Pathology* **1**, 151-157.

**Agrios G.N. (1988).** *Plant Pathology.* San Diego: Academic Press, pp. 622-657.

**Agrios G.N. (2005).** *Plant Pathology.* San Diego: Academic Press, pp. 724-799.

**Amin P.W., Reddy V.R., & Ghanekar A.M. (1981).** Transmission of tomato spotted wilt virus, the causative agent of bud necrosis of peanut, by *Scirtothrips* and *Frankliniella schultzei*. *Plant Disease* **65**, 663-665.

**Bandla M.D., Westcot D.M., Chenault K.D., Ullman D.E., German T.L., & Sherwood J.L. (1994).** Use of monoclonal antibody to the nonstructural protein encoded by the small RNA of tomato spotted wilt tospovirus to identify viruliferous thrips. *Phytopathology* **84**, 1477-1481.

**Bandla M.D., Campbell L.R., Ullman D.E., & Sherwood J.L. (1998).** Interaction of tomato spotted wilt tospovirus (TSWV) glycoproteins with a thrips midgut protein, a potential cellular receptor for TSWV. *Phytopathology* **88**, 98-104.

**Baulcombe D.C. (1996).** Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* **8**, 1833-1844.

**Best R.J. (1968).** Tomato spotted wilt virus. *Advances in Virus Research* **13**, 65-145.

**Bishop D.H.L. (1990).** Bunyaviridae and their replication, part 1. In: *Virology,* Fields B.N., Knipe D.M. (eds.). New York: Raven Press, pp. 1155-1169.

**Bos L. (1999).** *Plant Viruses: Unique and Intriguing Pathogens, A Textbook of Plant Virology.* Leiden: Backhuys, pp. 19-24.

**Brown S.L., & Brown J.E. (1992).** Effect of plastic mulch color and insecticides on thrips populations and damage to tomato. *Horticultural Technology* **2**, 208-210.

**Brown S.L., Todd J.W., & Culbreath A.K. (1996).** Effect of selected cultural practices on incidence of tomato spotted wilt virus and populations of thrips vectors in peanuts. *Acta Horticulturae* **431**, 491-498.

**Brittlebank C.C. (1919).** Tomato diseases. *Journal of Agriculture, Victoria* **17**, 231-235.

**Brunt A., Crabtree K., & Gibbs A. (1990).** *Viruses of Tropical Plants.* Walingford: CAB international, pp. 56-57.

**Brunt A., Crabtree K., Dallwitz M., Gibbs A., & Watson L. (1996).** *Viruses of Plants: Descriptions and Lists from the VIDE database.* Cambridge: CAB international, pp. 1312-1315.

**CAB/EPPO. (2005).** Distribution of Maps of Plant Diseases. *www.cab/eppo.co.za.*

**Cho J., Mau R.F.L., Gonsalves D., & Mitchell W.C. (1986).** Reservoir weed host of tomato spotted wilt virus. *Plant Disease* **70**, 1014-1017.

**Cho J.J., Mau R.F.L., Hamasaki R.T., & Gonsalves D. (1988).** Detection of tomato spotted wilt virus in individual thrips by enzyme-linked immunosorbent assay. *Phytopathology* **78**, 1348-1352.

**Cho J., Mau R.F.L., German T.L., Hartmann R.W., Yudin L.S., Gonsalves D., & Provvidenti P. (1989).** A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. *Plant Disease* **73**, 375-383.

**Cho J., Mau R.F.L., Pang S., Wang M., Gonsalves C., Watterson J., Custer D. M., Gonsalves D. (1998).** Approaches for controlling tomato spotted wilt virus. In: *Plant Virus Disease Control*, Hadidi A., Khetarpal R.K., & Kogenezawa B.D. (eds.). St. Paul: APS Press, pp. 547-563.

**Christie R.G., & Edwardson J.R. (1986).** Light microscope techniques for detection of plant virus inclusions. *Plant Disease* **70**, 273-279.

**Chu F., Chao C., Chung M., Chen C., & Yeh S. (2001).** Completion of the genome sequence of watermelon silver mottle virus and utilization of degenerate promoters for detecting tospoviruses in five serogroups. *Phytopathology* **91**, 361-368.

**Chu F., & Yeh S. (1998).** Comparison of ambisense M RNA of watermelon silver mottle virus with other tospoviruses. *Phytopathology* **88**, 351-358.

**Cook D.F., Houlding B.J., Steiner E.C., Hardie D. (1995).** The native anthocorid bug *Orius armatus* (heteroptera: anthocoridae) as a field predator of *Frankliniella occidentalis* (thysanoptera: thripidae) in western Australia. *International Symposium on Tospoviruses and Thrips of Floral and Vegetables Crops, Taichung,* p. 19.

**Culbreath A.K., Todd J.W., & Brown S.L. (2003).** Epidemiology and management of tomato spotted wilt virus in peanut. *Annual Review of Phytopathology* **43**, 53-75.

**de Haan P., Wagemakers L., Peters D., & Goldbach R. (1990).** The S RNA segment of tomato spotted wilt virus has an ambisense character. *Journal of General Virology* **71**, 1001-1007.

**de Haan P., Kormelink R., Resende R., van Poelwijk F., Peters D., & Goldbach R. (1991).** Tomato spotted wilt L RNA codes a putative RNA polymerase. *Journal of General Virology* **71**, 2207-2216.

**de Haan P. (1994).** Tospoviruses. In: *Encyclopedia of Virology*, Webster R.G., & Granoff A. (eds.). London: Academic Press.

**de Haan P., Ultzen T., Prins M., Gielen J., Goldbach R., & van Grinsven. (1996).** Transgenic tomato hybrids resistant to tomato spotted wilt virus infection. *Acta Horticulturae* **431**, 417-426.

**Dijkstra T. & de Jager C.P. (1998).** *Practical Plant Virology: Protocols and Exercises.* Germany: Springer-Verlag, pp. 221-297.

**Feldman J.M., & Boninsenga J.A. (1968).** Antiserum for tomato spotted wilt virus. *Nature* **219**, 183-184.

**Flint S.J., Enquist L.W., Krug R.M., Racaniello V.R., & Skalka A.M. (2000).** *Principles of Virology: Molecular Biology, Pathogenesis, and Control.* Washington: ASM Press, pp. 597-610.

**Francki R.I.B., & Hatta T. (1981).** Tomato spotted wilt virus. In: *Handbook of Plant Virus Infections and Comparative Diagnosis*, Kurstak E. (ed.). Amsterdam: Elsevier/North-Holland Biomedical Press, pp. 491-514.

**Francki R.I.B., Milne R.G., & Hatta, T. (1985).** *Atlas of Plant Viruses.* Boca Raton: CRC Press, pp. 72-75.

**Francki R.I.B., Fauquet C.M., Knudson D.L., & Brown F. (1991).** Classification and nomenclature of viruses: Fifth report of the International Committee on Taxonomy of Viruses. *Archives of Virology,* Supplement 2.

**German L.T., Ulman D.E., & Moyer J.W. (1992).** Tospoviruses: diagnosis, molecular biology, phylogeny, and vector relationships. *Annual Review of Phytopathology* **30**, 315-348.

**Gielen J.J.L., de Haan P., Kool A.J., Peters D., van Grinsven M.Q.J.M., & Goldbach R.W. (1991).** Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. *Bio/Technology* **9**, 1363-1367.

**Giovanna M., Piero R., & Luciana T. (2003).** Detection of tomato spotted wilt virus in its vector *Frankliniella occidentalis* by reverse transcription-polymerase chain reaction. *Journal of Virological Methods* **109**, 69 (abstract only).

**Goldbach R., & Kormelink R. (2002).** Tospovirus. In: *The Springer Index of Viruses,* Tidona C.A., & Gholamreza D. (eds.). Berlin: Springer-Verlag, pp. 169-174.

**Goldbach R., & Peters D. (1994).** Possible causes of the emergence of tospovirus diseases. *Seminars in Virology* **5**, 113-120.

**Goldbach R., & Peters D. (1996).** Molecular and biological aspects of tospoviruses. In: *The Bunyaviridae*, Elliot R.M. (eds.). New York: Plenum Press, pp. 129-157.

**Gonsalves D., & Trujillo E.E. (1986).** Tomato spotted wilt virus in Papaya and detection of the virus by ELISA. *Plant Disease* **70**, 501-506.

**Greenough D.R., Black L.L., & Bond W.P. (1990).** Aluminum-surfaced mulch: an approach to the control of tomato spotted wilt virus in solanaceous crops. *Plant Disease* **74**, 805-808.

**Gubba A., Gonsalves C., Stevens M.R., Tricoli D.M., & Gonsalves D. (2002).** Combining transgenic and natural resistance to obtain broad resistance to tospovirus infection in tomato (*Lycopersicon esculentum* Mill). *Molecular Breeding* **9**, 13-23.

**Hall D. (2003).** UCIPM Online Statewide Integrated Pest Management Program. *www.ipm.ucdavis.edu/PMG/T/D-PR-TSWV-FR.001.html.*

**Harris K.F., & Maramorosch K. (1980).** *Vectors of Plant Pathogens.* New York: Academic Press, pp. 149-161.

**Hill D.S. (1987).** *Agricultural Insect Pests of the Temperate Regions and their Control.* Cambridge: Cambridge University Press, pp. 252-257.

**Higgins C.J. (1992).** Western flower thrips (Thysanoptera: Thripidae) in glasshouses: population dynamics, distribution on plants, and associations with predators. *Journal of Economic Entomology* **85**, 1891-1903.

**Hobbs H.A., Johnson R.R., Story R.N., & Black L.L. (1996).** Weed hosts and thrips transmission of tomato spotted wilt virus in Louisiana. *Acta Horticulturae* **431**, 291-297.

**Hsu H.T. (1996).** Immunological detection and identification of tospoviruses. *Acta Horticulturae* **431**, 109-121.

**Hsu H.T., & Lawson R.H. (1991).** A serological distinct high temperature variant derived from a gloxinia isolate of impatiens necrotic spot virus. *Phytopathology*, **84**, 1132-1140.

**Hull R. (2001).** *Matthew's Plant Virology.* San deigo: Academic Press, pp. 75-90.

**Hussey N.W. (1985).** Thrips and their natural enemies. In: *Biological Pest Control, the Greenhouse Experience*, Hussey N.W., & Scopes N. (eds.). Poole: Blandford Press, pp. 53-57.

**Ie T.S. (1964).** An electron microscope study of tomato spotted wilt virus in the plant cell. *Netherlands Journal of Plant Pathology* **72**, 114-115.

**Ie T.S. (1971).** Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. *Virology* **43**, 468-479.

**Ie T.S. (1982).** A sap-transmissible defective form of tomato spotted wilt virus. *Journal of General Virology* **59**, 387-391.

**Jett L. (2003).** Problems in Tomato: tomato spotted wilt virus (TSWV). *www.agebb.missouri.edu/hort/meg.archives/v9n8/megic.*

**Kado C.I. (1972).** Mechanical and biological inoculation principles. In: *Principles and Techniques in Plant Virology,* Kado C.I., & Agrawal H.O. (eds.). New York: Van Nostrand Reinhold Company, pp. 3-30.

**Kikkert M., Meurs C., van de Wetering F., Dorfmuller D.P., Kormelink R., & Goldbach R. (1998).** Binding of tomato spotted wilt virus to a 94-kDa thrips protein. *Phytopathology* **88**, 63-69.

**Kikkert M., van Lent J., Storms M., Bodegom P., Kormelink P., & Goldbach R. (1999).** Tomato spotted wilt virus particle morphogenesis in plant cells. *Journal of Virology* **73**, 2288-2297.

**Kitajima E.W. (1965).** Electron microscopy of vira cabeca (Brazilian tomato spotted wilt virus) within the host cell. *Virology* **26**, 89-99.

**Kormelink R., Kitajima E.W., de Haan P., Zuidema D., Peters D., & Goldbach R. (1991).** The nonstructural protein (NSs) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structures in infected plant cells. *Virology* **181**, 459-468.

**Kormelink R., de Haan P., Meurs C., Peters D., & Goldbach R. (1992a).** The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. *Journal of General Virology* **73**, 2795-2804.

**Kormelink R., van Poelwijk F., Peters D., & Goldbach R. (1992b).** Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus mRNAs. *Journal of General Virology* **73**, 2125-2128.

**Law M.D., & Moyer J.W. (1990).** A tomato spotted wilt virus-like virus with a serologically distinct N protein. *Journal of General Virology*, **71**, 933-938.

**Law M.D., Speck J., & Moyer J.W. (1991).** Nucleotide sequence of the 3' non-coding region and the N gene of the S RNA of a serologically distinct tospovirus. *Journal of General Virology*, **72**, 2597-2601.

**Lawson R.H., Dienelt M.M., & Hsu H.T. (1996).** The cytopathology of tospovirus – host-plant interactions. *Acta Horticulturae* **431**, 267-289.

**Lazarowitz S.G. (2001).** Plant Viruses. In: *Fundamental Virology,* Knipe D.M., Howley P.M., & Griffin D.E. (eds.). Philadelphia: Lippincott Williams and Wilkins, pp. 378-432.

**Levy J.A., Fraenkel-Conrat H., & Owens R.A. (1994).** *Virology.* New Jersey: Prentice-Hall, Inc, pp. 100-109.

**Lewis T. (1973).** *Thrips: their biology, ecology and economic importance.* London: Academic Press, pp. 236-238.

**Lucas G.B., Campbell C.L., & Lucas C.L. (1985).** *Introduction to Plant Diseases: Identification and Management.* Westport: Avi Publishing Company, Inc, pp. 3-17.

**MacKenzie D.J., & Ellis P.J. (1992).** Resistance to tomato spotted wilt virus infection in transgenic tobacco expressing the viral nucleocapsid gene. *Molecular Plant-Microbe Interactions* **5**, 34-40.

**Mandahar C.L. (1999).** *Molecular Biology of Plant Viruses.* Boston: Kluwer Academic Publishers, pp. 78-80.

**Matthews R.E.F. (1982).** Classification and nomenclature of viruses: Fourth report of the International Committee on Taxonomy of Viruses. *Intervirology* **17**, 1-200.

**Matthews R.E.F. (1992).** *Fundamentals of Plant Virology.* New York: Academic Press, pp. 259.

**Matthews R.E.F. (1993).** *Diagnosis of Plant Virus Diseases.* Boca Raton: CRC Press, pp. 115-116.

**Mertelik J., & Mokra V. (1999).** Occurrence of tomato spotted wilt tospovirus in horticultural crops and weeds in the Czech Republic in 1992/1997. *OEPP/EPPO Bulletin* **29**, 81-83.

**Milne R.G. (1970).** An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. *Journal of General Virology* **6**, 267-276.

**Mohamed N.A. (1981).** Isolation and characterization of subviral particles of tomato spotted wilt virus. *Virology* **56**, 12-21.

**Moyer J.W., German T., Sherwood J.L., & Ullman D. (1999).** An Update of Tomato Spotted Wilt Virus and Related Tospoviruses. *www.scisoc.org/feature/tospovirus.com.*

**Mumford R.A., Barker I., & Wood K.R. (1996).** The biology of tospoviruses. *Annual Applied Biology* **128**, 159-183.

**Narayanasamy P. (2001).** *Plant Pathogen Detection and Disease Diagnosis.* New York: Marcel Dekker, pp. 167-191, 39-100.

**Paliwal Y.C. (1975).** Some characteristics of the thrip vector relationship of tomato spotted wilt virus in Canada. *Canadian Journal of Botany* **54**, 402-405.

**Pang S., Nagpala P., Wang M., Slightom J.L., & Gonsalves D. (1992).** Resistance to heterologous isolates of tomato spotted wilt virus in transgenic tobacco expressing its nucleocapsid protein gene. *Phytopathology* **82**, 1223-1229.

**Paape M., Solovyev A.G., Erokhina T.N., Minina E.A., Schepetilnikov M.V., Lesemann D.E., Schiemann J., Morozov S.Y., & Kellmann J.W. (2006).** At-4/1, an interactor of the tomato spotted wilt virus movement protein, belongs to a new family of plant proteins capable of directed intra-and intercellular trafficking. *Molecular Plant Microbe Interactions* **19**, 874-883.

**Peters D. (2003).** Tospoviruses. In: *Viruses and Virus-like Diseases of Major Crops in Developing Countries*, Loebenstein G., & Thottappilly G., eds. USA: Kluwer Academic Publishers, pp. 719-737.

**Prins M., & Goldbach R. (1998).** The emerging problem of tospovirus infection and non-conventional methods of control. *Trends in Microbiology* **32**, 31-35.

**Reddy D.V.R., & Wightman J.A. (1988).** Tomato spotted wilt virus: Thrips transmission and control. *Advances in Disease Vector Research* **5**, 203-220.

**Rice D.J., German T.L., Mau R.F.L., & Fujimoto F.M. (1990).** Dot blot detection of tomato spotted wilt virus RNA on plant and thrips tissues by cDNA clones. *Plant Disease* **74**, 274-276.

**Roselló S., Diez, M.J., & Nuez F. (1996).** Viral diseases causing the greatest economic losses to the tomato crop. I. The tomato spotted wilt virus- a review. *Scientia Horticulturae* **67**, 117-150.

**Rubio-Huertos M. (1972).** Inclusion bodies. In: *Principles and Techniques in Plant Virology,* Kado C.I., & Agrawal H.O. (eds.). New York: Van Nostrand Reinhold Company, pp. 62-75.

**Rudolph C., Schreier P.H., & Uhrig J.F. (2003).** Peptide-mediated broad-spectrum plant resistance to tospoviruses. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 4429-4434.

**Sakimura K. (1962).** The present status of thrips-borne viruses. In: *Biological Transmission of Disease Agents*, Maramorosch K. (ed.). New York: Academic Press, pp. 33-40.

**Sakimura K. (1963).** *Frankliniella fusca*, an additional vector for the tomato spotted wilt virus, with notes on *Thrips tabaci*, another vector. *Phytopathology* **53**, 412-415.

**Samuel B., Bald B.A., & Pittman K. (1972).** Tomato spotted wilt virus. In: *A Textbook of Plant Virus Diseases*, Smith K.M. (ed.). New York: Academic Press, pp. 545-549.

**Sanford J.C., & Johnston S.A. (1985).** The concept of pathogen-derived resistance - deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology* **113**, 395-405.

**Sin S-H., McNulty B.C., Kennedy G.G., Moyer J. W. (2005).** Viral genetic determinants for thrips transmission of tomato spotted wilt virus. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5168-5173.

**Smith K.M. (1937).** *A Textbook of Plant Virus Diseases.* London: J & A Churchill Ltd, pp. 296-315.

**Stevens M.R., Scott S.J., Gergerich R.C. (1992).** Inheritance of a gene for resistance to tomato spotted wilt virus (TSWV) from *Lycopersicon peruvianum* Mill. *Euphytica* **59**, 9-17.

**Sutic D.D., Ford R.E., & Tosic M.T. (1999).** *Handbook of Plant Virus Disease.* London: CRC Press, pp. 548-560.

**Tas P.W.L., Boerjan M.L., & Peters D. (1977).** The Structural Proteins of Tomato Spotted Wilt Virus. *Journal of General Virology* **36**, 267-279.

**Thompson G.J., & van Zijl J.J.B. (1996).** Control of tomato spotted wilt virus in tomatoes in South Africa. *Acta Horticulturae* **431**, 379-384.

**Thresh J.M. (2003).** The impact of plant virus diseases in developing countries. In: *Viruses and Virus-like Diseases of Major Crops in Developing Countries*, Loebenstein G., & Thottappilly G., eds. USA: Kluwer Academic Publishers, pp. 1-30.

**Tisserat N. (2003).** Virus Diseases of Tomato: tomato spotted wilt. *www.oznet.ksu.edu/dp -htrr/extension/problems/tomswv.htm*

**Ullman D.E. (1996).** Thrips and tospoviruses: advances and future directions. *Acta Horticulturae* **431**, 310-324.

**Ullman D.E., Cho J.H., Mau R.F.L., Wescot D.M., & Custer D.M. (1992).** A midgut barrier to tomato spotted wilt virus acquisition by adult western flower thrips. *Phytopathology* **82**, 1333-1342.

**Ullman D.E., Sherwood J.L., & German T.L. (1997).** Thrips as vectors of plant pathogens. In: *Thrips as Crop Pests*, Lewis T. (ed.). New York: CAB International.

**Ullman D.E., Whitfield A.E., & German T. L. (2005).** Thrips and tospoviruses come of age: Mapping determinants of insect transmission. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4931-4932.

**Urban L.A., Huang P., & Moyer J. W. (1991).** Cytoplasmic inclusions in cells infected with isolates of L and I serogroups of tomato spotted wilt virus. *Phytopathology* **81**, 525-529.

**Uys M.D.R., Thompson A.H., & Holz G. (1996).** Diseases associated with tomato in the main tomato-growing regions of South Africa. *Journal of South Africa Society of Horticultural Science* **6**, 78-80.

**van den Hurk J., Tas PW L., & Peters D. (1977).** The ribonucleic acid of tomato spotted wilt virus. *Journal of General Virology* **36**, 81-91.

**van Kammen A., Haustra S., & le T.S. (1966).** Morphology of tomato spotted wilt RNA. *Virology* **30**, 574-577.

**van Regenmortel M.H.V., Fraquet C.M., Bishop D.H.L., Carstens E.B., Estes M.K., Lemon S.M., Maniloff J., Mayo M.A., McGeoch D.J., Pringle C.R., & Wickner R.B. (2000).** *Virus Taxonomy: Classification and Nomenclature of Viruses.* San Deigo: Academic Press, pp. 599-619.

**Verkleij F.N., de Vries P., & Peters D. (1982).** Evidence that tomato spotted wilt virus RNA is a positive strand. *Journal of General Virology* **58**, 329-338.

**Voller A., Bartlett A., Bidwell D.E., Clark M.F., & Adams A.N. (1976).** The detection of viruses of enzyme-linked immunosorbent assay (ELISA). *Journal of General Virology* **33**, 165-167.

**Wang M., & Gonsalves D. (1990).** ELISA detection of various tomato spotted wilt virus isolates using specific antisera to structural proteins of the virus. *Plant Disease* **74**, 154-158.

**Wagner E.K., & Hewlett M.J. (1999).** *Basic Virology.* Massachusetts: Blackwell Science, Inc, pp. 294-297.

**Weekes R., Barker I., & Wood K.R. (1996a).** A RT-PCR test for the detection of tomato spotted wilt tospovirus incorporating immuncapture and colorimetric estimation. *Journal of Phytopathology* **144**, 575-580.

**Weekes R.J., Mumford., R.A., Barker I., & Wood K.R. (1996b).** Diagnosis of tospoviruses by reverse transcription polymerase chain reaction. *Acta Horticulturae* **431**, 159-166.

**Wijkamp I., & Peters D. (1993).** Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis*, using a novel leaf disk assay. *Phytopathology* **83**, 986-991.

**Wijkamp I., van Lent J., Kormelink R., Goldbach R., & Peters D. (1993).** Multiplication of tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*. *Journal of General Virology* **74**, 341-349.

**Wijkamp I., Almarza N., Goldbach R., & Peters D. (1995).** Distinct levels of specificity in thrips transmission of tospoviruses. *Phytopathology* **85**, 1069-1074.

# CHAPTER 2

# ISOLATION AND PARTIAL CHARACTERISATION OF A TOMATO SPOTTED WILT VIRUS (TSWV) ISOLATE INFECTING PEPPER (*Capsicum* sp.) IN KWAZULU-NATAL


## ABSTRACT

Leaf samples exhibiting virus-like symptoms and collected from a pepper-(*Capsicum* sp.) growing farm in KwaZulu-Natal were tested for TSWV using the enzyme-linked immunosorbent assay (ELISA). Samples testing positive were mechanically inoculated onto *Nicotinia rustica* L., a propagation host for TSWV. Three weeks post inoculation, typical necrotic TSWV symptoms were observed. Symptomatic leaves were harvested and the virus was partially purified using standard procedures. Under the transmission electron microscope (TEM), typical quasi-spherical and dumbbell-shaped particles of 80-100nm in diameter were observed in negatively stained preparations of both crude and partially purified virus samples. In negatively stained ultra-thin virus-infected leaf sections, an abundance of mature viral particles (100nm) housed in the cisternae of the endoplasmic reticulum (ER) were observed among typical viroplasm inclusions (30nm) and hollow tubules (200-300nm). Viral proteins migrating as a 29kDa band, which correspond to the TSWV nucleocapsid (N) proteins, were observed after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Total plant RNA, isolated from symptomatic *N. rustica*, was subjected to a reverse-transcription polymerase chain reaction (RT-PCR) using primers specific to the nucleocapsid (N) gene of TSWV. An expected 760bp product was amplified. The results obtained in this study confirm the presence of a Tospovirus in infected pepper plants from KZN. The Tospovirus has been positively identified as TSWV.

## 2.1 INTRODUCTION

The production of chilli and sweet pepper (*Capsicum annuum* L.) is a highly lucrative industry worldwide (Bosland & Votava, 2000). In South Africa (SA), KwaZulu-Natal (KZN) province boasts the highest consumption of chilli peppers (Thompson, 1980). This growing demand has spurred an incentive for capsicum production on a commercial scale in KZN (Budnik, 1995).

Viral diseases are a major limiting factor to pepper production worldwide. While some 45 viruses have been reported to infect peppers worldwide (Green & Kim, 1991), Doidge *et al.* (1953) identified tomato spotted wilt virus (TSWV) as one of the more predominant causes of viral disease on peppers in SA.

Reliable and rapid diagnosis of viral infections is essential for differential diagnosis and implementation of effective control measures (Chantler & Clayton, 1988). Plant viruses induce characteristic macroscopic or external symptoms that may be of diagnostic value (Narayanasamy, 2001). Symptoms are the observable effects that a virus has on the growth, development and metabolism of an infected host plant. In the early days of plant virology, symptoms were of major importance, for they were the only means by which a viral disease could be diagnosed and named. This not only accelerates the process of virus identification, but also enables us to avoid confusion of virus-induced symptoms with those caused by other disease agents. In the field symptoms give the first clue to a virus's identity, and in the laboratory the symptoms produced in a range of test plants may often be of considerable diagnostic value (Walkey, 1991).

It has long been known that most plant pathogens possess, as part of their structure, specific antigenic determinants in the form of proteins or other antigenic moieties. Recognition of the diagnostic potential of such determinants for both experimental and applied investigations in plant pathology has resulted in a bewildering array of techniques being developed, which can collectively be referred to as immunoassays

One such assay, ELISA (enzyme-linked immunosorbent assay), developed in the late 1970s, has been widely used by pathologists of all kinds. It has increased tremendously the ability of plant pathologists to detect and study plant viruses and other pathogens and the diseases they cause (Agrios, 2005). Several variations of the ELISA are currently in use. In the double antibody sandwich ELISA, usually referred to as direct ELISA, the wells (capacity 0.4ml) of a microtiter plate are first half-filled with and then emptied of, sequentially, (a) antibodies to the virus, (b) virus preparation or sap from an infected plant, (c) antibodies to the virus to which molecules of a particular enzyme have been attached, and (d) a substrate for the enzyme, that is, a substance that the enzyme can break down and cause a colour change (Agrios, 1983).

Tospoviruses are the only spherical enveloped plant-infecting viruses, thus members of this group can be readily identified by electron microscopy of thin sections of infected plant material or leaf-dip preparations (German *et al.*, 1992). Plant cells infected with Tospoviruses display numerous and variable arrays of viral inclusions. Cytopathological studies on ultra-thin sections of infected plant cells reveal the presence of numerous virus particles (Kitajima, 1965; Milne, 1970; Ie, 1971). In addition, the presence of viroplasms, hollow tubules and filamentous material is characteristic of a Tospovirus infection (Francki & Hatta, 1981). These characteristic cytopathological features reflect the complexity of the viral genome and particle structure (Lawson *et al.*, 1996). As additional Tospovirus species and isolates are identified, new variations in symptomatology and cytopathology can be expected (Urban *et al.*, 1991; Lawson *et al.*, 1996).

Proteins and nucleic acids are major structural components of most viruses (Bos, 1999). Therefore, for complete characterisation and identification of any new virus, it is necessary to analyse its nucleic acid and protein components (Walkey, 1991). Such information is imperative in establishing a basic idea of the virus's genetic complexity. Electrophoresis in either a polyacrylamide (PAGE) or agarose gel is now a widely used procedure for estimating the molecular weight of the viral nucleic acids

or protein, by reference to the mobilities of standard nucleic acid or protein of known molecular weights (Hull, 2001). Viral nucleic acid hybridisation techniques have been developed for their use in the detection and diagnosis of Tospoviruses (Mumford *et al.*, 1996). The reverse transcriptase polymerase chain reaction (RT-PCR) is an effective new technique for detecting small amounts of plant viral nucleic acids (German *et al.*, 1992).

For devising effective and sustainable control measures, it is important that the viruses present in the particular geographic area are accurately identified and properly characterised (Bosland & Votava, 2000). Many viruses infecting peppers in SA have not been identified or characterised. The objective of the present study was to positively identify a Tospovirus infecting peppers in KZN using symptomatology, electron microscopy, protein banding patterns and amplification of the N gene using RT-PCR. Our results show that the virus isolated from peppers is TSWV.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Virus Isolation, Detection and Propagation

#### *2.2.1.1 Field survey and inoculum collection*

Leaf samples exhibiting typical TSWV-like symptoms were collected from pepper-(*Capsicum* sp.) growing farms in KwaZulu-Natal. The samples were placed in plastic bags, labelled and stored at -80°C.

#### *2.2.1.2 Detection of TSWV by ELISA*

The leaf samples were tested for the presence of TSWV using a standard double antibody sandwich-(DAS) enzyme-linked immunosorbent assay (ELISA) (Clark & Adams, 1977). A Plantest TSWV ELISA kit (Bio-Rad laboratories, Phyto-Diagnostics,

France) was used in this test. Positive controls were rehydrated in 1ml distilled water whilst negative controls consisted of crude sap from uninoculated, healthy control plants. The buffers used are shown in Appendix A and dilution of antibodies was performed according to the manufacturers' instructions.

An ELISA plan was used to set out the ELISA procedure (Fig. 2.1A). Each well was coated with 100µl of coating antibody, diluted 1/250 with coating buffer. The plate was then incubated at 37°C for two hours. After incubation, the plate was washed three times in PBS-Tween buffer. The washing procedure consisted of the following:

(1) excess liquid was flicked out of the plate
(2) wells were then filled with PBS-Tween buffer
(3) the plate was allowed to stand for 3min
(4) liquid was flicked out of the plate
(5) the plate was then 'spanked' on a wad of paper towels until the plate contained no more droplets.

Samples were prepared by grinding a small piece of the leaf material in 500µl of sample extraction buffer. The samples were ground in a 1.5ml eppendorf tube using an AEG percussion drill (ACC, SA) fitted with sterile plastic drill bits. Between samples, bits were replaced and used bits were washed with soapy water.

A volume of 100µl of each leaf extract, positive and negative control was then added to the designated wells, according to the ELISA plan (Fig. 2.1A). Plates were incubated overnight at 4°C. After incubation, plates were emptied and washed as before using PBS-Tween buffer.

Each well was then coated with 100µl of enzyme-conjugated antiserum, diluted 1/250 with enzyme-conjugate buffer. The plate was then incubated at 37°C for 2 hours. After incubation, the plate was washed using PBS-Tween buffer as previously described. A p-Nitrophenyl phosphate (pNPP) substrate solution was prepared at a

concentration of 1mg/ml in substrate buffer and 100ul of this solution was added to each well. Plates were then incubated at 37°C for 30min and then at room temperature for 15min. Wells were observed for the development of a yellow colour which is indicative of a positive reaction.

#### *2.2.1.3 Virus propagation*

*Test plants*

*Nicotinia rustica* L. seeds were sown in seedling mix (Growmor, Cato Ridge). Three weeks post germination, seedlings were transplanted into individual pots. Transplants were then grown for a further two weeks before inoculation. Plants were kept at a constant temperature of 20-25°C in the Jolly Roger tunnel, University of KwaZulu-Natal, Pietermaritzburg (PMB) for the duration of the study.

*Inoculum preparation*

Samples testing positive for the presence of TSWV in the ELISA procedure were used as inoculum in the mechanical inoculation of *N. rustica*, a propagation host for the virus. Frozen infected leaf samples collected from the field were ground in freshly prepared ice-cold 0.5M potassium phosphate buffer (pH 7.0) using a chilled pestle and mortar. The crude sap was maintained on ice until the inoculation procedure was completed.

*Mechanical inoculation*

Mechanical inoculation of test plants was performed as described by Mandal *et al.* (2001), with modification. The mortars and pestles were maintained in a freezer at -20°C overnight, before use. In addition to adding 500-mesh caborundum to the inoculum, fully expanded leaves of test plants were also dusted with caborundum. The chilled sap was then rubbed onto the surface of the primary leaves to expose cell damage so that the virus may enter the plant via damaged plant cells. Leaves were then rinsed with chilled distilled water to facilitate healing of the wounded leaves. The inoculum was replaced with freshly ground leaf tissue every 10min to ensure virus

viability. Control plants were mock inoculated with inoculation buffer. All inoculated plants were maintained in the tunnel and observed for virus symptom development.

### 2.2.2 Virus Purification

#### *2.2.2.1 Purification procedure*

The virus was purified according to the method described by Gonsalves & Trujillo (1986) with modifications as described by Dijkstra & de Jager (1998). *Nicotinia rustica* L. leaves showing prominent symptoms were used as the virus source during the purification procedure. Samples collected after the partial and further purification procedures were stored at 4°C. The entire purification procedure was carried out at 4°C in pre-cooled lab-ware.

*Partial purification*

A total of 136.51 grams of freshly harvested symptomatic *N. rustica* leaves were ground in a Waring blender in 409.53ml of extraction buffer A (0.1M potassium phosphate, 0.01M $Na_2SO_3$, pH 7.0; 3ml/g). The homogenate was then filtered through muslin cloth, which had been pre-boiled for 30min in distilled water containing some $Na_2$-EDTA. The filtrate was then centrifuged at 6500g for 15min in a Bechman J2 HS centrifuge using a JA-10 rotor. The supernatant was discarded and the resulting pellet was gently and thoroughly resuspended in 136.51ml of resuspension buffer A (0.01M $Na_2SO_3$; 1ml/g). The suspension was allowed to set for 30min before it was centrifuged at 6500g for 15min. The resulting supernatant was then centrifuged at 73500g for 30min in a Bechman Coulter Optima™ L-90K ultracentrifuge using a SW 40 Ti rotor. The pellet was gently resuspended in 3.4ml of resuspension buffer A (2.5ml/100g) and centrifuged at 8000g for 15min in a Bechman J2 HS centrifuge using a JA-10 rotor. The crude preparation was stored at 4°C.

*Further purification*

The crude extract was further purified on a 10 and 40% sucrose cushion and centrifuged at 73000g for 90min in a Bechman Coulter Optima™ L-90K ultracentrifuge using a SW 40 Ti rotor. The resulting pellet was resuspended in a small amount of resuspension buffer A then layered onto 2ml gradients of a 10-40% sucrose gradient in resuspension buffer A using a micropipette. The suspension was then centrifuged at 75000g for one hour in a Bechman Coulter Optima™ L-90K ultracentrifuge using a SW 40 Ti rotor. The virus band was collected using a syringe and transferred to a fresh Eppendorf tube. All samples were stored at 4°C.

### *2.2.2.2 Detection of TSWV in purified samples*

*ELISA*

Samples collected after the partial and further purification procedures were tested for the presence of TSWV using a standard double antibody sandwich-(DAS) enzyme-linked immunosorbent assay (ELISA) (Clark & Adams, 1977). The procedure was done as previously described in section 2.2.1.2 with the omission of the sample preparation step.

*Electron microscopy*

Partially purified samples were removed from the storage at 4°C and maintained on ice prior to use. A drop of each sample was placed onto formvar-coated grids and allowed to stand for 30s. Excess liquid was removed by touching the edge of a filter paper wedge under each grid. Grids were negatively stained with 3% uranyl acetate for 30s. Excess stain was slowly removed with the edge of a filter paper wedge. The grids were then viewed under the Jeol 100 CX transmission electron microscope (TEM).

### 2.2.3 Identification of TSWV using Electron Microscopy

To verify the presence of TSWV in propagation host material, crude leaf sap and embedded infected leaf tissues were viewed under the TEM.

#### *2.2.3.1 Leaf-dip method*

A small section of infected leaf material was cut into a 5mm by 5mm section. The crude virus sap was prepared by macerating a leaf section in a drop of distilled water. Formvar-coated grids were then placed on the crude sap and allowed to stand for 30s. Excess liquid was removed from the grid using the edge of a filter paper wedge. Grids were negatively stained with 3% uranyl acetate for 10s. Excess stain was slowly removed with the edge of a filter paper wedge. Leaf material taken from healthy mock-inoculated plants was used as a control. The grids were then viewed under the Jeol 100 CX TEM.

#### *2.2.3.2 Tissue embedding and ultra-thin sectioning*

A standard procedure provided by the Electron Microscopy Centre, University of KwaZulu-Natal (PMB) was followed. The detailed steps are described in Appendix B. Leaf slices (2mm by 2mm) from chlorotic spots of *N. rustica* infected with TSWV were prefixed for 48h in 3% glutaraldehyde in 0.05M sodium cacodylate buffer (pH 6.88), washed twice in that buffer and then postfixed with 2% osmium tetroxide in 0.05M sodium cacodylate buffer (pH 6.88) for 1.5h. After dehydration with a graded ethanol series (10-100%) for a minimum of 10min per solution, the samples were imbedded in Epon-Araldite resin. Ultra-thin sectioning was performed with glass knives mounted on a microtone. Sections were placed on 200-mesh copper grids, stained with 2% uranyl acetate and viewed under a Jeol 100 CX TEM. All images were captured using a digital MegiViewBIII camera.

### 2.2.4 Analysis of Viral Proteins using SDS-PAGE

#### *2.2.4.1 SDS-PAGE procedure*

Viral proteins of partially purified virus preparations were dissociated and separated by electrophoresis in a 12.5% sodium dodecyl sulphate (SDS) polyacrylamide gel, using a Hoefer SE 250 Mighty Small II vertical slab electrophoresis unit, as described by Laemmli (1970). Stock solutions used in the gel preparation and staining are described in Appendix C. Volumes of solutions used to prepare the running and stacking gel are given in Table 2.1. Virus proteins were dissolved in reducing treatment buffer (Solution G) and heated for 2min in boiling water prior to loading onto the gel. A Benchmark pre-stained protein marker (Life Technologies, UK) was used as the molecular weight marker (MWM). Tobacco mosaic virus (TMV) coat protein was run as a control. Proteins were visualised by Coomassie blue staining.

#### *2.2.4.2 Coomassie blue staining*

Following SDS-PAGE, gels were placed in the Coomassie staining solution overnight with slight agitation in a Red Rotor automated shaker (Hoefer Scientific, USA). The staining solution was removed and the gel was placed into Destain I for four hours and then into Destain II until the gel background was clear. The gel was photographed using a gel documenting system (UVP's GDS 5000).

**Table 2.1** Reagents used in the preparation of Laemmli running and stacking gels.

| Reagent | Volume (ml) | |
|---|---|---|
| | Running gel (12.5%) | Stacking gel (4.0%) |
| A (Monomer solution) | 6.25 | 0.94 |
| B (4x Running gel buffer) | 3.75 | 0 |
| C(4x Stacking gel buffer) | 0 | 1.75 |
| D (10% (w/v) SDS) | 0.15 | 0.07 |
| E (10% (w/v) APS) | 0.075 | 0.035 |
| Distilled water | 4.75 | 4.3 |
| TEMED | 0.0075 | 0.015 |

### 2.2.5 Detection of TSWV by RT-PCR

#### *2.2.5.1 Total plant RNA isolation*

Total RNA was extracted from symptomatic *N. rustica* plant material according to the method of Napoli *et al.* (1990). Fresh leaf material was ground into a fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a sterile 2ml Eppendorf tube containing 150µl of phenol (saturated with Tris buffer pH 7.5) and 500µl of solution R (100mM NaCl, 10mM Tris-HCl, 1mM EDTA, 1 (w/v) % SDS). The solution was vortexed after the addition of 250µl chloroform: isoamyl alcohol (24:1). Vortexing was done twice. The mixture was centrifuged at 14000 rpm at 4°C for 3min in a Bechman J2-21M centrifuge and the upper aqueous phase transferred to a sterile Eppendorf tube. An equal volume of 4M lithium acetate was added and tubes were thoroughly mixed before incubation on ice for 24h to allow for RNA precipitation. The tubes were centrifuged at 14000 rpm at 4°C for 15min and the supernatant discarded. The RNA pellet was resuspended in 90µl DEPC-treated water. A volume of 10µl of 3M sodium acetate (pH 5.2) and 250µl of 100% cold ethanol was added to the resuspended pellet. The mixture was then centrifuged at 14000 rpm at 4°C for 15min and the supernatant was discarded. The pellet was air-dried in a fume-hood for

10min and then resuspended in 35µl of DEPC-treated water. Total RNA was also extracted from leaf samples of uninfected healthy control plants. All RNA preparations were stored at -80°C.

### *2.2.5.2 Quantification of total RNA by spectrophotometry*

The quantity of total extracted RNA was measured using a Beckman DU600 spectrophotometer. RNA samples were diluted 1:100 in DEPC-treated water. The optical density at $A_{260}$ and $A_{280}$ were determined. The ratio between the readings at $A_{260}$ and $A_{280}$ ($A_{260}/A_{280}$) provided an estimate of RNA purity. Pure preparations of RNA are regarded at ratios from 1.9. The RNA concentration (ug/ml) was calculated as follows:

$$A_{260} \text{ x dilution factor x } 50 = z \text{ ug/ml}$$

### *2.2.5.3 Analysis of total RNA by agarose gel electrophoresis*

The integrity of RNA was examined on a 1% (w/v) agarose gel containing formaldehyde prepared in 1X formaldehyde gel-running buffer (0.1M MOPS (pH 7.0), 40mM sodium acetate, 5mM EDTA (pH 8.0)) and stained with ethidium bromide (1ug/ml). Samples were prepared by adding 10-15ug RNA to a mixture consisting of 2µl 5X formaldehyde gel-running buffer, 3.5µl formaldehyde, 10µl formamide and 2µl formaldehyde gel-loading buffer (50% (v/v) glycerol, 1mM EDTA (pH 8.0); 0.25% (w/v) bromophenol blue, and 0.25% (v/v) xylene cyanol FF). Electrophoresis was done at 100V for 90min. RNA bands were visualised under an ultraviolet (UV) transilluminator and photographed using a gel documenting system (UVP's GDS 5000).

### *2.2.5.4 RT-PCR of total plant RNA*

Total RNA from infected leaf material of *N. rustica* was used as a template for amplification in the reverse transcription polymerase chain reaction (RT-PCR)

procedure. RT-PCR was performed using a TITANIUM™ One-Step RT-PCR Kit (Clontech,CA) according to manufacturers' instructions. The primer pair derived from the nucleocapsid protein (N) gene sequence of TSWV was used and an amplification product of 760bp was expected (Pang *et al.*, 1992):

JLS90-46 5'-AGCTAACCATGGTTAAGCTCACTAAGGAAAGC-3' (Forward)
JLS90-47 5'-AGCATTCCATGGTTAACACACTAAGCAAGCAC-3' (Reverse)

Amplification was performed in an automated thermal cycler programmed for one cycle of 50°C for 1h and 94°C for 5min and 30 cycles of amplification with 30s of denaturation at 94°C, 30s of annealing at 65°C and 1min of extension at 68°C followed by one cycle of final extension for 2min at 68°C. Negative controls consisted of DEPC-treated water and total RNA extracted from an uninfected healthy control plant. An amplified 540bp mouse ß-actin fragment, provided in the kit, was used as the positive control reaction.

#### *2.2.5.5 Analysis of RT-PCR products by agarose gel electrophoresis*

Amplified products of the RT-PCR was examined on a 1% (w/v) agarose gel prepared in 1X TAE buffer (Promega, USA) and stained with ethidium bromide (1ug/ml). Samples were prepared by adding 10µl product to 2µl DNA loading dye (Promega, USA). The GeneRuler™ 100bp DNA ladder (Fermantas, UK) was used as a MWM. Electrophoresis was done at 100V for 90min. DNA bands were visualised under an ultraviolet (UV) transilluminator and photographed using a gel documenting system (UVP's GDS 5000).

## 2.3 RESULTS

### 2.3.1 Detection of TSWV by ELISA

Leaf samples collected from pepper growing farms in central KZN tested positive for the presence of TSWV using ELISA. Yellow colour developments, which are indicative of a positive reaction, were observed in wells containing the leaf sample sap (Fig. 2.1B). The negative control yielded no reaction whilst the positive control gave a yellow colour reaction.

### 2.3.2 Symptomatology

*Nicotinia rustica* L. plants inoculated with crude sap from ELISA-positive samples developed a typical sequence of TSWV-like symptoms 15 days post inoculation. Chlorotic spots, followed by necrotic spots and vein necrosis (Fig. 2.2A/B) were observed on uninoculated leaves. This resulted in stem necrosis (Fig. 2.2C), severe necrosis, wilting and dieback of young leaves (Fig. 2.2D). When compared to healthy uninfected control plants, infected plants appeared stunted and wilted (Fig. 2.3). These observations in addition to the positive reaction seen in ELISA, confirm the identity of the virus as being TSWV.

TEST NO.: 01 DATE: 17-05-2004 VIRUS: TSWV

Coating--$A_s$--ID:_____ Concn.: 1/126 Time: 2 hrs Temp: 37°

Antigen Prep: Pepper leaves in extraction buffer Time: 24hrs Temp: 4°C

$A_s$ Conjugate ID:_____ Concn.: 1/125 Time: 2hrs Temp: 37°C

Substrate: Type: PNPP Concn.: 1mg/ml Time: 15min Temp: 37°C

Plate Type:_____

PLAN

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | +ve control | | | | sample 1 | | | Buffer ↓ | | |
| D | | | | | | | | | | | | |
| E | | | -ve control | | | | sample 2 | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

$OD_{405}$

RESULT

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | + | | | | + | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | + | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

$OD_{405}$

**(A)**

**(B)**

**Figure 2.1** Enzyme-linked immunosorbent assay (ELISA), used to detect the presence of the tomato spotted wilt virus (TSWV) in leaf samples collected from a pepper-growing farm in KwaZulu-Natal. (A) Plan and layout of ELISA plate including controls (+ve control = positive control, -ve control = negative control). (B) Photographic representation of the ELISA plate. Positive reactions are indicated by a yellow colour.


**Figure 2.2** Typical Tospovirus symptoms seen on uninoculated leaves of a *Nicotinia rustica* L. plant, a propagation host mechanically inoculated with those leaf samples testing positive for tomato spotted wilt virus (TSWV) in the enzyme-linked immunosorbent assay (ELISA). (A/B) necrotic spots and vein necrosis. (C) Stem necrosis. (D) severe necrosis, wilting, and dieback of young leaves.

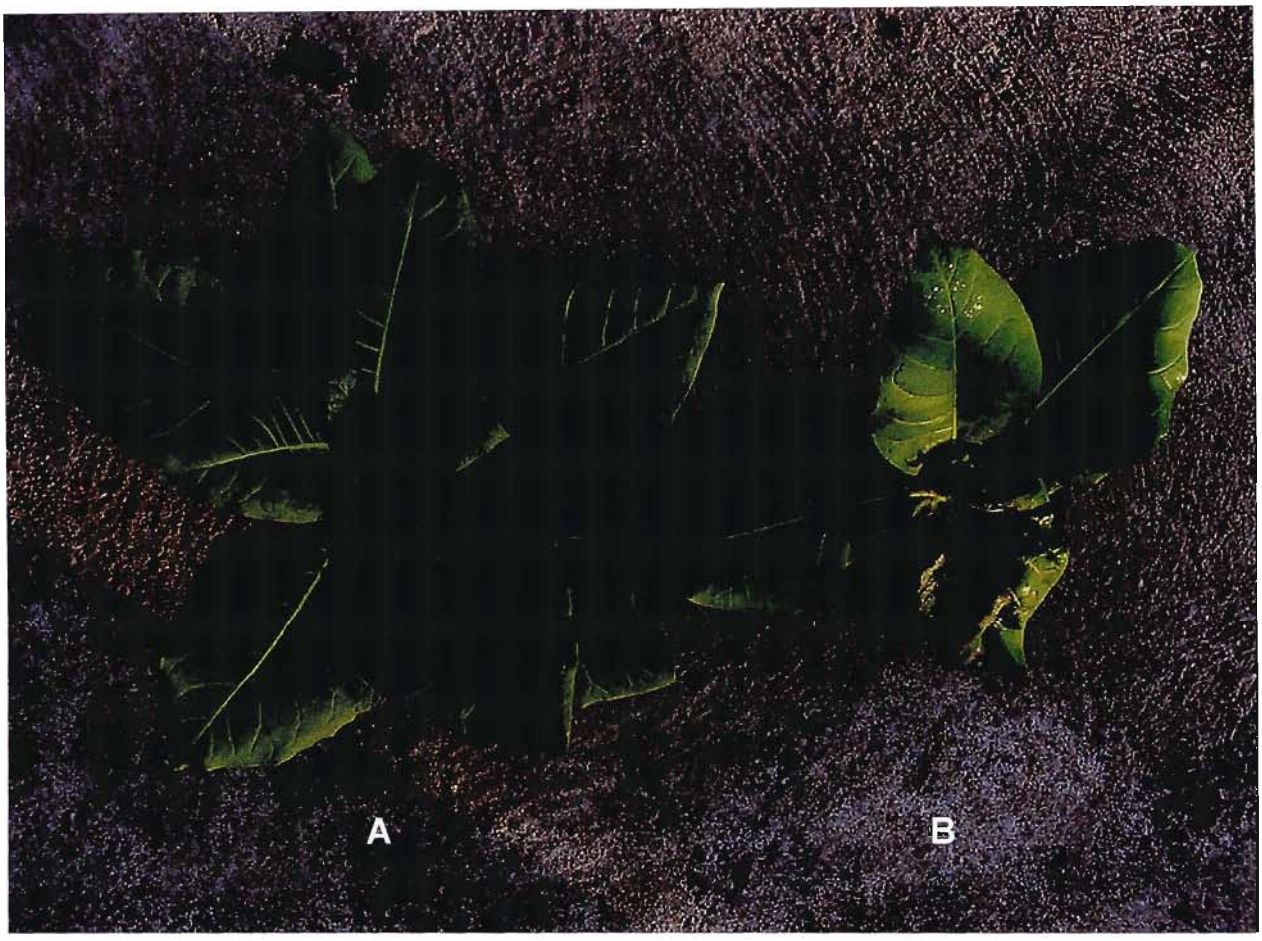



**Figure 2.3** Comparison of (A) healthy uninfected control plant with (B) tomato spotted wilt virus (TSWV) infected plant. Infected plant appears stunted and wilted.

### 2.3.3 Identification of TSWV using Electron Microscopy

The partially pure samples taken before subjection to the further purification procedure tested positive for TSWV using ELISA. Samples consisting of viral bands collected after the final purification procedure tested negative for the presence of TSWV. This indicates that TSWV was only partially purified from infected leaf material.

Under the TEM, typical spherical TSWV-like particles (80-110nm) were observed in negatively stained preparations of crude (Fig. 2.4 A/B) and partially purified (Fig. 2.4C/D) preparations from symptomatic *N. rustica* leaves. A few particles appeared distorted giving a characteristic 'dumbbell' or germinate form. There was clear evidence of the presence of an outer viral envelope, a key distinguishing feature of Tospoviruses (Fig. 2.4B). No virus particles were observed in samples subjected to the further purification procedure or negative controls.

In negatively stained ultra-thin sections of infected plant material, typical mature virus particles measuring 100nm in diameter were observed in clusters surrounded by enveloping membranes, which may be the cisternae of the endoplasmic reticulum (Fig. 2.5A). These structures were present only in the cytoplasm and not in nuclei, chloroplasts or mitochondria. Dense amorphous masses known as viroplasms measuring 30nm in diameter occurred in tight association with mature virion clusters (Fig. 2.5B). Singular hollow tubule-like particles (200-300nm) were observed rarely (Fig. 2.5C). All structures are typical of TSWV cytopathology. No comparable structures were found in leaf sections of healthy control plants (Fig. 2.5D).

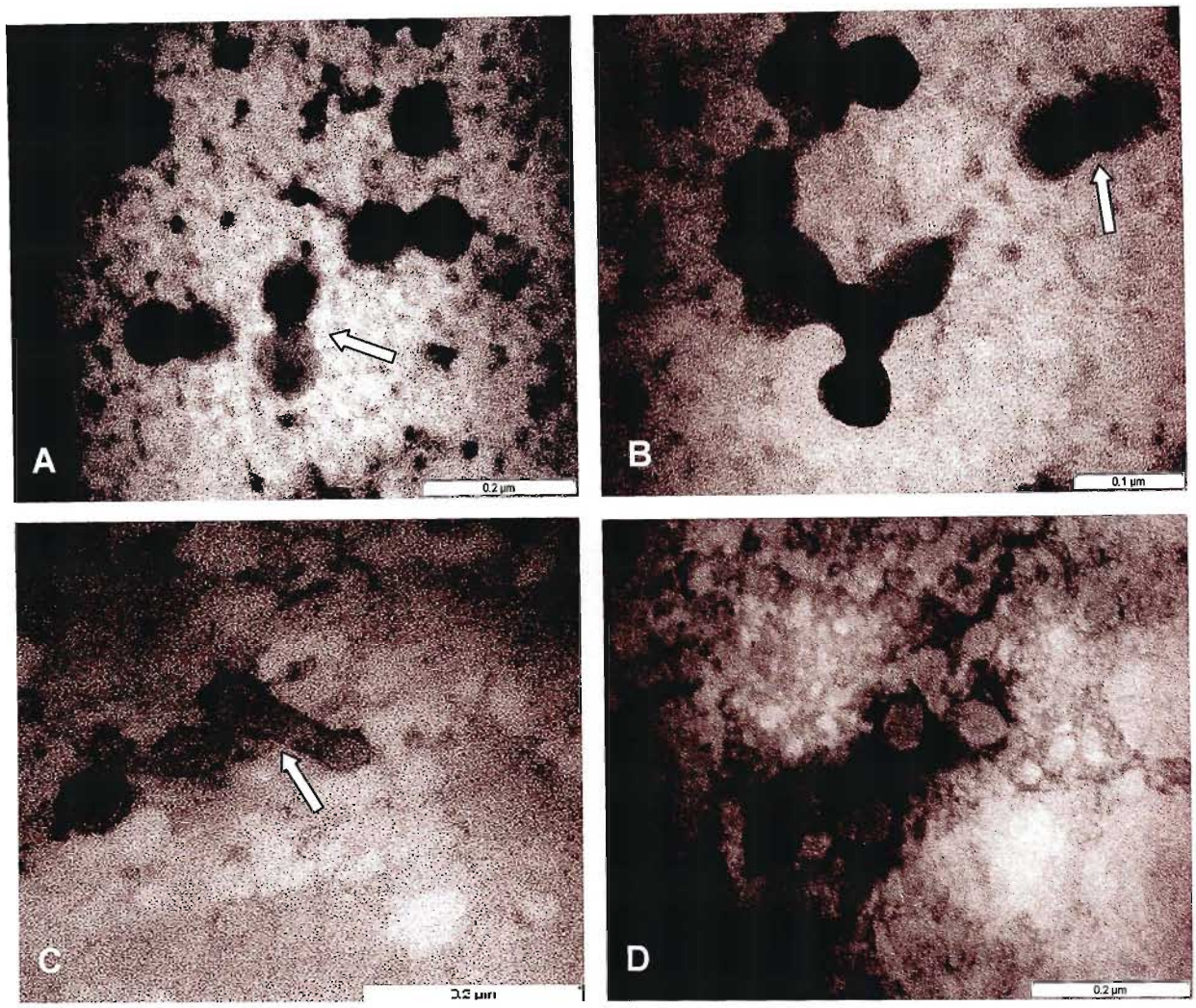



**Figure 2.4** Transmission electron micrographs of tomato spotted wilt virus (TSWV) particles in (A/B) negatively stained crude leaf sap from infected *Nicotinia rustica* L. plants and (C/D) negatively stained partially purified preparations. The characteristic outer-envelope of TSWV is visible in B. Arrow indicates the presence of a 'dumbbell' like particle.

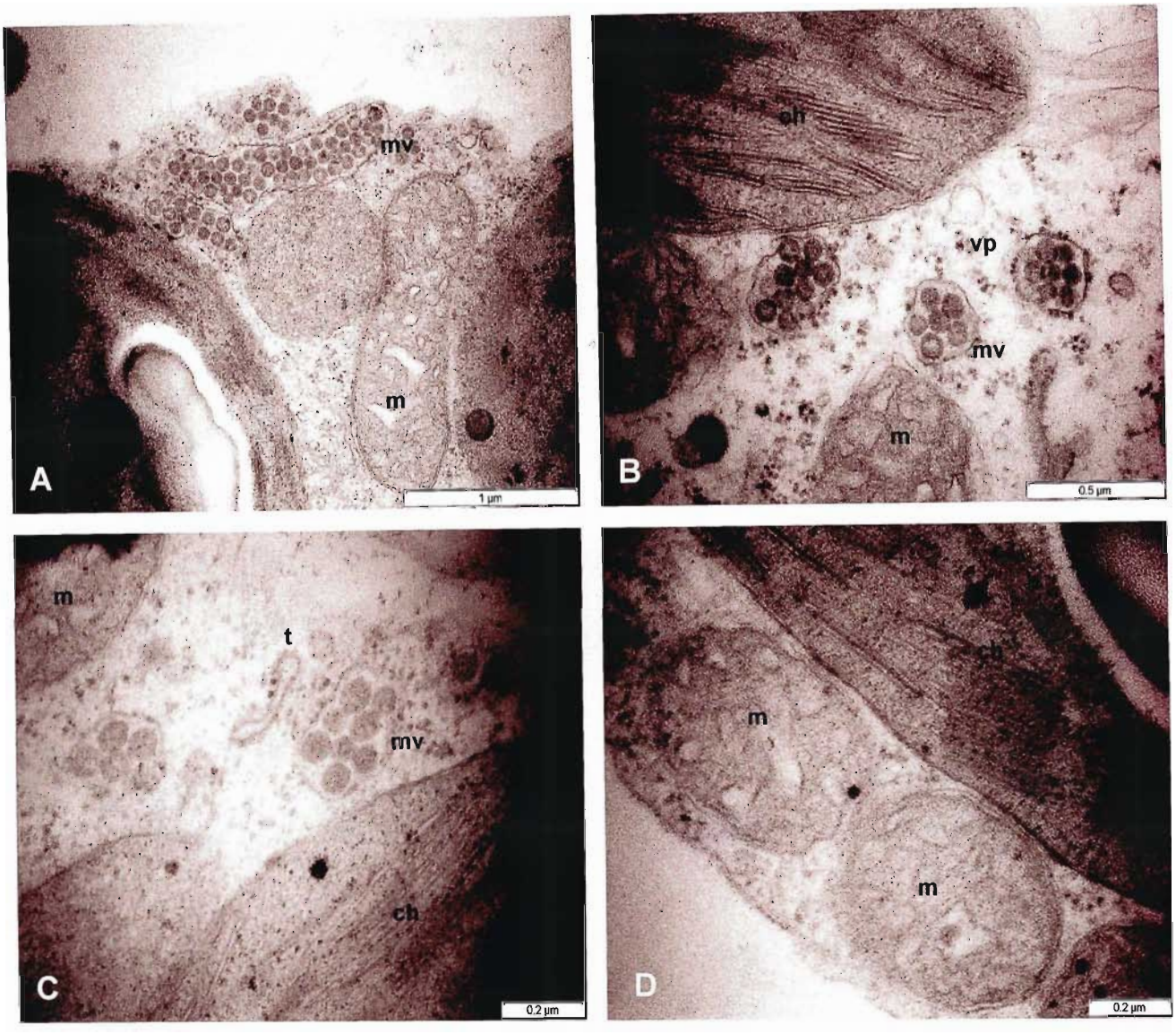



**Figure 2.5** Transmission electron micrographs of negatively stained ultra-thin leaf sections from *Nicotinia rustica* L. plants infected with the tomato spotted wilt virus (TSWV). (A) An abundance of TSWV mature virus particles (mv), housed in membrane-bound cisternae of the endoplasmic recticulum. (B) Typical viroplasm (vp) inclusion bodies scattered amongst mature TSWV particles (mv). (C) Hollow tubule structures (t). (D) Control leaf section taken from a healthy uninoculated plant. ch = chloroplast, m = mitochondrion.

### 2.3.4 Analysis of Viral Proteins by SDS-PAGE

The procedure revealed the migration of numerous bands, one of which corresponds to a typical TSWV protein. Viral protein migrated as a 29kDa band, which corresponds to the TSWV nucleocapsid (N) protein. The molecular weight of the control TMV coat protein was found to be 17.8kDa (Fig. 2.6).

### 2.3.5 Analysis of total RNA by agarose gel electrophoresis

The integrity of total plant RNA isolations was confirmed by the characteristic presence of three RNA bands (Fig. 2.7).

### 2.3.6 RT-PCR of total plant RNA

An amplification product of 760bp was detected after agarose gel electrophoresis of RT-PCR of the total plant RNA isolations. The positive control yielded an expected 540bp band whilst negative controls showed no products (Fig. 2.8).

## 2.4 DISCUSSION

Our results show that the identity of the virus isolated from pepper exhibiting virus-like symptoms in KZN is TSWV. Although TSWV is present as a major limiting factor to pepper production in the rest of SA, no reports have been made of its infection on pepper in KZN (Thompson, 1980; Budnik *et al.*, 1996). This is the first report of TSWV on pepper in KZN.

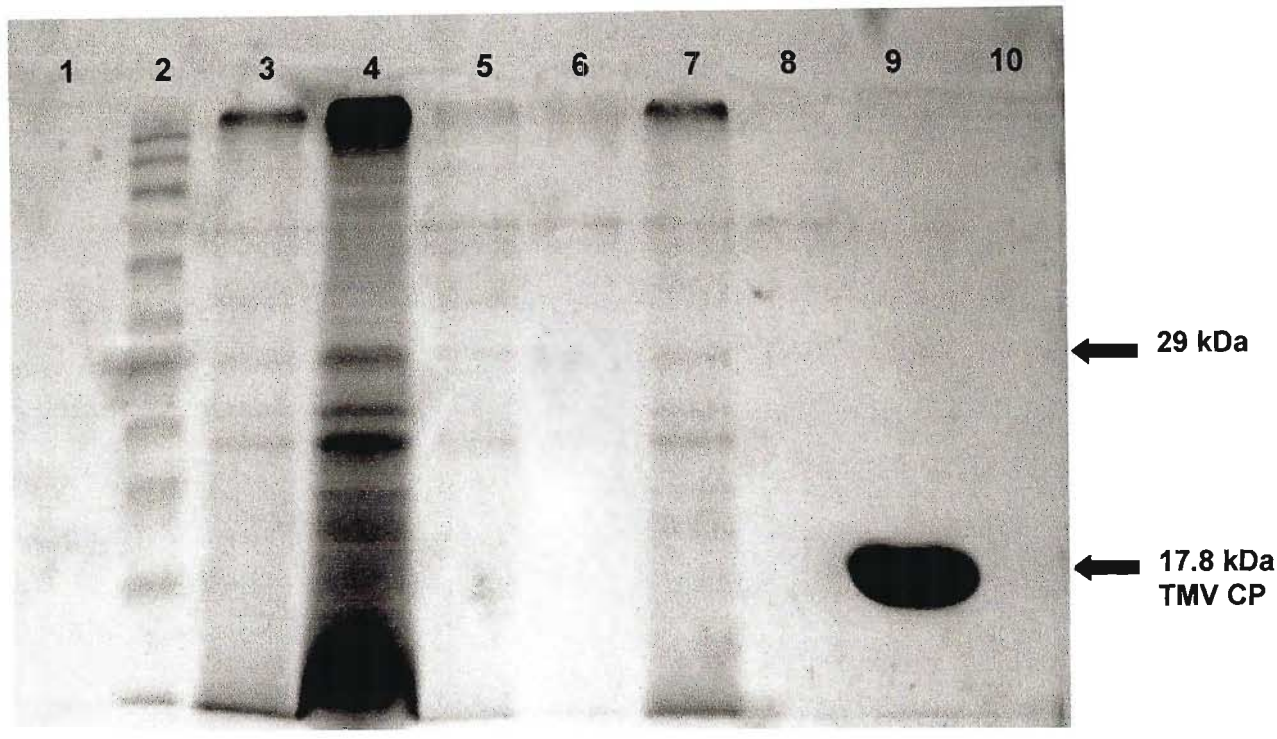



**Figure 2.6** Coomassie stain of partially purified preparations of the tomato spotted wilt virus (TSWV) on a 12.5% (w/v) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Band corresponds to the 29kDa nucleocapsid (N) protein. The control tobacco mosaic virus (TMV) coat protein (CP) migrated as a 17.8kDa band. Lanes 1 and 10 are blank. Lane 2 shows the protein molecular weight marker. Lanes 3 to 5 show partially purified samples. Lanes 6 to 8 show diluted (1:5) samples. Lane 9 shows the 17.8kDa TMV CP.

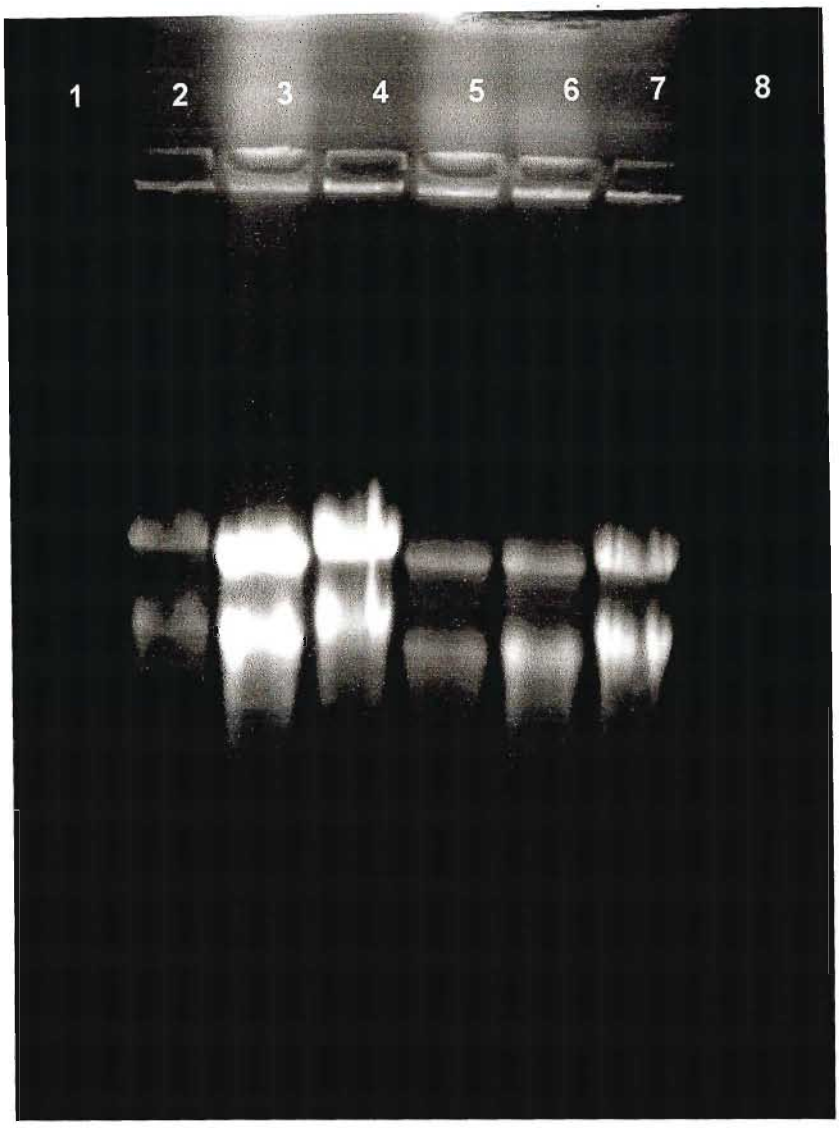



**Figure 2.7** Agarose gel containing formaldehyde, showing integrity of total plant RNA isolated from *Nicotinia rustica* L. inoculated with the tomato spotted wilt virus (TSWV). Lanes 1 and 8 are blank. Lanes 2 to 6 show total RNA samples, isolated from symptomatic *N. rustica*. Lane 7 shows total RNA isolated from healthy control *N. rustica* plants.

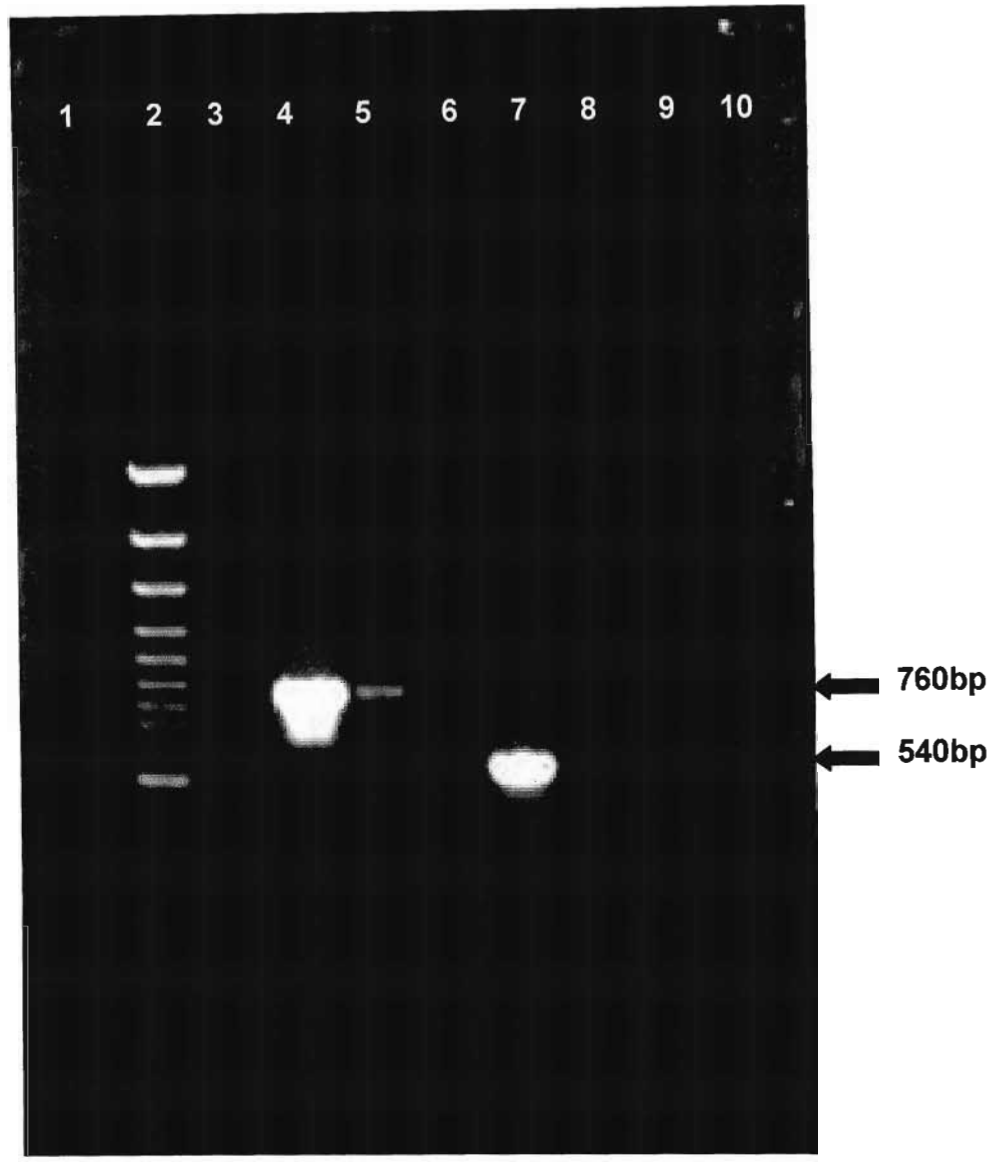



**Figure 2.8** Agarose gel showing the reverse-transcription polymerase chain reaction (RT-PCR) product of total plant RNA extracted from *Nicotinia rustica* L. infected with tomato spotted wilt virus (TSWV). Lanes 1, 3, 6 and 10 are blank. Lane 2 shows the molecular weight marker. Lanes 4 and 5 show the 760bp amplification products of the total RNA sample. Lane 7 shows the positive mouse liver (540bp) control reaction. Lanes 8 and 9 show the negative control reactions which contain water and total plant RNA from healthy uninfected plants respectively.

The presence of TSWV in pepper leaf samples from infected field was confirmed using DAS-ELISA (Fig. 2.1). Typical TSWV-like symptoms were observed on the propagation host *N. rustica*, after inoculation with ELISA positive samples (Fig. 2.2). The predominant symptoms of vein and stem necrosis, observed on *N. rustica,* are described (Best, 1968; Francki & Hatta, 1981; German *et al.*, 1992) as being typical of a TSWV infection.

Samples collected after the partial purification procedure tested positive for the presence of TSWV whilst samples collected after the further purification tested negative. This would indicate that the virus was conserved throughout the partial purification procedure but lost after being subjected to the further purification steps. An explanation for the failure of the further purification procedure could be due to the fact that partially purified preparations were stored at 4°C before being subjected to the further purification. Numerous authors have noted the instability of the virus. Black *et al.* (1963) observed that purification schemes requiring more than 8 to 10 hours seldom gave good results. After such time, the virus is subject to oxidation and subsequent degradation (Best, 1968). It can therefore be concluded that a substantial amount of virus was lost during the storage period which contributed to the absence of viral particles in the samples taken from steps in the further purification procedure.

Electron microscopy of negative stained preparations revealed the presence of typical spherical TSWV-like particles in crude (Fig. 2.4A/B) and partially pure (Fig. 2.4C/D) viral suspensions. Distortion in particle shape was also observed where particles occurred as 'twins', to form a dumbbell-like figure consisting of two particles joined by a connecting tube of variable length (Fig. 2.4). In some instances these forms seem to appear coalesced, forming quadruplet and septuplet arrangements (Fig. 2.4B). This occurrence has been noted by numerous authors who attribute this manifestation to an artifact of the negative staining procedure (Best, 1968; Joubert *et al.*, 1974; Francki & Hatta, 1981; Matthews, 1981). Black *et al.* (1963) and Martin (1964) suspected that the variation in particle shape and arrangement is due to the

characteristic instability of the virus. The presence of these forms is typical of TSWV. Another key distinguishing factor of all Tospoviruses is the presence of the double-outer envelope that surrounds the virus particle. The presence of these membrane-bound particles was shown (Fig. 2.4B). All observations point to the identity of the virus as being TSWV.

The cytopathological structures seen in section are characteristic of TSWV. Mature virion particles (Fig. 2.5A-C) were relatively uniform and occurred exclusively in the cisternae of the ER. Viroplasms (Fig. 2.5B), typical products of TSWV replication, were also observed in tight association with mature virion clusters. Viral-induced movement structures known as tubules were observed near mature virion clusters (Fig. 2.5C). The location, size and form of all cytopathological structures are characteristic to TSWV and could not be mistaken for any other virus (Milne, 1970; Ie, 1971; Francki & Hatta, 1981).

The formation of filamentous structures was not observed in ultra-thin sections of infected plant material. Although its presence could have contributed to the evidence supporting the presence of TSWV, its absence can be explained. Lawson *et al.* (1996) observed that the formation of the filamentous structures in infected tissue is quite variable, which has led to speculation that development might depend on the stage of infection and the host. Samples collected from the same plant at different times may contain filamentous inclusions at one time, but not at others. The NSs proteins which make up the fibrous structures in infected cells are thought to be produced only in the latter stages of infection. It follows that since leaves were collected in the initial stages, one would not expect the presence of such structures (Kormelink *et al.*, 1991).

Electrophoresis of a partially purified preparation of TSWV in the SDS-PAGE analysis revealed the migration of numerous bands, one of which corresponds to typical TSWV 29kDa nucleocapsid protein (N) (Fig. 2.6). The migration of this band has been noted by numerous authors who have carried out similar protein analysis

experiments (Mohamed *et al.*, 1973; Tas *et al.*, 1977; Law & Moyer, 1990). The result confirms the presence of TSWV in partially purified preparations. The migration of other bands is probably due to the partially pure nature of the virus preparation. Without further purification, many plant constituents may still be present in the virus preparation. These constituents may be of a proteinaecous nature and may have migrated as bands in the SDS-PAGE analysis. In addition, proteolytic degradation, which is enhanced when the virus is kept in prolonged contact with plant sap, is probably responsible for the migration of numerous bands (van Regenmortel, 1982).

The integrity of total RNA, isolated from infected and uninfected *N. rustica* plants, was confirmed in a formaldehyde agarose gel (Fig. 2.7). The presence of three characteristic RNA bands indicated that the total RNA procedure was successful and that total RNA samples was deemed fit to be subjected to RT-PCR.

Total RNA from healthy and symptomatic *N. rustica* plants was successfully subjected to RT-PCR. The procedure revealed the amplification of an expected 760bp product from total RNA extracted from infected plants and the lack of amplification in total RNA extracted from healthy control plants (Fig. 2.8). This confirms the presence of TSWV in symptomatic *N. rustica*.

The results of a single test by itself cannot provide a reliable means of virus identification. However, when our results are taken together, they complement each other to positively identify a virus. In this study: the positive reaction in ELISA; characteristic symptomatology; size and shape of virus particles; and cytopathological effects seen under the electron microscope; protein analysis and the amplification of the N gene using RT-PCR, all provide compatible evidence that confirms the identity of the virus infecting pepper as being TSWV.

In a survey of pepper-infecting viruses in KZN, Budnik *et al.* (1996) identified the presence of TSWV at a low incidence in some weeds, but not on peppers. They predicted that given time, TSWV could become an important viral disease of peppers

in KZN. From recent surveys conducted in this study, TSWV is now a widespread problem in pepper production in KZN.

Developing a proper control strategy for a viral disease requires early and correct virus identification. In such a characterisation study, fundamental knowledge is gained on virus molecular biology, epidemiology and pathogenicity. This knowledge will allow for the formulation of effective control measures and its swift implementation. With the presence of TSWV being identified in KZN, attempts can be made to control this disease in the early stages of its infection cycle. In addition, the detection of TSWV in KZN is crucial in understanding the variation and distribution of TSWV in SA. Accurate identification and proper characterization of viruses will allow the formulation of effective and sustainable control measures for the diseases they cause.

## 2.5 REFERENCES


**Best R.J. (1968).** Tomato spotted wilt virus. *Advances in Virus Research* **13**, 65-145.

**Black L.M., Brakke M.K., & Vatter A.E. (1963).** Purification and electron microscopy of tomato spotted-wilt virus. *Virology* **20**, 120-130.

**Bos L. (1999).** *Plant Viruses: Unique and Intriguing Pathogens, A Textbook of Plant Virology.* Leiden: Backhuys, pp. 42-79.

**Bosland P.W., & Votova E.J. (2000).** *Peppers: vegetable and spice capsicums.* CABI publishing, USA, pp. 8-10.

**Budnik K., (1995).** The epidemiology and control of capsicum viruses in Natal. *MSc. Thesis.* University of Natal, Pietermaritzburg, South Africa.

**Budnik K., Laing M.D., & Da Graca J.V. (1996).** An epidemiological study of viruses of pepper (*Capsicum annuum* L.) in KwaZulu-Natal, South Africa. *African Plant Protection* **2**, 9-14.

**Chantler S.M., & Clayton A.L. (1988).** The use of ELISA for rapid viral diagnosis: Viral antigen detection in clinical antigen detection in clinical specimens. In: *ELISA and Other Rapid Immunoassays*, Kemeny M.D., & Challacombe S.J (eds.). Florida: John Wiley and Sons (Ltd), pp. 279-301.

**Clark M.F., & Adams A.N. (1977).** Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475-483.

**Dijkstra T., & de Jager C.P. (1998).** *Practical Plant Virology: Protocols and Exercises*. Germany: Springer-Verlag, pp. 221-237.

**Diodge E.M., Bottomely A.M., Van der Plank J.E., & Pauer G.D. (1953).** A revised list of plant diseases in South Africa. *Department of Agriculture Technical Science Bulletin* no. 346.

**Francki R.I.B., & Hatta T. (1981).** Tomato spotted wilt virus. In: *Handbook of Plant Virus Infections and Comparative Diagnosis*, Kurstak E. (ed.). Amsterdam: Elsevier/North-Holland Biomedical Press, pp. 491-512.

**German L.T., Ulman D.E., & Moyer J.W. (1992).** Tospoviruses: diagnosis, molecular biology, phylogeny, and vector relationships. *Annual Review of Phytopathology* **30**, 315-348.

**Gonsalves D., & Trujillo E.E. (1986).** Tomato spotted wilt virus in Papaya and detection of the virus by ELISA. *Plant Disease* **70**, 501-506.

**Green S.K., & Kim J.S. (1991).** Characteristics and control of viruses infecting peppers: a literature review. *Asian Vegetable Research Development Centre Technical Bulletin* no. 18.

**Hull R. (2001).** *Matthew's Plant Virology*. San Diego: Academic Press, pp. 47-51.

**Ie T.S. (1971).** Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. *Virology* **43**, 468-479.

**Joubert J.J., Hahn J.S., von Wechmar M.B., & van Regenmortel M.H.V. (1974).** Purification and properties of tomato spotted wilt virus. *Virology* **57**, 11-19.

**Kitajima E.W. (1965).** Electron microscopy of vira cabeca (Brazilian tomato spotted wilt virus) within the host cell. *Virology* **26**, 89-99.

**Kormelink R., Kitajima E.W., de Haan P., Zuidema D., Peters D., & Goldbach R. (1991).** The nonstructural protein (NSs) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structures in infected plant cells. *Virology* **181**, 459-468.

**Laemmli U.K. (1970).** Cleavage of structural proteins during the assembly of the head of bacterial phage T4. *Nature* **227**, 680-685.

**Law M.D., & Moyer J.W. (1990).** A tomato spotted wilt virus-like virus with a serologically distinct N protein. *Journal of General Virology*, **71**, 933-938.

**Lawson R.H., Dienelt M.M., & Hsu H.T. (1996).** The cytopathology of tospovirus – host-plant interactions. *Acta Horticulturae* **431**, 267-289.

**Mandal B., Pappu H.R., Culbreath A.K. (2001).** Factors affecting mechanical transmission of tomato spotted wilt virus to peanut (*Arachis hypogaea*). *Plant Disease* **85**, 1259-1263.

**Martin M.M. (1964).** Purification and electron microscopy studies of tomato spotted wilt virus (TSWV) from tomato roots. *Virology* **22**, 645-649.

**Matthews R.E.F. (1981).** *Fundamentals of Plant Virology.* New York: Academic Press, pp. 166-168.

**Milne R.G. (1970).** An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. *Journal of General Virology* **6**, 267-276.

**Mohamed N.A., Randles J.W., & Francki R.I.B. (1973).** Protein composition of tomato spotted wilt virus. *Virology* **56**, 12-21.

**Mumford R.A., Barker I., & Wood K.R. (1996).** The biology of tospoviruses. *Annual Applied Biology* **128**, 159-183.

**Napoli C., Lemieux C., & Jorgensen R. (1990).** Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *The Plant Cell* **2**, 279-289.

**Narayanasamy P. (2001).** *Plant Pathogen Detection and Disease Diagnosis.* New York: Marcel Dekker, pp. 70.

**Pang S-Z., Nagpala P., Wang M., Slightom J.L., & Gonsalves D. (1992).** Resistance to heterologous isolates of tomato spotted wilt virus in transgenic plants expressing its nucleocapsid protein. *Phytopathology* **82**, 1223-1229.

**Tas P.W.L., Boerjan M.L., & Peters D. (1977).** The Structural Proteins of Tomato Spotted Wilt Virus. *Journal of General Virology* **36**, 267-279.

**Thompson G.J. (1980).** Plant host-virus interactions: the characterization of a mosaic disease of *Capsicum* and the relationship between tomato spotted wilt virus infection and the cytokinin content of tomato. *MSc. Thesis.* University of Natal, Pietermaritzburg, South Africa.

**Urban L.A., Huang P., & Moyer J. W. (1991).** Cytoplasmic inclusions in cells infected with isolates of L and I serogroups of tomato spotted wilt virus. *Phytopathology* **81**, 525-529.

**van Regenmortel M.H.V. (1982).** *Serology and Immunochemistry of Plant Viruses.* New York: Academic Press, pp. 112-120.

**Walkey L. (1991).** *Applied Plant Virology.* London: Chapman & Hall, pp. 103-133.

# CHAPTER 3

# PHYLOGENETIC ANALYSIS OF TOMATO SPOTTED WILT VIRUS (TSWV) ISOLATES OCCURING IN SOUTH AFRICA (SA) BY CLONING AND SEQUENCING OF N GENES


## ABSTRACT

Tomato spotted wilt virus (TSWV) has become an economically important virus in South Africa (SA). The objective of the study was to examine the genetic diversity of TSWV isolates occurring in SA. A total of six TSWV isolates originating from Gauteng, KwaZulu-Natal, North West, Limpopo and Mpumulanga provinces were used in the study. Total plant RNA, isolated from *Nicotinia rustica* L. infected with TSWV was subjected to reverse-transcription polymerase chain reaction (RT-PCR) using primers specific to the N gene of TSWV. The amplified 760bp product was then cloned into the pCR®2.1 vector. Positive recombinant clones were identified by endonuclease EcoR1 restriction analysis, and the nucleotide (nt) sequence was determined. Nucleotide sequence comparisons of the N gene revealed high similarity between the SA isolates and TSWV isolates from Asia and Europe. SA isolates showed a high degree of sequence similarity (99-100%). The information generated in this study will be useful in formulating effective control measures using genetic engineering approaches for this economically important virus.


## 3.1 INTRODUCTION

The molecular characteristics of the Tospoviruses have received considerable attention since the mid-1980s because of the increasing economic importance of the virus-induced diseases (Moyer & Qui, 1996). These diseases were considered to be caused by a single virus, TSWV. It was not until later that multiple distinct viruses were identified (Francki *et al.*, 1991; German *et al.*, 1992). Today, the diversity of the Tospovirus is a characteristic of the genus. In addition, diversity also exists within a

given virus as natural populations of TSWV may occur as heterogeneous complexes of distinct isolates (Moyer & Qui, 1996).

Heterogeneity and rapid adaptability are important characteristics that distinguish TSWV from other plant viruses. Genetic heterogeneity in TSWV populations is increased by the occurrence of genetic reassortment (Qui *et al.*, 1998) and the multiplication of the virus within its vector. These features make TSWV a good model for elucidating the genetic structure of viral populations that exist in a defined geographical area (Tspompana *et al.*, 2005).

A set of descriptors has been established to distinguish species within the Tospovirus genus. These are based on the nt similarity of the nucleocapsid (N) gene. In general, less than 90% nt similarity is considered sufficient to consider the two viruses to be distinct (Goldbach & Kuo, 1996).

The N gene protein is very conserved with nearly identical function in all members of the Tospovirus genus. This was assumed based on the crucial role played by the N protein in viral life cycle processes such as regulating the switching of the replicative machinery from transcription to replication, functioning in the replication complex and encapsidating genomic and anti-genomic RNA (Beaton & Krug, 1984; 1986). Therefore, phylogenetic patterns obtained by N gene comparisons reflect more closely the actual common ancestry of the group than working with more variable genes (Dewey *et al.*, 1997).

The use of resistant cultivars provides the most effective and durable way to minimise crop losses due to TSWV infection (Pappu *et al.*, 1998). As natural sources of resistance are limited and often strain-specific, there is a need to look for alternative methods to control the virus. To this end, crops with transgenic expression of the TSWV N gene have been developed (Mackenzie & Ellis, 1992; Pang *et al.*, 1992). However, the enormous diversity that exists among the isolates of TSWV has had a significant, negative impact on these control efforts.

In general, the resistance conferred by the N gene is effective only against viral isolates with a high degree of sequence similarity to the transgene. Thus knowledge of the relatedness of N gene sequences of the various isolates of TSWV is essential for the success of genetic engineering resistance strategies (Pappu *et al.*, 1998). However, little or no information is available on the genetic makeup of the virus isolates that are prevalent in SA. Here, we report the N gene sequences of TSWV isolates from SA. In order to understand the sequence diversity of the TSWV isolates, this study also investigates the sequence divergence of the N gene of the TSWV isolates from SA when compared to isolates occurring in different geographical locations.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Virus Isolates

A total of six TSWV isolates originating from different locations in SA were used in this study (Fig. 3.1). TSWV isolates from the former Transvaal (Limpopo province) and Kranskop (KwaZulu-Natal province) were identified using ELISA. Details of the identification procedure for the KZN isolate are described in Chapter 2. Desiccated and freeze-dried TSWV sources from North West, Limpopo and Mpumulanga provinces were provided by Jacolene Meyer[1]. Details of TSWV isolates used in this study are given in Table 3.1.

### 3.2.2 Virus Propagation and Maintenance

Mechanical inoculation of *N. rustica* plants using the different TSWV isolates was performed as previously described (section 2.2.1.3) with a minor modification. Inoculum from desiccated and whole-leaf material was prepared in 0.01M potassium phosphate buffer (pH 7) containing 0.01M sodium sulfite. Freeze-dried samples were

[1] Jacolene Meyer, Research Scientist, Plant Protection and Research Institute, Agricultural Research Council, Private Bag x134, Pretoria 0001, SA.

rehydrated in 1ml of the buffer to reconstitute the virus before inoculation. Plants were kept at constant temperature of 20-25°C in the Jolly Roger tunnel, University of KwaZulu-Natal, Pietermaritzburg (PMB) for the duration of the study.

Three weeks post inoculation, leaves exhibiting typical TSWV symptoms were harvested and used for total RNA extraction. A portion of infected leaf material was freeze-dried in storage buffer (0.08M Tris-HCl (pH 8) containing 8% (w/v) glucose-d and 6% Na-glutaminate) for long term storage. Freeze-dried samples were then kept at -80°C until further use.

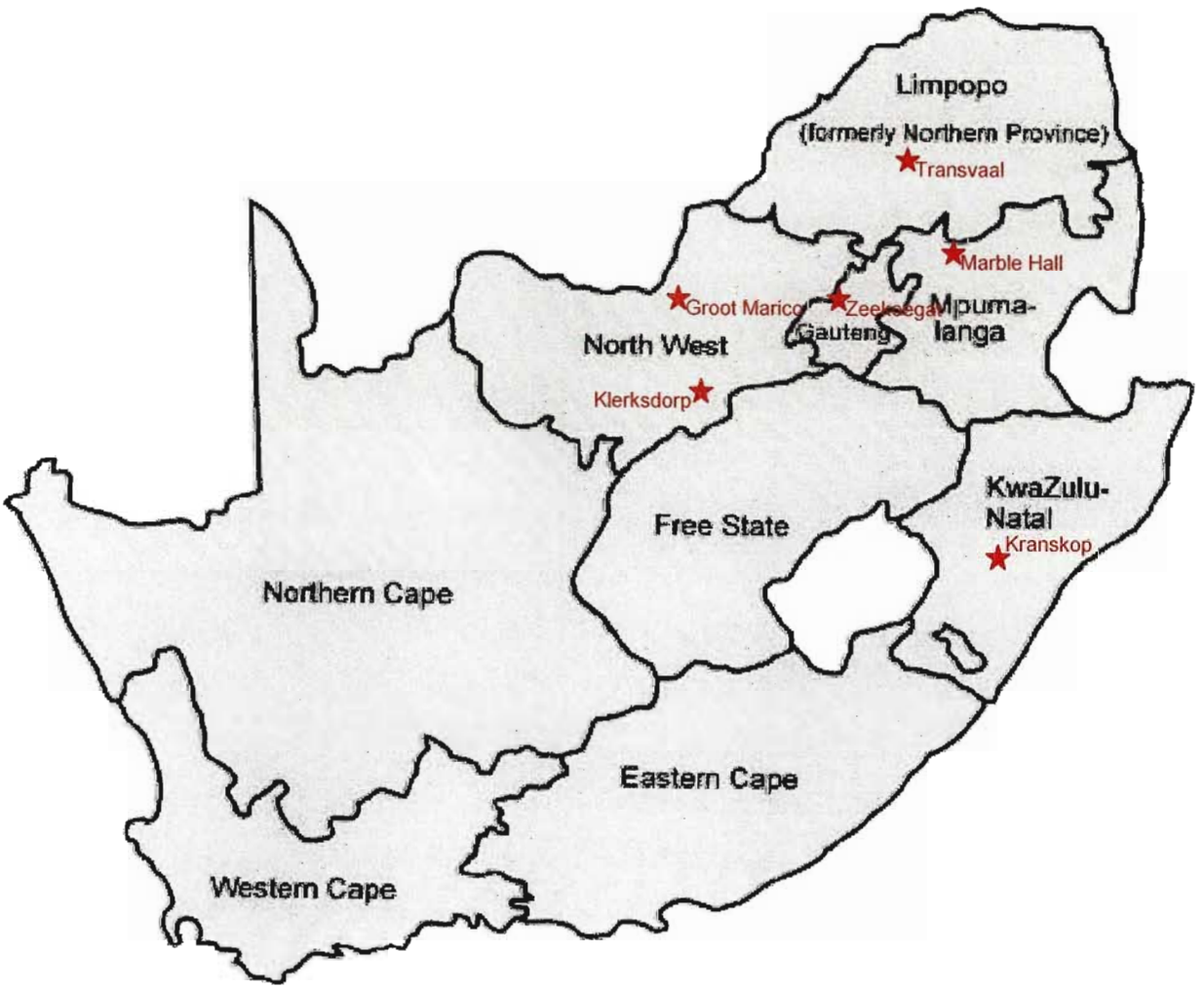



**Figure 3.1** Geographical locations of South African tomato spotted wilt virus (TSWV) isolates used in this study.

**Table 3.1** Details of the tomato spotted wilt virus (TSWV) isolates used in this study.

| Locality | Province | Plant source | Date accessed |
|---|---|---|---|
| Kranskop | KwaZulu-Natal | *Capsicum* sp. | 2004 |
| former 'Transvaal' | Limpopo | *Capsicum* sp. | 2003 |
| Zeekoegat | Gauteng | *Solanum tubersosum* | 1992 |
| Marble Hall | Mpumulanga | *Pisum sativum* | 1991 |
| Klerksdorp | North West | *Arachis hypogaea* | 1989 |
| Groot Marico | North West | *Pisum sativum* | 1988 |

### 3.2.3 Total Plant RNA Extraction

Total RNA from *N. rustica* leaves exhibiting typical TSWV symptoms was extracted using the SV Total RNA Isolation System (Promega, USA) according to manufacturers' instructions. Fresh leaf material was ground into a fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a sterile 2ml Eppendorf tube containing 175µl of SV RNA lysis buffer and then mixed by inversion. The solution was mixed again by inversion (3-4 times) after the addition of 350µl SV RNA dilution buffer. The mixture was heated at 70°C for 3min in water bath and then centrifuged (14000rpm) for 10min in a Bechman J2-21M centrifuge. The clear lysate was transferred to a sterile Eppendorf tube containing 200µl 95% ethanol. The solution was mixed well by pipetting and transferred to the spin basket assembly. The eluate present in the collection tube was discarded after each centrifugation step. After 1min of centrifugation, 600µl of SV RNA wash solution was added to the membrane of the assembly and centrifuged again for 1min. A DNase incubation mix containing 40µl yellow core buffer, 5µl 0.09M $MnCl_2$ and 5µl DNase I was prepared and added to each sample. The solution was then incubated at room temperature (RT) for 15min and after the addition of 200µl of SV DNase stop solution, the mixture was centrifuged for 1min. To each sample, 600µl of SV RNA wash solution was added and centrifuged for 1min. An additional 250µl of the SV RNA wash solution was added and the mixture was centrifuged for 2min. The spin basket was then

transferred to the elution tube. A 100µl of nuclease-free water was added to each tube and then centrifuged for 1min to elute the RNA. Total RNA was also extracted from leaf samples of uninfected healthy control plants. All RNA samples were labelled at stored at -80°C until further use.

### 3.2.4 RT-PCR of Total Plant RNA

Total RNA from TSWV-infected leaf material of *N. rustica* was used as a template for amplification in the reverse transcription polymerase chain reaction (RT-PCR) procedure. RT-PCR was performed using the RobusT I RT-PCR Kit (Finnzymes, Finland). The primer pair derived from the nucleocapsid protein (N) gene sequence of TSWV was used and an amplification product of 760bp was expected (Pang *et al.*, 1992):

JLS90-46 5'-AGCTAACCATGGTTAAGCTCACTAAGGAAAGC-3' (Forward)
JLS90-47 5'-AGCATTCCATGGTTAACACACTAAGCAAGCAC-3' (Reverse)

Amplification was performed in an automated thermal cycler programmed for one cycle of 48°C for 30min and 94°C for 2min and 25 cycles of amplification with 30s of denaturation at 94°C, 30s of annealing at 60°C and 1min of extension at 72°C followed by one cycle of final extension for 7min at 72°C. Negative controls consisted of DEPC-treated water and total RNA extracted from an uninfected healthy control plant. For the positive control reaction, control RNA and upstream and downstream primers supplied in the kit were used. Control RNA is MS2 viral RNA with carrier (*Escherichia coli* ribosomal RNA). With the positive control primers, a 1011bp product was expected.

### 3.2.5 Analysis of RT-PCR Products by Agarose Gel Electrophoresis

Products of the RT-PCR procedure were visualised by agarose gel electrophoresis as previously described (section 2.2.5.5). A 100bp DNA ladder was used as the MWM (Promega, USA).

### 3.2.6 Cloning Procedure

The cloning of the RT-PCR products was done using the TA Cloning® Kit according to manufacturers' instructions with minor modifications (Invitrogen, CA).

#### *3.2.6.1 Cloning of RT-PCR product into pCR®2.1*

The RT-PCR product was cloned into the plasmid cloning vector pCR®2.1 according to manufacturers' instructions (Invitrogen, CA). For optimal ligation efficiencies, fresh (less than one day old) RT-PCR product was used.

The linearised vector supplied in the kit has 3' deoxythymidine (T) residues. *Taq* polymerase, which has a non-template-dependent activity, adds a single deoxyadenosine (A) to the 3' ends of the RT-PCR product. This would allow for the RT-PCR insert to ligate efficiently to the vector (Fig. 3.2).

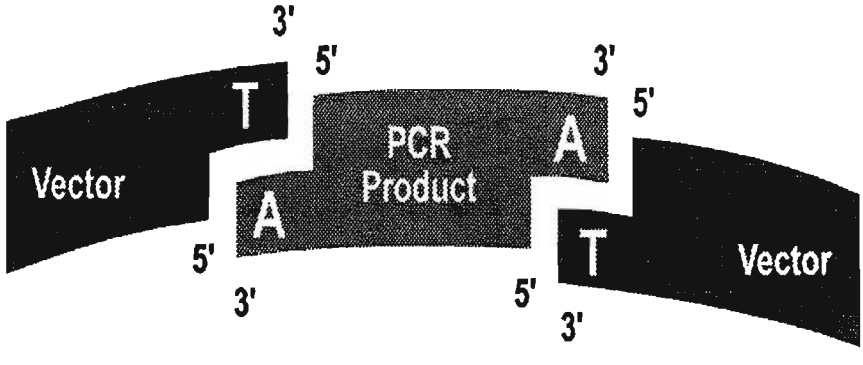



**Figure 3.2** Illustration showing the concept behind the TA Cloning® method (Invitrogen, 2004).

To ensure ligation efficiency, 0.5µl *Taq* DNA polymerase (Promega, USA) was added to 35µl of RT-PCR product. The mixture was incubated in a heating block at 72°C for 9min. Thereafter, the products were placed on ice.

Ligation reaction samples were prepared as described in Table 3.2 and incubated overnight at 14°C in an automated thermal cycler.

**Table 3.2** Preparation of reaction samples for ligation of RT-PCR product to pCR®2.1. vector

| Reagent | Amount (µl) |
|---|---|
| Fresh RT-PCR insert | 6 |
| 10X Ligation Buffer | 1 |
| pCR®2.1 vector (25ng/µl) | 2 |
| T4 DNA Ligase (4.0 Weiss units) | 1 |
| TOTAL VOLUME | 10 |

### *3.2.6.2 Transformation of competent cells*

The construct (vector with insert) was transformed into TOP10F competent *E.coli* One Shot® cells according to manufacturers' instructions, with minor modifications (Invitrogen, CA).

A volume of 2µl for each ligation reaction was added directly into one 50µl vial of competent cells and mixed gently with the pipette tip. Vials were then incubated on ice for 30min and then heat shocked at 42°C for 30s in an automated heating block. To each vial, 250µl of RT S.O.C medium was added and vials were shaken horizontally at 37°C for 1h at 225rpm in a shaking incubator. Luria Bertani (LB) plates containing 50ug/ml kanamycin and 40mg/ml X-Gal (Promega, USA) were prepared and equilibrated at 37°C for 30min. Two plates per transformation vial were plated

with 50µl and 250µl respectively using the hockey stick method. Two different volumes per transformation were plated to ensure that at least one plate would show well-spaced colonies. Plates were incubated overnight at 37°C and then at 4°C for 3h to allow for proper colour development.

### *3.2.6.3 Screening for positive recombinants*

Ten white colonies for each reaction were selected for plasmid isolation and restriction analysis.

*Plasmid DNA purification*

Plasmid DNA of the 10 independently transformed bacterial cells was purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). Each colony was transferred aseptically into tubes containing 2ml of LB broth containing 50ug/ml kanamycin. The cultures were grown overnight at 37°C in a shaking incubator at 225rpm. Bacterial cells were pelleted by centrifugation at 13000rpm for 10min. The pellet was resuspended thoroughly in 250µl of buffer P1 and transferred to a fresh Eppendorf tube. A volume of 250µl of buffer P2 and 350µl of buffer N3 was added respectively to each tube with gentle mixing by inversion after each buffer addition. The solution was centrifuged for 10min at 13000rpm in a Bechman J2-21M centrifuge. The supernatant was decanted into the QIAprep spin column and centrifuged for 1min. The eluate present in the collection tube was discarded after each centrifugation step. The QIAprep spin column was washed by adding 0.5ml buffer PB and centrifuged for 1min. An additional wash was done by adding 0.75ml buffer PE and centrifugation for 1min. Tubes were again centrifuged for a further 1min to remove any residual wash buffer. The QIAprep column was transferred to a clean 1.5ml Eppendorf tube. To eluate the DNA, 50µl buffer EB was added to the centre of each column, allowed to stand for 1min and then centrifuged for 1min. DNA preparations were then stored at -20°C until further use.

*Restriction analysis*

Recombinant clones were identified by EcoR1 restriction endonuclease digestion. Restriction reaction samples were prepared as described (Table 3.3). Digests were incubated at 37°C for 2h in a heating block. Reaction products were visualised by agarose gel electrophoresis as described in section 2.2.5.5, with minor modifications. A 1.2% (w/v) agarose gel was prepared and a 1kb DNA ladder (Promega, USA) was used as the MWM.

**Table 3.3** Preparation of reaction samples for restriction analysis of plasmid DNA.

| Reagent | Amount (µl) |
|---|---|
| Plasmid DNA | 15 |
| 10X NEBuffer EcoR1 (BioLabs, New England) | 2 |
| EcoR1 (BioLabs, New England) | 0.5 |
| Distilled water | 2.5 |
| TOTAL VOLUME | 20 |

### 3.2.7 Sequencing of N Gene

Two positively identified recombinant clones per transformation were selected and sent to Inqaba Biotech[2] for sequencing with the M13 forward and reverse primers.

### 3.2.8 Phylogenetic Analysis

The phylogenetic status of TSWV isolates from SA relative to isolates occurring in different parts of the world was assessed by employing a compilation of phylogenetic software programs in the PHYLIP package version 3.65 (Felsenstein, 1985). An overview of steps taken in the phylogenetic analysis of TSWV isolates is outlined (Figure 3.3).

[2] Inqaba Biotechnical Industries (Pty) Ltd, P.O. Box 14356, Hatfield 0028, SA

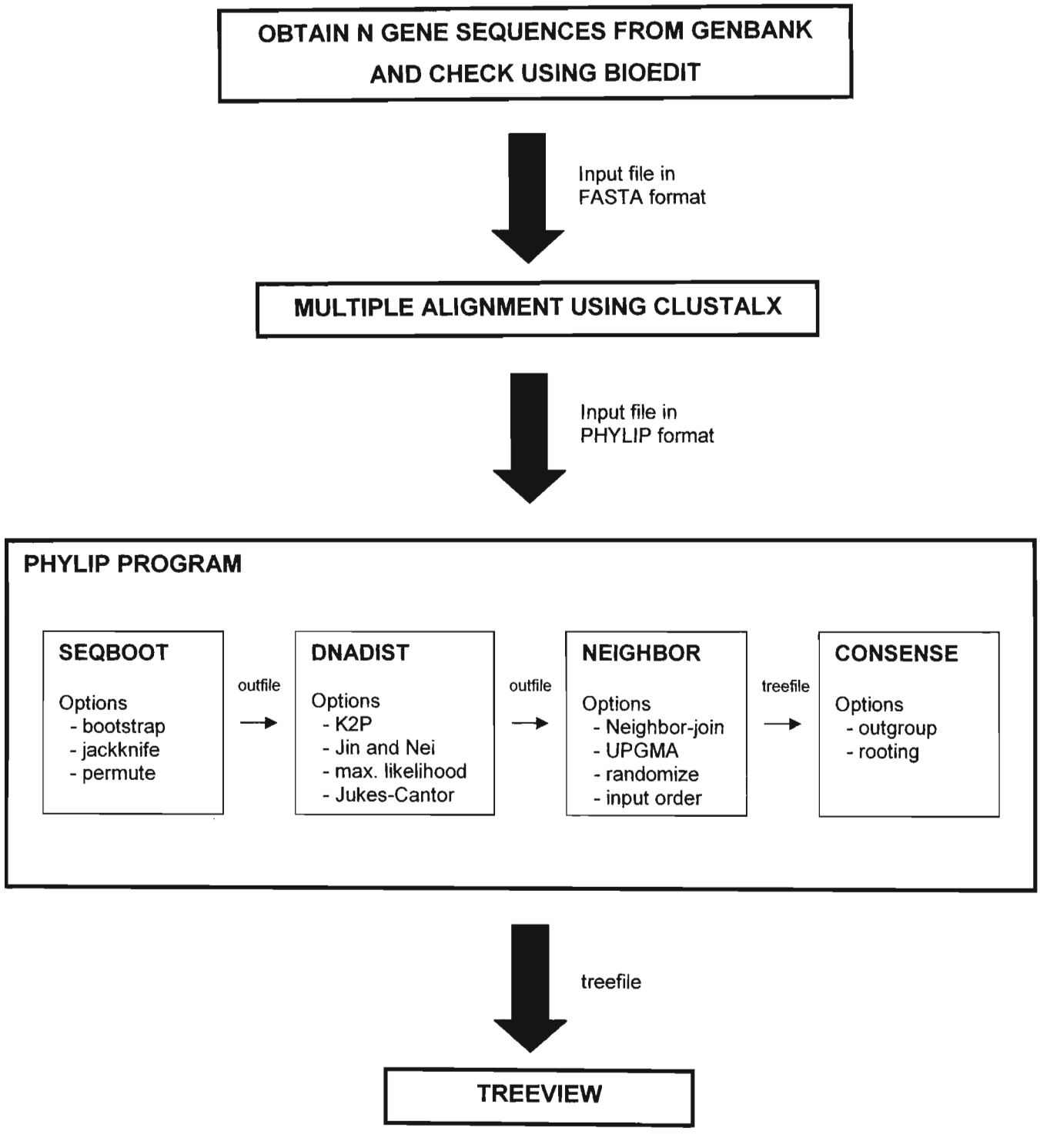



**Figure 3.3** Flow diagram of the steps involved in the phylogenetic analysis of tomato spotted wilt virus (TSWV) isolates occurring in South Africa.

#### *3.2.8.1 Virus sequences*

The N gene sequences reported in this study were deposited into GenBank (Benson *et al.*, 1996). The details of the sequences used in the analysis are presented (Table 3.4.). Only sequences from published references were selected in this study. The BLAST program (Altschul *et al.*, 1990) was used to obtain related TSWV N gene sequences from different geographical locations. These sequences were obtained from the public database of the National Centre for Biotechnology Information (NCBI) (www.ncbi.nml.nih.gov). Sequences were manually verified and edited to ensure optimal alignment using BioEdit Sequence Alignment Editor version 6.0 (Hall, 1999).

#### *3.2.8.2 Multiple alignments*

Percentage sequence homologies and comparisons via multiple alignments were computed using CLUSTALX version 1.81 (Thompson *et al.*, 1997). The sequences to be aligned were placed into a text file in FASTA format and loaded onto the CLUSTALX program. The alignment outfile was saved in phylip and clustal formats.

#### *3.2.8.3 Bootstrap analysis*

The reliability of different phylogenetic groupings was evaluated using bootstrap analysis of the SEQBOOT program in the PHYLIP package. SEQBOOT reads data in phylip format and produces multiple data sets by bootstrap resampling (Blank, 2002). A 1000 replicates with a random odd seed number of 3 were chosen for the analysis. Figure 3.4 provides an outline of parameters used in the bootstrap analysis.

**Table 3.4** Sources of tomato spotted wilt virus (TSWV) N gene sequences used in this study.

| Designation | Origin | Reference | Source of sequence |
|---|---|---|---|
| TSWV-BULG | Bulgaria | Heinze *et al.*, 2003 | GenBank, AJ418779 |
| TSWV-CZECH | Czech Republic | Heinze *et al.*, 2001 | GenBank, AJ296599 |
| TSWV-GER | Germany | Heinze *et al.*, 2003 | GenBank, AJ418781 |
| TSWV-ITALY | Italy | Ciuffo *et al.*, 2005 | GenBank, AY848922 |
| TSWV-JAPAN | Japan | Takeda *et al.*, 2002 | GenBank, AB088385 |
| TSWV-GP | SA, Gauteng | This study | This study, EF059705 |
| TSWV-KZN | SA, KwaZulu-Natal | This study | This study, DQ834847 |
| TSWV-LP | SA, Limpopo | This study | This study, EF059704 |
| TSWV-MP | SA, Mpumulanga | This study | This study, EF059706 |
| TSWV-NW1 | SA, North West | This study | This study, EF059702 |
| TSWV-NW2 | SA, North West | This study | This study, EF059703 |
| TSWV-SPAIN | Spain | Tsompana *et al.*, 2005 | GenBank, AY744480 |
| TSWV-CAL | USA, California | Tsompana *et al.*, 2005 | GenBank, AY744474 |
| TSWV-COL | USA, Colorado | Tsompana *et al.*, 2005 | GenBank, AY744475 |
| TSWV-BL | USA, Hawaii | Pang *et al.*, 1992 | GenBank, L20953 |
| TSWV-NC | USA, North Carolina | Tsompana *et al.*, 2005 | GenBank, AY744477 |
| RiftValley[a] | - | Giorgi *et al.*, 1991 | GenBank, NC002045 |

[a] Rift Valley Fever Virus (*Bunyaviridae*, Phlebovirus) was used as the outgroup

**Bootstrapping algorithm, version 3.65**

Settings for this run:

| | | |
|---|---|---|
| D | Sequence, Morph, Rest., Gene Freqs? | Molecular sequences |
| J | Bootstrap, Jackknife, Permute, Rewrite? | Bootstrap |
| % | Regular or altered sampling fraction? | regular |
| B | Block size for block-bootstrapping? | 1 (regular bootstrap) |
| R | How many replicates? | 1000 |
| W | Read weights of characters? | No |
| C | Read categories of sites? | No |
| S | Write out data sets or just weights? | Data sets |
| I | Input sequences interleaved? | Yes |
| 0 | Terminal type (IBM PC, ANSI, none)? | IBM PC |
| 1 | Print out the data at start of run | No |
| 2 | Print indications of progress of run | Yes |

Y to accept these or type the letter for one to change

y

Random number seed (must be odd)?

3

**Figure 3.4** Program parameters used in the SEQBOOT program for bootstrapping analysis.

### *3.2.8.4 Generation of distance matrices*

Using the output file created by SEQBOOT, distance matrices were calculated with the DNADIST program according to the Jukes-Cantor model. An outline of parameters used in the DNADIST program is shown (Figure 3.5).

**Nucleic acid sequence Distance Matrix program, version 3.65**

Settings for this run:

| | | |
|---|---|---|
| D | Distance (F84, Kimura, Jukes-Cantor, LogDet)? | Jukes-Cantor |
| G | Gamma distributed rates across sites? | No |
| C | One category of substitution rates? | Yes |
| W | Use weights for sites? | No |
| L | Form of distance matrix? | Square |
| M | Analyze multiple data sets? | Yes, 1000 data sets |
| I | Input sequences interleaved? | Yes |
| 0 | Terminal type (IBM PC, ANSI, none)? | IBM PC |
| 1 | Print out the data at start of run | No |
| 2 | Print indications of progress of run | Yes |

Y to accept these or type the letter for one to change

**Figure 3.5** Program parameters used in the DNADIST program for the calculation of distance matrices.

### *3.2.8.5 Generation of phylogenetic trees*

Phylogenetic trees were constructed using the neighbor-joining algorithm in the NEIGHBOR program. The rift valley fever virus (Phlebovirus, *Bunyaviridae*) was used as the outgroup. An outline of parameters used in the NEIGHBOR program is shown (Figure 3.6).

**Neighbor-Joining/UPGMA method version 3.65**

Settings for this run:

| | | |
|---|---|---|
| N | Neighbor-joining or UPGMA tree? | Neighbor-joining |
| O | Outgroup root? | Yes, at species number 17 |
| L | Lower-triangular data matrix? | No |
| R | Upper-triangular data matrix? | No |
| S | Subreplicates? | No |
| J | Randomize input order of species? | Yes (random number seed = 3) |
| M | Analyze multiple data sets? | Yes, 1000 sets |
| 0 | Terminal type (IBM PC, ANSI, none)? | ANSI |
| 1 | Print out the data at start of run | No |
| 2 | Print indications of progress of run | Yes |
| 3 | Print out tree | Yes |
| 4 | Write out trees onto tree file? | Yes |

Y to accept these or type the letter for one to change

**Figure 3.6** Program parameters used in the NEIGHBOR program for the generation of phylogenetic trees.

### *3.2.8.6 Generation of consensus tree*

The output treefile from the NEIGHBOR program was used as input in the CONSENSE program. A majority rule (extended) consensus tree was calculated. An outline of parameters used in the CONSENSE program is shown (Figure 3.7).

**Consensus tree program, version 3.65**

Settings for this run:

| | | |
|---|---|---|
| C | Consensus type (MRe, strict, MR, MI): | Majority rule (extended) |
| R | Trees to be treated as Rooted: | Yes |
| T | Terminal type (IBM PC, ANSI, none): | IBM PC |
| 1 | Print out the sets of species: | Yes |
| 2 | Print indications of progress of run: | Yes |
| 3 | Print out tree: | Yes |
| 4 | Write out trees onto tree file: | Yes |

Are these settings correct? (type Y or the letter for one to change)

**Figure 3.7** Program parameters used in the CONSENSE program for the generation of a consensus phylogenetic tree.

#### *3.2.8.7 Visualisation of consensus tree*

The TREEVIEWX version 0.4 was used to view the rooted consensus tree (Page, 1996).

## 3.3 RESULTS

### 3.3.1 RT-PCR of Total Plant RNA

An amplification product of 760bp was detected after agarose gel electrophoresis of RT-PCR products of total plant RNA isolations. The positive control yielded an expected 1100bp band whilst negative controls showed no products (Fig. 3.8).

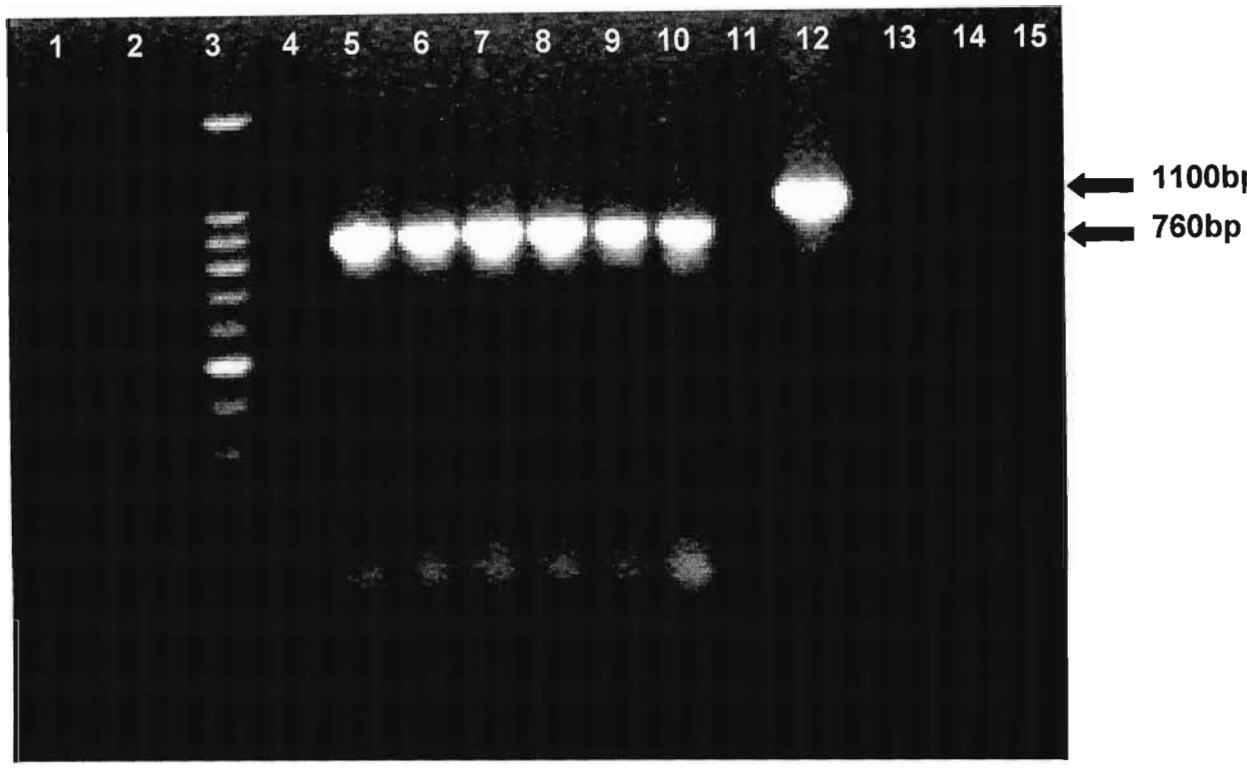



**Figure 3.8** Agarose gel showing the reverse-transcription polymerase chain reaction (RT-PCR) product of total plant RNA extracted from *Nicotinia rustica* L. infected with tomato spotted wilt virus (TSWV) isolates from South Africa. Lanes 1, 2, 4,11 and 15 are blank. Lane 3 shows the molecular weight marker. Lanes 5-10 show the 760bp amplification products of the total RNA from KwaZulu-Natal (KZN), Limpopo (LP), North West (NW1), North West (NW2), Mpumulanga (MP) and Gauteng (GP) TSWV isolates respectively. Lane 12 shows the MS2 viral RNA (1100bp) control reaction. Lanes 13 and 14 show the negative control reactions which contain water and total plant RNA from healthy uninfected plants respectively.

### 3.3.2 Screening for Positive Recombinants

#### *3.3.2.1 Picking of recombinant colonies*

Colonies produced by untransformed cells will be blue, whilst colonies produced by transformed cells will be white. Thus, white colonies were picked and used for further characterisation. An even distribution of blue and white colonies was observed on plates containing the transformation mixture from each reaction (Fig. 3.9).

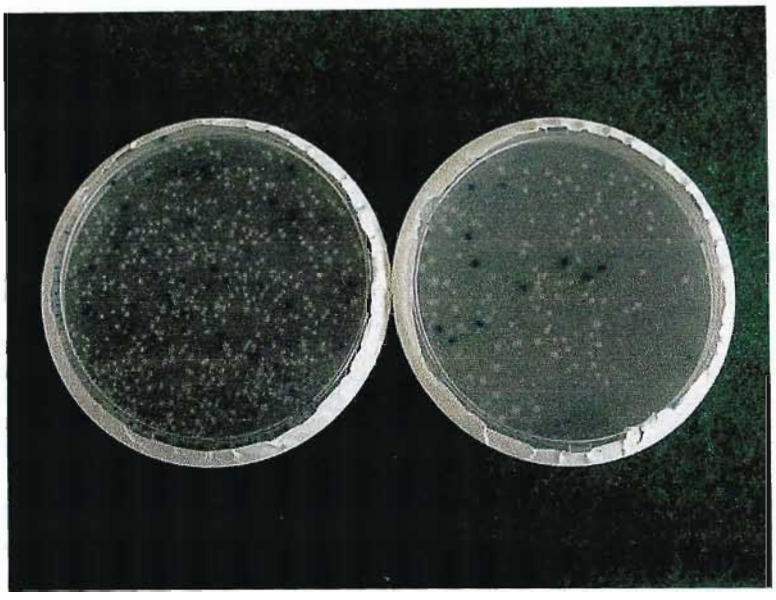


**Figure 3.9** Photograph showing the even distribution of blue and white colonies seen on LB plates plated with 250µl (left) and 50µl (right) of transformation mixture.

#### *3.3.2.2 Restriction analysis*

Positive recombinants containing the N gene insert were identified by restriction digestion of plasmid DNA into 760bp (insert) and 3.9Kb (plasmid) products (Fig. 3.10). Only those colonies that yielded the 760bp restriction product were selected for sequencing.

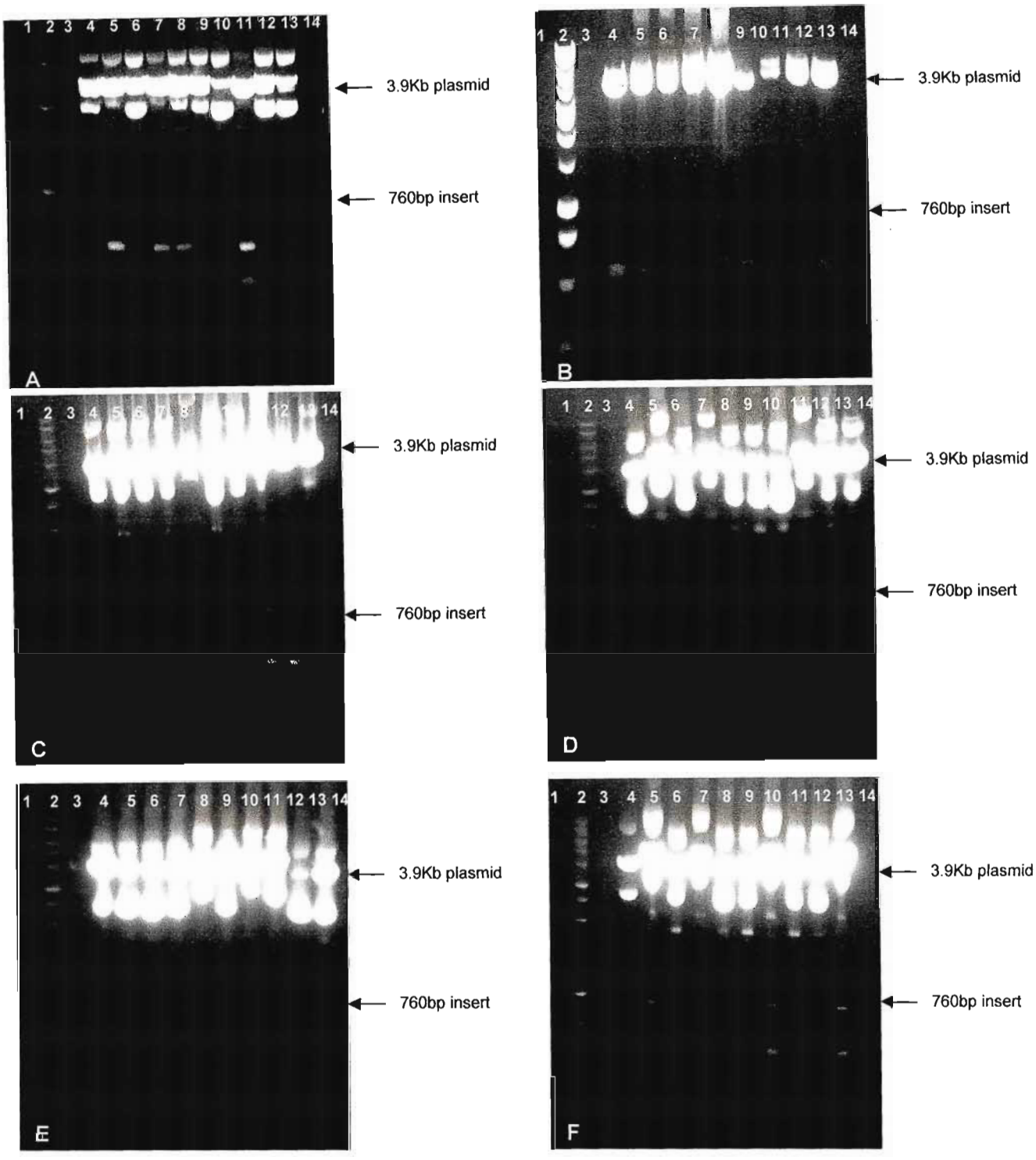



**Figure 3.10** Restriction analysis of plasmid DNA isolated from recombinant clones of (A) KwaZulu-Natal (KZN), (B) Limpopo (LP), (C) North West (NW1), (D) North West (NW2), (E) Mpumulanga (MP) and (F) Gauteng (GP) TSWV isolates. Lanes 1, 3 and 14 are blank. Lane 2 contains the MWM. Lanes 4-13 contain colonies 1-10.

### 3.3.3 Phylogenetic Analysis

The CLUSTAL multiple alignment (Appendix D) showed a large number of areas with high sequence similarity. A (*) indicates that the codon belongs to a fully conserved group. All TSWV isolates used in the comparison revealed a nucleotide identity of greater than 90%. Isolates from SA showed a 99-100% nt identity. Despite these high nucleotide percentage homologies, a distinct evolutionary clustering pattern was observed. The phylogenetic tree derived from the TSWV N gene sequences grouped the isolates into two distinct clusters, one composed of isolates from Bulgaria, Czech Republic, Germany, Italy and South Africa (Eurasian group) whilst the other cluster comprised of isolates from Japan, California, North Carolina, Hawaii, Spain and Colorado (American group) (Fig. 3.11). With the exception of the Japan and Spain isolates, the observed clustering pattern is in accordance with the findings of Silva *et al.* (2001), who tentatively designated the clusters as 'Eurasian' and 'American' groups.

## 3.4 DISCUSSION

The nt sequences and comparisons of six TSWV isolates from SA are reported here. This study is the first phylogenetic analysis of TSWV in SA.

The N gene of TSWV isolates occurring in SA was sequenced and compared to known N gene sequences from TSWV isolates occurring in different geographical locations. To consider a Tospovirus isolate as a distinct species, nt identity percentage should be below 90% (Goldbach & Kuo, 1996). Comparisons of the N gene sequences of TSWV isolates with SA isolates yielded nt identity percentages greater than 90% indicating that the all isolates are indeed the same strain of TSWV. Comparisons of nt sequences of SA isolates revealed a 99-100% sequence similarity. An almost 100% degree of sequence similarity would indicate that TSWV in SA has not evolved on a molecular level.

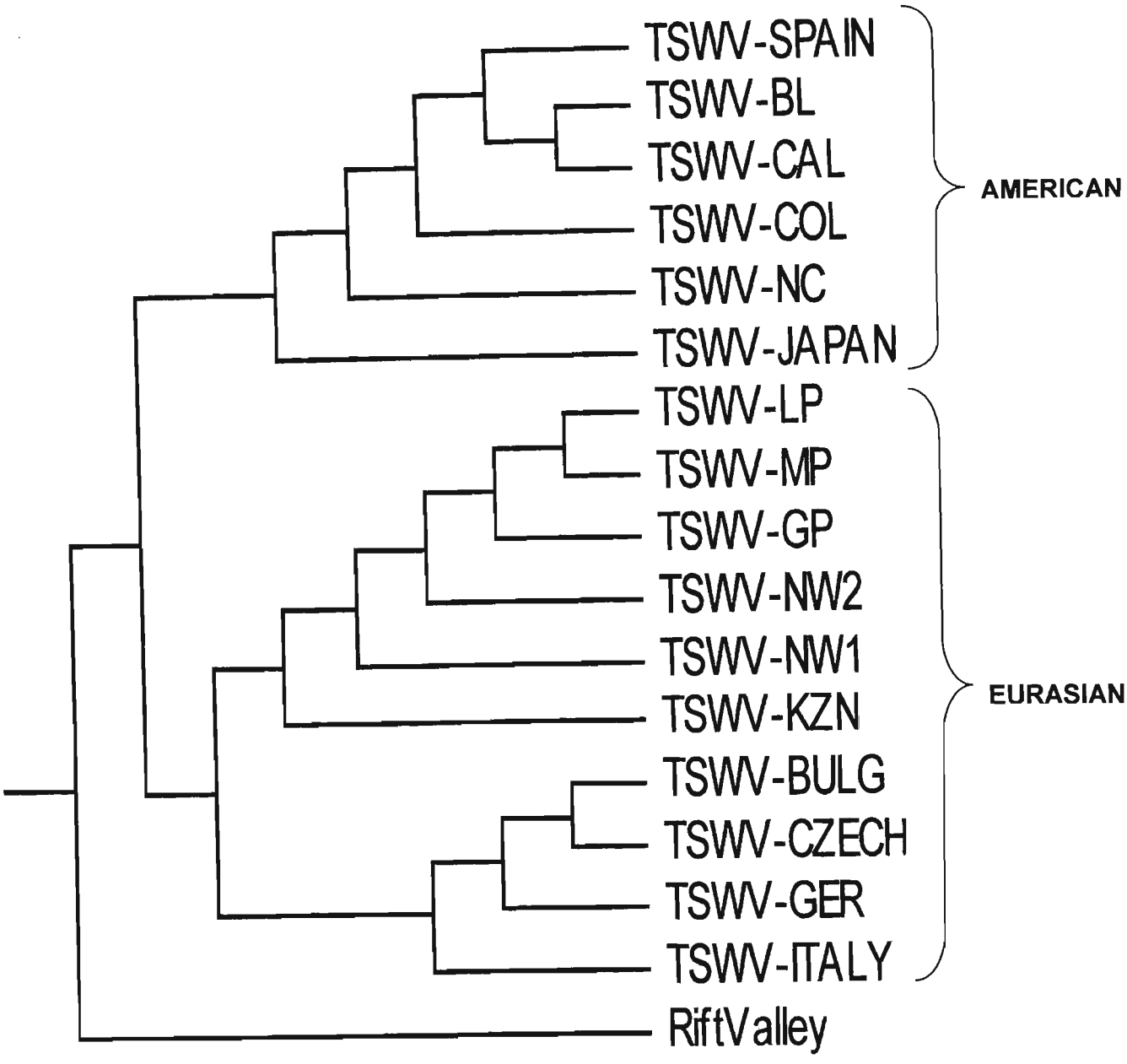



**Figure 3.11** Phylogram based on nucleocapsid gene sequences of tomato spotted wilt virus (TSWV) isolates. The TSWV isolates from South Africa sequenced in this study were compared with TSWV isolates reported from other parts of the world. Sources and designations given to each TSWV isolate used in the study were given in Table 3.4. The Rift Valley Fever Virus was used as an outgroup. Vertical length of the branches was arbitrary.

The multiple alignment of sequences plays a central role in the analysis of heterogeneous data (Blank, 2002). By placing the SA TSWV sequences in the context of known TSWV sequences, evolutionary trends in the genetic structure of these sequences can be determined (Appendix D). Although sequence comparisons showed a high degree of codon conservation (indicated by (*)), codons that were not conserved displayed an evolutionary trend similar to that depicted in the phylogenetic tree. The trend of codon conservation separated the TSWV sequences into two clusters: American and Eurasian groups.

Considering the geographical prevalence of phylogenetic patterns of the N and NSm proteins, Silva *et al.* (2001) proposed the evolution of two geographically distinct sequence variants in the natural population of TSWV. The authors suggest that although TSWV is widespread, it might have originated in the American continent due to the high similarity of N and NSm proteins with other Tospovirus species from the American continent. The clusters were tentatively designated as 'American' and 'Eurasian' groups. The latter group comprised of isolates from Europe and Asia. This clustering pattern was confirmed by glycoprotein comparison of Tospoviruses demonstrated by Lovato *et al.* (2004). These authors speculated that the role of the glycoproteins in transmission efficiency and specificity could explain this grouping according to the geographical distribution of co-existing thrips populations.

With the exception of the Japan and Spain isolates, the phylogenetic analysis is in agreement with the classification proposed by Silva *et al.* (2001) and Lovato *et al.* (2004). One cluster comprised isolates from Bulgaria, Czech Republic, Germany, and Italy (Eurasian group) whilst the other cluster comprised isolates from Japan, California, North Carolina, Hawaii, Spain and Colorado (American group). The SA isolates showed high sequence similarity with members of the Eurasian group. The Japan and Spain isolates formed tight associations with the American group though having originated in 'Eurasian' countries. This report of Tospovirus species belonging to one specific cluster though being detected in different continents has previously been reported (Verthoeven *et al.*, 1996; Cortez *et al.*, 1998; Pozzer *et al.*, 1999). This

could be explained by plant material exchanges and/or the introduction of efficient thrips vectors into these distinct geographical regions (Silva *et al.*, 2001).

Although the SA isolates form a cluster with the Eurasian isolates, they formed a distinct clade within the cluster. The grouping of the SA isolates apart from the Eurasian isolates may reflect that these isolates represent a divergent TSWV group that has not yet acquired phenotypic or genotypic differences that allow its discrimination by serology and molecular methods. This distinct clustering among the SA isolates could be a representative evolutionary trend of TSWV in Africa. To confirm this speculation, TSWV isolates from the rest of the African continent need to be characterised.

Discrimination among TSWV isolates is essential to improve existing control strategies. These include the generation of N gene transgenic plants resistant to TSWV infection. It has been demonstrated that depending on the isolate used as a source of the N gene to develop the transgenic plant, the resistance against different Tospovirus species varies from non-detectable to a high degree of protection (Gielen *et al.*, 1991). Therefore, in order to devise effective and sustainable strategies to control TSWV, knowledge of the diversity of the virus becomes indispensable (Dewey *et al.*, 1995).

In view of the world-wide spread of TSWV, its ability to infect a large host range and the variety of ecological niches in which it exists, an extended survey of TSWV isolates occurring in Africa should be undertaken. Knowledge of the occurrence and diversity of TSWV isolates in the country will be used as a basis for developing transgenic plants with resistance to infection by the virus.

## 3.5 REFERENCES

**Altschul S.F., Gish W., Miller W., Myers E.W., & Lipman D.J. (1990).** Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

**Beaton A.R., & Krug R.M. (1984).** Synthesis of the templates for influenza virion RNA replication in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 4682-4686.

**Beaton A.R., & Krug R.M. (1986).** Transcription, antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 6282-6286.

**Benson D.A., Boguski M., Lipman D.J., & Ostell J. (1996).** GenBank. *Nucleic Acids Research* **24**, 1-5.

**Blank E. (2002).** Phylogenetic and Evolutionary Analysis of the Borna Disease Virus. *MSc. Thesis.* University of Natal, Pietermaritzburg, South Africa.

**Ciuffo M., Finetti-Sialer M.M., Gallitelli D., & Turina M. (2005).** First report in Italy of a resistance-breaking strain of tomato spotted wilt virus infecting tomato cultivars carrying the sw5 resistance gene. *Plant Pathology* **54**, 564-570.

**Cortez I., Livieratos I.C., Derks A., Peters D., & Kormelink R. (1998).** Molecular and serological characterization of iris yellow spot virus, a new and distinct tospovirus species. *Phytopathology* **88**, 2801-2807.

**Dewey R.A., Semorile L., Grau O., De Avila A.C., & Kitajima E.W. (1995).** Characterization of the N gene of a Groundnut Ringspot Virus (GRSV) isolate from Argentina. *Journal of Phytopathology* **143**, 713-717.

**Dewey R.A., Semorile L., Grao O., & Criscill J.V. (1997).** Cladistic analysis of tospovirus using molecular characters. *Molecular Phylogenetics and Evolution* **8**, 11-32.

**Felsenstein J. (1985).** Confidence limits on phylogenies: An approach using bootstrap. *Evolution* **39**, 783-791.

**Francki R.I.B., Fauquet C.M., Knudson D.L., & Brown F. (1991).** Classification and nomenclature of viruses: fifth report of the international committee on taxonomy of viruses. *Archives of Virology,* Supplement 2.

**German L.T., Ulman D.E., & Moyer J.W. (1992).** Tospoviruses: diagnosis, molecular biology, phylogeny, and vector relationships. *Annual Review of Phytopathology* **30**, 315-348.

**Gielen J.J.L., de Haan P., Kool A.J., Peters D., van Grinsven M.Q.J.M., & Goldbach R.W. (1991).** Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. *Bio/Technology* **9**, 1363-1367.

**Giorgi C., Accardi L., Nicoletti L., Gro M.C., Takehara K., Hilditch C., Morikawa S., & Bishop D.H. (1991).** Sequences and coding strategies of the S RNAs of Toscana and Rift valley fever viruses compared to those of Punto toro, Sicilian sandfly fever, and Uukuniemi viruses. *Virology* **180**, 758-753.

**Goldbach R., & Kuo G. (1996).** Introduction. *Acta Horticulturae* **431**, 21-26.

**Hall T.A. (1999).** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT. *Nucleic acids Symposium Series* **41**, 95-98.

**Heinze C., Letschert B., Hristova D., Yankulova M., Kauadjouor O., Willingmann P., Atanassov A., & Adam G. (2001).** Variability of the N protein and the intergenic region of the S RNA of tomato spotted wilt tospovirus (TSWV). *New Microbiology* **24**, 175-187.

**Heinze C., Willingmann P., Schwach F., & Adam G. (2003).** An unusual large intergenic region in the S RNA of a Bulgarian tomato spotted wilt virus isolate. *Archives of Virology* **148**, 199-205.

**Invitrogen. (2004).** TA Cloning® Kit Instruction Manual. Invitrogen Corporation, California.

**Lovato F.A., Nagata T., Resende O.R., De Avila A.C., & Inoue-Nagata A.K. (2004).** Sequence analysis of the glycoprotein of tomato chlorotic spot virus and groundnut ringspot virus and comparison with other tospoviruses. *Virus Genes* **29**, 321-328.

**Mackenzie D.J., & Ellis P.J. (1992).** Resistance to tomato spotted wilt virus infection in transgenic tobacco expressing the viral nucleocapsid gene. *Molecular Plant-Microbe Interactions* **5**, 34-50.

**Moyer J.W., & Qui W. (1996).** Molecular and genetic determinants of diversity in tomato spotted wilt virus (TSWV). *Acta Horticulturae* **431**, 219-227.

**Page R.D.M. (1996).** Treeview: An application to display phylogenetic trees on personal computers. *Computer Application in Bioscience* **12**, 357-358.

**Pang S-Z., Nagpala P., Wang M., Slightom J.L., & Gonsalves D. (1992).** Resistance to heterologous isolates of tomato spotted wilt virus in transgenic plants expressing its nucleocapsid protein. *Phytopathology* **82**, 1223-1229.

**Pappu H., Pappu S., Jain R., Bertrand P., Culbreath A., Mcpherson R., & Csinos A. (1998).** Sequence characteristics of natural populations of tomato spotted wilt tospovirus infecting flue-cured tobacco in Georgia. *Virus Genes* **17**, 169-177.

**Pozzer L., Bezerra I.C., Kormelink R., Prins M., Peters, D., Resende R.O., & De Avila A. (1999).** Characterization of a distinct isolate of iris yellow spot virus associated with a disease in onion fields in Brazil. *Plant Disease* **83**, 345-350.

**Qui W.P., Geske S.M., Hickey C.M., & Moyer J.W. (1998).** Tomato spotted wilt Tospovirus genome reassortment and genome segment-specific adaptation. *Virology* **244**, 186-194.

**Silva M.S., Martins C.R.F., Bezerra I.C., Nagata T., De Avila A.C., & Resende R.O. (2001).** Sequence diversity of NSm movement protein of tospoviruses. *Archives of Virology* **146**, 1267-1281.

**Takeda A., Sugiyama K., Nagano H., Mori M., Kaido M., Mise K., & Tsuda S. (2002).** Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. *FEBS Letters* **532**, 72-79.

**Thompson J.D., Gibson T.J., Plewnaik F., Jeanmougin F., & Higgins D.G. (1997).** The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876-4882.

**Tspompana M., Abad J., Purugganan M., & Moyer J.W. (2005).** The molecular population genetics of the tomato spotted wilt virus (TSWV) genome. *Molecular Ecology* **14**, 53-66.

**Verthoeven J.T.J., Roenhorst J.W., Cortes I., & Peters D. (1996).** Detection of a novel tospovirus in chrysanthemum. *Acta Horticulturae* **431**, 159-160.

# CHAPTER 4

# EVALUATION OF TOMATO (*Lycopersicon esculentum* Mill.) PLANTS WITH NATURAL AND TRANSGENIC RESISTANCE AGAINST TOMATO SPOTTED WILT VIRUS (TSWV) ISOLATES OCCURING IN SOUTH AFRICA


## ABSTRACT

Tomato spotted wilt virus (TSWV) infections cause significant economic losses in the commercial production of tomato (*Lycopersicon esculentum* Mill.). This study was undertaken to evaluate tomato with natural and transgenic resistance when inoculated with TSWV isolates occurring in South Africa (SA). The Stevens cultivar which has natural resistance to TSWV conferred by the Sw-5 gene and the transgenic 13-1 line which expresses the nucleocapsid (N) protein gene of the TSWV-BL isolate were used as test plants. Six TSWV isolates collected from Gauteng, KwaZulu-Natal, North West, Limpopo and Mpumulanga provinces were mechanically inoculated onto the test plants. The trial was arranged in a general treatment structure with randomised block design and repeated once. Plants were assessed for TSWV resistance based on a disease severity rating scale and measurements of virion accumulation levels ($A_{405}$nm). There were no significant differences among the reactions produced by the six TSWV isolates on the test plants. Although both plants were susceptible to the SA TSWV isolates by exhibiting similar high viral accumulation levels, the transgenic tomato line showed milder disease severity than the natural resistant cultivar. Results suggest that transgenic resistance is a more viable approach in the control of TSWV in SA.


## 4.1 INTRODUCTION

Tomato spotted wilt virus (TSWV) is an important pathogen infecting many crops on a worldwide scale. Over 1090 plant species including vegetables, ornamentals, fruit

trees and industrial crops are susceptible to TSWV, thus it has one of the largest host ranges of any plant virus (German *et al.*, 1992; Llamas-Llamas *et al.*, 1998; Peters, 2003). Considerable economic losses can occur due to infection rates of up to 90% in commercial crops (Cho *et al.*, 1989).

In South Africa (SA), the emergence of TSWV is a significant problem in crop cultivation (Thompson & van Zijl, 1996). A disease survey has ranked TSWV as the most prevalent viral disease in tomato (*Lycopersicon esculentum* Mill.) in SA. The tomato yields in each province in SA are reduced each year by TSWV (Uys *et al.*, 1996). The great economic loss caused by TSWV in tomato crops in SA has spurred interest into research on TSWV disease management.

The control of TSWV has been extremely difficult due to its extensive host range and resistance of the thrips vector to insecticides (Boiteux & Giordano, 1993). Consequently, resistant cultivars have proven to be the most effective method for controlling TSWV (Fraser, 1990).

Host-plant resistance to TSWV may be the most promising means of controlling the disease in the long term (de Haan *et al.*, 1996). Research has led to the identification and characterisation of several genes for TSWV resistance in tomato (Finlay, 1953; Stevens *et al.*, 1992; Roselló *et al.*, 1998). The Sw-5 gene, first identified in *L. peruvianum*, was found to be the more stable and less isolate-specific (Stevens *et al.*, 1992). Therefore, the Sw-5 gene has been used widely in breeding programmes (Cho *et al.*, 1989). However, in the field plants carrying the Sw-5 gene still accumulate virus resulting in the development of disease symptoms (Ultzen *et al.*, 1995). In addition, despite offering the most promising natural form of TSWV resistance, TSWV isolates virulent to Sw-5 have been identified in SA (Thompson & van Zijl, 1996), Hawaii (Canady *et al.*, 2001) and Australia (Latham & Jones, 1998). Therefore there is a need to identify and develop new sources of resistance to be incorporated into crop breeding programmes.

When host resistance against TSWV is overcome, pathogen-derived resistance (PDR) for virus control may provide a significant alternative to traditional strategies (Hoffmann *et al.*, 2001). Transgenic resistance to TSWV was first introduced into tobacco by Gielen *et al.* (1991) with the use of the nucleocapsid (N) gene. Engineered TSWV resistance has been introduced into tomato plants (Kim *et al.*, 1994; Ultzen *et al.*, 1995; Gonsalves *et al.*, 1996). This type of resistance is known as RNA-mediated resistance; where untranslatable constructs containing full length or segments of the N gene are able to confer resistance via a post-transcriptional gene silencing (PTGS) mechanism (Baulcombe, 1996; Pang *et al.*, 1996). Resistance is only effective against viral sequences with a high degree of sequence similarity to the transgene (Pang *et al.*, 1996).

It is well known that TSWV shows the capacity to generate new phenotypes more readily than other viruses (Moyer & Qui, 1996). This attribute makes it difficult to develop cultivars that exhibit long-term durable resistance. This study was conducted to evaluate the resistance of transgenic tomato expressing the N gene and natural resistant tomato carrying the Sw-5 gene to TSWV isolates occurring in SA.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Virus Isolates

Six TSWV isolates from different geographical locations in SA were used in this study. Isolates were tentatively named after the province in which they were sourced. These are: TSWV-GP (Zeekoegat, Gauteng), TSWV-KZN (Kranskop, KwaZulu-Natal), TSWV-LP (former 'Transvaal', Limpopo), TSWV-MP (Marble Hall, Mpumulanga), TSWV-NW1 (Klerksdorp, North West) and TSWV-NW2 (Groot Marico, North West). The details of these isolates are given in Chapter 3.

### 4.2.2 Test Plants

Two accessions of tomato were evaluated for TSWV resistance. Seeds of the transgenic line '13-1' and the natural resistant cultivar 'Stevens' were provided by C. Gonsalves[3]. The transgenic line had been produced by transferring the N gene of the TSWV-BL (Pang *et al.*, 1992) into a tobacco mosaic virus (TMV) resistant tomato line (G-80) (Gonsalves *et al.*, 1996). The 'Stevens' cultivar confers natural resistance through the expression of the Sw-5 dominant gene (Stevens *et al.*, 1992). The Geneva 80 (G-80) line was used as a susceptible control. The G-80 line was chosen because it possesses the Tm-$2^2$ gene which confers resistance to TMV, *Verticillium wilt*, and *Phytophthora infestans* (Race 0) (Provvidenti & Gonsalves, 1995).

Seeds were germinated in Speedling® 24 trays containing sterilised seedling mix (Growmor, Cato Ridge). Three weeks post germination, seedlings were transplanted into individual pots (12cm) filled with potting medium (Growmor, Cato Ridge). Irrigation was done three times daily for durations of 5min. Water was supplemented with soluble NPK fertilizer [3:1:3(38)]. Plants were kept at a constant temperature of 20-25°C in the Jolly Roger tunnel, University of KwaZulu-Natal, Pietermaritzburg (PMB) for the duration of the study.

### 4.2.3 Trial Design

A total of 18 treatments comprising of the three tomato lines inoculated with the six TSWV isolates was used. The pots were arranged in a general treatment structure with randomised block design. Each treatment comprised five replicates. The trial was repeated once.

[3] C. Gonsalves, Department of Plant Pathology, Cornell University, Geneva, NY 14456, USA.

### 4.2.4 Mechanical Inoculation

TSWV inoculum was obtained from fresh leaf material of infected *Nicotinia rustica* L. plants. At least 10 plants served as the inoculum source for each block. The inoculation procedure was carried out as previously described in section 2.2.1.3 (Chapter 2) with minor modifications. Three weeks post germination, the first two true leaves of the plant were mechanically inoculated. The upper leaves were reinoculated one week later to ensure maximum disease incidence. Negative controls were mock-inoculated with inoculation buffer only.

### 4.2.5 Screening for Resistance

Two weeks after the second inoculation, plants were assessed for resistance to TSWV. TSWV infection of plants was confirmed by ELISA. Samples with absorbance values higher than two-fold the value of the negative control were presumed to be positive for TSWV. The severity of TSWV disease and the amount of virion accumulation was used as an assessment of TSWV resistance.

#### *4.2.5.1 Disease rating*

Disease severity was assessed visually using the rating scale described by Canady *et al.* (2001) (Fig. 4.1). The visual rating scale:

1 = no visible symptoms

2 = mild chlorosis and limited leaf distortion

3 = moderate chlorosis, leaf distortion with some plant stunting

4 = severe chlorosis, leaf distortion and plant stunting

5 = severe chlorosis, leaf distortion and extreme stunting.


**Figure 4.1** Rating scale used for the assessment of disease severity caused by tomato spotted wilt virus (TSWV) on tomato (*Lycopersicon esculentum* Mill.). The visual rating scale: 1 = no visible symptoms; 2 = mild chlorosis and limited leaf distortion; 3 = moderate chlorosis, leaf distortion with some plant stunting; 4 = severe chlorosis, leaf distortion and plant stunting; 5 = severe chlorosis, leaf distortion and extreme stunting.

#### *4.2.5.2 Accumulation of TSWV virions*

Enzyme-linked immunosorbent assay (ELISA) was used to determine the amount of virion accumulation in infected plant tissue. The procedure was carried out as previously described in section 2.2.1.2 (Chapter 2). Plant tissue was collected from the apical leaves nearest to the growing point using an Eppendorf tube. To obtain a uniform sample size, the Eppendorf tube cap was snapped down on the leaf to punch out a leaf sample of approximately 10mm in diameter. Care was taken to ensure that the sample did not have excess non-affected tissue. Dead tissue was not tested. The absorbance value at $A_{405}$nm, which reflects viral particle accumulation in each sample, was read on an Anthos 2001 photometer (Anthos Labtec Instruments, Austria). Expressed sap from uninfected healthy control plants was used as the negative controls. Positive controls, provided in the kit, were rehydrated in 1ml distilled water. Each sample was loaded in duplicate wells and the mean absorbance value for the two duplicate wells was used for analysis.

### 4.2.6 Statistical Analysis

The experiment was repeated once and results were pooled together for statistical analysis. Data were subjected to analysis of variance (ANOVA) using GenStat 7$^{th}$ Edition statistical analysis software program. Mean separations were based on the Fiseur's least significant differences (LSD) at $P<0.05$.

## 4.3 RESULTS

All inoculated test plants tested positive for the presence of TSWV using ELISA. This indicated a disease incidence of 100%.

### 4.3.1 Disease Severity

There were no significant differences among the reactions produced by the six TSWV isolates on the Stevens cultivar and 13-1 line. Between the two resistant plants, the Stevens cultivar proved more susceptible to all six TSWV isolates with a mean disease severity of 2.9. The transgenic 13-1 line proved resistant to infection with a mean severity of 1.75. The susceptible G-80 line showed a high disease severity with an average mean of 4.78 (Table 4.1, Fig. 4.2, 4.3).

### 4.3.2 Accumulation of TSWV Virions

All six TSWV isolates showed no significant difference in the amount of virion accumulation in the Stevens cultivar and 13-1 line. The Stevens cultivar and 13-1 line also showed no significant difference in the amounts of virion accumulation with absorbance means of 0.444 and 0.413 respectively. The susceptible G-80 line showed a high virion accumulation with an absorbance mean of 1.828 (Table 4.1, Fig 4.4).

**Table 4.1** ANOVA of disease severity and virion accumulation in the evaluation of natural (Stevens) and transgenic (13-1) resistant tomato (*Lycopersicon esculentum* Mill.) plants against tomato spotted wilt virus (TSWV) isolates from South Africa.

| TSWV isolate<br>Test Plant | Severity[x] | Virion accumulation ($A_{405}$nm)[y] |
|---|---|---|
| TSWV-GP | | |
| G-80 | 4.7[a] | 1.79[a] |
| 13-1 | 1.6[b] | 0.40[b] |
| Stevens | 2.9[c] | 0.48[b] |
| TSWV-KZN | | |
| G-80 | 4.8[a] | 1.87[a] |
| 13-1 | 1.7[b] | 0.39[b] |
| Stevens | 2.8[c] | 0.47[b] |
| TSWV-LP | | |
| G-80 | 4.8[a] | 1.95[a] |
| 13-1 | 1.6[b] | 0.40[b] |
| Stevens | 2.7[c] | 0.45[b] |
| TSWV-MP | | |
| G-80 | 4.8[a] | 1.84[a] |
| 13-1 | 1.6[b] | 0.40[b] |
| Stevens | 2.7[c] | 0.40[b] |
| TSWV-NW1 | | |
| G-80 | 4.8[a] | 1.88[a] |
| 13-1 | 1.6[b] | 0.40[b] |
| Stevens | 2.7[c] | 0.43[b] |
| TSWV-NW2 | | |
| G-80 | 4.8[a] | 1.78[a] |
| 13-1 | 1.7[b] | 0.47[b] |
| Stevens | 2.9[c] | 0.40[b] |
| F-test | | |
| Test plant | <0.001 | <0.001 |
| Isolate | 0.350 | 0.431 |
| Test plant * Isolate | 0.700 | 0.139 |
| l.s.d | 0.316 | 0.166 |
| s.e.d | 0.155 | 0.081 |
| cv% | 6.1 | 11.2 |

-means followed by a different letter in the same column are significantly different at P = 0.05.
[x] disease severity assessed from visual rating scale of 1-5 (Fig. 4.1).
[y] TSWV virion accumulation measured by absorbance ($A_{405}$nm).

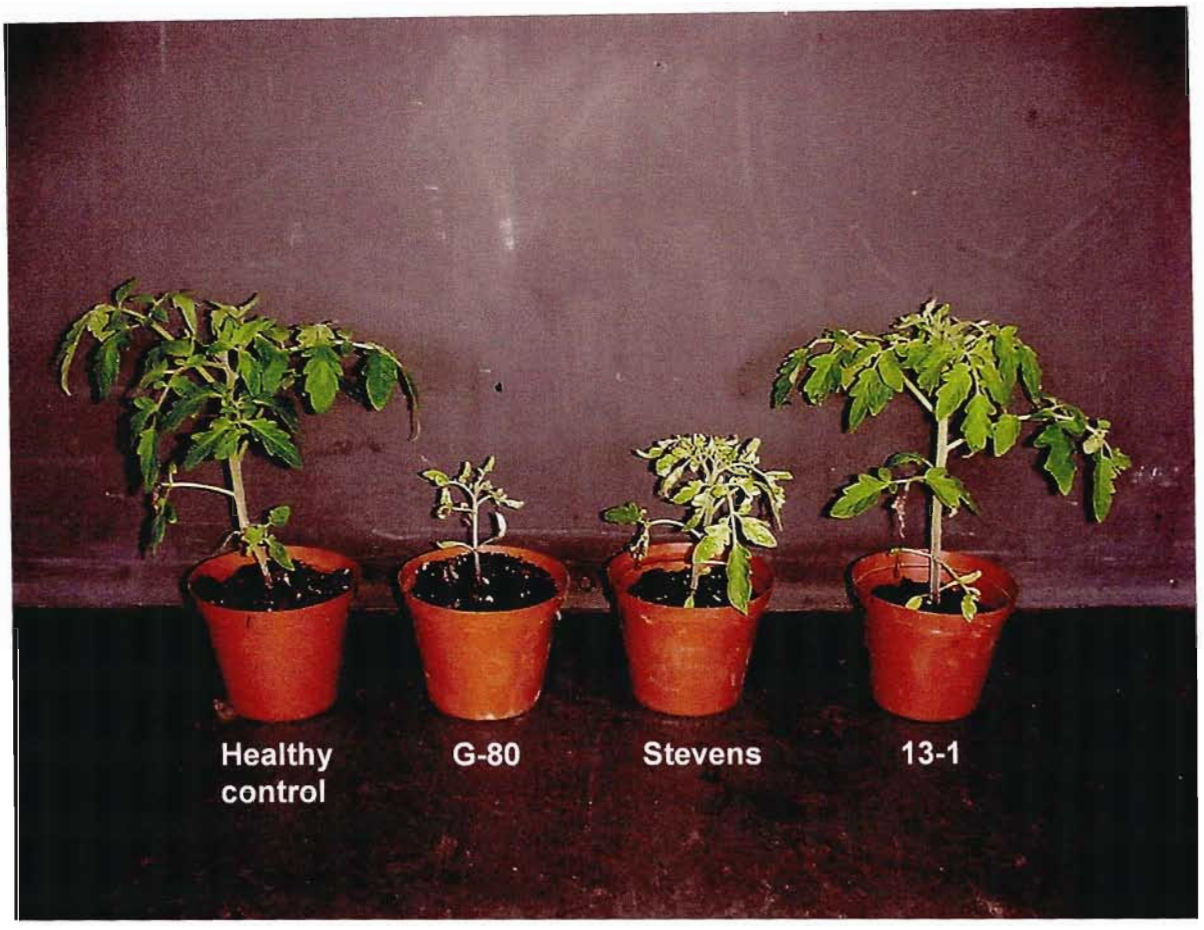



**Figure 4.2** Differential response of natural (Stevens) and transgenic (13-1) resistant tomato (*Lycopersicon esculentum* Mill.) plants against tomato spotted wilt virus (TSWV) isolates from South Africa. The Geneva 80 (G-80) line was used as a susceptible control. Healthy control was mock-inoculated with inoculation buffer.

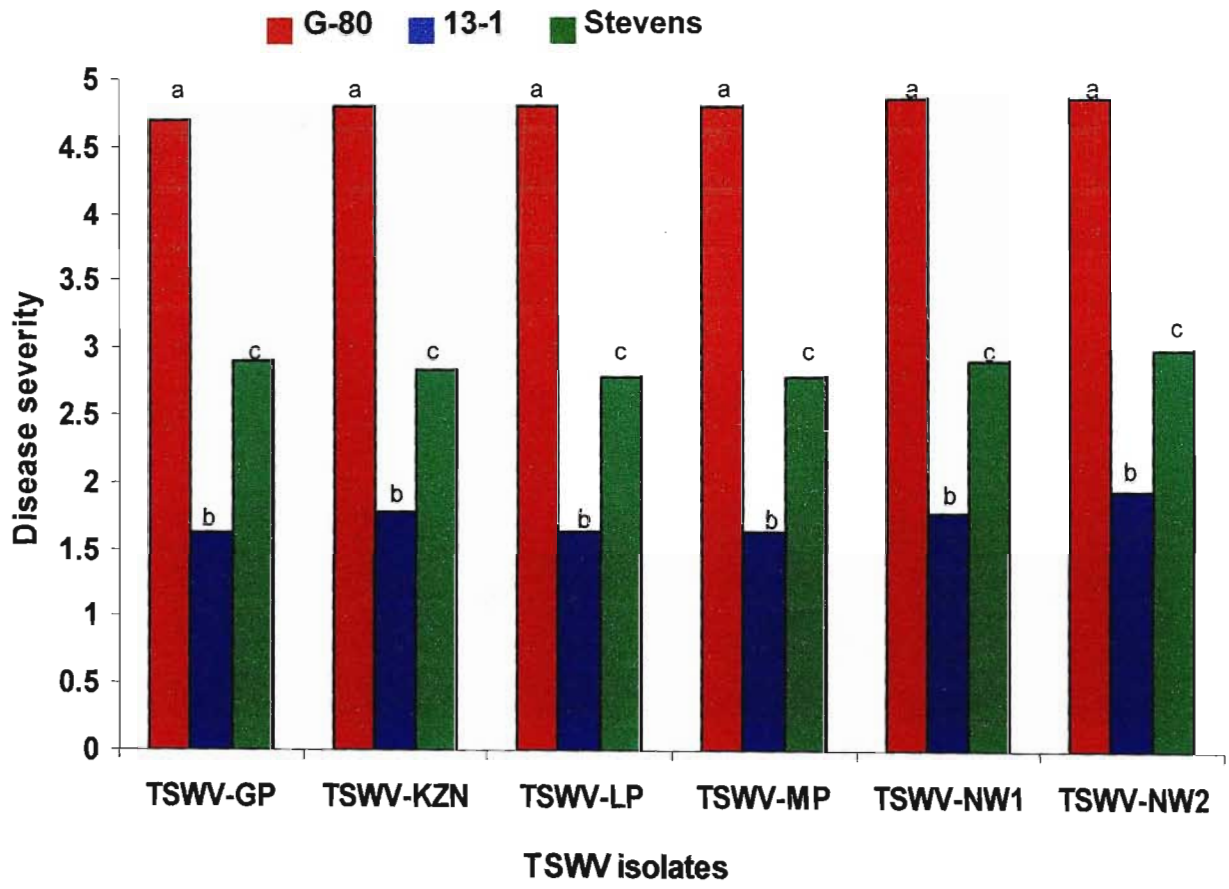



**Figure 4.3** Disease severity reactions in the evaluation of natural (Stevens) and transgenic (13-1) resistant tomato (*Lycopersicon esculentum* Mill.) plants against tomato spotted wilt virus (TSWV) isolates from South Africa.

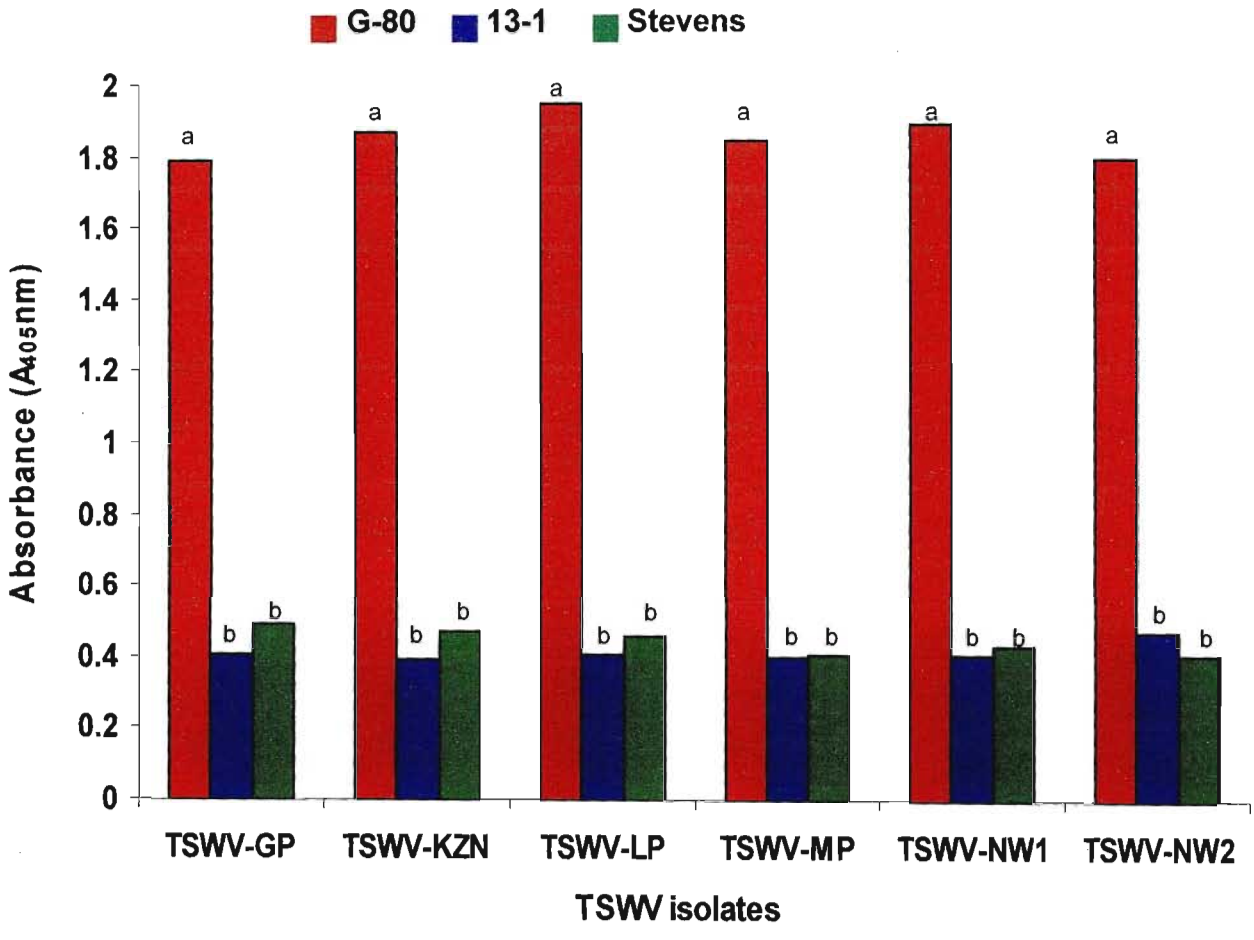



**Figure 4.4** Measurements of virion accumulation ($A_{405}$nm) in the evaluation of natural (Stevens) and transgenic (13-1) resistant tomato (*Lycopersicon esculentum* Mill.) plants against tomato spotted wilt virus (TSWV) isolates from South Africa.

## 4.4 DISCUSSION

Results of this study showed no significant differences in the reactions produced by the six SA TSWV isolates on the test plants evaluated. All isolates showed the same degree of virulence in that they produced comparable disease severity reactions and virion accumulation levels in all lines evaluated. Therefore, this degree of aggressiveness is a phenotypic trait that is common to all SA TSWV isolates used in this study. This phenotypic uniformity among isolates is reflected genotypically in the phylogenetic analysis of these isolates (Chapter 3). The phylogenetic analysis showed that N gene sequence homologies of 99-100% of SA isolates indicated that TSWV in SA has not evolved on a molecular level. The uniformity of reactions produced by SA TSWV isolates on test plants confirms the findings of the phylogenetic analysis.

All lines evaluated tested positive for the presence of TSWV by ELISA. Disease incidence of 100% would indicate that neither cultivar Stevens (natural) nor the 13-1 (transgenic) line exhibited immunity to TSWV isolates from SA. However, resistance encompasses a wide variety of host-pathogen interactions. A resistant phenotype reduces the growth, replication or disease-producing activities of the pathogen. Disease symptoms are less severe on resistant hosts than on susceptible hosts (Pataky & Carson, 2004). The Geneva 80 (G-80) line was used as a susceptible control as its phenotype was unable to restrict the replication and disease-producing properties of TSWV. Therefore, symptoms produced on G-80 were severe (disease severity mean of 4.78) and virion accumulation levels was high (absorbance value mean of 1.828). Resistant reactions produced on Stevens and 13-1 plants varied in degree and kind. The Stevens cultivar proved more susceptible to infection by SA TSWV isolates by exhibiting disease severity reactions in the range of 2-3, whilst the transgenic 13-1 line showed milder disease in the range of 1-2. The difference in host reaction is the basis for the differentiation of the two plants into categories of resistance. The Stevens cultivar can be seen as moderately resistant whilst the 13-1 line is seen as tolerant resistant.

Both test plants showed a decline in virion accumulation when compared to the level seen in the susceptible G-80 line. This is indicative of the mechanisms that govern both Sw-5 and N gene mediated resistance. In natural resistance, it was proposed that reduced virion accumulation levels may be due to the inhibition of virion disassembly in initially infected cells (Baulcombe, 1996). In the case of N gene mediated resistance, the reduction in virion accumulation levels is due to post-transcriptional gene silencing (PTGS) (Pang *et al.*, 1992).

The varied disease severity reactions produced on the Stevens and 13-1 plants were not reflected in the levels of virion accumulation. Both test plants exhibited similar levels of virion accumulation despite showing significantly different disease severity reactions. Although the 13-1 line showed the same level of virion accumulation, it exhibited milder disease symptoms than seen on the Stevens cultivar. Absorbance readings detect levels of N protein which indirectly show the amount of virion accumulation. Research has shown that transgenic resistance conferred by the N gene is RNA-mediated. Therefore transgenic plants expressing intact N gene showing resistance to homologous or closely related TSWV isolates is due to the presence of the N gene transcript and not the N protein. A high amount of N protein detected by ELISA that would cause disease in the Stevens cultivar does not result in severe disease in the transgenic 13-1 line.

Generally, resistance to TSWV from tomato containing the Sw-5 gene has been non-isolate dependent and stable (Bioteux & Giordano, 1992). However in this study, the Stevens cultivar which contains the Sw-5 gene was overcome by the SA TSWV isolates. TSWV isolates that overcome the Sw-5 gene have also been identified in SA ((Thompson & van Zijl, 1996), Hawaii (Canady *et al.*, 2001) and Australia (Latham & Jones, 1998). In SA, Thompson & van Zijl (1996) reported that four samples of symptomatic Stevens plants from the Klapmuts district of the Western Cape were positive for TSWV. The isolates were referred to as the JF strains. At the time, they found that the JF strains were not able to establish themselves and spread beyond the original field in which they were isolated from. The ability of the SA TSWV

isolates reported in this study to overcome the Stevens cultivar, extend these initial reports, by suggesting that the JF isolates might have spread from the Western Cape to other provinces in SA. Considering the current rapid expansion of one of its major vectors (*Frankliniella occidentalis*) and the recent upsurge of agricultural activity in SA, it is not surprising that these strains have spread and established themselves in other provinces in SA.

The tolerant phenotype observed in the transgenic 13-1 line is indicative of a milder form of plant infection in which the virus has accumulated without causing significant damage to the plant. This phenotype is of commercial value as it reduces economic losses that result from severe symptom expression. Results indicate that resistance conferred by the N gene is similarity-dependent (Baulcombe, 1996). The transgenic 13-1 line was transformed with N gene from the TSWV-BL isolate. Sequence comparisons reported in Chapter 3 reveal a >90% similarity between the N genes of the SA isolates and the TSWV-BL isolate.

Field evaluations will be more useful in this study than mechanical inoculation of tomato cultivars in evaluating TSWV resistance. The potential for multiple introductions of a virus across an entire leaf surface during the mechanical inoculation procedure may impart a higher level of disease pressure than natural thrips inoculation. As a result, mechanical inoculations may overwhelm slight variations in disease resistance mechanisms, which may be more apparent under field conditions (Garcia *et al.*, 2000). In addition, field environments present a more diverse array of challenging TSWV strains that occur as a result genomic reassortment in nature.

Information presented here clearly illustrates the devastating effects of TSWV infection on tomato. Data suggests that transgenic resistance is a more viable approach to disease control when natural host-resistance is overcome. Knowledge of uniform virulence among TSWV strains from SA together with information on disease severity on transgenic and natural resistant cultivars could be used as a tool to make strategic decisions in integrated control programmes for TSWV.

## 4.5 REFERENCES


**Baulcombe D.C. (1996).** Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* **8**, 1833-1844.

**Boiteux L.S., & Giordano L.B. (1992).** Screening *Lycopersicon* germplasm for resistance to a Brazilian isolate of tomato spotted wilt virus (TSWV). *Tomato Genetics Cooperation Reporter* **42**, 13-14.

**Boiteux L.S., & Giordano L.B. (1993).** Genetic basis of resistance against two Tospovirus species in tomato (*Lycopersicon esculentum*). *Euphytica* **71**, 151-154.

**Canady M.A., Stevens M.R., Barineau M.S., & Scott J.W. (2001).** Tomato spotted wilt virus (TSWV) resistance in tomato derived from *Lycopersicon chilense* Dun. LA 1938. *Euphytica* **117**, 19-25.

**Cho J., Mau R.F.L., German T.L., Hartmann R.W., Yudin L.S., Gonsalves D., & Provvidenti P. (1989).** A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. *Plant Disease* **73**, 375-383.

**de Haan P., Ultzen T., Prins M., Gielen J., Goldbach R., & van Grinsven. (1996).** Transgenic tomato hybrids resistant to tomato spotted wilt virus infection. *Acta Horticulturae* **431**, 417-426.

**Finlay K.W. (1953).** Inheritance of spotted wilt resistance in the tomato II. Five genes controlling spotted wilt resistance in four tomato types. *Australian Journal of Biological Sciences* **6**, 153-163.

**Fraser R.S.S. (1990).** The genetics of resistance to plant viruses. *Annual Review of Phytopathology* **28**, 179-200.

**Garcia L.E., Brandenburg R.L., & Bailey J.E. (2000).** Incidence of tomato spotted wilt virus (Bunyavirus) and tobacco thrips in Virginia-type peanut in North Carolina. *Plant Disease* **84**, 459-464.

**German L.T., Ulman D.E., & Moyer J.W. (1992).** Tospoviruses: diagnosis, molecular biology, phylogeny, and vector relationships. *Annual Review of Phytopathology* **30**, 315-348.

**Gielen J.J.L., de Haan P., Kool A.J., Peters D., van Grinsven M.Q.J.M., & Goldbach R.W. (1991).** Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. *Bio/Technology* **9**, 1363-1367.

**Gonsalves C., Xue B., Pang S-Z., Provvidenti R., Slightom J. L., & Gonsalves D. (1996).** Breeding transgenic tomatoes for resistance to tomato spotted wilt virus and cucumber mosaic virus. *Acta Horticulturae* **431**, 442-448.

**Hoffmann K., Qui W.P., & Moyer J.W. (2001).** Incoming host-and pathogen-mediated resistance in tomato and tobacco maps to the M RNA of tomato spotted wilt virus. *Molecular Plant-Microbe Interactions* **14**, 242-249.

**Kim J.W., Sun S.S.M., & German T. L. (1994).** Disease resistance in tobacco and tomato plants transformed with the tomato spotted wilt virus nucleocapsid gene. *Plant Disease* **78**, 615-621.

**Latham L.J., & Jones R.A.C. (1998).** Selection of resistance breaking strains of tomato spotted wilt tospovirus. *Annals of Applied Biology* **133**, 385-402.

**Llamas-Llamas M.E., Zavaleta-Mejia E., Gonzalez-Hernandez V.A., Cervantes-Diaz L., Santizo-Rincon J.A., & Ochoa-Martinez D.L. (1998).** Effect of temperature on symptom expression and accumulation of TSWV in different host species. *Plant Pathology* **47**, 341-347.

**Moyer J.W., & Qui W. (1996).** Molecular and genetic determinants of diversity in tomato spotted wilt virus (TSWV). *Acta Horticulturae* **431**, 219-227.

**Pang S.Z., Nagpala P., Wang M., Slightom J.L., & Gonsalves D. (1992).** Resistance to heterologous isolates of tomato spotted wilt virus in transgenic tobacco expressing its nucleocapsid protein gene. *Phytopathology* **82**, 1223-1229.

**Pang S.Z., Jan F.L., Carney K., Stout J., Tricoli D.M., Quemada H.D., & Gonsalves D. (1996).** Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. *Plant Journal* **9**, 899-909.

**Pataky J.K., & Carson M.L. (2004).** Host Resistance. In: *Plant Pathology: Concepts and Laboratory Excercises,* Trigiano R.N., Windham M.T., & Windham A.S., (eds.). New York: CRC Press, pp. 295-233.

**Peters D. (2003).** Tospoviruses. In: *Viruses and Virus-like Diseases of Major Crops in Developing Countries*, Loebenstein G., & Thottappilly G., eds. USA: Kluwer Academic Publishers, pp. 719-737.

**Provvidenti R., & Gonsalves D. (1995).** Inheritance of resistance to cucumber mosaic virus in a transgenic tomato line expressing the coat protein gene of the white leaf strain. *Journal of Hereditary* **86**, 85-88.

**Roselló S., Soler S., Diez M.J., Rambla J.L., Richarte C., & Nuez F. (1998).** New sources for high resistance of tomato to the tomato spotted wilt virus from *Lycopersicon peruvianum*. *Plant Breeding* **118**, 425-429.

**Stevens M.R., Scott S.J., Gergerich R.C. (1992).** Inheritance of a gene for resistance to tomato spotted wilt virus (TSWV) from *Lycopersicon peruvianum* Mill. *Euphytica* **59**, 9-17.

**Thompson G.J., & van Zijl J.J.B. (1996).** Control of tomato spotted wilt virus in tomatoes in South Africa. *Acta Horticulturae* **431**, 379-384.

**Ultzen T., Gielen J., Venema F., Westerbroek A., de Haan P., Tan M-L., Schram A., van Grinsven M., & Goldbach R. (1995).** Resistance to tomato spotted wilt virus in transgenic tomato hybrids. *Euphytica* **85**, 159-168.

**Uys M.D.R., Thompson A.H., & Holz G. (1996).** Diseases associated with tomato in the main tomato-growing regions of South Africa. *Journal of South Africa Society of Horticultural Science* **6**, 78-80.

# CHAPTER 5
# GENERAL OVERVIEW

It is well documented that the tomato spotted wilt virus (TSWV) is responsible for considerable economic losses in crop productivity on a world-wide basis (Cho *et al.*, 1989; Goldbach & Peters, 1994; Mumford *et al.*, 1996). Yet despite years of investigation and research, control measures are still not entirely effective (Cho *et al.*, 1998).

The lack of tangible progress in devising effective control measures continues to be a severe impediment to the comprehensive assessment of the impact of TSWV in SA and in the implementation of sustainable control measures (Thresh, 2003). Contributions of molecular and characterisation studies provide immeasurable value in the development of control for any plant pathogen. Therefore, the purpose of this investigation was to provide information on the basic characterisation and genetic diversity of TSWV in SA.

The findings presented in this study result from the isolation and partial characterisation of a TSWV isolate from KwaZulu-Natal (KZN), phylogenetic analysis of TSWV isolates occurring in SA, and the evaluation of plants with natural and transgenic resistance against the SA TSWV isolates. It was established that:

- TSWV is present in KZN. The characteristic symptoms; size, shape and cytopathological effects seen under the electron microscope; positive reaction in ELISA; protein analysis via SDS-PAGE and the amplification of the N gene using RT-PCR confirm this isolate to be a typical Tospovirus.
- All SA TSWV isolates shared a high degree of sequence similarity that is reflected in their distinct clustering pattern. This clustering pattern might be indicative of an evolutionary trend of TSWV in Africa.
- The genotypic uniformity of TSWV isolates is reflected in their resistance phenotypes when inoculated on plants with transgenic and natural resistance,

where all isolates showed no significant differences in virion accumulation levels and disease severity reactions on all test plants.

- The transgenic 13-1 line proved to be more effective in controlling TSWV compared to the natural resistant Stevens cultivar which was overcome by TSWV isolates from SA.

## 5.1 The Emergence of TSWV in KZN

TSWV was long believed to be found only in the Eastern Cape, Free State and throughout the former 'Transvaal'. Early failures to detect TSWV in other provinces can be attributed partly to the use of inadequate detection and identification techniques (Peters, 2003). In this study, a synergistic combination of techniques that explore the macroscopic and microscopic effects of virus replication, virus size and shape, serology, protein and nucleic acid composition were used to tentatively identify the Tospovirus isolate as TSWV.

The extremely wide host range and the ability to propagate in its insect vector, a capacity which increases the efficiency of transmission, demonstrates the great potential of TSWV (and other Tospoviruses) to become a major limiting factor to crop production in KZN. The rapid and reliable diagnosis of TSWV is therefore essential in the implementation of effective control measures. Having established the presence of TSWV in KZN, attempts can be made to implement control measures in the early stages of the virus infection cycle before it becomes a widespread problem throughout the province.

Still one may wonder why this virus, although first identified in SA in 1939, has only now been identified in KZN. It may have been that the disease and its causal agent had previously been overlooked or not correctly identified. A more probable explanation is that TSWV, or its vector, has spread recently due to natural and/or human influence. The impact of changing agricultural practices and the increase of international and national exchange of plant material such as cuttings, seedlings and

vegetative plant parts may have catalysed the expansion and spread of TSWV into KZN. Another major cause underlying the emergence of TSWV in KZN is undoubtedly the worldwide spread of the major TSWV vector *Frankliniella occidentalis*, which is due to increased transcontinental trading and the indiscriminate use of chemicals to control it. The increase in thrips population coincides with an increasing resistance of these insects to the chemicals that have traditionally been used to control them. The use of chlorated hydrocarbon compounds has been partially replaced by artificial pyrethriods. This change, together with the build up of pesticide resistance, corroborates the evidence of the dramatic outbreaks of thrips and in turn TSWV (Goldbach & Peters, 1994).

Purified virus samples are essential for conducting detailed virus studies. The best illustration of this is that although numerous viruses were recognised, and studied extensively for many years, their nature remained a mystery until tobacco mosaic virus (TMV) was purified by Stanley (1953). Its clear that very pure virus preparations are essential for chemical, physical, and certain biochemical studies. In addition, purification of a virus is a significant step in its identification. Hence, during the past three decades much effort has been directed towards the development of techniques for the purification of plant viruses (Francki, 1972). The ultimate goal of purification is a high yield of virus with the lowest amount of contaminates and highest degree of integrity, including infectivity (Bos, 1999). However the final result of the purification procedure used in this study was disappointing because the virus was lost after the further purification procedure. Although the partially pure suspension proved useful in the characterisation procedures, a suspension of higher purity could have been more beneficial in revealing precise protein banding patterns in SDS-PAGE procedure.

Many purification procedures for TSWV have been reported in the available literature (Black *et al.*, 1963; Joubert *et al.*, 1974; Gonsalves & Trujillo, 1986). The difficulty in successfully applying a published procedure that was formulated in one laboratory in purifying TSWV in our laboratory reflects the different intrinsic properties of virus culture in each investigation. The different properties of virus isolates from different

geographical locations during purification may represent different isolates of TSWV being studied (Hsu, 1996). Hence, a purification procedure that encompasses the specific intrinsic properties of the SA TSWV isolates should be formulated.

## 5.2 Genetic Diversity of TSWV in SA

TSWV has long been known to exist as complex populations of stable isolates in nature (Best & Gallus, 1953; Best, 1968). Their rapid adaptability and the ability of TSWV to multiply in its thrips vector increase the opportunity for genetic diversification in virus populations (Hoffmann *et al.*, 2001). Given these attributes, it was logical to investigate the existence of variations among isolates of TSWV from SA. Knowledge of the genetic variability of TSWV isolates is essential in the success of genetic engineering resistance strategies of control. This is due to the fact that resistance conferred by the N gene is only effective against isolates with a high degree of sequence similarity to the transgene (Pang *et al.*, 1996).

Surprisingly, no genetic variation was found among TSWV isolates in SA was found. This result indicated that TSWV in SA has not evolved at a molecular level. Evolution is a process in which genetic variation in a population changes over time (Snustad & Simmons, 1997). It could be that since the emergence of TSWV in SA dates back to as early as 1939 (Thompson & van Zijl, 1996), insufficient time has passed for evolutionary forces to sift and shape mutational variation in SA TSWV populations. However, given the necessary length of evolutionary time, natural selection will allow viral mutants with impaired survival and replication abilities to decrease in frequency, and mutants with superior survival and replication abilities to increase in frequency (Snustad & Simmons, 1997). It may be that the generation of new resistant varieties could impose selection pressure that could favour the selection of variants with the ability to overcome current resistance strategies for control. Therefore, population genetic studies should be carried out on SA TSWV populations to investigate the systemic and random forces of evolution, and in doing so, attempts can be made to

elucidate the fundamental mechanisms that will cause genetic changes in TSWV populations over time.

The fact that TSWV was first discovered in Australia, and that at least 10 years had passed before it was detected in any other country, does not necessarily imply that TSWV originated in Australia. But until valid evidence is presented in favour of its origin in some other country, it is presumed that TSWV may have been indigenous to Australia, being maintained there in symptomless or near symptomless native plants as hosts, and then spread to other countries from these host plants. The United Kingdom can be ruled out as the original source, because to explain the export of TSWV from any country would require that country to have a good reservoir for TSWV. The UK did not have the virus when it was rampant in Australia in the second and third decades of this century. The same could be said for the rest of Europe, which can be likewise ruled out. It is more likely that the virus entered the UK from Australia than the reverse, since the rapid spread in England post-dates that of Australia by 10 to 15 years (Best, 1968).

The question still remains - could TSWV have originated in another country: Africa, perhaps? In this study, a distinct clustering pattern was observed amongst all SA TSWV isolates and it is believed that this pattern could be indicative of an evolutionary trend of TSWV in Africa. Taking into consideration the limited amount of sequence data from TSWV isolates in Africa, further isolates from the rest of the African continent need to be identified and characterised. The nucleotide sequence data could provide the foundation to elucidate any phylogenetic relationships between isolates. An inference of these relationships could be used to explore the possibility of Africa being the source of TSWV.

## 5.3 Transgenic vs. Natural Resistance

Since there are very few natural resistant genes effective against TSWV infections, biotechnological approaches may become of great value in the control of TSWV. To

this end, transforming plants with the N gene of TSWV has been shown impart some resistance to infection by TSWV (Gielen *et al.*, 1991; Kim *et al.*, 1994; Ultzen *et al.*, 1995; Gonsalves *et al.*, 1996). This study illustrates the value of transgenic resistance. Whilst the natural resistant Stevens cultivar was overcome by the SA TSWV isolates, high levels of resistance were shown by transgenic plants with the nucleoprotein (N) gene.

The complexity of virus-host interactions and the great variability of TSWV condition the appearance of more virulent isolates (Latham & Jones, 1998) or the increase in the aggressiveness of those already present (Roselló *et al.*, 1999). These attributes have been a major impediment to those developing resistant cultivars. It should be stated that virtually no cultivars of major crops that carry significant levels of resistance to TSWV have been sufficiently durable to last more than a mere few years. Frequently, the resistance has been broken by the time the new cultivar has been passably field tested. TSWV has repeatedly overcome resistance genes deployed in these crops (Moyer & Qui, 1996). Therefore, the results of this study are anecdotal as it is too soon to know if transgenic resistance will be a more effective control approach in the future. Any long-term, stable effects, genetic or epidemiological, are amenable to direct experimentation and more accurate risk assessment using an extensive range of plant species transformed with a wide range of viral sequences (Wilson, 1993). More studies such as this should be carried out as they provide valuable information in the assessment of the durability of transgenic resistance in the long term. The results of this study can be extended and confirmed by further evaluation of the cultivars under field conditions. A field study will provide a more accurate array of challenging viral isolates that arise in a natural environment.

## 5.4 Future Research

Any attempt to provide an adequate characterisation of a virus must include information on its transmission. Vector transmission of TSWV is critical in determining the development of the virus (Boiteux *et al.*, 1993). Specific

understanding of the insect-virus interactions leading to successful transmission is a central problem in vector biology and crucial to developing effective control strategies (Ullman *et al.*, 2005). Our understanding of the intermediate relationship between TSWV and thrips is poor. Therefore, studies on thrips biology, viral determinants of transmission, the feeding behaviour of thrips on plants and the genetic diversity of thrips in SA should be undertaken.

It still remains to be seen whether the uniformity among TSWV isolates in SA will remain stable in future. The present study provides a preliminary account of the characterisation and genetic diversity of TSWV in SA. Such information could be integrated into the data generated by possible future studies on virus-vector relationships, characterisation of African TSWV isolates, population genetics of SA isolates, and field evaluations of transgenic lines to lay a more substantial and durable foundation in the formulation of effective control measures.

## 5.5 References


**Best R.J. (1968).** Tomato spotted wilt virus. *Advances in Virus Research* **13**, 65-145.

**Best R.J., & Gallus H.P.C. (1953).** Strains of tomato spotted-wilt virus. *Australian Journal of Science* **15**, 212-214.

**Black L.M., Brakke M.K., & Vatter A.E. (1963).** Purification and electron microscopy of tomato spotted-wilt virus. *Virology* **20**, 120-130.

**Bioteux L.S., Nagata T., Dutra W.P., & Fonseca M.E.N. (1993).** Sources of resistance to tomato spotted wilt virus (TSWV) in cultivated and wild species of Capsicum. *Euphytica* **67**, 89-93.

**Bos L. (1999).** *Plant Viruses: Unique and Intriguing Pathogens, A Textbook of Plant Virology.* Leiden: Backhuys, pp. 42-79.

**Cho J., Mau R.F.L., German T.L., Hartmann R.W., Yudin L.S., Gonsalves D., & Provvidenti P. (1989).** A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. *Plant Disease* **73**, 375-383.

**Cho J., Mau R.F.L., Pang S., Wang M., Gonsalves C., Watterson J., Custer D. M., Gonsalves D. (1998).** Approaches for controlling tomato spotted wilt virus. In: *Plant Virus Disease Control*, Hadidi A., Khetarpal R.K., & Kogenezawa B.D. (eds.). St. Paul: APS Press, pp. 547-563.

**Gielen J.J.L., de Haan P., Kool A.J., Peters D., van Grinsven M.Q.J.M., & Goldbach R.W. (1991).** Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. *Bio/Technology* **9**, 1363-1367.

**Goldbach R., & Peters D. (1994).** Possible causes of the emergence of tospovirus diseases. *Seminars in Virology* **5**, 113-120.

**Gonsalves D., & Trujillo E.E. (1986).** Tomato spotted wilt virus in Papaya and detection of the virus by ELISA. *Plant Disease* **70**, 501-506.

**Gonsalves C., Xue B., Pang S-Z., Provvidenti R., Slightom J. L., & Gonsalves D. (1996).** Breeding transgenic tomatoes for resistance to tomato spotted wilt virus and cucumber mosaic virus. *Acta Horticulturae* **431**, 442-448.

**Hoffmann K., Qui W.P., & Moyer J.W. (2001).** Incoming host-and pathogen-mediated resistance in tomato and tobacco maps to the M RNA of tomato spotted wilt virus. *Molecular Plant-Microbe Interactions* **14**, 242-249.

**Hsu H.T. (1996).** Immunological detection and identification of tospoviruses. *Acta Horticulturae* **431**, 109-121.

**Joubert J.J., Hahn J.S., von Wechmar M.B., & van Regenmortel M.H.V. (1974).** Purification and properties of tomato spotted wilt virus. *Virology* **57**, 11-19.

**Francki R.I.B. (1972).** Purification of viruses. In: *Principles and Techniques in Plant Virology*, Kado C.I., & Agrawal H.O. (eds.). New York: Van Nostrand Reinhold Company, pp. 295-344.

**Kim J.W., Sun S.S.M., & German T. L. (1994).** Disease resistance in tobacco and tomato plants transformed with the tomato spotted wilt virus nucleocapsid gene. *Plant Disease* **78**, 615-621.

**Latham L.J., & Jones R.A.C. (1998).** Selection of resistance breaking strains of tomato spotted wilt tospovirus. *Annals of Applied Biology* **133**, 385-402.

**Moyer J.W., & Qui W. (1996)** Molecular and genetic determinants of diversity in tomato spotted wilt virus (TSWV). *Acta Horticulturae* **431**, 219-227.

**Mumford R.A., Barker I., & Wood K.R. (1996).** The biology of tospoviruses. *Annual Applied Biology* **128**, 159-183.

**Pang S.Z., Jan F.L., Carney K., Stout J., Tricoli D.M., Quemada H.D., & Gonsalves D. (1996).** Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. *Plant Journal* **9**, 899-909.

**Peters D. (2003).** Tospoviruses. In: *Viruses and Virus-like Diseases of Major Crops in Developing Countries*, Loebenstein G., & Thottappilly G., eds. USA: Kluwer Academic Publishers, pp. 719-737.

**Roselló S., Soler S., Diez M.J., Rambla J.L., Richarte C., & Nuez F. (1999).** New sources for high resistance of tomato to the tomato spotted wilt virus from *Lycopersicon peruvianum*. *Plant Breeding* **118**, 425-429.

**Stanley W.M. (1935).** Isolation of a crystalline protein possessing the properties of tobacco mosaic virus. *Science* **81**, 644-700.

**Snustad P., & Simmons M.J. (1997).** Principles of Genetics. New York: John Wiley & Sons, Inc, pp. 774-777.

**Thompson G.J., & van Zijl J.J.B. (1996).** Control of tomato spotted wilt virus in tomatoes in South Africa. *Acta Horticulturae* **431**, 379-384.

**Thresh J.M. (2003).** The impact of plant virus diseases in developing countries. In: *Viruses and Virus-like Diseases of Major Crops in Developing Countries*, Loebenstein G., & Thottappilly G., eds. USA: Kluwer Academic Publishers, pp. 1-30.

**Ullman D.E., Whitfield A.E., & German T. L. (2005).** Thrips and tospoviruses come of age: Mapping determinants of insect transmission. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4931-4932.

**Ultzen T., Gielen J., Venema F., Westerbroek A., de Haan P., Tan M-L., Schram A., van Grinsven M., & Goldbach R. (1995).** Resistance to tomato spotted wilt virus in transgenic tomato hybrids. *Euphytica* **85**, 159-168.

**Wilson T.M.A. (1993).** Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 3134-3141.

# APPENDIX A
# ELISA BUFFERS

**PBS (pH 7.4)**

8.0g NaCl

0.2g $KH_2PO_4$

2.9g $Na_2HPO_4.12H_2O$ (1.15g anhydrous)

0.2g KCl

0.2g $NaN_3$

Make up to 1 liter

**PBS-Tween**

PBS + 0.5ml Tween 20 per liter

**Coating Buffer (pH 9.6)**

1.59g $Na_2CO_3$

2.93g $NaHCO_3$

0.2g $NaN_3$

Make up to 1 liter

**Sample Extraction Buffer**

PBS-Tween + 2% PVP-40 polyvinyl pyrrolidone

**Enzyme Conjugate Buffer**

PBST + 2% PVP-40 polyvinyl pyrrolidone + 0.2% ovalbumin

**Substrate Buffer (pH 9.8)**

97ml Diethanolamine

800ml water

0.2g $NaN_3$

Make up to 1 liter

# APPENDIX B
# TISSUE EMBEDDING AND ULTRA-THIN SECTIONING PROCEDURE

**Step 1**

5mm by 5mm sections were cut around chlorotic spots from leaves of infected *N. rustica* plants.

**Step 2**

Samples were fixed in 3% Glutaraldehyde in 0.05M buffer (sodium cacodylate buffer, pH 6.88) for 48 hours.

**Step 3**

The leaf sections were washed in 0.05M buffer. Two washes of 30 minutes each were done with the caps of vile taken off. The sections were then placed in a post-fixation solution consisting of 2% Osmium tetroxide in 0.05 M buffer for 1, 5 hours.

**Step 4**

The sections were washed in 0.05M buffer twice, 30 minutes per washed, before dehydration in a graded ethanol series (10-100%) for a minimum of 10 minutes per ethanol solution.

**Step 5**

Leaf sections were washed twice in propylene oxide (30 minutes per wash) and embedded in Epon/Araldite using the following EPON-propylene oxide mixes:

25% EPON: 75% propylene oxide + one drop DMP-30 (1 hour with cap on)
50% EPON: 50% propylene oxide + two drops DMP-30 (1 hour with cap on)
75% EPON: 25% propylene oxide + two drops DMP-30 (overnight with cap off)
100% EPON: EPON + two drops DMP-30 (24 hours with caps on) x 2

**Step 6**

The samples were placed in a foil dish containing a 100% EPON plus DMP mix. The dish was baked in the oven at 70°C for 48 hours.

**Step 7**

The embedded samples were then cut out of the resin using a handsaw. The blocks were then mounted onto plastic stubs and cut into ultra-thin sections with glass knives in a microtone. The thin sections were carefully placed on formvar coated grids, negatively stained with uranyl acetate and viewed under the TEM. Control leaf material taken from healthy uninoculated plants was also prepared in the same way.

# APPENDIX C
# SDS-PAGE STOCK SOLUTIONS

**Solution A: Monomer Solution [30% (m/v) acrylamide, 2.7% (m/v) Bis-acrylamide]**

Acrylamide (14.0g)

Bis-acrylamide (0.4g)

Dissolved and made up to 50ml with distilled water

Stored in an amber coloured bottle at 4°C.

Filtered through Whatman No. 1 filter paper before use

**Solution B: 4x Running Gel Buffer [1.5M Tris-HCl, pH 8.8]**

Tris (9.070g)

Dissolved in 40ml distilled water

Adjust to pH 8.8 with HCl

Made up to 50ml

Filtered through Whatman No. 1 filter paper before use

**Solution C: 4x Stacking Gel Buffer [500mM Tris-HCl, pH 6.8]**

Tris (3g)

Dissolved in 20ml distilled water

Adjust to pH 6.8

Made up to 25ml

Filtered through Whatman No. 1 filter paper before use

**Solution D: 10% (m/v) SDS**

SDS (2.5g)

Dissolved in 25ml distilled water with gentle heating

**Solution E: Initiator [10% (m/v) ammonium persulfate]**

Ammonium persulfate (0.1g)

Made up to 1 ml just before use

**Solution F: Tank Buffer [250mM Tris-HCl, 192mM glycine, 0.1% (m/v) SDS, pH 8.3]**

Tris (6g)

Glycine (14.4g)

Made up to 1 liter with distilled water

2.5ml of solution D was added to 250ml prior to use

**Solution G: Reducing Treatment Buffer [125mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]**

Solution C (0.25ml)

Solution D (0.4ml)

Glycerol (0.2ml)

2-mercaptoethanol (0.1ml)

Made up to 1ml in distilled water

**Stain stock solution [1% (m/v) Coosmassie blue R-250]**

Coomassie blue R-250 (0.5g)

Dissolved in 50ml of distilled water, magnetic stirring for 1 hour at room temp.

Filter through Whatman No. 1 filter paper

**Staining solution [0.125% (m/v) Coosmassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]**

Stain stock (31.25ml)

Methanol (125ml)

Acetic acid (25ml)

Made up to 250ml with distilled water

**Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]**

Methanol (250ml)

Acetic acid (50ml)

Made up to 500ml with distilled water

**Destaining solution II [5% (v/v) methanol, 7% (v/v) acetic acid]**

Methanol (25ml)

Acetic acid (35ml)

Made up to 500ml with distilled water

# APPENDIX D
# CLUSTALX (1.81) MULTIPLE SEQUENCE ALIGNMENT

```
TSWV-NW2        AAGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-KZN        AAGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-NW1        AAGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-MP         AAGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-LP         AAGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-GP         AAGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-ITALY      AAGCAAGTTCTGCGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-GER        AAGCAAGTTCTGCGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-CZECH      AAGCAAGTTCTGCGAGTTTTGCCTGTTTTTTAACCCCGAACATTTCATAGAACTTGTTAA
TSWV-BULG       AAGCAAGTTCTGCGAGTTTTGCCTGTTTTTTAACCCCAAACATTTCATAGAACTTGTTAA
TSWV-JAPAN      AAGCAAGTTCTGCAAGTTTTGCCTGTTTTTTAACCCCAAACATTTCATAGAACTTGTTAA
TSWV-NC         AGGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCAAACATTTCATAGAACTTGTTAA
TSWV-CAL        AAGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCAAACATTTCATAGAACTTGTTAA
TSWV-BL         AAGCAAGTTCTGTGAGTTTTGCCTGTTTTTTAACCCCAAACATTTCATAGAACTTGTTAA
TSWV-SPAIN      AAGCAAGTTCTGTGAGTTTTGCTTGTTTTTTAACCCCAAACATTTCATAGAACTTGTTAA
TSWV-COL        AAGCAAGTTCTGTGAGTTTTACCTGTTTTTTAACCCCAAACATTTCATAGAACTTGTTAA
                * **********  ****** * ************** **********************

TSWV-NW2        GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-KZN        GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-NW1        GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-MP         GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-LP         GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-GP         GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-ITALY      GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-GER        GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-CZECH      GAGTTTCACTGTAATGTTCCAATAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-BULG       GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTG
TSWV-JAPAN      GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTA
TSWV-NC         GAGTTTCACTGTAATGTTCCA-TAGCAACACTTCCTTTAGCATTAGGATTGCTGGAACTA
TSWV-CAL        GAGTTTCGCTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTA
TSWV-BL         GGGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTA
TSWV-SPAIN      GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTA
TSWV-COL        GAGTTTCACTGTAATGTTCCA-TAGCAATACTTCCTTTAGCATTAGGATTGCTGGAGCTA
                * ***** ************* ****** *************************** **

TSWV-NW2        AGTATAGCAGCATACTCTTTCCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-KZN        AGTATAGCAGCATACTCTTTCCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-NW1        AGTATAGCAGCATACTCTTTCCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-MP         AGTATAGCAGCATACTCTTTCCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-LP         AGTATAGCAGCATACTCTTTCCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-GP         AGTATAGCAGCATACTCTTTCCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-ITALY      AGTATAGCAGCATACTCTTTCCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-GER        AGTATAGCAGCATACTCTTTCCCTTTCTTCACCTGATCTTCATTCATTTCGAATGCTTTG
TSWV-CZECH      AGTATAGCAGCATACTCTTTTCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-BULG       AGTATAGCAGCATACTCTTTTCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-JAPAN      AGTATAGCAGCATACTCTTTTCCTTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-NC         AGTATAGCAGCATACTCTTTCCCCTTCTTCACCTGATCTTCAGTCATTTCAAATGCTTTG
TSWV-CAL        AGTATAGCAGCATACTCTTTCCCCTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
TSWV-BL         AGTATAGCAGCATACTCTTTCCCCTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTT
```

```
TSWV-SPAIN      AGTATAGCAGCATACTCTTTCCCCTTCTTCACCTGATCTTCATTCATCTCAAATGCTTTG
TSWV-COL        AGTATAGCGGCATACTCTTTCCCCTTCTTCACCTGATCTTCATTCATTTCAAATGCTTTG
                ******** *********** ** ****************** **** ** ********

TSWV-NW2        CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTATCATACTTCTTTGGGTCA
TSWV-KZN        CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTATCATACTTCTTTGGGTCA
TSWV-NW1        CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTATCATACTTCTTTGGGTCA
TSWV-MP         CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTATCATACTTCTTTGGGTCA
TSWV-LP         CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTATCATACTTCTTTGGGTCA
TSWV-GP         CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTATCATACTTCTTTGGGTCA
TSWV-ITALY      CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTGTCATACTTCTTTGGGTCA
TSWV-GER        CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTGTCATACTTCTTTGGGTCA
TSWV-CZECH      CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCTCTGGTGTCATACTTCTTTGGGTCA
TSWV-BULG       CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCCCTGGTGTCATACTTCTTTGGGTCA
TSWV-JAPAN      CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCCTTAGTGTCATACTTCTTTGGGTCG
TSWV-NC         CTTTTCAGCACAGTGCAAACTTTCCCTAAGGCTTCCCTGGTGTCATACTTCTTCGGGTCA
TSWV-CAL        CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCCCTGGTGTCATACTTCTTTGGGTCG
TSWV-BL         CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCCCTGGTGTCATACTTCTTTGGGTCG
TSWV-SPAIN      CTTTTCAGCACAGTGCAAACTTTTCCTAAAGCTTCCCTGGTGTCATACTTCTTTGGGTCG
TSWV-COL        CTTTTCAGCACAGTGCAAACTTTTCCTAAGGCTTCCCTGGTGTCATACTTCTTTGGGTCG
                ********************** ***** *****  * ** ********** *****

TSWV-NW2        ATCCCAAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-KZN        ATCCCAAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-NW1        ATCCCAAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-MP         ATCCCAAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-LP         ATCCCAAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-GP         ATCCCAAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-ITALY      ATCCCGAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-GER        ATCCCGAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-CZECH      ATCCCGAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-BULG       ATCCCGAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-JAPAN      ATCCCGAGGTCTTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-NC         ATCCCGAGGTCCTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-CAL        ATCCCGAGGTCCTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-BL         ATCCCGAGATCCTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-SPAIN      ATCCCGAGGTCCTTGTATTTTGCATCTTGATATATAGCCAAGACAACACTGATCATCTCA
TSWV-COL        ATCCCGAGGTCCTTGTATTTTGCATCCTGATATATAGCCAAGACAACACTGATCATCTCA
                ***** ** ** ************** ********************************

TSWV-NW2        AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-KZN        AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-NW1        AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-MP         AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-LP         AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-GP         AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-ITALY      AAGCTATCAACTGAAGCAATAAGAGGTAAACTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-GER        AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-CZECH      AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-BULG       AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGTCTCACA
TSWV-JAPAN      AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-NC         AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCTAGCATTATGGCAAGCCTCACA
TSWV-CAL        AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCTAGCATTATGGCAAGCCTCACA
TSWV-BL         AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-SPAIN      AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
TSWV-COL        AAGCTATCAACTGAAGCAATAAGAGGTAAGCTACCTCCCAGCATTATGGCAAGCCTCACA
                **************************** ******** ************** ******
```

```
TSWV-NW2        GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAACCAAAGGGTGGGAAGCAATCTTA
TSWV-KZN        GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAACCAAAGGGTGGGAAGCAATCTTA
TSWV-NW1        GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAACCAAAGGGTGGGAAGCAATCTTA
TSWV-MP         GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAACCAAAGGGTGGGAAGCAATCTTA
TSWV-LP         GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAACCAAAGGGTGGGAAGCAATCTTA
TSWV-GP         GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAACCAAAGGGTGGGAAGCAATCTTA
TSWV-ITALY      GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAATCAAAGGGTGGGAAGCAATCTTA
TSWV-GER        GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAATCAAAGGGTGGGAAGCAATCTTA
TSWV-CZECH      GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAATCAAAGGGTGGGAAGCAATCTTA
TSWV-BULG       GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGGATCAAAGGGTGGGAAGCAATCTTA
TSWV-JAPAN      GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAATCAAAGGGTGAGAAGCAATCTTA
TSWV-NC         GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAATCAAAGGGTGGGAAGCAATCTTA
TSWV-CAL        GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAATCAAAGGGTGGGAAGCAATCTTA
TSWV-BL         GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGACTCAAAGGGTGGGAAGCAATCTTA
TSWV-SPAIN      GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAATCAAAGGGTGGGAAGCAATCTTA
TSWV-COL        GACTTTGCATCATCAAGAGGTAATCCATAGGCTTGAACCAAAGGGTGGGAAGCAATCTTA
                ************************************   ********* ************

TSWV-NW2        GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-KZN        GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-NW1        GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-MP         GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-LP         GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-GP         GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-ITALY      GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-GER        GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-CZECH      GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-BULG       GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCTACAAGCCTGACCCTGATC
TSWV-JAPAN      GATTTGATAGTATTGAGATTCTCTGAATTCCCAGTTTCCTCGACAAGCCTGACCCTGATC
TSWV-NC         GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCAACAAGCCTGACCCTGATC
TSWV-CAL        GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCAACAAGCCTGACCCTGATC
TSWV-BL         GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCAACAAGCCTGACCCTGATC
TSWV-SPAIN      GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCAACAAGCCTGACCCTGATC
TSWV-COL        GATTTGATAGTATTGAGATTCTCAGAATTCCCAGTTTCCTCAACAAGCCTGACCCTGATC
                ************************ ***************** ******************

TSWV-NW2        AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-KZN        AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-NW1        AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-MP         AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-LP         AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-GP         AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-ITALY      AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-GER        AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-CZECH      AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-BULG       AAGCTATCAAGCCTTCTGGAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-JAPAN      AAGCTATCAAGCCTTCTGAAGGTCATTTCAGTGGCTCCAATCCTGTTTGAAGTTTTCTTT
TSWV-NC         AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-CAL        AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATTCTGTCTGAAGTTTTCTTT
TSWV-BL         AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-SPAIN      AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
TSWV-COL        AAGCTATCAAGCCTTCTGAAGGTCATGTCAGTGGCTCCAATCCTGTCTGAAGTTTTCTTT
                ****************** ******* ************** **** ************

TSWV-NW2        ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTGATAACCTTCATTATACTCTGACGA
TSWV-KZN        ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTGATAACCTTCATTATACTCTGACGA
```

```
TSWV-NW1        ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTGATAACCTTCATTATACTCTGACGA
TSWV-MP         ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTGATAACCTTCATTATACTCTGACGA
TSWV-LP         ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTGATAACCTTCATTATACTCTGACGA
TSWV-GP         ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTGATAACCTTCATTATACTCTGACGA
TSWV-ITALY      ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTAATAACCTTCATTATACTCTGACGA
TSWV-GER        ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTAATAACCTTCATTATACTCTGACGA
TSWV-CZECH      ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTAATAACCTTCATTATACTCTGACGA
TSWV-BULG       ATGGTAATTTTACCAAAAGTAAAATCACTTTGTTTAATAACCTTCATTATACTCTGACGA
TSWV-JAPAN      ATGGTAATTTTACCAAAAGTAAAATCACTTTGCTTAATAACCTTCATTATACTCTGACGA
TSWV-NC         ATGGTAATTTTACCAAAAGTAAAATCACTTTGCTTAATAACCTTCATTATGCTCTGACGA
TSWV-CAL        ATGGTAATTCTACCAAAAGTAAAATCACTTTGCTTAATAACCTTCATTATGCTCTGACGA
TSWV-BL         ATGGTAATTTTACCAAAAGTAAAATCGCTTTGCTTAATAACCTTCATTATGCTCTGACGA
TSWV-SPAIN      ATGGTAATTTTACCAAAAGTAAAATCACTCTGCTTAATAACCTTCATTATGCTCTGACGA
TSWV-COL        ATGGTAATTCTACCAAAAGTAAAATCACTTTGCTTAATAACCTTCATTATGCTCTGACGA
                ********* ********************* ** ** ** ************** *********

TSWV-NW2        TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-KZN        TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-NW1        TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-MP         TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-LP         TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-GP         TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-ITALY      TTCTTCAGGAATGTCAGACATGAAATAATGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-GER        TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-CZECH      TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTTTTCC
TSWV-BULG       TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTCTTGATCTGGTCGAGGTCTTCC
TSWV-JAPAN      TTCTTTAGGAACGTCAGACACGAAATAATGCTCATCTTCTTGATCTGGTCAAGGTTTTCC
TSWV-NC         TTCTTCAGGAATGTCAGACATGAAATAATGCTCATCTTTTTGATCTGGTCAAGGTTTCCT
TSWV-CAL        TTCTTCAGGAATGTCAGACATGAAATAATGCTCATCTTTTTGATCTGGTCAAGGTTTTCC
TSWV-BL         TTCTTCAGGAATGTCAGACATGAAATAATGCTCATCTTTTTGATCTGGTCAAGGTTTTCC
TSWV-SPAIN      TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTTTTGATCTGGTCAAGGTTTTCC
TSWV-COL        TTCTTCAGGAATGTCAGACATGAAATAACGCTCATCTTTTTGATCTGGTCAAGGTTTTCC
                ***** ***** ******** ******* ********* *********** **** * *

TSWV-NW2        AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTTTGATCTTCCTCAAACTCAAGGTCT
TSWV-KZN        AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTTTGATCTTCCTCAAACTCAAGGTCT
TSWV-NW1        AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTTTGATCTTCCTCAAACTCAAGGTCT
TSWV-MP         AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTTTGATCTTCCTCAAACTCAAGGTCT
TSWV-LP         AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTTTGATCTTCCTCAAACTCAAGGTCT
TSWV-GP         AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTTTGATCTTCCTCAAACTCAAGGTCT
TSWV-ITALY      AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAACTCAAGGTCT
TSWV-GER        AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAACTCAAGGTCT
TSWV-CZECH      AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAACTCAAGGTCT
TSWV-BULG       AAACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAACTCAAGGTCT
TSWV-JAPAN      AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAATTCAAGGTCT
TSWV-NC         AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCCTCCTCAAACTCAAGGTCT
TSWV-CAL        AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAACTCAAGGTCT
TSWV-BL         AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAACTCAAGGTCT
TSWV-SPAIN      AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAACTCAAGGTCT
TSWV-COL        AGACAAAAAGTCTTGAAGTTGAATGCTACCAGATTCTGATCTTCCTCAAACTCAAGGTCT
                * ********************************** ***** ******** *********

TSWV-NW2        TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-KZN        TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-NW1        TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-MP         TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-LP         TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
```

```
TSWV-GP          TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-ITALY       TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-GER         TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-CZECH       TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-BULG        TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-JAPAN       TTGCCTTGTGTCAACAAAGCAACAATGTTTTCCTTAGTGAG
TSWV-NC          TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-CAL         TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-BL          TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-SPAIN       TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
TSWV-COL         TTGCCTTGTGTCAACAAAGCAACAATGCTTTCCTTAGTGAG
                 *************************** ************
```

## CONFERENCES TO DATE

**Sivparsad, B.J., and Gubba, A.** Isolation and Partial Characterisation of a Tomato Spotted Wilt Virus (TSWV) isolate from KwaZulu-Natal. *17th Annual Symposium of the South African Society for Microbiology (SASM, KZN branch).* Durban Institute of Technology, Durban, South Africa, 22nd October 2004.

**Sivparsad, B.J., and Gubba, A.** Isolation and Partial Characterisation of a Tomato Spotted Wilt Virus (TSWV) isolate from KwaZulu-Natal. *Postgraduate Research Symposium.* University of KwaZulu-Natal, Pietermaritzburg, South Africa, 19th October 2005. (poster presentation)

**Sivparsad, B.J., and Gubba, A.** Electron Microscopy Studies of a Tomato Spotted Wilt Virus (TSWV) isolate from KwaZulu-Natal. *44th Annual Conference of the Microscopy Society of Southern Africa (MSSA).* University of KwaZulu-Natal Pietermaritzburg, South Africa, 5th - 7th December 2005. (attached)

**Sivparsad, B.J., and Gubba, A.** Isolation and Molecular Characterisation of Tomato Spotted Wilt Virus (TSWV) isolates occurring in South Africa. *Postgraduate Research Symposium.* University of KwaZulu-Natal, Pietermaritzburg, South Africa, 7th November 2006.

# ELECTRON MICROSCOPY STUDIES OF A TOMATO SPOTTED WILT VIRUS (TSWV) ISOLATE FROM KWAZULU-NATAL

B.J. Sivparsad and A. Gubba

Department of Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg

Tomato spotted wilt virus (TSWV), the type species of the genus Tospovirus within the family Bunyaviridae, is one of the ten most economically destructive plant viruses worldwide, causing losses exceeding one billion dollars annually in several important crops[1, 2]. More than 500 species in 50 plant families of ornamentals, vegetables and fruit are susceptible to TSWV[3]. The virus is exclusively transmitted by thrips in a persistent manner[4]. In South Africa (SA), the emergence of TSWV is a significant problem in crop cultivation, where it can seriously limit crop production with infection rates of up to 90% in most commercial crops[5]. Since the introduction of *Frankliniella occidentalis* into SA in 1988, and its subsequent spread throughout the country, the incidence of TSWV has increased in many provinces including KwaZulu-Natal (KZN)[6].

The overall objective of this study was to partially characterize a TSWV isolate occurring in KZN using electron microscopy. This information will be useful in laying a foundation for the formulation of effective control measures for this economically destructive plant virus.

Transmission electron microscopy was one of the techniques employed to identify and characterize the presence of TSWV in infected leaf samples and purified viral suspensions. Symptomatic *Nicotinia rustica* leaves were harvested and purified using standard procedures to yield a partially pure viral suspension. A crude viral suspension was also prepared by crushing slices of symptomatic leaf tissue in droplets of distilled water. Droplets of both crude and partially pure suspensions were then placed on formvar-coated copper grids and negatively stained with 2% uranyl acetate. For ultra-thin sectioning, leaf slices from chlorotic spots of *N. rustica* were prefixed with 3% glutaraldehyde in 0.5M phosphate buffer and postfixed with 2% osmium tetroxide for 1.5 hours. After dehydration with a graded ethanol series, the samples were imbedded in Epon resin. Ultra-thin sectioning was performed with glass knives mounted on a microtone. Sections were placed on grids, stained with 2% uranyl acetate and viewed under the transmission electron microscope (TEM).

Under the TEM, typical quasi-spherical and dumbbell-shaped particles of 80-100nm in diameter were observed in stained preparations of both crude (Fig. 1A) and partially pure (Fig. 1B) suspensions. In negatively stained ultra-thin TSWV infected leaf sections, an abundance of mature viral particles and typical viroplasm inclusions were observed (Fig. 2).

Additional techniques such as ELISA, SDS-PAGE and RT-PCR were also used to identify and characterise the TSWV isolate. The summation of results presented in this study confirms the presence of TSWV as a typical tospovirus in KZN. This is the first report of TSWV in KZN. More comprehensive surveys for the virus are currently being conducted to determine the status of TSWV in the country.

References

1. Adkins, S. (2000) Mol. Plant Pathol. 1, 151.
2. Mumford, R.A., Barker, I. and Wood, K.R. (1996) Ann. Appl. Biol. 128, 159.
3. Kormelink, R. *et al.* (1992) J. Gen. Virol. 73, 2795.
4. Paliwal, Y.C. (1975) Can. J. Bot. 54, 402.
5. Thompson, G.J. and van Zijl, J.J.B. (1996). Acta. Hortic. 431, 379.
6. Giliomee, J.H. (1989) J. Ent. Soc. Sth. Afr. 52, 179.

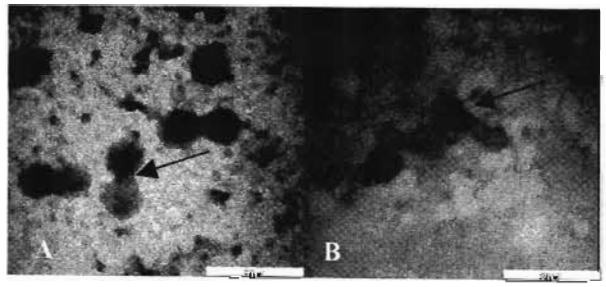



Figure 1. TEM of TSWV particles in negatively stained (A) crude leaf sap and (B) partially purified preparations. Arrow indicates the presence of a dumbbell like particle.

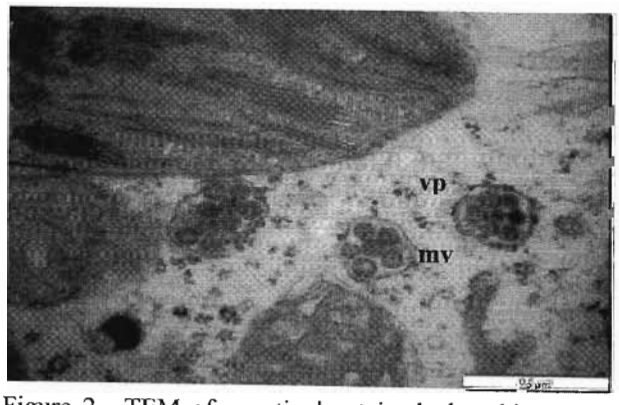



Figure 2. TEM of negatively stained ultra-thin TSWV infected leaf sections. mv – mature virus particles and vp – viroplasm inclusions.

e-mail: 201297209@ukzn.ac.za