PREVALENCE, DETECTION AND CHARACTERIZATION OF TOMATO SPOTTED WILT AND IRIS YELLOW SPOT TOSPOVIRUSES OCCURING IN ZIMBABWE

By

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THESIS ABSTRACT

Tospoviruses (genus *Orthotospovirus*, family *Tospoviridae*) are emerging plant pathogens capable of causing serious losses in field and greenhouse crops worldwide. Currently, 11 confirmed and at least 18 tentative tospoviral species have been reported worldwide, most of them in tropical and sub-tropical regions. While Asia has the greatest tospoviral species diversity with 21 species, only five species are reported in Africa. In Zimbabwe, only *Tomato spotted wilt orthotospovirus* (TSWV) was known to occur prior to this study. A study was undertaken to ascertain the prevalence and characteristics of tospoviruses in Zimbabwe. Prior to sampling for tospoviruses, a survey was undertaken to assess different agricultural stakeholders' knowledge and perceptions of plant viruses. The study showed that plant viruses and virus diseases are poorly known and appreciated by most stakeholders in the agricultural sector. Most (72%) agricultural technical staff rated *Maize streak virus* as the most important plant virus in Zimbabwe. Only 23.7% of farmers had heard about tospoviruses and none of the respondents could name tospovirus species other than TSWV. The study revealed the need to train stakeholders in plant virology as an intervention in virus disease management.

In the studies to ascertain the prevalence and characteristics of tospoviruses, plant leaves exhibiting virus-like symptoms were sampled from open fields and protected environments in 18 districts across six provinces. Immunostrips were used for field detection of the tospoviruses. Symptomatic leaves from tospovirus-positive plants were collected in RNA*later*® solution and FTA cards and transported to South Africa for further characterization by serology and molecular assays. TSWV was detected in 50% of the surveyed districts, with disease incidence below 15% in the surveyed fields. For the first time, TSWV was reported infecting butternut squash (*Cucurbita moschata*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita pepo*) and baby's breath (*Gypsophila elegans*) in Zimbabwe. Molecular analysis of the partial nucleocapsid (N) gene showed that Zimbabwean TSWV isolates were at least 93.99% similar and identical to each other at nucleotide and amino acid levels and were most closely related to TSWV isolates from Montenegro, New Zealand and Italy.

Iris yellow spot orthotospovirus (IYSV) was reported for the first time in Zimbabwe, with the disease detected in all the districts that were surveyed. High disease incidence of 78.3% and disease

severity of 3.7 were recorded in some districts. IYSV was detected in *Allium cepa, A. ampeloprasum, A. sativum* and *A. cepa* var. *aggregatum*. Besides the alliums, IYSV was also detected in *Amaranthus hybridus, Amaranthus spinosus* and *Eleusine indica*. Zimbabwean IYSV isolates were at least 94.89% identical and similar at the nucleotide and amino levels, and were most closely related to the onion-infecting South African isolate (EF579801). The study confirmed the widespread occurrence of IYSV in Zimbabwe though farmers were not aware of its presence. There is need to roll out extension programs to educate farmers about this disease to reduce its impact on alliums production. The survival of the virus and its vectors on weeds highlighted the need to control weeds so as to break the pathogen's life cycle.

In order to get better understanding of the characteristics of IYSV in Zimbabwe, a garlic-infecting isolate was subjected to Next-Generation Sequencing (NGS). Three full genes i.e. the nucleocapsid (N), the non-structural (NSs) and movement (NSm) were recovered by both *de novo* and reference-based mapping and subjected to phylogenetic analyses. The N gene was 822 nucleotides (nt) long (273 amino acids), while the NSs and NSm were 1332 nt and 983 nt long, respectively. The N gene clustered with isolates from different regions of the world, indicating the possibilities that recombination, long-distance migration and reassortment events might have occurred in this isolate. The NSs and NSm proteins clustered with the few homologous IYSV proteins that were retrieved from public databases.

Zimbabwean tomato germplasm was screened for the *Sw-5b* resistance gene using marker assisted selection. DNA was extracted from four-week old greenhouse-grown seedlings and used to perform polymerase chain reaction using gene-specific and single nucleotide polymorphism (SNP) primers. Results showed that 21 out of 23 (91.3%) of commercial cultivars and 3 out of 7 (42.9%) accession lines had the *Sw-5b* gene. This could partly explain the low incidence and severity of TSWV in Zimbabwean tomatoes. Information on the resistance/susceptibility of tomato commercial cultivars to TSWV should be included on seed labels to assist farmers with choosing varieties to grow. The accession lines with *Sw-5b* gene could be used in breeding for resistance to TSWV should the need arise.

DECLARATION

I, **Charles Karavina**, declare that:

i. The research reported in this thesis, except where otherwise indicated, is my original work.

ii. This thesis has not been submitted for any degree or examination at any other university.

iii. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Signed: …… Date: ……... (Supervisor)

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DEDICATION

To my wife Grace, our children Emmaculate Keyla and Kegan Emmanuel, and my parents John (late) and Jenny.

CONTENTS

THESIS INTRODUCTION

Global perspective on plant viruses

Plants are susceptible to infection by many disease-causing agents resulting in significant reduction in quality, yield and lifespan. Globally, plant diseases cause billions of dollars' worth of losses in agricultural production annually (Agrios, 2005). There have been complete crop failures due to disease outbreaks, resulting in famines. For example, the Irish famine of the 1840s that killed about two million people (Vidhyasekaran, 2007) and resulted in the migration of many more to the United States of America, was due to the outbreak of late blight disease caused by *Phytopthora infestans* in potatoes (*Solanum tuberosum*).

Plant pathogenic viruses are capable of causing serious losses in crop production worldwide. Globally, losses due to plant viral diseases are estimated at US$60 billion annually (Wei *et al*., 2010). The losses are particularly severe in developing countries due to several reasons. Firstly, pesticide use for virus disease control is less extensive and less effective. Secondly, farmers often grow unimproved varieties that are low-yielding and not disease-resistant. Such varieties are more likely to be severely affected by disease outbreaks than improved varieties (Rybicki and Pietersen, 1999). Thirdly, disease surveillance is poor. Many virus diseases continue to cause severe yield losses and yet are unreported and so, not controlled. This greatly affects most countries' national economic growth as they depend on agriculture for export earnings, employment opportunities and food security.

There are at least one thousand reported phytopathogenic virus species worldwide (Astier *et al*., 2007). A survey by Scholthof *et al*. (2011) ranked phytopathogenic viruses in order of economic importance as follows: (1) *Tobacco mosaic virus* (TMV); (2) *Tomato spotted wilt virus* (TSWV); (3) *Tomato yellow leaf curl virus* (TYLCV); (4) *Cucumber mosaic virus* (CMV); (5) *Potato virus Y* (PVY); (6) *Cauliflower mosaic virus* (CaMV); (7) *African cassava mosaic virus* (ACMV); (8) *Plum pox virus* (PPV); (9) *Brome mosaic virus* (BMV) and (10) *Potato virus X* (PVX).

The tospovirus problem

Since the 1980s, many plant viruses have emerged as threats to world agriculture. Amongst these are the begomoviruses (Navas-Castillo *et al*., 2011), criniviruses and torradoviruses (Hanssen *et al*., 2010), and tospoviruses (Pappu *et al*., 2009). The tospoviruses have been gaining in prominence since the 1990s and there has been a phenomenal increase in the number of species reported worldwide (Soler *et al*., 2003). To date, there are 11 confirmed and at least 18 tentative tospovial species (Table 1.1). Yield losses attributed to tospoviruses amount to over US$1 billion annually for several important crops worldwide (Goldbach and Peters, 1994). Most tospoviral species have been reported in the tropics and subtropics.

The tospoviruses have a wide host range of over 1300 plant species in more than 92 plant families (Parrella *et al*., 2003). They are transmitted by highly polyphagous thrips. Being RNA viruses, they readily mutate to produce resistance-breaking strains (Hoffmann *et al*., 2001). The emergence of new tospoviral species has also been exacerbated by the growing intensification and diversification of agricultural systems (Marchoux *et al*., 2001). In addition, climate change has increased tospovirus epidemic instability and unpredictability through its influences on vector distribution and behavior, effects on crop susceptibility, effects on rainfall patterns, temperature and wind speed, all of which influence virus population dynamics (Goldbach and Peters, 1994; Marchoux *et al*., 2001).

Tospoviral disease management has been effected through the utilization of biological, chemical, cultural, host-plant resistance and physical methods. Of these, host-plant resistance has been the most successful strategy in the medium- to long-term. Several resistance genes have been identified (Saidi and Warade, 2008), with the *Sw-5b* gene in tomatoes and *Tsw* gene in peppers, being the most durable and effective against multiple tospovirus species (Soler *et al*., 2003; Gordillo *et al*., 2008). Breeding for tospovirus resistance can be speeded up by using molecular markers to identify resistant lines based on genotype instead of phenotype (Hnetkovsky *et al*., 1996).

Rationale of this study

While many tospovirus studies have been undertaken worldwide, this has not been the case in most African countries. For example, no tospovirus studies have been undertaken in Zimbabwe in the last two decades. This is largely attributed to a decline in agricultural research exacerbated by the flight of highly skilled and experienced researchers from the country. While most new tospovirus species have been reported in the last two and half decades, it is not known if any of them occur in Zimbabwe. Only TSWV, first reported in 1940 (Hopkins, 1940), is known to occur in Zimbabwe. TSWV is reported to infect several ornamental and solanaceous crops and some weeds (Masuka *et al*., 1998; Nyamupingidza and Machakaire, 2003). The ornamental and solanaceous hosts are important as food and cash crops to Zimbabwe's agro-based economy. TSWV was rated as an important pathogen in Zimbabwe (Nyamupingidza and Machakaire, 2003), but there are no records of how detection and characterization studies were conducted.

Given that Zimbabwe has a subtropical climate, there is high likelihood of more tospovirus species being present than currently reported. Crops are grown all-year-round, and this favours tospovirus survival. International trade in plants and plant products further increases the likelihood that tospoviruses and their vectors have been introduced into the country. Like other countries, Zimbabwe is experiencing climate change, a major factor in the development of new tospovirus species and the outbreaks of tospoviral epidemics.

Study objectives

The overall objective of this study was to generate information on tospoviruses in Zimbabwe in order to develop effective disease management strategies for improved crop productivity. Specifically, the study aimed at:

i. Determining agricultural stakeholders' knowledge and perceptions of plant viral diseases in Zimbabwe.

ii. Assessing the geographical distribution, incidence and severity of tospoviruses in Zimbabwe.

iii. Detecting and characterizing tospoviruses infecting different hosts in Zimbabwe.

iv. Carrying out phylogenetic studies of tospoviruses infecting crops in Zimbabwe.

v. Sequencing the whole gene sequences of a garlic-infecting *Iris yellow spot orthotospovirus* isolate from Zimbabwe.

vi. Screening Zimbabwean tomato genotypes for the *Sw-5b* resistance gene using molecular markers.

Thesis organization

This thesis is made up of the following chapters:

Chapter 1: Literature Review

Chapter 2: Knowledge and perceptions of plant virus diseases by different stakeholders in Zimbabwe's agricultural sector: Implications for disease management.

Chapter 3: Detection and characterization of *Tomato spotted wilt orthotospovirus* infecting field and greenhouse-grown crops in Zimbabwe.

Chapter 4: *Iris yellow spot orthotospovirus* in Zimbabwe: Incidence, severity and characterization of *Allium*-infecting isolates.

Chapter 5: Alternative hosts of *Iris yellow spot orthotospovirus* in Zimbabwe.

Chapter 6: Characterization of three full *Iris yellow spot orthotospovirus* genes of a garlic-infecting isolate from Zimbabwe using Next-Generation Sequencing.

Chapter 7: Marker-assisted identification of *Tomato spotted wilt orthotospovirus*-resistant tomato genotypes in commercial and accession lines.

Chapter 8: Thesis Overview.

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CHAPTER 1: LITERATURE REVIEW

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1.1 Origin and distribution of tospoviruses

The origin of tospoviruses has not been satisfactorily determined. Though the first-ever tospovirus species was reported in Australia, it is most likely that they did not originate from that continent. Australian tospoviruses have been recorded predominantly in non-native plants, with only one native floral species, *Calectasia cyanea*, host to tospoviruses (Latham and Jones, 1997). Also, the tospoviruses are vectored exclusively by non-native thrips species (Mound and Gillespie, 1997). So, tospoviruses are most likely to be introductions in Australia (Jones, 2005).

Phylogenetic studies based on nucleocapsid (N) and non-structural movement (NSm) proteins have also been undertaken to determine tospovirus origin. Based on these studies, tospoviruses are said to have originated from the Americas, Asia or Eurasia (Hassani-Mehraban *et al*., 2011). So, they are classified in the "American", "Eurasian" or "Asian" clades. The Asian clade has more species than the other clades. This shows that there is greater genetic diversity of tospoviruses in Asia than anywhere else. Therefore, Asia is most likely the center of tospovirus origin.

Tospoviruses are present in all continents except Antarctica (Figure 1.1). In temperate regions, tospoviruses are prevalent in greenhouses (Soler *et al*., 2003). Tropical and sub-tropical regions have the greatest tospoviral diversity, with Asia and South America having 20 and 11 reported species, respectively (Table 1.1). To date, Africa has five recorded species namely; TSWV, *Groundnut ringspot orthotospovirus* (GRSV), *Iris yellow spot orthotospovirus* (IYSV), *Tomato yellow ring orthotospovirus* (TYRV) and *Impatiens necrotic spot orthotospovirus* (INSV) (Pappu *et al*., 2009; Birithia *et al*., 2012).

The global floriculture industry has greatly contributed to tospoviral spread. TSWV and INSV, with wide ornamental host ranges (Sether and DeAngelis, 1992; EPPO, 2004), have been detected at international ports of entry. Thrips species, some of which transmit these tospoviruses, have also been detected hitch-hiking on ornamentals moving in international trade. The cut flower trade has largely been implicated in spreading tospoviruses and their vectors (Jones, 2005). In addition, shifts in tomato production from temperate to tropical regions due to lower labor costs, better transportation, and treaties like the North American Free Trade Agreement and the World Trade Organization Agreement, have coincided with the tospovirus problem in the tropics and sub-tropics (Robbins *et al*., 2010).

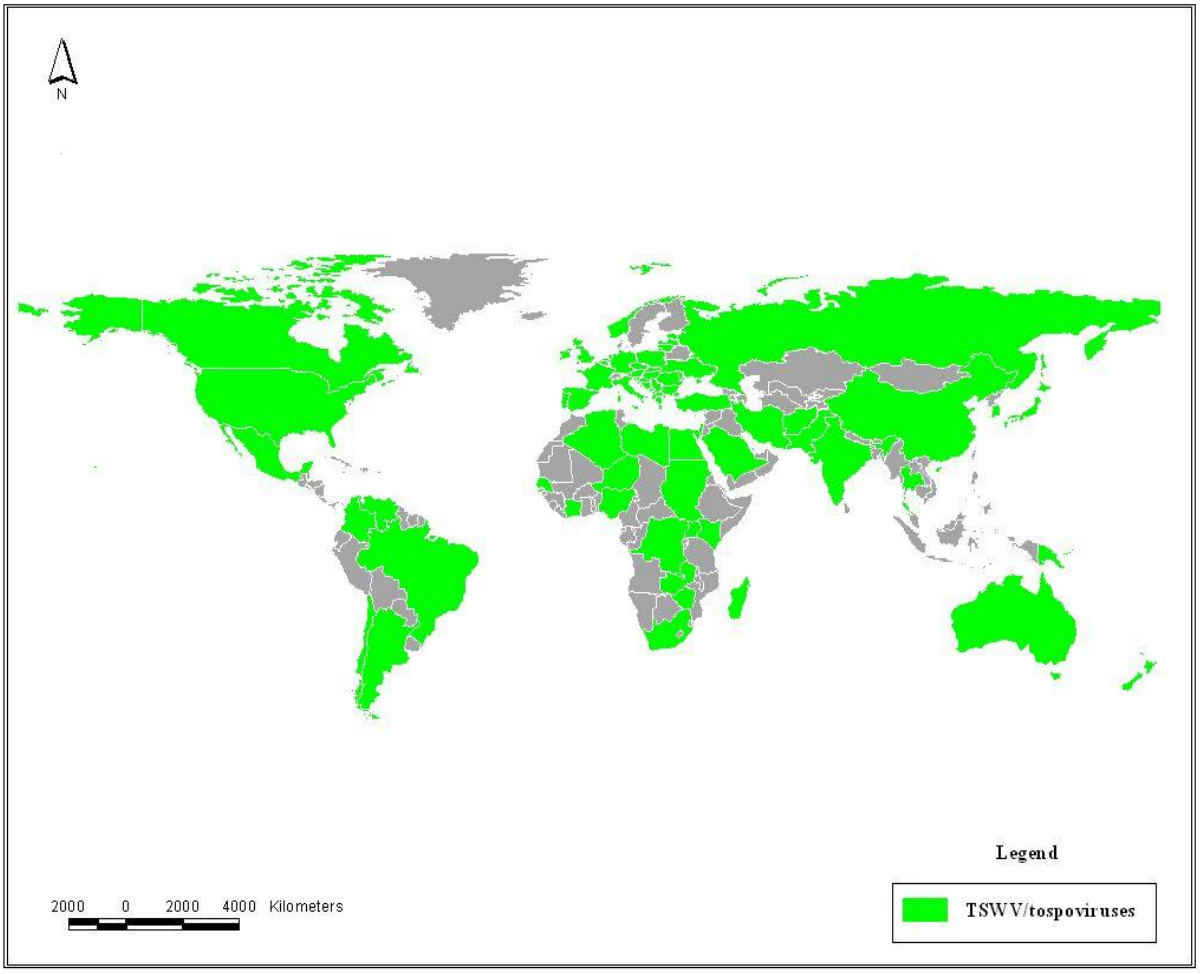

Figure 1.1 Worldwide distribution of tospoviruses. Tospoviruses have been reported in countries shaded in green.

1.2 Taxonomy

Tospoviruses belong to the genus *Orthotospovirus* in the family *Tospoviridae* and order *Bunyavirales*. They are one of only three plant-infecting viruses in this new virus order that mainly contains animal- and human-infecting virus species (Tsompana *et al*., 2005; Rotenberg *et al*., 2015). Tospoviruses derive their name from the *Tomato spotted wilt virus*, first reported in Victoria, Australia in 1915 (Brittlebank, 1919). TSWV was classified as the type member of the "tomato spotted wilt virus group" in 1970 based on particle morphology, host range and transmission by thrips. The virus group was later designated as a genus by the International Committee on Taxonomy of Viruses in 1991 (Francki *et al*., 1991). Until the early 1990s, the genus *Orthotospovirus* was considered monotypic. However, more species have since been discovered, starting with INSV in 1990 (Law and Moyer, 1990). There are now 11 confirmed and at least 18 tentative tospoviruses (Table 1.1), with TSWV the most characterized species (Naidu *et al*., 2008).

Tospoviruses have high genetic diversity attributed to error-prone replication, high replication rates, short generation times and a large population size (Tsompana *et al*., 2005; Dimmock *et al*., 2007). The high diversity also explains the emergence of new strains from old viruses and the ability to rapidly adapt to new or resistant hosts. Species are demarcated by host range, vector specificity, genome structure and organization, serological and molecular relationships of N genes (Dong *et al.*, 2008; Pappu *et al*., 2009; King *et al*., 2012; Oliver and Whitfield, 2016). The N gene sequence is now the primary basis for tospovirus species demarcation, with N protein identity of at least 90% denoting members of the same species, while N protein identity of less than 80% denoting distinct species (Fauquet *et al*., 2005). Viruses with N protein identity of 80-89% may be considered as different strains or different species depending on biological properties that include host range and vector thrips (Jones, 2005; Mahy and van Regenmortel, 2008). Tospoviruses were formerly subdivided into serogroups I through V, but this system has been replaced by a classification system that refers to phylogenetic clades. Each clade is named based on the type species of the major seropgroups: TSWV, *Watermelon silver mottle orthotospovirus* (WSMoV), IYSV, *Groundnut yellow spot orthotospovirus* (GYSV) and Soybean vein necrosis virus (SVNV) (Knierim *et al*., 2006; Oliver and Whitfield, 2016).

1.3 Characteristics of tospoviruses

1.3.1 Morphological and physicochemical properties

Tospoviruses have unique quasi-spherical particle morphology of 80-120 nm diameter. The virions display surface glycoprotein projections of 5–10 nm which are embedded in a lipid bilayer envelope 5 nm thick. Viral nucleocapsids are 200-300 nm long and 2-2.5 nm in diameter (Whitfield *et al*., 2005; King *et al*., 2012).

The virion has 5% nucleic acid, 5% carbohydrate, 20% lipid and 70% protein (Adkins, 2000; Mukhopadhyay, 2011). Its molecular weight is 300×10^6 to 400×10^6 and it has a sedimentation coefficient ($S_{20,w}$) of 350-500. Virion buoyant densities in sucrose and cesium chloride are 1.16-1.18 gcm^{-3} and 1.20-1.12 gcm^{-3}, respectively. Virions are sensitive to heat, lipid solvents, detergents and formaldehyde (Khan and Dijkstra, 2006; King *et al*., 2012). In plant sap, tospoviruses have a thermal inactivation point (TIP) of 45^{o}C, longevity *in vitro* (LIV) of five hours and a dilution end point (DEP) of $\log_{10}$ minus 3 (Adkins, 2000).

1.3.2 Genomic organization

The genome consists of three single-stranded RNAs: one negative-sense large (L) RNA and two ambisense medium (M) and small (S) RNAs (Adkins, 2000; Whitfield *et al*., 2005). The L RNA (~8.9kb) encodes the RNA-dependent RNA polymerase (RdRp) (331k) in the viral complementary (vc) strand. The M and S segments each encode two proteins. The M RNA (~4.8kb) encodes the precursor to the envelope membrane glycoprotein in the vc sense, and the NSm in the viral (v) sense. The NSm is essential for systemic infection of plants by tospoviruses while the glycoproteins are required for virus acquisition and transmission by thrips vectors (Turina *et al*., 2016). The S RNA (~2.9kb) encodes the N protein in the vc sense and a nonstructural protein (NSs) (52.4k) in the v sense (de Haan *et al*., 1990). The NSs behaves as the suppressor of gene silencing to counteract plant innate defense (Margaria *et al*., 2007; Chen *et al*., 2012). It has a bi-function as ATPase and phosphatase, as shown from *Groundnut bud necrosis orthotospovirus* (GBNV) (Lokesh *et al*., 2010). The N protein tightly associates to genomic RNA and together with small amounts of viral RNA-dependent RNA polymerase (RdRp) form transcriptionally active ribonucleoproteins (Geerts-Dimitriadou *et al*., 2012).

The genomic RNAs have complementary 5' and 3' termini (AGAGCAAU at the 5'-end, UCUCGUUA at the 3'-end) and can assume a pseudo-circular panhandle structure. The M and S RNAs each possess an intergenic region (IGR) with high A-U content that is capable of forming a stable hairpin structure (Mahy and van Regenmortel, 2008). The three genomic RNAs are individually encapsidated by many copies of N protein and are surrounded by a host-derived lipid envelope in which two external glycoproteins (G_N and G_C) are integrated (King *et al*., 2012). The two glycoproteins are seen as spike-like projections covering the surface of the virus particle (Figure 1.2). In TSWV, the glycoproteins are modified by glycosylation. The G_C protein is N-glycosylated with high mannose- and complex-type oligosaccharides, whereas G_N protein is glycosylated with high-mannose type oligosaccharides (de Haan *et al*., 1990). Figure 1.2 shows the structure of a TSWV virion.

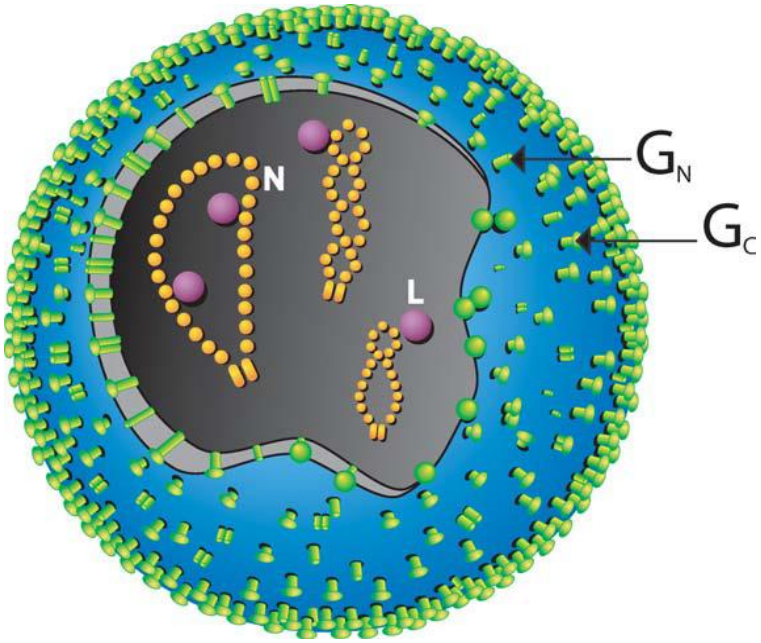

Figure 1.2: Diagrammatic representation of a TSWV virion. A double-layered membrane of host origin (*blue*) is shown with the viral-encoded proteins G_N and G_C (*green*) projecting from the surface in monomeric and dimeric configurations. The genomic RNA is presented as noncovalently closed circles in the form of a ribonucleoprotein (RNP) complex created by its association with many copies of N protein (*peach*). A few copies of the virion-associated RNA-dependent RNA polymerase (RdRp) are shown (*purple*) in association with the RNPs (Whitfield *et al*., 2005).

1.3.3 Host range

Over 1300 plant species from at least 92 families of monocotyledonous and dicotyledonous plants are hosts of tospoviruses (Parrella *et al*., 2003). Amongst them are important ornamental, food and cash crops, and weeds. Most common hosts belong to the Asteraceae, Curcubitaceae, Fabaceae and Solanaceae families (German *et al*., 1992; Parrella *et al*., 2003). Table 1.1 lists the known tospoviruses, continents where reported and main natural hosts.

Table 1.1 Main natural hosts and continents where tospoviruses have been reported

Tospovirus species	Continent where reported	Main natural hosts	Source(s)
ANSV	South America	*Alstroemeria*	Hassani-Mehraban *et al*. (2010).
BeNMV	South America	Bean	de Olieveira *et al*. (2012).
CaCV	Australia, Asia	*Capsicum*, tomato, groundnut	Jones (2005); Jones and Sharman (2005).
CCSV	Asia	Calla lily	Chen *et al*. (2012).
CSNV	Asia, Europe, South America	Chrysanthemum, *Gerbera*	Nagata and de Avila (2000).
GBNV	Asia	Groundnut, grain legumes, cucurbits, tomato, potato	Reddy *et al*. (2000); Jain *et al*. (2007); Mandal *et al*. (2012).
GCFV	South America	Groundnuts	Chu *et al*. (2001).
GRSV	Africa, South America	Groundnut, tomato	Pappu *et al*. (2009); Gopal *et al*. (2010)

GYSV	Asia	Groundnut	Pappu *et al.* (2009); Gopal *et al*. (2010).
HCRV	Asia	*Hippeastrum*	Dong *et al*. (2013).
INSV	All continents	Groundnut, ornamentals, *Capsicum*, potato	Naidu *et al*. (2005); Perry *et al*. (2005).
IYSV	All continents	*Allium*	Gent *et al*. (2006); Bag *et al*. (2015).
LiNRSV	Asia	*Lisianthus*	Shimomoto *et al*. (2014).
MeSMV	North America	Melon	Ciuffo *et al*. (2009).
MVBaV	Asia	Mulberry	Meng *et al*. (2015).
MYSV	Asia	Melon, watermelon	Kato *et al.* (2000); Peng *et al*. (2011).
PCSV	Asia	Pepper	Cheng *et al*. (2013).
PNSV	Asia, South America	Pepper, tomato	Torres *et al*. (2012).
PolRSV	Europe	Wild buckwheat	Ciuffo *et al*. (2008).
SVNaV	North America	Soybean	Zhou *et al*. (2011).
TCSV	North and South America	Tomato, sweet pepper	de Avila *et al*. (1990); Londono *et al.* (2012).
TNRV	Asia	Tomato	Chiemsombat *et al*. (2010).
TNSaV	Asia	Tomato	Zheng *et al*. (2016).

TSWV	All continents	Many plants	Sether and De Angelis (1992).
TYRV	Africa, Asia, Europe	Tomato, *Alstroemeria*	Beikzadeh *et al*. (2012); Birithia *et al*. (2012).
TZSV	Asia	Tomato	Dong *et al*. (2008).
WBNV	Asia	Watermelon	Singh and Krishnareddy (1996).
WSMoV	Asia	Cucurbits, tomato	Rao *et al*. (2011); Suresh *et al*. (2013).
ZLCV	Americas	Zucchini, cucumber	Nagata *et al*. (1998); Giampan *et al*. (2007).

Alstroemeria necrotic streak virus (ANSV); Bean necrotic mosaic virus (BeNMV); Calla lily chlorotic spot virus (CCSV); Capsicum chlorosis virus (CaCV); Chrysanthemum stem necrosis virus (CSNV); *Groundnut bud necrosis orthotospovirus* (GBNV); Groundnut chlorotic fan-spot virus (GCFV); *Groundnut ringspot orthotospovirus (*GRSV); *Groundnut yellow spot orthotospovirus* (GYSV); Hippeastrum chlorotic ringspot virus (HCRV); *Impatiens necrotic spot orthotospovirus* (INSV); *Iris yellow spot orthotospovirus* (IYSV); Lisianthus necrotic ringspot virus (LiNRSV); Melon severe mosaic virus (MeSMV); Melon yellow spot virus (MYSV); Mulberry vein banding associated virus (MVBaV); Pepper necrotic spot virus (PNSV); Pepper chlorotic spot virus (PCSV); *Polygonum ringspot orthotospovirus* (PolRSV); Soybean vein necrosis-associated virus (SVNaV); *Tomato chlorotic spot orthotospovirus* (TCSV); Tomato necrotic ringspot virus (TNRV); Tomato necrotic spot-associated virus (TNSaV), *Tomato spotted wilt orthotospovirus* (TSWV); Tomato yellow ring virus (TYRV); Tomato zonate spot virus (TZSV); *Watermelon bud necrosis orthotospovirus* (WBNV); *Watermelon silver mottle orthotospovirus* (WSMoV); *Zucchini lethal chlorosis orthotospovirus* (ZLCV).

1.4 Tospoviral replication

All stages of viral replication and virion assembly occur in plant host cytoplasm and insect vector. The virus enters the plant cell during probing or feeding by viruliferous thrips (Whitfield *et al*., 2005). Once in the cell, the virus particle loses its nucleocapsid into the cytoplasm. Depending on N protein concentration, viral RNA is either transcribed or replicated. At low N concentration, the polymerase will produce mRNAs, resulting, after translation, in accumulation of various viral-encoded proteins. As the concentration of the N proteins increases, the polymerase switches to replicase mode and viral genomic RNAs are multiplied (Peters, 2003).

The translation of proteins from the S and M ambisense RNAs occurs from the subgenomic mRNAs. The S and M subgenomic mRNAs are capped at the 5' terminus with 10-20 nucleotides of nonviral origin, indicating that tospoviruses utilize a cap-snatching mechanism to regulate transcription (Adkins, 2000). TSWV transcriptase has a reported preference for caps with multiple base complementarity with the viral template (van Knippenberg *et al*., 2005).

Replicated viral RNAs form ribonucleoproteins (RNPs) that can presumably associate with the NSm protein for movement through plasmodesmata to adjacent plant cells. Alternatively, the RNPs form virions by associating with glycoproteins and budding through the Golgi membranes (Kikkert *et al*., 1999). The virions are initially double-membraned, but soon they coalesce to form groups of virions with a single membrane surrounded by another membrane (Mahy and van Regenmortel, 2008).

1.5 Transmission

Tospoviruses are transmitted in a circulative-propagative mode exclusively by thrips (Thysanoptera: Thripidae). Only 15 thrips species are currently known to be vectors, with most belonging to the genera *Frankliniella* (8) and *Thrips* (3) (Riley *et al*., 2011). Adult thrips that would have acquired the viruses at the larval stage and retained them through moulting, pupation and emergence to adult stage transmit tospoviruses (Naidu *et al*., 2008; Peters, 2008; Riley *et al*., 2011). Those adult thrips that acquire the viruses for the first time during feeding cannot be vectors even if they are allowed lengthy feeding on tospovirus-infected plants (de Assis Filho *et al*., 2004;

Schmaljohn and Nichol, 2007). This is because the viruses are not able to complete their life cycle within the adult insects to allow for transmission. Such adults represent an epidemiological "dead-end" for the virus (Pappu *et al*., 2009). Similarly, plant species that do not support thrips development are "dead-ends" for the viruses irrespective of the number of infected thrips species that feed on them (Whitfield *et al.*, 2005).

The first and second instar larvae are efficient in acquiring the virus, with acquisition rates decreasing as larval thrips develop. For example, when the first instar larvae were allowed to acquire TSWV, 47% of the concomitant adults transmitted the virus. This compared to only 12% of adults from cohorts that acquired the virus at the second instar larval stage (Nagata *et al*., 2002). About five days are required from virus acquisition by larval thrips to transmission to another plant. This time allows the virus to move and multiply in the insect gut and salivary glands (Ullman *et al*., 2005). The thrips remain infective for life, usually 20-40 days. Males exhibit higher transmission ability than females, but females take the greatest share in virus spread because they live longer (Peters, 2008). Short feeding periods of 5-10 minutes are adequate for efficient virus transmission (Naidu *et al*., 2008).

Tospovirus infections usually occur through primary spread from outside sources into a clean crop. There is usually little or no secondary viral spread within a crop (Pappu *et al*., 2009). Virus-infected weeds and volunteer plants on which thrips can multiply can act as "green bridges" within which the viruses survive in the absence of susceptible host crops (Szostek and Schwartz, 2015). There has been no evidence of seed and transovarial transmission for most tospoviruses (Pappu *et al*., 2009; Mandal *et al*., 2012), except for SVNV which has recently been reported to have low seed transmissibility (Groves *et al*., 2016). Neither are the viruses spread by pruning, cultivation equipment, nor plant handling. However, they can be vegetatively disseminated in cuttings and bulbs.

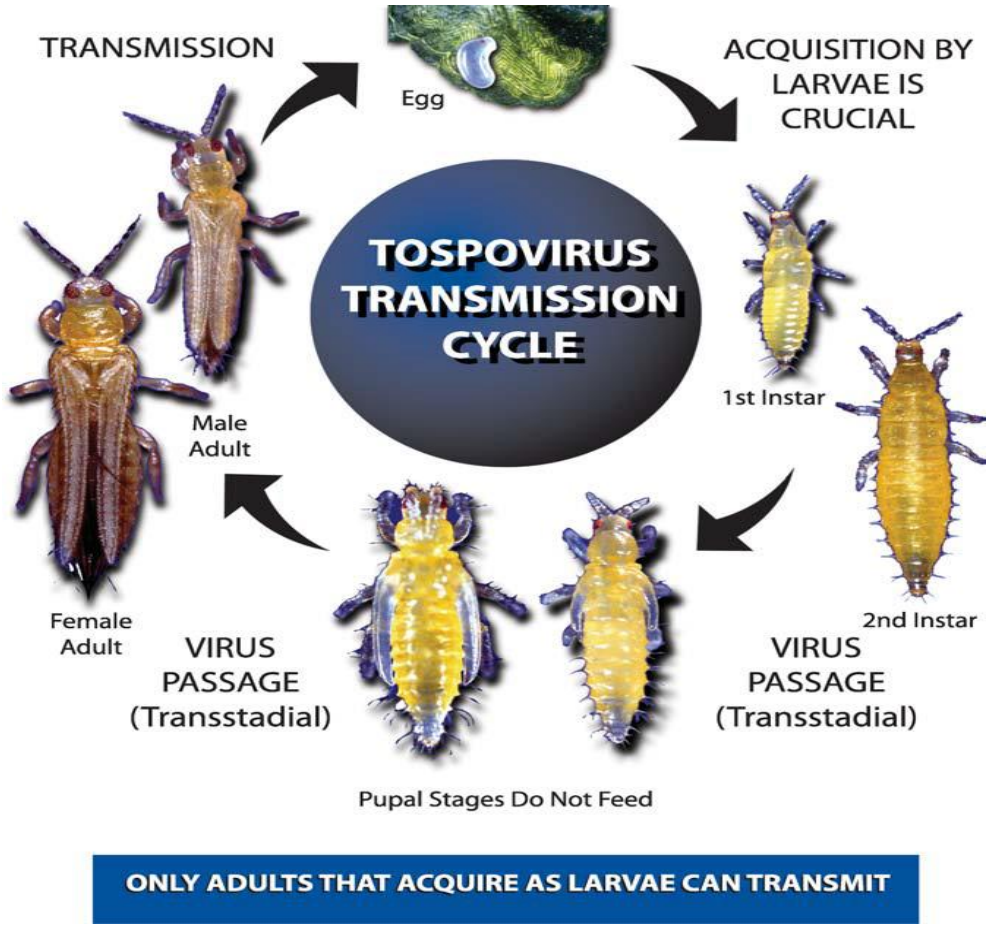

Figure 1.3 Graphic representation of the thrips life cycle and the tospovirus transmission cycle. Thrips eggs are oviposited into plant tissue and within a few days the first instar larvae emerge. Virus acquisition occurs solely during the larval stages after which the virus is passed transstadially to the adult. The pupal stages are nonfeeding and do not move, although they do maintain virus infection. Pupation takes place either in the soil or in the foliage. Adults emerge and have a tendency to disperse widely. Only adult thrips that acquired the virus during their larval stages can transmit tospoviruses. Graphic design by Eileen Rendahl. Thrips and kalanchoe photographs produced by Jack Kelley Clark (Whitfield *et al*., 2005).

1.6 Effects of tospovirus infection

The effects of infection depend on whether the plant is susceptible, immune or resistant. An immune plant will not support tospovirus development even when infection is successful. Resistant

plants allow the pathogen to develop, but at a reduced rate. Susceptible plants readily support the development of the viruses. Some cytopathological effects and symptoms manifest if susceptible and resistant hosts are infected.

1.6.1 Cytopathological effects

Tospoviruses can establish widespread systemic infections in plants. They invade roots, stems, leaves, flowers and fruits. In leaves, they infect all cell types, including immature xylem vessels and sieve tube elements. Infected cells display numerous cytopathological features that reflect the structural and genomic complexity of tospoviruses and their widespread interaction with cellular membranes during virion synthesis. They produce microscopic cellular inclusion bodies that vary in morphology and composition. Mature virus particles occur in the cisternae of the endoplasmic reticulum. Several or many particles may occur in a single cavity (Lawson *et al*., 1996).

1.6.2 Symptom expression

Tospoviruses induce many symptoms on their hosts including necrosis, chlorosis, concentric ring patterns, mottling, silvering, sunken spots, etches, wilting, leaf speckling, poor flowering, "oak-leaf" patterns, stunting, local lesions and death. These symptoms vary with host species, cultivar, virus strain and/or isolate, plant age, time of year and the environment (German *et al*., 1992; Sevik and Arli-Sokmen, 2012). Sometimes, the same virus strain may induce a variety of symptoms within the same plant host. In other instances, symptoms that are expressed may be mimics of other pathogens or environmental stress. Symptom expression is usually more pronounced when plants are infected at a young age (EPPO, 2004). Some hosts may be infected but remain symptomless.

1.7 Detection and diagnosis of tospoviruses

1.7.1 Sampling and field detection

Successful tospovirus diagnosis is dependent upon the sampling technique employed. As with other RNA viruses, collected samples must be processed immediately before they deteriorate. In the tropics and subtropics, it is sometimes difficult to deliver collected plant samples to laboratories in the right conditions before they deteriorate. So, techniques of quick field detection and/or storage for later processing have been developed.

Nucleic acid stabilization solutions

For storage and later processing of tospoviruses, stabilization buffers or solutions are available. The solutions preserve and protect the viruses in fresh samples from degradation by rapidly penetrating and inactivating nuclease enzymes. Depending on the solution, samples can be stored for up to one week at room temperature, one month at 2-8^{o}C, or indefinitely at -20^{o}C or lower before virus isolation. According Steward and Culley (2010), viral nucleic acid maintains its infectivity if stored at room temperature for 72 hours in some of these solution. Examples of commercially available stabilization solutions are RNA*later*™ (Qiagen) and RNA Shield™ (Zymo Research).

FTA® (Whatman) cards

The FTA® card is a paper-based technology designed for the collection and archiving of nucleic acids, either in their purified form or within pressed samples of fresh tissues (Ndunguru *et al*., 2005). The card contains chemicals that lyse cells, denature proteins and protect nucleic acids from nucleases, oxidative and UV damage (Predajna *et al*., 2012). After a short drying period, pressed samples can be stored at room temperature for extended periods and processed when required. The nucleic acids stored on the cards are eluted by a simple extraction buffer and used as template for amplification by reverse transcription polymerase chain reaction, cloning and sequencing (Grund *et al*., 2010; Kouassi *et al*., 2010).

Tospovirus immunostrips

Quick, on-site virus detection can be achieved by using immunostrips. These are lateral flow devices that have antibodies for particular tospoviruses impregnated within an absorbent pad (www.agdia.com). Plants showing virus-like symptoms are targeted for sampling and tissues from such plants collected into a small plastic bottle containing a buffer solution. The tissues are ground up using a pestle. Thereafter, 2-3 drops of the resultant sap is added to the test device window and observed for a color reaction in a viewing window. A control line (B) appears in the viewing window within a minute of applying the sap, while the test line (A) should be visible within 5-10 minutes if the test is positive. Immunostrips only show positive reaction when virus levels in the tissues are very high.

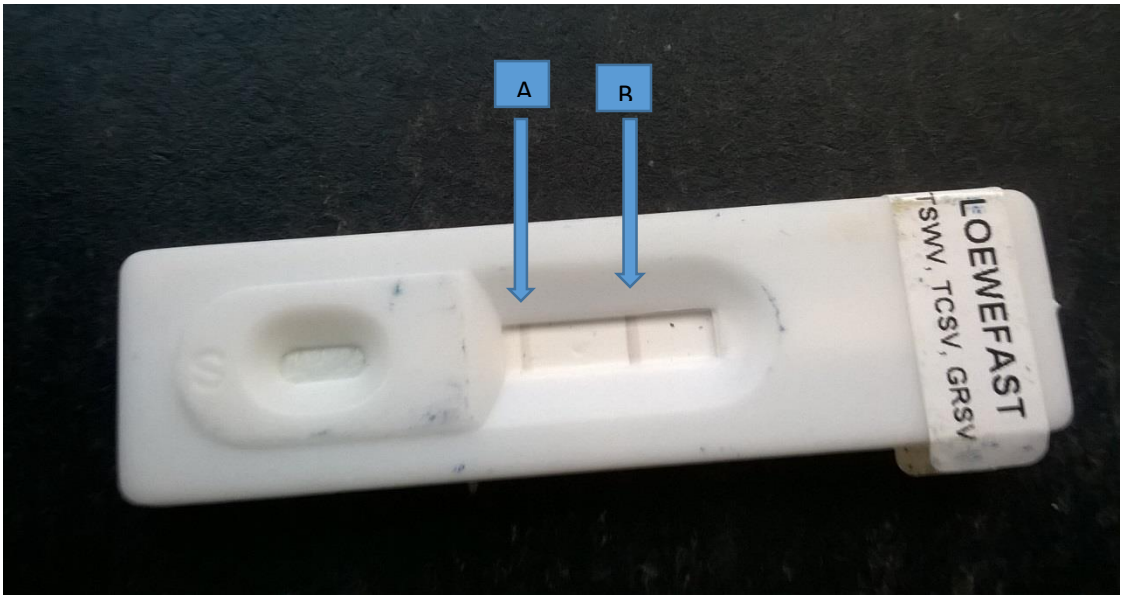

Figure 1.4 Tospovirus immunostrip used during field detection of tospoviruses in Zimbabwe. This immunostrip can detect *Tomato spotted wilt orthotospovirus*, *Groundnut ringspot orthotospovirus* and *Tomato chlorotic spot orthotospovirus*.

Tissue blot immunoassay

Tissue blotting utilizes antibodies raised against viruses. Plant sap is pressed onto blotting paper (nitrocellulose or nylon membrane) and the virus is detected by labeled probes. The procedure is less labor-intensive than ELISA. It is rapid, sensitive, simple and inexpensive. It can be used for surveys where up to 2000 samples are handled per day (Webster *et al*., 2004). The samples can later be taken for processing. The main disadvantage of this technique is the possible interference of sap components with the subsequent diagnostics reactions. Sometimes, the color of the sap will prevent weak positive reactions from being observed and results cannot be readily quantified (Naidu and Hughes, 2003).

1.7.2 Diagnosis

Several tospoviral diagnostic techniques are available, and more continue to be developed. Basically, the techniques can be classified into symptomatology, host indexing, serology, electron microscopy and molecular techniques.

Host indexing

Indexing is any test that reproducibly assesses the presence or absence of transmissible pathogens, or identifies a disease on the basis of reaction induced on specific indicator plants. Tospoviruses can be detected on the basis of symptom production on indicator plants. The indicator plants must be virus-free and easy to grow. They must react rapidly and specifically to a particular virus species, exhibit diagnostic symptoms under different conditions, and be readily available during the growing seasons (Jan *et al*., 2012). There are a number of indicator plants for tospoviruses (Table 1.2). Though it has high sensitivity, indexing is time-consuming and does not identify the actual virus.

Table 1.2 Some indicator hosts for tospoviruses

Botanical name	Common name	Family
Arachis hypogaea	Groundnut	Fabaceae
Capsicum annuum	Pepper	Solanaceae
Chenopodium album	Fat hen	Amaranthaceae
Chenopodium quinoa	Quinoa	Amaranthaceae
Cucumis sativus	Cucumber	Cucurbitaceae
Datura stramonium	Apple of Peru	Solanaceae
Impatiens	Snapweed	Balsaminaceae
Solanum lycopersicum	Tomato	Solanaceae
Spinacia oleracea	Spinach	Amaranthaceae
Nicotiana tabacum	Common tobacco	Solanaceae
Nicotiana glutinosa	Tobacco	Solanaceae
Nicotiana clevelandii	Cleveland's tobacco	Solanaceae
Nicotiana benthamiana	Tobacco	Solanaceae
Petunia hybrida	Petunia	Solanaceae
Phaseolus vulgaris	Field bean	Fabaceae
Pisum sativum	Peas	Fabaceae
Vigna unguiculata	Cowpea	Fabaceae

Symptomatology

Symptoms assessment is the initial step in tospovirus diagnosis. While some tospoviral diseases are readily diagnosed by visual examination of host symptoms, other hosts though infected, are often symptomless and require further diagnostic tests. Sometimes, different viruses can cause similar symptoms in a plant (Webster *et al*., 2004). In other instances, different strains of the same virus may induce different symptoms in the same host. Some symptoms are cultivar-dependent and also vary with environmental conditions (Naidu and Hughes, 2003).

Electron microscopy

The electron microscope uses a beam of highly energetic electrons to illuminate objects on a very fine scale to yield the topography, morphology, composition and crystallographic information. For viruses to be visualized, their concentration must be at least $10^{5\text{-}6}$ particles per ml (Biel *et al*., 2004). Compared to other techniques, electron microscopy is expensive, and requires highly technical and experienced staff for operation. It cannot detect viruses with much higher sensitivity and specificity. However, as the only spherical and enveloped plant viruses, tospoviruses are readily identified by electron microscopy (German *et al*., 1992; Sivparsad and Gubba, 2008).

Serology

Serological detection, or immunochemical diagnostics, is based on the use of monoclonal or polyclonal antibodies for virus detection. The antibodies are capable of binding to specific virus antigens (Strange, 2003). The most commonly used serological method is enzyme-linked immunosorbent assay (ELISA). It has two main variants, namely double antibody sandwich ELISA (DAS-ELISA) and triple antibody sandwich ELISA (TAS-ELISA) (Naidu and Hughes, 2003; Strange, 2003).

ELISA can be used to test multiple plants for a single tospovirus using a well per plant sample. Also, a single plant can be simultaneously tested for many tospoviruses on a single plate with different antibodies coated to each well in duplicate or triplicate. It is accurate, cheap, highly

sensitive, simple, and can quantify pathogens. A major limitation of ELISA is that it requires monoclonal or polyclonal antibody sera specific for each virus of interest that does not cross-react with plant proteins. As such, one must to know the virus to be detected. It often fails if virus titre in the test sample is low (Webster *et al*., 2004; Jan *et al*., 2012). Despite advances made in virus diagnostics, serology remains very important in tospovirus identification.

Molecular diagnostics

They are also called nucleic-acid based methods. They rely on specific complementary association of the different bases that make up the nucleic acid molecules. Molecular assays are better alternatives to the other methods in terms of sensitivity, rapidity, specificity or in situations where no suitable serological tests are available. Specificity is directly related both to the design of primers and amplification protocols (Strange, 2003; Olmos *et al*., 2007). Commonly used molecular techniques are the reverse transcription polymerase chain reaction (RT-PCR) and next generation sequencing (NGS).

RT-PCR involves an initial step of reverse transcription that converts single strand RNA to complementary DNA (cDNA). This is accomplished by using the reverse transcriptase enzyme. Subsequently, the newly synthesized cDNA is amplified using conventional PCR. RT-PCR is very sensitive and requires minimum skill to perform. The efficiency of virus detection depends on quality of template nucleic acid, polymerase enzyme, buffer composition, stability, purity and concentration of deoxynucleotide triphosphates (dNTPs) and cycling parameters (Jan *et al*., 2012).

Real-time RT-PCR, also called quantitative RT-PCR, is a quantitative and highly sensitive method for tospovirus detection. It does not need downstream analysis of PCR amplicons by gel electrophoresis, Southern blotting or sequencing as is the case with routine PCR assays (Vidhyasekaran, 2007).

Next-Generation Sequencing (NGS)

NGS, also called high-throughput sequencing, is a term used to describe a number of different modern sequencing technologies that allow the sequencing of nucleic acids much more quickly, accurately and cheaply than Sanger sequencing. This is accomplished by fragmenting entire genomes into small pieces and then ligating those small pieces to designated adapters for random read during DNA synthesis (Grada and Weinbrecht, 2013). Massive amounts of data, usually tens or hundreds of gigabytes per single run or terabytes per experiment, are generated in NGS (Xuan *et al*., 2013). Rare variant sequences can be identified (Reis-Filho, 2009) and complete genome sequences are identified in an unbiased fashion (Kreuze *et al*., 2009).

Several NGS platforms are available including; Roche GS-FLX 454, Illumina Genome Analyzer, ABI SOLiD Analyzer, Polonator and Helicos HeliScope. These platforms generate different base read lengths, error rates, and error profiles relative to Sanger sequencing and to each other. The average read length is 35-500 continous base-pair reads, compared to 1000-1200 bp for Sanger sequencing (Zhang *et al*. 2011). Since NGS technologies produce different short reads, coverage is very important if accurate genomic assembies are to be obtained.

The massive data generated needs to be properly stored and managed to successfully enjoy the benefits of NGS. Many software tools are available for NGS data analysis (Magi *et al*., 2010; Zhang *et al*., 2013). The functions of the data analysis tools fit into several categories of alignment, assembly, base calling, genome annotation and data analysis utilities. Some of the software packages provide a user-friendly interface, easy-to-use data input and output formats, and integrated multiple computing programs into one software package. Examples of such end-user packages are the CLC Genomics Workbench, NextGENe and SeqMan Ngen (Zhang *et al*., 2011).

1.8 Management of tospovirus diseases

It is very difficult to eradicate tospoviruses in regions where they have infected crops because these viruses have a wide host range and efficient vectors. As with all viruses, once the pathogen has entered the plant, it is practically impossible to break the host-pathogen relationship or even just reduce disease development (Pappu *et al*., 2009; Riley *et al*., 2011). Tactics employed in tospovirus

management must be based on thorough understanding of epidemiological principles and must fall within Integrated Disease Management (IDM) approaches that include biological, chemical, cultural, host-plant resistance and physical control (Culbreath *et al*., 2003).

1.8.1 Biological control

Biocontrol relies on the use of thrips' natural enemies like the predatory mites (*Neoseiulus cucumeris*, *Stratiolaelaps miles* and *Gaeolaelaps aculeifer*), minute pirate bug (*Orius insidiosus*), entomopathogenic nematode (*Steinerma feltiae*) and entomopathogenic fungi (*Beauveria bassiana* and *Metarhizium anisopliae*) to control vectors (Bennison *et al*., 2001; Krishnamoorthy *et al*., 2007; Cloyd, 2009). Biocontrol can be difficult to implement as synthetic pesticides and antagonists are likely to interfere with the biocontrol agent. The key to successful biocontrol is releasing the natural enemies early enough in the cropping cycle. Biocontrol is more long-term, and will work best on perennial crops.

1.8.2 Chemical control

Chemical control aims to reduce thrips vector population so as to reduce virus spread. Systemic insecticides are most ideal since thrips like to inhabit areas that are difficult for contact insecticides to penetrate (Melzer *et al*., 2012). Both foliar and soil-applied insecticides can be used. Insecticides are relatively ineffective in suppressing tospovirus species like TSWV where *Frankliniella occidentalis* is present because this vector can quickly develop resistance to insecticides (Kay and Herron, 2010). However, the use of newer insecticides and the inclusion of insecticides with different chemistries and modes of action in an insecticide rotation system, can effectively manage thrips vectors (Herron and James, 2007; Kay and Herron, 2010). Insecticides that have been used with some degree of success include imidacloprid, thiamethoxam, abamectin, spinosad, fipronil, spirotetramat, carbamates, organophosphates and synthetic pyrethroids (Csinos *et al*., 2001; Kay and Herron, 2010; Awondo *et al*., 2012). Insecticide application should be done judiciously so that there are no toxic residues, insecticide resistance and destruction of biological control agents (Pappu *et al*., 2009).

1.8.3 Cultural control

Old infected crops are a major source of tospoviruses. As such, they should be sprayed for thrips and removed as soon as possible, especially if young crops are to be planted nearby. This is also true for volunteer plants and weeds, especially from the Asteraceae and Solanaceae families, and those with yellow flowers (Cloyd, 2009; Melzer *et al*., 2012).

Farmers should avoid overlapping sowing of susceptible crops and sequential plantings side by side. This tends to create a "green-bridge" as infected thrips vectors can migrate from the old crop to the new crop and spread the viruses. During fallow periods, greenhouses should be heated for 4-5 hours at 30^{o}C daily to speed up pupation of any overwintering pupae which then starve to death (Cloyd, 2009). Fallowing or growing a non-susceptible crop allows thrips to migrate out of the area. Thereafter, a susceptible crop can be grown (Cho *et al*., 1998).

Tospovirus disease build-up can also be managed by adjusting crop planting dates such that susceptible stages of crop development do not coincide with peak or increasing thrips populations. Intercropping susceptible and non-susceptible crops also reduces tospovirus incidence (Reddy *et al*., 2000). Planting at high density also dilutes the overall numbers of infected plants and helps healthy plants to shade out neighboring infected plants (Brown *et al*., 1996). It also allows for compensation for yield loss of diseased plants by adjacent healthy plants (Culbreath *et al*., 2003). In greenhouses, overhead irrigation or misting decreases thrips population by creating an environment less favorable for their development.

Tospoviruses can be introduced in infected seedling plants which then provide a virus source throughout the crop life. Therefore, seedlings bought from nurseries should be inspected for signs of thrips feeding injury or tospoviral disease symptoms. Nurseries must be located away from production areas. They must also be kept weed-free and systematically monitored for pests and diseases. Monitoring can be done by hanging yellow sticky traps vertically above plants to monitor adult thrips.

Trap or lure crops like Transvaal daisy (*Gerbera jamesonii*) can also be grown away from the main crop. These should be periodically sprayed with an insecticide or inoculated with thrips' biocontrol agents (Blumthal *et al*., 2005).

1.8.4 Physical control

Insect vectors can be controlled by growing crops in protected environments like thrips-proof nets and cladding tunnel houses with plastics that absorb ultra-violet light (Costa and Robb, 1999; Jones, 2004). Silver reflective mulches and row covers hinder thrips infestation by decreasing thrips landing rates (Momol *et al*., 2001). These are most effective when plants are young, with small canopy (Melzer *et al*., 2012). Screen meshes on greenhouse air vents and sidewalls reduce the population of thrips entering the greenhouse. In the field, covering the ground with straw mulches allows the build-up of thrips predators. Application of horticultural oils and film-forming products to plants also decreases the spread of viruses by vectors. The oils cause behavioral changes in the vector. In addition, other non-behavioral factors may also be responsible for the oil's inhibitory effect on transmission (Khan and Dijkstra, 2006). However, such treatments may be phytotoxic and expensive because they need to be applied repeatedly.

1.8.5 Host plant resistance

The biological, chemical, cultural and physical methods employed in tospoviral management have only achieved limited success (Soler *et al*., 2003). Thus, the development of genetic resistance appears to be the most effective strategy of managing tospoviruses. Both natural and engineered resistance have been applied to manage tospoviruses.

Natural resistance

Tomato and pepper varieties that are resistant to tospoviruses are commercially available (Soler *et al*., 2003; Sivparsad and Gubba, 2011). The resistance operates by leaf cells around the point of inoculation dying, thereby preventing virus movement from infected areas. Such resistance is controlled by single dominant genes (*Sw-5b* in tomato and *Tsw* in pepper). In India, two groundnut varieties with resistance to GBNV have been released. The resistance in these genotypes may be due to resistance to the vector combined with viral tolerance (Mandal *et al*., 2012). In Kenya, Birithia *et al*. (2014) evaluated onion varieties for resistance to IYSV and *Thrips tabaci*. Resistance

in the onion varieties was attributed to morphological and structural traits like foliage color, amount of leaf wax and leaf tightness which interfered with the feeding behavior of the vector.

Natural resistance is vulnerable to the development of pathogen resistance-breaking strains (Thomas-Carroll and Jones, 2003). It has been overcome in situations of high inoculum pressure. There have been TSWV resistant-breaking strains in Australia, Hawaii, Hungary (Gabor *et al*., 2012), Italy, Spain, South Africa (Melzer *et al*., 2012; Spano *et al*., 2011) and South Korea (Chung *et al*., 2012). Thus, farmers can no longer rely solely on growing resistant varieties as they may still suffer losses. However, resistant cultivars must be part of IDM.

Engineered resistance

Conventional breeding efforts have not yielded genes in crops and their close relatives able to counter resistance-breaking virus strains. The limited utility of natural resistance, and the amount of time required to produce resistant plants through conventional breeding, led scientists to investigate genetically-engineered resistance strategies as alternatives (Mumford *et al*., 1996; Prins *et al*. 2008). This has resulted in the development of tospovirus-resistant transgenic plants (Gubba *et al*., 2002; Uhrig, 2007).

The high costs and socio-political debates associated with the production of transgenic plants are impediments to the adoption of tospovirus-resistant varieties in some countries. Thus, there is need for alternatives to the genetic engineering strategy for tospovirus management. It is possible to combine natural resistance with transgenic resistance by sexual crossing to achieve broad resistance to various tospoviruses (Gubba *et al*., 2002).

1.9 Challenges in managing tospoviruses

The management of tospoviruses is very difficult due to several reasons. The natural host range of tospoviruses is ever-increasing. For example, INSV, once considered to be primarily a pathogen of ornamental plants, now infects other crops such as groundnuts, tobacco, tomato, potato and pepper (Naidu *et al*., 2005; Perry *et al*., 2005). Similar host range expansions have also been

reported for GBNV (Jain *et al*., 2007; Mandal *et al*., 2012), CaCV (Sharma and Kulshrestha, 2014), TSWV (Adkins and Baker, 2005) and IYSV (Gent *et al*., 2006; Bag *et al*., 2015). Tospoviruses are generalist and opportunistic pathogens surviving by infecting many plant species. They can invade new geographical areas and quickly exploit suitable ecological niches (Pappu and Subramanian, 2013).

Tospoviruses have multiple thrips vectors that exhibit high polyphagy without compromising their fitness and reproductive output (Pappu *et al*., 2009; Riley *et al*., 2011). The vectors can efficiently colonize non-host crops and competently transmit the viruses. In TSWV studies, transmission efficiency ranged from 10-80% (Srinivasan *et al*., 2013). Both the viruses and the vectors can overwinter on a broad range of host plant species. This makes it difficult to deal with the diseases.

The excessive and continuous use of low-cost carbamate, organophosphate and pyrethroid insecticides has resulted in the built up of insecticide-resistant vector thrips populations, thereby exacerbating tospovirus spread (Pappu *et al*., 2009). While alternative insecticides are available (Awondo *et al*., 2012), they are expensive to farmers.

The development of resistance-breaking virus strains is another big threat to tospovirus management efforts (Persley *et al*., 2006). Tospovirus resistance traits governed by multigenes need to be employed in breeding programs. More effort must go towards screening germplasm and breeding lines to identify such resistance sources.

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CHAPTER 2: KNOWLEDGE AND PERCEPTIONS OF PLANT VIRAL DISEASES BY DIFFERENT STAKEHOLDERS IN ZIMBABWE'S AGRICULTURAL SECTOR: IMPLICATIONS FOR DISEASE MANAGEMENT

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Abstract

Plant viruses are major constraints to crop production worldwide, causing US$60 billion losses annually. This study assessed various agricultural sector stakeholders' knowledge and perceptions of plant viruses in Zimbabwe. Data was collected from six provinces using surveys and participatory rural appraisal methodologies between December 2013 and October 2014. *Maize streak virus*, *Tobacco mosaic virus*, *Cucumber mosaic virus*, *Tomato mosaic virus* and *Groundnut rosette virus* were ranked as the country's five most important plant viruses by agricultural technical staff. Most (72%) technical staff rated *Maize streak virus* as the most important plant virus in Zimbabwe. Over 30% of farmers were self-taught to identify diseases, while only 15.3% were trained by agricultural extension staff. Most (95.8%) technical staff trained people in disease identification through running short courses, use of demonstration plots and field days. The majority (41.9%) of farmers recommended the use of radio/TV/newspaper broadcasts to improve virus awareness. Only 23.7% of farmers and 41.6% of technical staff had heard about TSWV/tospoviruses. While most (97.2%) technical staff rated TSWV/tospoviruses as "fairly important" to "very important" plant pathogens, only 15.7% were able to correctly identify tospoviral vectors. The study showed that there is poor knowledge of plant viruses by stakeholders in the agricultural sector. There is need to train the technical staff in plant virology so that they can disseminate their knowledge to farmers for improved virus disease management.

Keywords: awareness, disease identification, tospoviruses, training.

2.1 Introduction

Zimbabwe has an agro-based economy, with over 70% of the population either directly or indirectly dependent on agriculture for a living (Marongwe et al., 2012). The major stakeholders in Zimbabwean agriculture are the farmers, input (seed, pesticide and fertilizer) suppliers, researchers, extension staff, and agricultural teachers and lecturers. All of them play significant roles in ensuring successful agricultural productivity which is seriously constrained by many abiotic and biotic factors. Amongst the biotic factors are plant pathogenic viruses that cause about US$60 billion losses annually worldwide (Wei et al., 2010).

Plant pathogenic viruses cause huge agricultural losses especially in the developing world where most farmers have poor knowledge of these pathogens. This can be attributed to the fact that unlike insects, fungal mycelia and rodents that can be seen with the naked eye, viruses are microscopic entities. In addition, viruses may incite symptoms similar to those by other pathogens, nutritional and/or environmental disorders (Astier et al., 2007). So, farmers tend to apply the wrong control measures in virus-infected plants. Furthermore, plant virus studies require highly specialized equipment and study techniques which are not readily available in most developing countries (Kaitisha, 2003).

Zimbabwe is a developing country reported to have impressive agricultural training, research and extension systems for improved agricultural productivity (Mutambara et al., 2013). A common perception is that stakeholders in Zimbabwe's agricultural sector are highly knowledgeable about all farming aspects, including disease and pest identification and management. However, this may not be the case with plant viral diseases due to the reasons mentioned earlier. Furthermore, changes in Zimbabwe's economy and education system since the year 2000 may have had an impact on knowledge and perceptions of viral diseases by agricultural sector stakeholders. Globally, climate change, trade and genetic mutations have contributed to the emergence of new variants of viruses like begomoviruses, criniviruses, carlaviruses, torradoviruses and tospoviruses in the last 30 years (Navas-Castillo et al., 2012; Pappu et al., 2009). The tospoviruses, in particular, have become very important in tropical and subtropical regions. One tospovirus species, *Tomato spotted wilt orthotospovirus*, is estimated to cause US$1 billion losses annually for several important food and

ornamental crops worldwide (Parella et al., 2003). This virus has previously been reported infecting weeds, ornamentals, and food and industrial crops in Zimbabwe (Dobson et al., 2002).

In light of these pointers, a survey was conducted to capture the understanding and perceptions of plant viral diseases by key stakeholders within Zimbabwe's agricultural sector. The survey provided a useful way to canvas ideas and opinions of the respondents about plant virus diseases. This would form the basis for identifying potential intervention points in developing viral disease management strategies. Results of the study will also assist policy makers in the Agriculture and Higher Education ministries during policy formulation on curricula development, research and extension services on plant viral diseases.

The objectives of this study were to: (i) identify ten plant viruses that agricultural technical staff rank as the most important in Zimbabwe, (ii) determine farmers' trainers and methods of training for disease identification, (iii) assess respondents' perceptions of plant viruses, (iv) gather respondents' opinions on how to improve awareness of plant virus diseases, and (v) evaluate respondents' knowledge of *Tomato spotted wilt orthotospovirus* (TSWV)/tospoviruses (TSWV/tospoviruses).

2.2 Materials and Methods

2.2.1 Study area

The study was carried out in Zimbabwe (latitudes 15^{o}13'S and 22^{o}30'S; longitudes 25^{o}E and 33^{o}E), a country bordered by South Africa to the south, Mozambique to the east, Zambia to the north and Botswana to the west. Zimbabwe has five natural farming regions (NFRs) delineated primarily on the basis of rainfall, soil quality and vegetation (Chiremba and Masters, 2003). The best rainfall and land resources occur in NFR 1, while NFR 5 is very hot and unsuitable for most crops, except traditional small grains and sugarcane. Up to 80% of Zimbabwe's crops are grown in the Mashonaland Provinces, which are mainly in NFR 2. Mid-season dry spells and high temperatures occur in NFR 3 which receives 500-750 mm rainfall annually. NFRs 4 and 5 are low-lying, receiving not more than 650 mm rainfall per annum.

2.2.2 Sampling procedure and selection of participants

The study was conducted between December 2013 and October 2014. A multistage sampling process was conducted to select provinces, districts and respondents. Six provinces, namely: Harare, Manicaland, Mashonaland Central, Mashonaland East, Mashonaland West and Masvingo, were selected. From each province, three districts were chosen for surveys. The chosen districts represented the country's NFRs as follows: NFR 1: Nyanga and Chimanimani; NFR 2: Bindura, Chegutu, Chinhoyi Urban, Goromonzi, Harare districts, Mazowe, Mutare, Seke, Shamva and Zvimba; NFR 3: Gutu and Mutoko; NFR 4: Masvingo Urban; and NFR 5: Chiredzi (Figure 2.1). At each district, the Principal Investigator (PI) engaged the District Agricultural Extension Officer (DAEO) who recommended wards (cluster of villages) for assessments, and agricultural extension staff (Agricultural Extension Officers and Agricultural Extension Workers) who assisted in identifying interviewed farmers. Three wards were selected per district, and fifteen farmers per ward were interviewed with questionnaires. In addition, ten farmers per district were interviewed in Farmer Group Discussions (FGDs). Respondents from agricultural colleges, high schools, input suppliers, research stations, non-governmental and private organizations involved in agriculture in the study areas were also interviewed. In total, 810 farmers and 214 technical staff (composed of agricultural extension staff, research and training officers, agricultural teachers and lecturers, and input suppliers) were interviewed using questionnaires, and another 180 farmers were interviewed in FGDs. All protocols were followed in regards of research ethics, which included securing government permission to conduct surveys and allowing free choice of participation in the interviews.

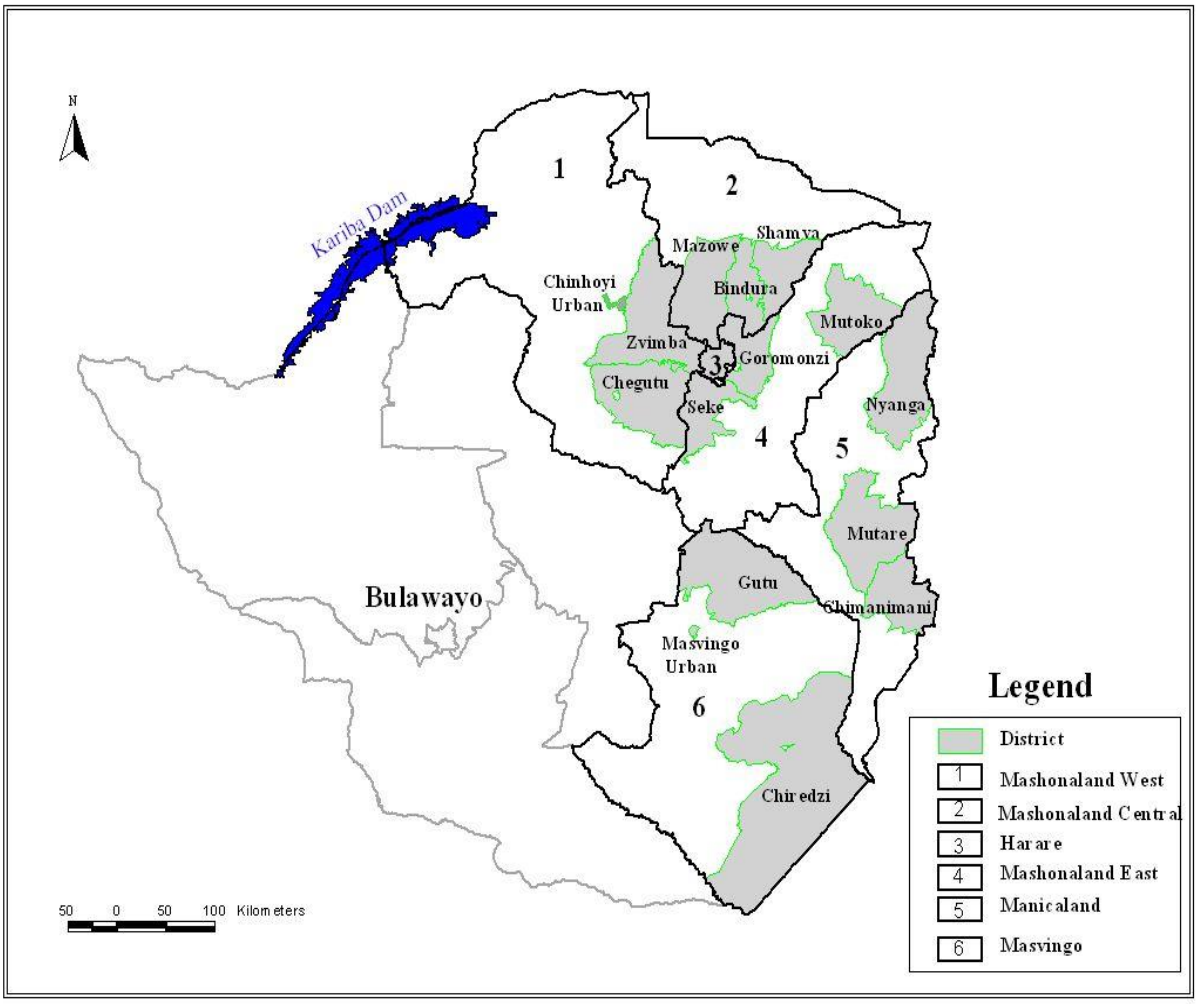

Figure 2.1: Provinces and districts chosen for the studies on plant virus knowledge and perceptions by stakeholders in Zimbabwean agriculture. The provinces are numbered 1 to 6 while districts are shaded grey.

2.2.3 Data collection

Two questionnaires were designed, one for farmers and another for the technical staff. Both questionnaires were designed in English and had closed and open-ended questions. For illiterate farmers, questionnaires were administered in Shona (a local language) and completed by the PI and his assistants. The questionnaires were pretested with fifteen farmers and eight technical staff, and modified to ensure that meanings were unambiguous. Some interviews and FGDs were

recorded on audio tapes and later processed to extract information. To ensure maximum data collection, some probing and interactive sessions outside the formal data collection sessions were carried out. Printed color photographs of virus-infected plants were shown to respondents to assist with disease identification.

The questionnaires captured respondents' general knowledge of plant viruses, including major plant viruses in Zimbabwe, rating of viruses as plant pathogens and methods of improving virus awareness. Perceptions on viruses were captured as categorized variables using a scale of 1 to 5 where, 1 = Not important; 2 = Fairly important; 3 = Important; 4 = Very important and 5 = Don't know. For TSWV/tospoviruses, respondents provided the following information: virus knowledge source, rating alongside other viruses, vectors and control measures. Respondents' socio-economic characteristics captured on the questionnaires included province, district, gender, age, educational level, marital status, land tenure system and agricultural experience.

2.2.4 Data analysis

Statistical analysis for quantitative survey data was done using the Statistical Package for Social Sciences (SPSS) Version 16.0. Survey data was coded and entered into the SPSS spreadsheet and checked before analysis. Both descriptive statistics and econometric models were used in data analysis. The logistic (logit) regression model was used to assess the respondents' awareness about TSWV/tospoviruses. The logit model is found in random utility theory and built around a latent regression.

$$Y^* = \beta x + \varepsilon_i$$

Y^*= is an underlying latent variable that indexes respondents' knowledge on TSWV/tospoviruses. β is a column vector of unknown parameters to be estimated. X is a row vector of respondent characteristics and ε is the stochastic error term. The dependent variable that was used for the model is the respondents' awareness of the viruses. This was chosen because the logistic model can be used in binary data; 1 = those who were aware of the viruses and 0 = those who were not aware of the viruses. The explanatory variables for the farmers' questionnaire were age, educational level, farming experience, land tenure system and land area (Table 2.1). For technical

staff, the explanatory variables were age, gender, employer, education level and agricultural experience.

Table 2.1: Explanatory variables used in assessing farmers' awareness to TSWV/tospoviruses

Variable Description	Variable type	Units
Awareness of farmers on topoviruses	Dummy	1=aware, 0= not aware
Age of farmer	Years	Continuous
Education level	Category	1=None; 2=Primary; 3=Secondary; 3=Post-secondary
Farming experience	Years	Continuous
Tenure system	Category	1=Communal; 2=A1; 3=A2, 4=Large scale commercial; 5=Plot/Nursery; 6=Other
Land area	Continuous	Continuous

To calculate the odds ratios (which represents the constant effect of the explanatory variables on the likelihood that the respondents were aware of TSWV/tospoviruses), the formula ODDS = e^{a+bX} was used; while the probabilities from the odds ratio were calculated using the formula:

$$Y=\frac{ODDS}{1+ODDS}$$

The analysis used both the odds ratio and probabilities because the odds ratio is a single summary score of the effect and the probabilities are more intuitive.

2.3 Results

2.3.1 Socio-economic characteristics of respondents

The proportion of male to female farmers was 60:40. Most (65.1%) farmers were from NRF 2, with only 3.7% from NR 1 and 5.6% from NR 5. The literacy rate amongst the farmers was 97.8%, and 29.6% of them had post-secondary education. All illiterate farmers were females; the literacy rate of female farmers was higher than that of their male counterparts only at primary school level. Most (76.8%) farmers were married, with 10.2% widowed and 12% single. The youngest farmer was 18 years old, while the oldest was 79 years. The largest proportion (37.9%) of farmers were communal, while 18% and 5.4% were A1 and large scale commercial farmers, respectively. Farmers with no more than 10 years' farming experience accounted for 45.9% of the respondents.

For the technical staff, the male to female ratio was 55:45. The government employed 77.1% of the technical staff, while only 20.1% were employed in the private sector. Agricultural extension staff constituted 50.5% of the technical staff. The majority (67.1%) of the agricultural extension workers (AEWs) had diplomas, while 91.4% of the agricultural extension officers (AEOs) had agriculture bachelor's degrees as their highest relevant qualifications. Only 15% of the technical staff had postgraduate degrees, with 1.9% having doctoral degrees. Of the lecturers and teachers, 22.4% had agricultural diplomas as their highest qualification. Most (87.9%) technical staff were married. Those with 2-10 years' work experience accounted for 77.1% of the technical staff, while only 3.3% had more than 20 years' experience.

2.3.2 Major plant viruses in Zimbabwe

According to the technical staff, the major plant viruses that occur in Zimbabwe are as shown in Table 2.2. *Maize streak virus* (MSV) was rated as Zimbabwe's most important plant virus by 72% of the technical staff. All agricultural extension staff highlighted the importance of MSV in maize production. *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) were ranked as the second and third most important plant pathogenic viruses, respectively (Table 2.2). GRV was reported mainly by extension staff working with smallholder groundnut farmers from NFRs 3 and 4.

Table 2.2: Top ten most economically important viruses affecting crops in Zimbabwe.

Rank	Acronym	Virus name	Genus
1	MSV	*Maize streak virus*	*Mastrevirus*
2	TMV	*Tobacco mosaic virus*	*Tobamovirus*
3	CMV	*Cucumber mosaic virus*	*Cucumovirus*
4	ToMV	*Tomato mosaic virus*	*Tobamovirus*
5	GRV	*Groundnut rosette virus*	*Umbravirus*
6	PVY	*Potato virus Y*	*Potyvirus*
7	TBTV	*Tobacco bushy top virus*	*Umbravirus*
8	CTV	*Citrus tristeza virus*	*Closterovirus*
9	PVX	*Potato virus X*	*Potexvirus*
10	BCMV	*Bean common mosaic virus*	*Potyvirus*

2.3.3 Training for disease identification

The majority of farmers were self-taught to identify diseases (Figure 2.2). Another 20.2% were trained by agrochemical and seed company agents, while only 16% were trained by agricultural extension and research staff (AREX/research officers). Farmers who grew greenhouse flowers hired foreign experts to assist with disease identification and management.

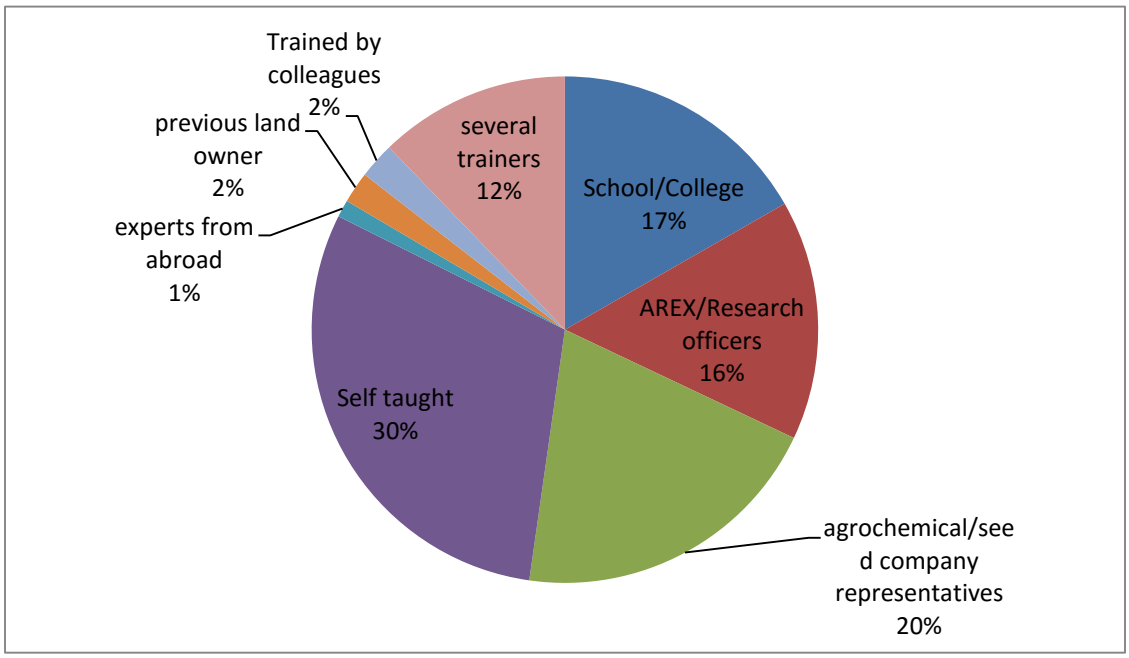

Figure 2.2: Farmers' trainers for disease identification

The main methods of farmer training for disease identification used by 51.9% of the technical staff included conducting of short courses, setting of demonstration plots and field days. Lectures/lessons and practicals were mainly used by teachers and lecturers to train students in disease identification.

2.3.4 Respondents' perceptions of plant viruses and methods used in virus disease identification.

Close to 29% of farmers did not know about plant pathogenic viruses. Only 3.1% of farmers and 2.8% of the technical staff rated viruses as "not important", while 22.7% of farmers and 41.1% of the technical staff rated them as "very important."

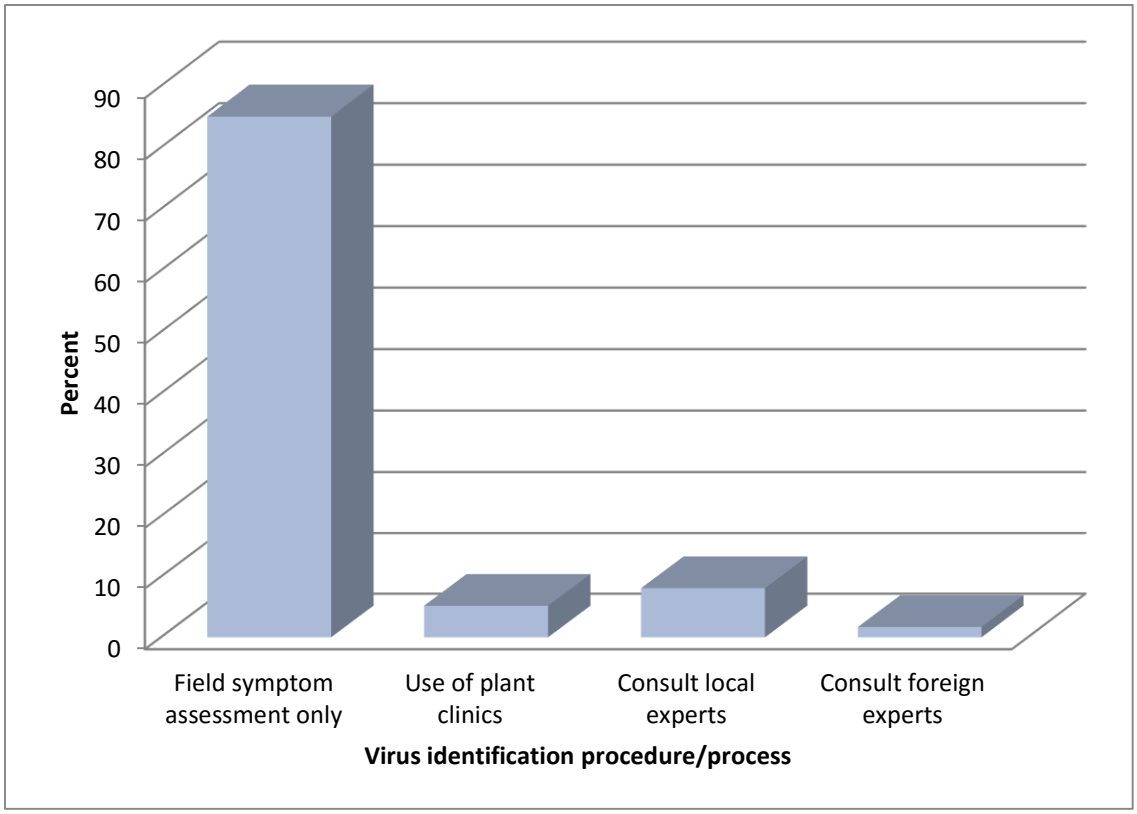

Figure 2.3: Methods of identifying virus diseases by farmers

The majority (85%) of farmers who were able to identify viruses relied on field symptom assessments only. Only 5.2% of farmers sent samples to plant clinics for disease diagnosis (Figure 2.3).

2.3.5 Opinions on improving virus diseases awareness

To improve virus disease awareness, 41.9% of farmers proposed the use of radio/TV/newspaper broadcasts, while 48.1% of the technical staff recommended farmer training. Another 22.3% of farmers were of the opinion that agricultural extension staff should train farmers, while 11.4% of farmers proposed distribution of color pamphlets of virus-infected plants as a method of improving virus awareness. About 6.5% of the technical staff proposed extension staff training through workshops and short courses as methods of improving virus diseases awareness (Figure 2.4).

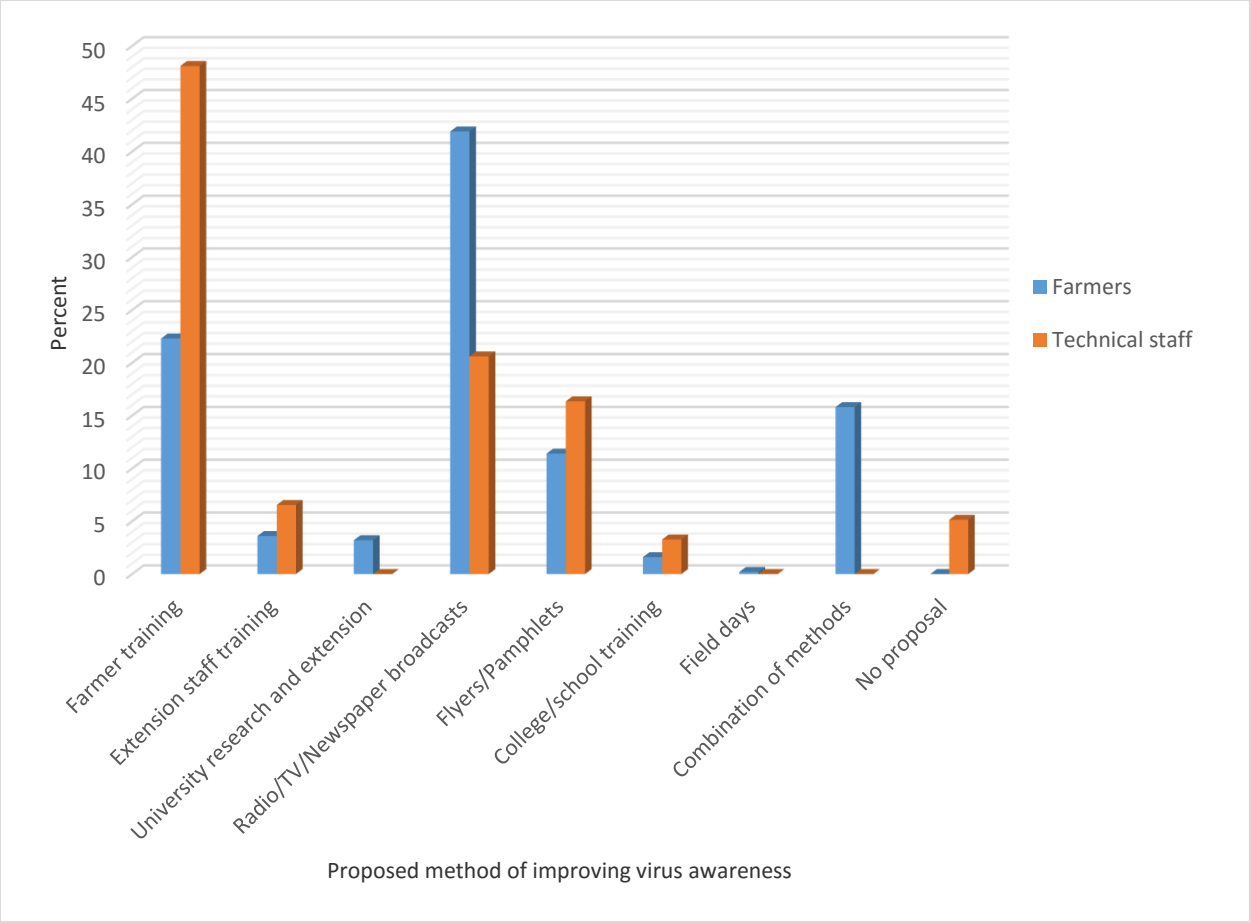

Figure 2.4: Respondents' opinions on how to improve virus diseases awareness

2.3.6 Knowledge of TSWV/tospoviruses by respondents

Education level (p=0.000), farmer age (p=0.011) and agricultural experience (p=0.020) had significant effects on respondents' knowledge of TSWV/tospoviruses (Tables 2.3 and 2.4). There were 1.042 chances that older farmers were aware of TSWV/tospoviruses, and only 0.124 chances that educated farmers were aware of the viruses (Table 2.3). There were 0.522 chances that technical staff respondents were aware of the viruses, and 0.541 more chances that experienced staff were aware of the viruses (Table 2.4). Only 23.7% of farmers and 41.6% of technical staff had heard about TSWV/tospoviruses, mainly from school/college. The majority (70.8%) of farmers that had heard about TSWV/tospoviruses had post-secondary education. Only 2.2% of the technical staff mentioned the electronic media as an information source for these viruses. Of those

who had heard about TSWV/tospoviruses, 43.8% of farmers and 70.8% of technical staff were able to correctly identify three plant hosts to the viruses, while 39.5% farmers and 18% technical staff could only identify the tomato as a host. Close to 7% of farmers and 11% of technical staff rated TSWV/tospoviruses as "not important," while 33.2% of farmers and 31.5% of technical staff rated them as "very important." Only 14.2% of farmers and 15.7% of technical staff were able to correctly name the TSWV/tospoviruses vectors. None of the respondents were able to name tospoviral species other than TSWV.

Table 2.3 Logit regression results on factors influencing farmers' knowledge of TSWV/tospoviruses

	B	S.E.	Wald	DF	Sig.	Exp(B)
Step 1a EducLevel	-2.085	0.179	136.235	1	0.000	0.124
MaritalStatus	-0.116	0.214	0.297	1	0.586	0.890
FarmExperience	-0.031	0.020	2.294	1	0.130	0.970
FarmType	-0.101	0.062	2.673	1	0.102	0.904
Age	0.041	0.016	6.487	1	0.011	1.042
Constant	5.286	0.633	69.662	1	0.000	197.611

Table 2.4 Logit regression results on factors influencing technical staff's knowledge of TSWV/tospoviruses

	B	S.E.	Wald	DF	Sig.	Exp(B)
Step 1a EducLevel	-0.649	180	12.964	1	0.000	0.552
AgricExperience	-0.615	0.265	5.374	1	0.020	0.541
Age	0.064	0.035	1.692	1	0.093	1.047
Gender	0.529	0.308	2.951	1	0.086	1.696
Employer	0.342	0.321	1.135	1	0.287	1.407
Constant	1.025	1.124	0.831	1	0.362	2.787

For TSWV/tospoviruses control, insecticide use was recommended by 68.9% of farmers and 40.7% of the technical staff. The use of certified seeds and fumigation were proposed by 5.8% of farmers and 14.8% of the technical staff. To improve TSWV/tospoviruses awareness, 27.1% of the technical staff recommended "college/university/school training," while 19.2% recommended "workshops/short courses for research/technical staff" and 20.6% proposed "print and electronic media campaigns."

2.4 Discussion

There were more male than female farmer respondents because males, as household heads, were generally more willing to come forward and give information to the researchers. This is despite the fact that women constitute the majority of workers on most farms. Similar findings were reported by Khan et al. (2014). Also, the higher literacy rate among males meant that they could confidently participate during the surveys.

There were fewer female than male agricultural technical staff respondents because fewer females graduate with agricultural professional qualifications in Zimbabwe. Historically, fewer female students study science-oriented subjects in high school and this translates to a smaller number of females who enroll for professional agricultural courses. In addition, agricultural extension is generally considered a masculine profession (Mutambara et al., 2013).

The study confirmed the changes in land demographics brought about by the country's land reform program that started in the year AD2000. The large scale commercial farming sector, previously the backbone of Zimbabwe's agriculture, has been decimated and replaced mainly by A1 and A2 farms. The fact that most farmers had no more than 10 years farming experience shows that they ventured into farming after the land reform program. Most such farmers either did not receive formal agricultural training or were poorly trained, and so are likely to be poorly knowledgeable about plant viruses, their effects and management.

Most respondents rated MSV as the most important plant virus in Zimbabwe. MSV is endemic to Zimbabwe and the sub-Saharan Africa region (Shepherd et al., 2010; Karavina, 2014). Therefore, most maize breeding and extension programs incorporate MSV researches and knowledge dissemination, respectively.

Some farmers either did not know about plant viruses or the different groups of plant pathogens that attack crops. During the FGDs, farmers talked more about insect pests and fungal diseases than plant viruses. This observation was similar to results reported by Sibiya et al., (2013) who found that plant diseases were lowly ranked by farmers in KwaZulu-Natal, South Africa. The major contributory factor, it appears, is poor education and training about plant viruses since even the technical staff had poor knowledge of plant viruses. While most technical staff were sufficiently trained to assist farmers to improve agricultural productivity, most could not distinguish viruses from other pathogens. A major reason for this was that most of them had diplomas as their highest relevant qualifications and so were not adequately trained in plant pathology. Even amongst the technical staff with degrees, viral diseases appreciation was poor probably because as students, most of them were poorly trained in plant pathology. The lack of qualified lecturers and training facilities in the last decade, and the "Open Distance Learning" system now in operation in the country compromised agricultural training.

The majority of respondents relied on visual symptoms assessment for disease diagnosis. This is not totally reliable, as symptom expression is influenced by the environment, host species, plant nutritional status, season, and pathogen strain (Sevik and Arli-Sokmen, 2012). It was noted that wherever maize is grown, most respondents attributed almost all mosaics, streaking and chlorosis to MSV, yet pathogens that cause similar symptoms like *Maize dwarf mosaic virus, Sugarcane mosaic virus, Maize stripe tenuivirus* and *Maize chlorotic mottle virus,* occur in Zimbabwe (Bonga and Cole, 1997). This highlights the need to employ several diagnostic tests to confirm pathogen identity. Where farmers sent diseased samples to plant clinics, the absence of qualified virologists and well-resourced laboratories also compromised viral disease diagnosis and ultimately, virus disease control.

Amongst the four major plant pathogen groups, viruses were the least appreciated by AEWs. This means the AEWs are less likely to talk about plant viruses to farmers than the other pathogens. Therefore, viruses will remain largely unknown to farmers. To remedy this situation, AEWs ought to be trained in plant virology so that they can disseminate correct information about pathogen biology, epidemiology and control. To achieve that, the agricultural training curricula must incorporate a significantly bigger section on plant virology in which emerging and re-emerging virus diseases are also taught.

Of the respondents who said they knew TSWV/tospoviruses, the large proportions of those who only knew tomato as a host crop and those who could not name any other tospoviruses besides TSWV, raise suspicions as to whether they really knew the pathogens. It also questions the seriousness accorded to the plant virology discipline in the country given that tospoviruses are an emerging problem worldwide (Scholthof et al., 2011). Currently, there are at least 28 tospovirus species causing serious yield losses worldwide (Margaria et al., 2014). The fact that wrong vectors were named and wrong control methods recommended showed that respondents had poor knowledge of pathogen biology and epidemiology. This means wrong control measures are likely to be implemented against the pathogens. According to Mehle and Trdan (2012), correct vector diagnosis is the first key step in tospovirus management. The observation that most respondents recommended insecticide use to control TSWV/tospoviruses reinforces an observation by

Nagaraju et al. (2002) that there is a “pesticide culture” that has been created by agrochemical companies through their extension programs and aggressive product promotion.

Only a small proportion of the technical staff mentioned the electronic media as a source of information for TSWV/tospoviruses, showing that traditional media platforms still dominate information dissemination in agricultural extension, research and training in Zimbabwe. While the country has an Information and Communication Technology (ICT) Policy that promotes the use of modern ICT tools, the agriculture sector has not adequately embraced it. Most AEWs lacked ICT resources to enable them to do their work effectively. The majority of research and tertiary education institutions have internet connectivity challenges that further limit internet use by students, academics and researchers.

In conclusion, the study showed that plant viral diseases are poorly appreciated by stakeholders in Zimbabwe’s agricultural sector. Besides MSV, other viral disease remain largely unknown by most people. This is worrying given the fact that there are currently many emerging and re-emerging plant viruses worldwide that are causing significant crop yield losses. The survey revealed the need for concerted and multifaceted approaches to increase knowledge of plant viruses in Zimbabwe through training of all stakeholders and conveyance of information by the media. This will then enable better plant viral disease management.

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CHAPTER 3: DETECTION AND CHARACTERIZATION OF *TOMATO SPOTTED WILT ORTHOTOSPOVIRUS* INFECTING FIELD AND GREENHOUSE-GROWN CROPS IN ZIMBABWE

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Abstract

Tomato spotted wilt orthotospovirus (TSWV), the type species of the genus *Orthotospospovirus* in the family *Tospoviridae*, is one of the most economically important emerging plant viruses worldwide. It causes over US$1 billion losses annually in open field and greenhouse-grown crops. A study was carried out to determine the geographical distribution, host range and phylogeny of TSWV in Zimbabwe. Disease surveys were conducted in 18 districts over a three-year period using tospovirus immunostrips. Virus-infected leaf samples were collected on FTA cards and in RNA*later* solution. TSWV was characterized by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription polymerase chain reaction (RT-PCR) followed by sequencing and phylogenetic analysis. The virus was detected in 50% of the districts surveyed, mostly in the country's prime agricultural region. It was confirmed to be present by DAS-ELISA and RT-PCR. In addition to some previously reported hosts, TSWV was detected for the first time in *Cucurbita moschata*, *Cucurbita pepo*, *Cucumis sativus* and *Gyposphila elegans*. Molecular analysis of the TSWV nucleocapsid gene showed that Zimbabwean TSWV isolates were highly similar (≥93.99%) and identical (99.02%) to each other at nucleotide and amino acid sequence levels. When compared to isolates from the rest of the world, Zimbabwean TSWV isolates were most closely related to isolates from Italy, Montenegro, New Zealand and Serbia. The study lays the foundation for future TSWV studies by providing protocols and procedures that could be used for pathogen detection and characterization. Information on TSWV host range generated in this study will be useful in designing cropping programs that do not allow for the growing of TSWV hosts either in succession or in close proximity to each other.

Keywords: Immunostrip, host, phylogenetic analysis, RT-PCR, *Orthotospovirus*

3.1 Introduction

Tomato spotted wilt orthotospovirus (TSWV), the type species of the genus *Orthotospovirus* in the family *Tospoviridae*, is one of the most economically important emerging plant-infecting viruses worldwide (Tsompana *et al*. 2005; Pappu *et al*. 2009; Scholthof *et al*. 2011). It causes losses estimated at US$1 billion annually for several important ornamental, food and cash crops worldwide (Goldbach and Peters 1994; Parrella *et al*. 2003). It infects over 1300 species in more than 92 plant families (Parrella *et al*. 2003; EFSA 2012). Since its first report in Australia in 1915 (Brittlebank 1919), TSWV has been reported in over 60 countries worldwide.

TSWV has a tripartite single-stranded RNA genome consisting of the large (L), medium (M) and small (S) RNA segments. The L RNA encodes the RNA-dependent RNA polymerase in the negative sense. The M RNA encodes two proteins: the non-structural movement protein (NSm) in the viral sense and the Gn-Gc glycoprotein in the nonviral sense. The S RNA encodes the non-structural protein (NSs) in the viral sense and the nucleocapsid (N) protein in the nonviral sense (Whitfield *et al*. 2005; Pappu *et al*. 2009). The TSWV virion is quasi-spherical, 80-120 nm diameter, and enveloped by a double membrane (German *et al*. 1992). Currently, at least nine thrips (Thysanoptera: Thripidae) species are known to transmit TSWV in a persistent-propagative manner, with *Frankliniella occidentalis* Pergande being the primary vector (Riley *et al*. 2011; Rotenberg *et al*. 2015).

The virus induces a wide range of symptoms in its hosts depending on environmental factors, host cultivar, pathogen strain and stage of host development (Sevik and Arli-Sokmen 2012). Common symptoms include ringspots, line patterns, mottling and chlorotic blotches on leaves, severe stunting, wilting and even plant death. TSWV-infected young tomato (*Solanum lycopersicum* L.) plants are characterized by inward cupping of leaves, bronzing and deformed fruits which show uneven ripening and raised bumps on the surface (Sether and DeAngelis 1992). In pepper (*Capsicum annuum* L.), severe stunting and chlorotic or mosaic yellow flecking of leaves are observed in infected plants. Necrotic spots are also present on pepper fruits, which often display ring patterns (Turina *et al*. 2012).

In Zimbabwe, TSWV was first reported infecting tobacco (*Nicotiana tabacum* L.) in 1940 (Hopkins 1940). It was subsequently reported infecting at least 26 other vegetable, ornamental and

weed species (Masuka *et al*. 1998; Nyamupingidza and Machakaire 2003). Recent studies have pointed out that the worldwide TSWV host range is expanding (Pappu *et al*. 2009), in part due to agricultural diversification and intensification, climate change, mutations and virus introductions into new habitats due to global trade in agricultural commodities (Hoffman *et al*. 2001; Sharman and Persely 2006). New hosts are being reported mainly in tropical and sub-tropical environments where the disease is now prevalent (Robbins *et al*., 2010; Macharia *et al*. 2016). This necessitates the study of TSWV host range in a sub-tropical country like Zimbabwe.

Moreover, Zimbabwean TSWV records are outdated, with no new records since 1998 (Masuka *et al*. 1998). The available records do not indicate how TSWV detection and characterization were done. In the last three decades, advances in the discipline of virology have revolutionized plant virus diagnostics. For example, modern virological studies incorporating molecular assays followed by phylogenetic analyses have been employed to help avoid ambiguity in tospoviral identification (Okuda and Hanada 2001; Sivparsad and Gubba 2008). Correct TSWV identification is crucial for effective disease management.

In this study, we investigated the occurrence of TSWV in Zimbabwe. Specifically, we assessed the geographical distribution and host range, outlined protocols for the detection and characterization of Zimbabwean TSWV isolates, and assessed the phylogenetic relationship of Zimbabwean TSWV isolates to those isolates that occur worldwide.

3.2 Materials and Methods

3.2.1 Sampling and field detection of tospoviruses

TSWV disease surveys were conducted from December 2013 to October 2015 in 18 districts across six provinces of Zimbabwe (see Figure 2.1). For Harare Province, the three surveyed districts were Harare East, Harare West and Harare North. The surveys targeted agricultural research institutions, irrigation schemes, plots, nutrition gardens, commercial and communal farms where all-year-round cropping is practiced. Sampling was done in both open fields and greenhouses/protected environments. Leaves exhibiting virus-like symptoms were tested on-site for tospoviruses before

sample collection either onto FTA™ cards (Whatman International, USA) or in 2 mL microcentrifuge tubes in RNA*later*® solution (Life Technologies, USA). Plant sap pressed onto FTA cards was air-dried for 30 min and stored at room temperature until processing. RNA*later*® solution-preserved samples were initially stored at 2-8°C and later at -80°C. In total, 1075 samples representing 13 different plant families were collected as follows: 429 samples in 2013, 381 samples in 2014, and 265 samples in 2015. Of these, 612 samples were collected on FTA cards while 463 samples were collected in RNA*later*® solution. All samples were transported to the University of KwaZulu-Natal, Plant Virology Laboratory (Pietermaritzburg, South Africa) for processing.

On-site tospovirus detection was done using immunostrips (Loewe® Biochemica GmbH, Germany) that could simultaneously detect *Tomato spotted wilt orthotospovirus* (TSWV), *Tomato chlorotic spot orthotospovirus* (TCSV) and *Groundnut ringspot orthotospovirus* (GRSV), according to manufacturer's instructions. Briefly, 5 mm diameter leaf discs of plants displaying tospovirus-like symptoms were collected into a small plastic bottle containing a buffer solution. The discs were ground using a plastic pestle. Two drops of the resultant sap were added to the test device window and observed for a color reaction in a viewing window. A control line appeared in the viewing window within a minute of sap application, while the test line appeared within 5-10 min in positive samples.

3.2.2 Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

All samples collected in RNA*later* solution were analyzed with a commercial TSWV DAS-ELISA diagnostic kit (Loewe Biochemica GmbH, Germany) according to manufacturer's instructions. Briefly, 0.2 grams of leaf samples were retrieved from the RNA*later*® solution using a sterile forcep. The RNA*later*® solution was removed from leaf samples by wiping the samples on paper towel. The samples were then submerged in 800 µL extraction buffer solution (50 mM sodium phosphate containing 20 mM sodium sulphite) in a 2 mL sterile microcentrifuge and then homogenized with a beads beater for 1 min. The homogenate was centrifuged at 14 000 rpm for 2 min and the lysate used for DAS-ELISA. For every test, negative and positive controls were included.

3.2.3 Molecular characterization of TSWV

Nucleic acid extraction

Total nucleic acid was extracted from FTA card samples as follows: a Harris® punch was used to cut out four 2 mm diameter discs from the card. The discs were place into a 2 mL microcentrifuge in 500 µL nuclease-free water. The tube was pulse-vortexed for 30s. The discs were transferred to a new microcentrifuge tube to which 50 µL water was added and pulse-vortexed for a further 15s. Thereafter, the discs were heated at 95ºC for 30 min, pulse vortexed three times for 5s and then centrifuged at 14 800 rpm for 5 min. The discs were removed from the tube and the eluate was used to perform reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was extracted from leaf samples preserved in RNA*later*® solution using a Quick-RNA™ MiniPrep Kit (Zymo Research, Irvine, USA) following the manufacturer's instructions. For each sample, about 0.5g of leaf materials were ground in 2 mL microcentrifuge tubes using a bead beater. The RNA quality was assessed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and stored at -80ºC.

Reverse transcription-polymerase chain reaction

First strand complementary DNA (cDNA) synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) using both degenerate and TSWV N gene primers (Table 3.1). Briefly, for each sample, 10 µL RNA (42 ng/µL) was pre-heated at 65ºC for 10 min. Then, a 20 µL reaction consisting of 4 µL reaction buffer (250 mM Tris-HCl, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 1 µL RevertAid M-MuLV Reverse Transcriptase enzyme (200 nmol of dTMP/µL), 1 µL RiboLock RNase Inhibitor (20 u/µL), 2 µL dNTPs (10 mM mix), 2 µL reverse primer (10 µM) and the pre-heated template RNA, was performed. The reaction mixture was incubated at 42ºC for 60 min, followed by a 5 min termination step at 70ºC. The resultant cDNA was used to perform polymerase chain reaction (PCR).

PCR was undertaken using the primer sets listed in Table 3.1. The primer pair gM410/gM870C amplifies the NSm protein, while all the other primers amplify the N gene. One-step 20 µL PCR reactions consisting of 3 µL cDNA, 2 µL of each primer (10 µM), 10 µL KAPA 2G HotStart

Ready Mix (0.5 U/µL DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP) and nuclease-free water, were performed. Amplifications were carried out under the following cycling conditions: an initial denaturation at 95^{o}C for 2 min; 35 cycles of denaturation at 95^{o}C for 15s, annealing at the appropriate temperature for each primer pair (Table 3.1) for 15s and extension at 72^{o}C for 15s; followed by an incubation period at 72^{o}C for 5 min. The PCR products were visualized by electrophoresis on 1.5% agarose gel stained with SYBR Safe Gel Stain (Life Technologies, USA).

Table 3.1 Primers used for the successful detection of *Tomato spotted wilt virus* in Zimbabwe

Primer name	Target gene	Amplicon size (bp)	Primer sequence	Annealing temperature (^{o}C)	Source
gM410	NSm	500	AACTGGAAAAATGATTYNYTTGTTGG	53.3	Chen *et al.* (2012).
gM870c			ATTAGYTTGCAKGCTTCAATNAARGC		
TSW 1	N	628	TCTGGTAGCATTCAACTTCAA	51.7	Roberts *et al.* (2000).
TSW 2			GTTTCACTGTAATGTTCCATAG		
TSWV 722	N	620	GCTGGAGCTAAGTATAGCAGC	55.4	Adkins and Rosskopf (2002).
TSWV 723			CACAAGGCAAAGACCTTGAG		
TSWV-N-1F	N	777	ATGTCTAAGGTTAAGCTCACTA	51.3	Navarre *et al.* (2009).
TSWV-N-777c			TTAAGCAAGTTCTGTGAGTTTT		

Cloning, sequencing and phylogenetic analysis

PCR products corresponding to targeted genes were purified using a QIAQuick DNA Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Purified products were either directly sequenced or cloned to pCR®2.1 vector before sequencing at Inqaba Biotechnical Industries (Pretoria, South Africa). Cloning was performed using the TOPO-TA Cloning Kit (Invitrogen, USA) according to the manufacturer's instructions. The cloning vector with the ligated insert was used to transform TOP10 Chemically Competent *Escherichia coli* cells, according to manufacturer's instructions (Invitrogen, USA). Plasmid DNA of independently transformed bacterial cells were purified using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, USA). Recombinant clones were identified by *EcoR1* restriction endonuclease digestion. Three independent clones for each insert were selected and sequenced in both directions using M13 primers.

Raw sequence data were checked for quality, edited and consensus sequences were compiled using MEGA6 software. The Basic Local Alignment Search Tool (BLAST) embedded in MEGA6 program was used to search the National Centre for Biotechnology Information (NCBI) GenBank for related TSWV N gene sequences used in phylogenetic analysis. All sequences were aligned using ClustalW imbedded in the MEGA6 software (Tamura *et al*. 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model with a discrete Gamma distribution generated by MEGA 6.0 (Tamura 1992). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 75% bootstrap replicates were collapsed. *Iris yellow spot orthotospovirus* (Accession code AF001387.1) was used as an outgroup. Nucleotide identity and similarity were determined using the SIAS software program (www.imed.ucm.es/Tools/sias.html).

3.3 Results

3.3.1 Geographical distribution of TSWV in Zimbabwe

Out of the 18 districts surveyed, tospoviruses were detected in Harare (West, East and North), Seke, Goromonzi, Mutoko, Shamva, Mazoe and Zvimba (see Figure 2.1). The virus was not

detected in Bindura, Chegutu, Chimanimani, Chinhoyi Urban, Chiredzi, Gutu, Mutare, Nyanga and Masvingo Urban. TSWV was mainly detected at farms where there is all-year round cropping. Most of the TSWV-positive sites were within a 30 km radius of Harare Central Business District.

3.3.2 Field detection of tospoviruses using immunostrips

Tospoviruses were successfully detected from plants displaying chlorosis, leaf chlorosis, crinkling and reduced size using immunostrips. The time it took for the test line to appear in the test device window and the intensity of the line varied from sample to sample. Tospovirus incidence was highest in Solanaceae (11.8%) and Cucurbitaceae (6.3%) families. There were inconclusive test results from lettuce (*Lactuca sativa* L.), onion (*Allium cepa* L.), begonia (*Begonia*), baby's breath (*Gypsophila elegans*) and asters (*Callistephus chinensis*). Inconclusive results appeared either as faint test lines or after the standard 10 min viewing period. Tospoviruses were not detected in Fabaceae and Brassicaceae crops (Table 3.2) even though they supported high thrips populations and had virus-like infection symptoms.

3.3.3 Serological characterization of TSWV

Of the 663 samples analyzed by DAS-ELISA, only 96 (14.47%) were positive for TSWV. The majority (55.2%) of the positive samples belonged to the Solanaceae family, while 20.8% belonged to the Cucurbitaceae family. The alliums, basil, begonia, lettuce and peas samples that were inconclusive with immunostrips were negative by DAS-ELISA, while *Gypsophila elegans* samples were positive by DAS-ELISA. TSWV was not detected in Brassicaceae and Fabaceae samples by DAS-ELISA.

3.3.4 Molecular and phylogenetic analyses

Amplification products of the expected sizes were detected after agarose electrophoresis of the RT-PCR products of total RNA isolations from leaf material infected with TSWV. The negative controls showed no products. Because of high similarities amongst the TSWV sequences, only representative sequences were deposited into GenBank with the accession codes given in Table 3.3. None of the samples that had been negative by DAS-ELISA were positive by PCR even when different primers were used.

Table 3.2 List of crops and ornamental plants tested for tospoviruses using immunostrips

Family	Common name	Botanical name	Test result
Alliaceae	Onion	*Allium cepa*	NC
	Garlic	*A. sativum*	NC
	Leek	*A. ampeloprasum*	NC
	Shallot	*A. cepa* var. *aggregatum*	NC
Amaranthaceae	Spinach	*Spinacia oleracea*	-
Asteraceae	Chrysanthemum	*Dendranthema morifolium*	+
	Aster	*Callistephus chinensis*	-
Begoniaceae	Begonia	*Begonia*	NC
Brassicaceae	Cabbage	*Brassica oleracea*	-
	Rape	*B. napus*	-
	Broccoli	*B. oleracea*	-
	Cauliflower	*B. oleracea capitata*	-
Caryophyllaceae	Baby's breath	*Gypsophyla elegans*	NC
Cucurbitaceae	Cucumber	*Cucumis sativum*	+
	Butternut	*Cucurbita moschata*	+
	Baby marrow	*Cucurbita pepo*	-
	Pumpkin	*Cucurbita pepo*	+
	Watermelon	*Citrullus lanatus*	-

	Gourds	*Lagenaria siceraria*	-
Gesneriaceae	African violet	*Saintpaulia*	-
Lamiaceae	Basil	*Ocimum basilicum*	NC
Leguminosae	Peas	*Pisum sativum*	NC
	Beans	*Phaseolus vulgaris*	-
	Soyabean	*Glycine max*	-
	Groundnut	*Arachis hypogaea*	-
	Cowpea	*Vigna unguiculata*	-
Roseceae	Roses	*Rosa*	-
Rubiaceae	Pentas	*Pentas*	-
Solanaceae	Potato	*Solanum tuberosum*	+
	Tomato	*Solanum lycopersicum*	+
	Tobacco	*Nicotiana tabacum*	-
	Cherry pepper	*Capsicum annuum*	+
	Bell pepper	*Capsicum annuum*	+
	Eggplant	*Solanum melongena*	+
	Chilli pepper	*Capsicum frutescens*	NC
Violaceae	Viola	*Viola*	-

Key: '+' – positive; '-' – negative; 'NC' – inconclusive

Table 3.3 Accession codes of *Tomato spotted wilt virus* isolates detected in different hosts in this study.

Accession code	Isolate name	Host name	Family	Sequenced gene
KT732271	Butternut_Harare	*Cucurbita moschata*	Cucurbitaceae	Nucleocapsid
KU892656	92-tom1-zim	*Solanum lycopersicum*	Solanaceae	Nucleocapsid
KX192331	90-eggplant-zim	*S. melongena*	Solanaceae	Nucleocapsid
KX192332	86-potato-zim	*S. tuberosum*	Solanaceae	Nucleocapsid
KU884650	mum-a5	*Dendranthema morifolium*	Asteraceae	Non-structural movement
KU884651	tom16	*S. lycopersicum*	Solanaceae	Non-structural movement
KU671049	174_TSWV	*Capsicum annuum*	Solanaceae	Nucleocapsid
KU884648	42-pep-zim	*C. annuum*	Solanaceae	Nucleocapsid
KX192330	a5-mum	*D. morifolium*	Asteraceae	Nucleocapsid
KX273062	gyp-316-zim	*Gypsophila elegans*	Asteraceae	Nucleocapsid
KX192329	81-cherry-zim	*C. annuum*	Solanaceae	Nucleocapsid
KX273061	cuc-48-zim	*Cucumis sativus*	Cucurbitaceae	Nucleocapsid

The BLAST results confirmed that the identified isolates were TSWV, based on the high levels of sequence similarities with TSWV isolates from other countries. When compared amongst themselves, the partial N gene of the Zimbabwean isolates showed at least 99.02% and 93.99% nucleotide and amino acid sequence identities and similarities, respectively. Isolate 81-cherry-zim

(Accession code KX192329) had the lowest similarity (93.99) to the other Zimbabwean isolates (Tables 3.4a and 3.4b). When compared to isolates from elsewhere in the world (Supplementary Table 1), Zimbabwean isolates were most closely related to isolates from Italy (Accession code KM096540), New Zealand (Accession code KC494501) and Montenegro (Accession code GU355939) at 98.64 – 99.15% nucleotide sequence identity. The Zimbabwean isolates were most similar to the Montenegrin isolate (code GU355939) and Serbian isolate (code GU339805) at 99.36%. The partial NSm gene of the Zimbabwean isolates showed 97-99% similarities to isolates from Australia (HM015516), Serbia (GQ373174.1), USA (AY744486), Italy (HQ830185.1) and Spain (HM015511.1).

Table 3.4a Sequence similarity (%) of the partial nucleocapsid protein gene of Zimbabwean TSWV isolates at nucleotide and amino acid levels

KT732271	100										
KU671049	100	100									
KU884648	99.51	99.51	100								
KU884649	94.48	94.48	94.15	100							
KU892956	99.83	99.83	99.35	94.31	100						
KX192332	99.83	99.83	99.33	94.31	99.67	100					
KX192331	99.67	99.67	99.18	94.15	99.51	99.51	100				
KX192329	99.51	99.51	99.02	93.99	99.35	99.35	99.18	100			
KX273061	100.00	100.00	99.51	94.48	99.83	99.83	99.67	99.51	100		
KX273062	100.00	100.00	99.51	94.48	99.83	99.83	99.67	99.51	100	100	
KX192330	99.83	99.83	99.35	94.64	99.67	99.67	99.51	99.35	99.83	99.83	100
Accession	KT732271	KU671046	KU884648	KU884649	KU892956	KX192332	KX192331	KX192329	KX273061	KX273062	KX192330

Table 3.4b Sequence identity (%) of the partial nucleocapsid protein gene of Zimbabwean TSWV isolates at nucleotide and amino acid levels

KT732271	100										
KU671049	100	100									
KU884648	99.51	99.51	100								
KU884649	99.65	99.65	99.31	100							
KU892956	99.83	99.83	99.35	99.48	100						
KX192332	99.83	99.83	99.35	99.48	99.67	100					
KX192331	99.67	99.67	99.18	99.31	99.51	99.51	100				
KX192329	99.51	99.51	99.02	99.14	99.35	99.35	99.18	100			
KX273061	100	100	99.51	99.65	99.83	99.83	99.67	99.51	100		
KX273062	100	100	99.51	99.65	99.83	99.83	99.67	99.51	100	100	
KX192330	99.83	99.83	99.35	99.82	99.67	99.67	99.51	99.35	99.83	99.83	100
Accession	KT732271	KU671046	KU884648	KU884649	KU892956	KX192332	KX192331	KX192329	KX273061	KX273062	KX192330

Phylogenetic analysis with the Maximum Likelihood method separated all isolates into two clusters (Figure 3.1). All Zimbabwean isolates were in Cluster A with isolates from Algeria, Egypt, Kenya, some European, Asian, and the American countries. Cluster B had isolates from South

Africa, China, Syria, Bulgaria, Brazil, Turkey, South Korea and Texas (USA). The Zimbabwean isolates grouped into a distinct sub-cluster in Cluster A.

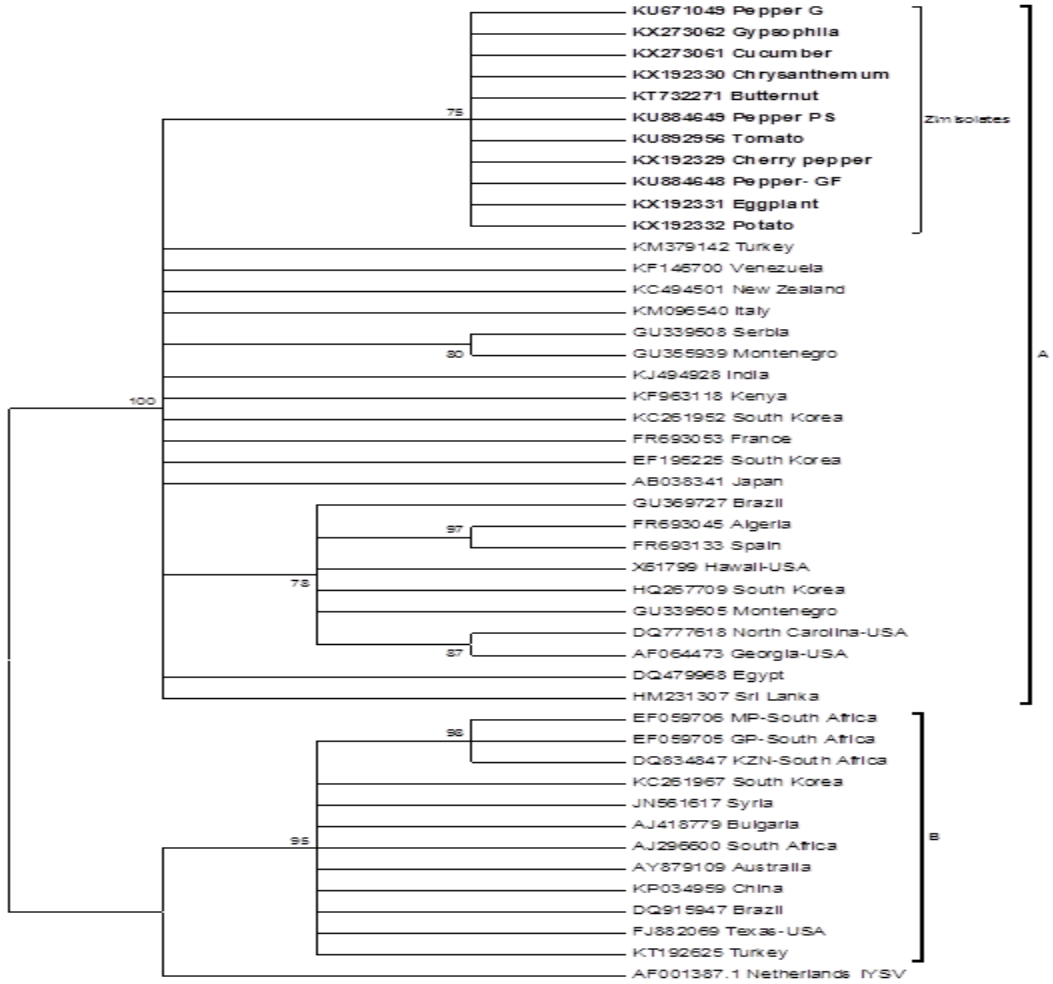

Figure 3.1 Phylogenetic relationships of partial N gene sequences from TSWV isolates collected from Zimbabwe and database sequences from other geographical regions of the world. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 75% bootstrap replicates are collapsed. Zimbabwean isolates (Zim isolates) are printed in bold font.

3.4 Discussion

The study confirmed the presence of TSWV in Zimbabwe, a pathogen first reported in 1940 (Hopkins 1940). It was detected in 50% of the surveyed districts. TSWV was not detected in some districts even though two of its vectors, *Frankliniella occidentalis* and *Thrips tabaci*, were detected (data not shown). This could be due to the fact that there has been limited movement of infected plant material and/or vectors within the country. Eight of the nine districts where TSWV was detected are in agro-ecological region II, the country's bread basket region. Any serious future TSWV disease outbreaks could be disastrous given the economic damage the disease causes (Cho *et al*. 1996; Pappu *et al*. 2009).

In this study, immunostrips were used to detect TSWV for the first time in Zimbabwe. This is important as immunostrips provide an easier-to-use and quicker detection technique than DAS-ELISA, electron microscopy and molecular assays. Immunostrip-use during field sampling ensured that mostly virus-positive samples were collected for further processing in the laboratory. The time variation in the appearance of the test line in the test device window was probably due to differences in virus titre within the samples. The test line quickly appears in those samples that have high virus titre and vice versa (Strange 2003). Similarly, inconclusive results could be due to low virus titre or cross-reaction of antibodies with plant sap (Naidu and Hughes 2003).

The use of RNA*later* solution and FTA cards during sampling was important as it enabled virus-infected samples to be conveniently transported to the laboratory for further characterization. In this case, there was no need for dry ice and/or refrigeration facilities to preserve RNA integrity in the collected samples. Crucially, RNA*later*-stored samples were successfully used in TSWV detection by DAS-ELISA. Stabilization buffers are said to contain enzyme inhibitors that potentially interfere with ELISA. We did not observe such interference either in this or the other studies where we screened samples for *Iris yellow spot orthotospovirus* (Karavina and Gubba 2017a, b) and other viruses (data not shown). RNA*later*-stored samples had previously been used to perform ELISA by Blacksell *et al*. (2004) and Rader *et al*. (2008).

The identity of the virus detected by immunostrips and DAS-ELISA was confirmed by molecular assays. The molecular assays also helped to resolve the TSWV status of samples that had been inconclusive and/or negative by serology. This is the first time molecular assays had been

employed in characterizing TSWV in Zimbabwe. Given that there were no records of how TSWV was characterized in Zimbabwe, this study provides protocols that could either be adapted or adopted by other researchers studying the pathogen. Crucially, the protocols could enable early detection and correct characterization of TSWV for effective disease management (Awondo *et al*. 2012).

Host range records indicate that Zimbabwean TSWV was prevalent mainly in ornamentals and solanaceous crops (Rothwell 1982; Masuka *et al*. 1998). Amongst the ornamentals, this study detected the virus in *Dendranthema morifolium* and *Gypsophila elegans* only. The narrow ornamental host range observed could be attributed to the fact most of the flowers previously reported to be TSWV hosts are no longer being grown in the country. Zimbabwe's land reform program negatively impacted the floriculture sector as "new farmers" concentrated on maize, soybean and tobacco production at the expense of flowers which are highly capital-intensive. For the first time, TSWV was detected in the cucurbits *Cucurbita moschata, C. pepo* and *Cucumis sativus*, an indication that our knowledge of the pathogen's host range in Zimbabwe is expanding. Worldwide, TSWV infects over 1300 plant species (Parella *et al*. 2003), and this host range is also expanding (Nischwitz *et al*. 2006; Pappu *et al*. 2009). Host range expansion is attributed to the generalist and opportunistic survival mode of TSWV (Pappu and Subramanian 2013). Though TSWV was not detected in some of the cucurbits grown in Zimbabwe, there is high likelihood of the virus infecting them in future, as has been reported elsewhere (Sether and de Angelis 1992; Massumi *et al*. 2007). So, there is need for regular surveys to monitor the TSWV host range.

TSWV was mostly detected in solanaceous crops, indicating that they are highly susceptible to the virus. Of the solanaceous crops, infected tomatoes did not display severe disease symptoms. This could be attributed to the fact that most tomato varieties grown in Zimbabwe have the *Sw-5b* resistance gene (data not shown). In some *Capsicum annuum* samples that were infested with both *Frankliniella occidentalis* and *Myzus persicae*, mixed infections of TSWV and *Potato virus Y* were detected. The effects of mixed infections on symptom development and crop growth need further investigations. TSWV was not detected in tobacco, a major cash crop in Zimbabwe. Local tobacco breeding programs incorporate TSWV resistance, and local TSWV isolates probably have not yet been able to break the resistance.

The fact that Zimbabwean TSWV isolates have high nucleotide and amino acid identity and similarities shows that minor evolutionary differences exist amongst the isolates. However, the minor isolate differences are evidence of some mutations taking place. There is need to carry out population genetics studies to investigate the systematic and random forces of evolution acting on the isolates.

Besides Zimbabwean isolates, the only other African TSWV isolate sequences that could be retrieved from sequence databases are from Kenya (Macharia *et al*. 2015), South Africa (Sivparsad and Gubba 2008), Egypt and Algeria. The Zimbabwean isolates clustered in the same group with Algerian, Egyptian and Kenyan isolates, but separately from South African isolates. This suggests that there may have been multiple introductions of TSWV into Africa (Sivparsad and Gubba 2008). The TSWV isolates in this study did not group according to geographical origin, as previously reported by Zindovic *et al*. (2014). This could be due to global trade in plant products which has resulted in TSWV strains spreading throughout the world.

In conclusion, the study revealed that TSWV in Zimbabwe is not as geographically widespread as its two worldwide vectors, *F. occidentalis* and *T. tabaci*. However, new Zimbabwean TSWV hosts have been reported in this study. The virus detection techniques and characterization protocols employed to study TSWV could be employed to characterize other plant viruses in Zimbabwe.

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CHAPTER 4: *IRIS YELLOW SPOT ORTHOTOSPOVIRUS* IN ZIMBABWE: INCICDENCE, SEVERITY AND CHARACTERIZATION OF *ALLIUM*-INFECTING ISOLATES

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Abstract

Iris yellow spot orthotospovirus (IYSV), an emerging pathogen of alliaceous crops, has been reported in many countries worldwide. The pathogen has recently been reported infecting garlic (*Allium sativum*), leek (*A. ampeloprasum*) and onion (*A. cepa*) in Zimbabwe. A study was carried to determine its incidence, severity and distribution in Zimbabwe. IYSV disease surveys were conducted in 18 districts across six provinces. Symptomatic alliaceous leaf samples were tested for IYSV by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Furthermore, the pathogen was characterized by transmission studies, transmission electron microscopy and molecular assays. The pathogen was detected in garlic, leek, shallot (*A. cepa* var. *aggregatum*) and onion crops in all districts, with disease incidences ranging from 26.7 – 78.8% and disease severities of up to 3.7 in some districts. Up to 70% of onion seedlings that were mechanically inoculated with sap from diseased onion plants produced typical IYSV symptoms characterized by green chlorotic tissues surrounded by tan-colored necrotic tissues. Electron microscopy studies revealed spherical enveloped particles that were 80-120 nm in diameter. Analysis of the partial nucleocapsid protein (N) gene of the Zimbabwean IYSV isolates revealed that they were at least 94.89% identical and similar to each other at nucleotide and amino acid levels. These isolates were closely related to isolates from Asia, Australia, Europe, South Africa and the USA, but were distinct from isolates from Brazil, The Netherlands and The UK. Further work to ascertain the pathogen's economic impact needs to be carried out. In addition, farmers need to be educated about the disease so that they can control it by growing resistant varieties and/or controlling the vector.

Keywords: detection; distribution; *Orthotospovirus*; phylogenetic analysis; symptoms.

4.1 Introduction

Iris yellow spot orthotospovirus, IYSV, an emerging pathogen of alliaceous crops, has been reported in many countries worldwide (Bag *et al*., 2015). The pathogen is responsible for causing significant yield losses in bulb and seed onion crops (Gent *et al*., 2006; Mandal *et al*., 2012). Besides infecting cultivated alliaceous crops, IYSV also infects wild *Allium* species, ornamental plants and several weeds (Smith *et al*., 2011; Bag *et al*., 2015). Since its detection and first report in iris (*Iris holandica*) in the Netherlands in 1998 (Cortês *et al*., 1998), IYSV has been reported on all continents (Pappu *et al*., 2009), and continues to be reported in many countries. In Africa, IYSV was first reported in Reunion (Robene-Soustrade *et al*., 2005). It has subsequently been reported in South Africa (du Toit *et al*., 2007), Kenya and Uganda (Birithia *et al*., 2011), Egypt (Hafez *et al*., 2012), Mauritius (Lobin *et al*., 2012) and Zimbabwe (Karavina *et al*., 2016a).

IYSV belongs to the genus *Orthotospovirus* in the family *Tospoviridae*. The IYSV virion consists of quasi-spherical enveloped particles that are 80 - 120nm in diameter. The virion has a tripartite single-stranded RNA genome consisting of large (L), medium (M) and small (S) segments. The L RNA (~8.9kb) has a single open reading frame (ORF) and encodes the RNA-dependent RNA polymerase in negative sense (Bag *et al*., 2010). The M and S segments are ambisense and each encodes two proteins. The M RNA (~4.8kb) encodes the non-structural movement (NSm) protein in the viral sense and the Gn-Gc protein in the viral complementary sense, while the S RNA (~3.1kb) encodes the nucleocapsid (N) protein in the viral complementary sense and the nonstructural protein (NSs) in the viral sense (Cortês *et al*., 1998). Like other tospoviruses, IYSV is transmitted exclusively by thrips (Thysanoptera: Thripidae) in a circulative-propagative mode (Whitfield *et al*., 2005). Currently, *Thrips tabaci* Lindeman and *Frankliniella fusca* (Hinds) are the known IYSV vectors (Srinivasan *et al*., 2012; Bag *et al*., 2015). In addition to transmitting IYSV, thrips damage alliaceous crops through their feeding activities.

Thrips are the primary pests of alliaceous crops in Zimbabwe, capable of causing up to 40% yield loss (Zindoga, 2015). Most of the thrips-induced yield loss is attributed to their feeding on the crop foliage. Typical thrips damage symptoms include silvery spots that turn into white blotches along the leaves, followed by the development of silvery patches and leaf curling. This reduces the plant's photosynthetic capacity, and ultimately decreases bulb size and yield (Waiganjo *et al*.,

2008; Munoz *et al*., 2014). On leafy *Allium* species like shallots (*A. cepa* var. *aggregatum*) and leeks (*A. ampeloprasum*), the presence of leaf lesions and necrotic spots reduces crop marketability.

In recent years, alliaceous crops with necrotic, irregularly-shaped and grey-to-bleached white leaf lesions have been found in Zimbabwe. The lesions grow and coalesce, eventually girdling and killing the plant leaf. Sometimes, the necrotic lesions surround islands of green and/or chlorotic tissues. Early and severe crop infection results in premature defoliation. These symptoms are typical of IYSV infection (Krauthausen *et al*., 2012; Buckland *et al*., 2013). Recent surveys have reported up to 60% Iris yellow spot disease (IYSD) incidence in alliaceous crops in Zimbabwe (Karavina *et al*., 2016a, b). However, the incidence figures were based on localized disease surveys. This study sought to determine the occurrence and distribution of IYSV in Zimbabwe. In pursuance of that, nationwide disease surveys were conducted and IYSV isolates were characterized based on symptomatology, serology, microscopy, transmissibility and molecular assays. This would provide critical information needed to develop control strategies against the IYSD.

4.2 Materials and Methods

4.2.1 Disease surveys

IYSD surveys were conducted from May 2014 to November 2015 in 18 districts across six Zimbabwean provinces (see Figure 2.1). In each season, 101 fields were surveyed and 608 *Allium* species samples displaying suspected IYSD symptoms were collected. In addition, a total of 93 non-alliaceous crop samples were collected from fields where *Allium* species were intercropped with brassicas (*Brassica* species), Irish potatoes (*Solanum tuberosum* L.) and roses (*Rosa* species). Sampled sites were located with global positioning system (GPS) coordinates in order to facilitate return visits. A field was considered IYSV-positive if at least one collected sample tested positive by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Young leaves displaying IYSD-like symptoms were sampled. Disease incidence and severity were visually assessed and recorded. Disease severity was scored on a scale of 0-5, based on the number and size of leaf lesions using a scale developed by Shock *et al*. (2008) where:

0 = No symptoms.

1 = 1% - 25% of foliage diseased.

2 = 26% - 50% of foliage diseased.

3 = 51% - 75% of foliage diseased.

4 = 76% - 99% of foliage diseased.

5 = 100% of foliage diseased (leaves completely dried).

Disease incidence (DI) was calculated as below:

$$DI = \frac{\text{Number infected plants}}{\text{Total field plant population}} \times 100$$

During the surveys, a short questionnaire on farmers' knowledge of IYSV, its vectors and control was administered.

4.2.2 Serological diagnosis

Leaf samples were tested for IYSV in duplicate wells using a commercial kit supplied by Loewe® Biochemica GmbH (Sauerlach, Germany) following the manufacturer's instructions. Briefly, a microtiter plate was coated with IYSV-specific coating antibody (IgG). About 0.5g of alliaceous tissue from the middle and bottom portions of youngest leaves showing disease symptoms were excised and ground in liquid nitrogen in a pestle and mortar. Macerated plant tissues were mixed with Conjugate Buffer at a 1:20 dilution, and 0.2 ml mixture added to each microtiter well and incubated overnight. After four washes, an enzyme-labelled antibody-AP-conjugate was applied to the plate wells. In the final step, 0.2 ml of the Substrate Buffer Solution containing the dissolved substrate tablet was applied to the microtiter plate. After two hours of incubation, the reaction was

visually assessed for yellow color development. Samples giving weak yellow color were subjected to reverse transcription polymerase chain reaction (RT-PCR) to confirm whether they were IYSV-infected or not.

4.2.3 Transmission studies

Commercial *Allium cepa* cv. Texas Grano seeds were sown in a 242-cell float tray in pine bark media, pH 5.5 ($CaCl_2$ scale). Three-week-old onion seedlings were individually transplanted to 15cm diameter plastic pots. A week later, the seedlings were mechanically inoculated with IYSV. Briefly, IYSV-infected onion leaves were homogenized in 10mM sodium phosphate buffer (pH 7.0), containing 0.1% sodium sulfite using sterilized pestle and mortar. Leaf debris were removed by squeezing the homogenate through a non-absorbent cotton pad. The extracted sap was gently rub-inoculated onto fully expanded onion transplant leaves dusted with carborandum. Inoculated plants were kept in an insect-proof greenhouse with 25/18^{o}C cycle, day/night temperatures and observed for virus symptom development over a three-week period. Twenty onion seedlings were mechanically inoculated with the virus each season and the experiment was duplicated per season. *Allium cepa* cv. Texas Grano control plants were mock-inoculated with distilled water.

4.2.4 Electron microscopy studies

These were carried out at the Microscopy and Microanalysis Unit of the University of KwaZulu-Natal, Pietermaritzburg, South Africa. Briefly, 2mm × 2mm onion leaf pieces were excised from young symptomatic leaves and fixed in 3% glutaraldehyde, for 12 hours. Samples were twice washed in 0.05M sodium cacodylate for 30 minutes each time and then fixed in a 50:50 solution of 2% osmium tetroxide and 0.05M sodium cacodylate for 2 hours. After two 30 minute washes in 0.05M sodium cacodylate, the samples were dehydrated in graded ethanol series (10%, 30%, 50% and 70% for 10 minutes each; 90% and 100% for 15 minutes each). Samples were twice washed with 100% propylene oxide for 15 minutes each time to remove residual ethanol, embedded in Epon-Araldite resin mixture, and polymerized. Sectioned material was stained with

uracyl acetate followed by lead nitrate and examined for virus particles under the JEOL JEM-1400 Transmission Electron Microscope (TEM).

4.2.5 Molecular detection

Total RNAs of healthy and IYSV-infected, ELISA-positive alliaceous plant tissues were extracted using the Quick-RNA™ MiniPrep Kit (Zymo Research, USA) following manufacturer's instructions. The RNA was subjected to reverse transcription-polymerase chain reaction (RT-PCR) using the RevertAid RT Reverse Transcription Kit (Thermo Fischer Scientific, USA). The resultant cDNA was used for PCR amplification using the KAPA2G Fast Hot Start ReadyMix Kit (KAPA Biosystems, USA) and nucleocapsid (N) gene-specific primers (IYSV_2F: 5'-GGCGGTCCTCTCATCTTACTG-3' and IYSV-NCP2_R: 5'-GAAGTTCCAGGAGTGCATTTAGTC-3') (Lee *et al.,* 2011) under the following cycling conditions: initial denaturation at 95ºC for 2 min; 35 cycles of 94ºC for 15 s (denaturation step), 57ºC for 15 s (annealing step), and 72ºC for 15 s (extension step), followed by a 5-min incubation period at 72ºC. PCR products were analyzed by 1.5% agarose gel electrophoresis. The amplicons were excised, purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA), and cloned into pCR™2.1 vector with One Shot® TOP10 Chemically Competent *E. coli* cells (Life Technologies, USA). Isolation of recombinant plasmid DNA was done using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions. The presence of an insert in transformants was confirmed by the restriction enzyme *ECoR1* digestion. Three independent clones were sequenced in both directions at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) using M13 forward and reverse primers. Consensus sequences generated from the three clones were blasted into MEGA6 program. The nucleotide sequences of the IYSV isolates reported in this study have been deposited into GenBank under the accession numbers given in Table 4.2.

4.2.6 Phylogenetic analysis of the partial IYSV N gene

The evolutionary history of isolates generated in this study was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). N gene sequences reported from elsewhere in the world

were obtained from GenBank and, together with Zimbabwean isolates, were aligned by ClustalW embedded in MEGA6 software (Tamura *et al*., 2013). The bootstrap consensus tree inferred 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Genetic distances between Zimbabwean IYSV isolates and those from the rest of the world were calculated based on the Tamura 3-parameter model (Tamura, 1992). Nucleotide sequence identity and similarity were computed using the SIAS program (www.imed.ucm.es/Tools/sias.html).

4.3 Results

4.3.1 Geographical distribution, incidence and severity of *Iris yellow spot orthotospovirus* in Zimbabwe

The study showed that the IYSD is widespread in Zimbabwe. IYSV was detected by DAS-ELISA and confirmed to be present in infected samples by RT-PCR in all districts that were surveyed. Disease incidence and severity figures in *Allium* species for 2014 and 2015 seasons are given in Table 4.1.

In 2014, the highest IYSV incidence and severity were recorded in Chinhoyi District, while in 2015, Shamva District had the highest disease incidence and severity. Gutu and Harare East districts had the lowest disease incidences in 2014 and 2015, respectively (Table 4.1). There was no clear pattern of disease progress in the study areas over the two seasons.

All farmers interviewed during the surveys did not know about the IYSD. Most (90%) did not know about the presence of thrips in their crops, and attributed thrips feeding damage and IYSD symptoms to water deficiency, red spider mite infestation, and/or crop maturity. Only 10% of farmers sprayed their crops with insecticides to control thrips.

4.3.2 *Iris yellow spot orthotospovirus* symptomatology in Zimbabwe

Two distinct IYSD symptoms were observed on *Allium* species in Zimbabwe. One symptom type consisted of irregularly-shaped, tan-to-bleached white or straw-colored, necrotic lesions on leaves.

Infected plants were affected by tip dieback and also by progressive necrosis of older leaf area. The other symptoms consisted of a series of concentric lighter and darker rings that diamond-shaped around the feeding point. Sometimes, there were green chlorotic islands of leaf tissues surrounded by white or straw-colored necrotic tissues (Figure 4.1). Both symptom types were present in some alliaceous fields.

Table 4.1 *Iris yellow spot orthotospovirus* incidence and severity on alliaceous crops in Zimbabwe

Province	District	2014		2015	
		Incidence (%)	Severity	Incidence (%)	Severity
Harare	Harare East	60.0	2.3	43.3	2.0
	Harare West	57.0	2.7	65.0	2.7
	Harare North	55.0	2.7	55.0	2.7
Mashonaland Central	Bindura	40.0	2.7	46.7	1.7
	Mazowe	43.3	2.3	58.3	3.3
	Shamva	70.0	2.7	78.3	3.7
Mashonaland East	Goromonzi	48.3	2.3	61.7	2.7
	Seke	46.7	2.3	56.7	2.0
	Marondera	63.3	3.0	45.0	2.0
Mashonaland West	Zvimba	43.3	1.7	66.7	3.0
	Chegutu	65.0	2.7	73.3	2.0
	Chinhoyi	75.0	3.5	50.0	2.0
Manicaland	Nyanga South	61.7	2.3	55.0	2.3

	Mutare	53.3	2.3	76.7	3.0
	Nyanga North	71.7	3.3	60.0	2.3
Masvingo	Gutu	26.7	1.0	60.0	2.7
	Masvingo	46.7	2.7	53.3	3.0
	Chiredzi	57.3	2.3	63.7	3.3

Figure 4.1. *Iris yellow spot orthotospovirus*-infected onion plants. Notice the white lesions and green chlorotic islands surrounded by necrotic tissues on the leaves.

4.3.3 IYSV natural host range and detection by serological, electron microscopy and transmission detection studies

The virus naturally infected sole onion, garlic, leek and shallot crops (Table 4.2). It was also detected in garlic and onion intercropped with roses, potatoes and *Brassica* species. However, the virus was not detected in the companion crops. Volunteer and out-of-season alliaceous crops collected from harvested fields and home gardens, respectively were also IYSV-infected.

IYSV was positively detected by serology, electron microscopy and mechanical transmission studies. DAS-ELISA detected IYSV in 85% (1 034) of alliaceous samples collected during the surveys. Fifty-two (4.3%) samples that gave weak positive DAS-ELISA reactions were subjected to RT-PCR and were all confirmed to be IYSV-positive. None of the non-alliaceous crop samples were ELISA-positive. In transmission studies, the virus was transmissible to 70% of the mechanically inoculated plants. White, necrotic lesions with green islands were observed on leaves seven days after mechanical inoculation of onion seedlings with IYSV. The lesions enlarged and spread to both newly formed and older leaves by the second week after inoculation (Figure 4.2). The presence of IYSV in the mechanically inoculated plants was confirmed by RT-PCR followed by sequencing. Ultra-thin sections of onion leaves that had IYSD symptoms revealed the presence of spherical, enveloped particles 80-120 nm diameter under TEM (data not shown).

4.3.4 Molecular detection and Phylogenetic analysis

When total RNAs of the ELISA-positive samples were subjected to RT-PCR amplification using the IYSV N gene-specific primers, amplicons of 236 bp were observed from infected tissues but not healthy controls. The partial N gene sequences of IYSV from garlic, leek, onion and shallot hosts were successfully amplified, sequenced and submitted to GenBank with the accession numbers given in Table 4.2. The Zimbabwean IYSV isolates shared 94.89-100% identity and 94.35-100% similarity at nucleotide and amino acid levels, respectively. Isolate T5-Ruwa (Accession code KX192317) was the least related to the other Zimbabwean isolates at nucleotide and amino acid levels. When compared to isolates from elsewhere in the world, the Iranian isolate HQ148174 shared the lowest identity (38.29%) with the Zimbabwean isolates, while the

Netherlands isolate AF001387 shared the lowest similarity (36.69%) with Zimbabwean isolates. The South African isolate (EF579801) was most closely related to the Zimbabwean isolates.

Figure 4.2: Necrotic lesions with green chlorotic islands that developed on onion plants mechanically inoculated with *Iris yellow spot orthotospovirus*.

Table 4.2: Representative *Iris yellow spot orthotospovirus* isolates generated in this study. Isolates were selected and sequenced based on host plants and geographical location.

Accession number	Host	Isolate	District	GPS coordinates
KT271469	Onion	5c_Harare	Harare West	30.95ºE; 17.82ºS
KU892653	Onion	N13-Manica-O	Nyanga	32.90º E; 18.03º S
KT732272	Garlic	Garlic_manicaland	Nyanga	32.90º E; 18.03º S
KT732273	Leek	Leek_manicaland	Mutare	32.62º E; 18.66º S
KU892655	Onion	N2_chere	Marondera	31.45º E; 18.11º S
KU892654	Onion	P16-MashC	Bindura	31.18º E; 17.32º S
KX192313	Garlic	R12-Mazoe-zim	Mazoe	30.96º E; 17.61º S
KX192314	Onion	R13-Mazoe-zim	Mazoe	31.05º E; 17.31º S
KX192316	Onion	Q11-chegutu-onion	Chegutu	30.15º E; 18.12º S
KX192317	Garlic	T5-ruwa	Goromonzi	31.24º E; 17.86º S
KX192318	Shallot	T4-ruwa	Goromonzi	31.24º E; 17.86º S
KX192319	Onion	Dema-onion	Seke	31.23ºE; 18.10ºS
KX192320	Garlic	Q12-chegutu	Chegutu	30.15º E; 18.12º S
KX192322	Leek	Q23-manresa-zim	Harare East	31.18º E; 17.80º S
KX192323	Garlic	Q5-garlic	Nyanga North	32.83º E; 17.57º S
KX192324	Onion	Q6-onion	Nyanga North	32.83º E; 17.57º S
KX192326	Onion	Q33-shamva-zim_2	Shamva	30.32º E; 17.68º S
KX192327	Onion	P2-onion	Zvimba	30.22º E; 17.73º S
KX192328	Onion	Q26-gutu	Gutu	30.98º E; 19.46º S

Phylogenetic analysis placed the Zimbabwean IYSV isolates into the same group (Figure 4.3). The Zimbabwean IYSV isolates clustered with isolates from Europe (Bosnia-Herzegovina, Greece, Italy, Israel, Spain, and Slovenia), the Americas (Brazil, Ecuador, Guatemala, Peru, and the USA), Canada, Asia (Japan, India, Sri Lanka, Tajikistan, and Pakistan), Australia, New Zealand and Africa (Egypt, Kenya, Mauritius, and South Africa). They were in a distinct cluster from isolates originating from Iran, The Netherlands and The UK. Serbian IYSV isolates were found in both clusters. Similar phylogenetic trees were obtained when Minimum Evolution, Maximum Parsimony and UPMGA analyses were performed (data not shown).

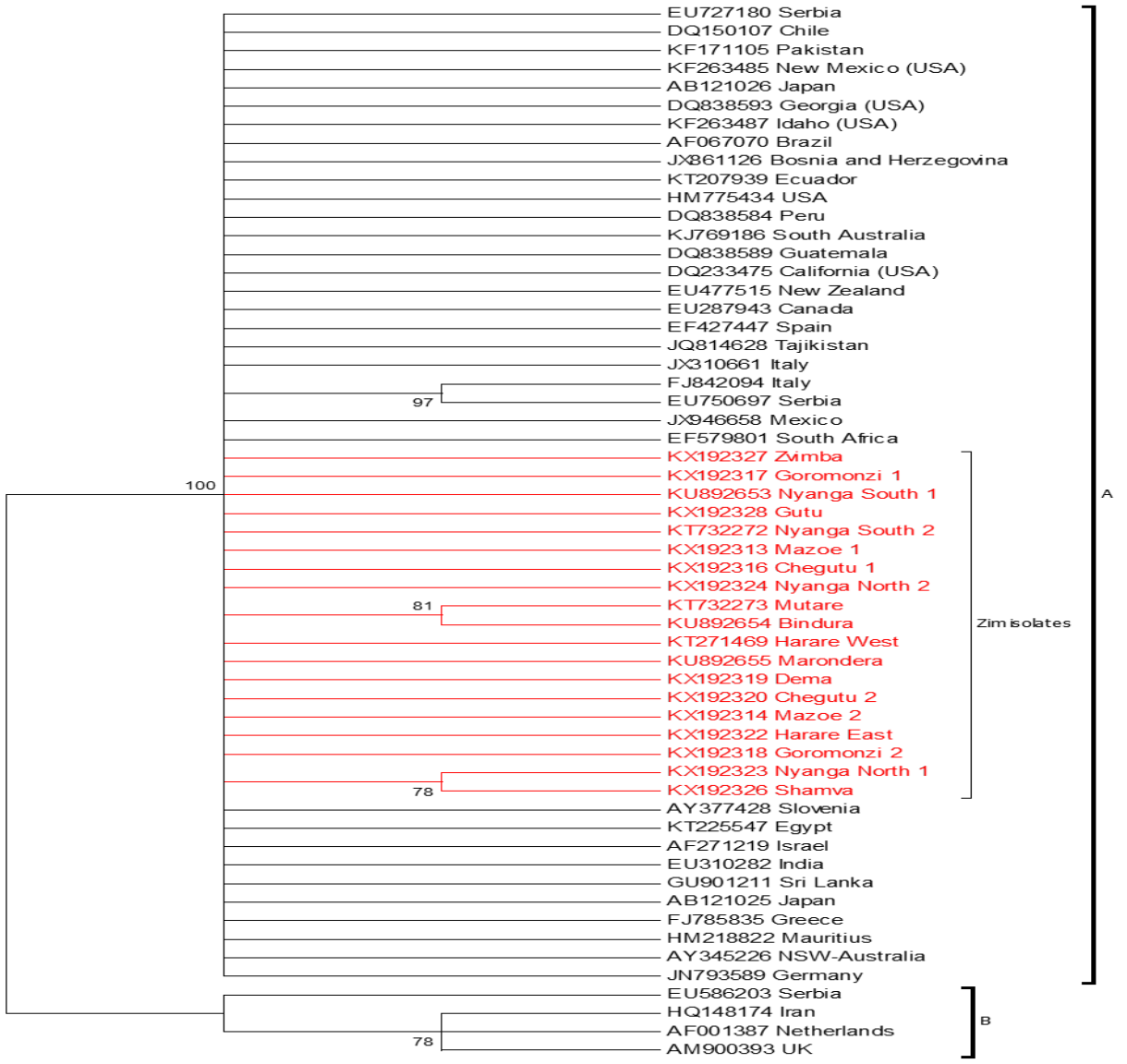

Figure 4.3: Phylogenetic relationships of partial N-gene sequences of Zimbabwean IYSV isolates and those in GenBank from different regions of the world. The evolutionary history was inferred using the Neighbor-Joining method based on the Tamura 3-parameter model with 1000 bootstrap replicates in MEGA 6. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Zimbabwean isolates (Zim isolates) are in red.

4.4 Discussion

The study revealed the widespread occurrence of IYSV in alliaceous crops in Zimbabwe. In some instances, the disease had attained both high incidence and severity. The fact that IYSV is widespread in Zimbabwe indicates that the disease has been present for much longer than has been reported. Even though figures on yield losses are not available, the high disease incidences and severities are causes for concern as they resulted in premature crop defoliation. This is likely to have contributed to reducing bulb size, yield and crop quality. In leeks and shallots, the presence of lesions on leaves negatively impacts on marketability and edibility, as leaves with lesions are discarded (Krauthausen *et al*., 2012). In studies by Munoz *et al*. (2014), economic losses of 50% and 60% were observed in onion fields with 68% and 90% disease incidences, respectively. Such severe yield losses were observed in early IYSV-infected crops. The fact that similar disease incidences were observed in Zimbabwe (Table 1) meant that high yield losses probably occurred in those crops. Besides direct losses, the virus weakens plants, making them susceptible to other pests and pathogens (Bag *et al*., 2012).

Lack of knowledge about the IYSD amongst Zimbabwean farmers is a major contributor to its high prevalence in the country. Currently, there are no attempts by most farmers to control either the disease or its vectors. Besides not knowing about the disease, most farmers believe that *Allium* species are pest- and disease-resistant. Therefore, they do not implement any control measures against *Allium* pests and diseases. There is need to educate farmers about the disease through extension outreach programs and both electronic and print media campaigns so that they know about it and implement control measures. Where IYSV was unmanaged, Birithia *et al*. (2014) reported 75-100% onion leaf drying and high yield loss in Kenya.

In addition to garlic, leek and onion which were already known to be IYSV natural hosts in Zimbabwe (Karavina *et al*., 2016a, b), the study detected the virus in shallots for the first time (Table 4.2). This shows that our knowledge of the IYSV host range is expanding in the country. The detection of IYSV in volunteer and out-of-season *Allium* species highlights their importance in providing epidemiological bridges for the virus. Therefore, farmers must destroy volunteer plants to prevent IYSV epidemics (Munoz *et al*., 2014). Also, they should not grow *Allium* species in the off-season. The detection of the virus on alliaceous crops intercropped with other crops

means that farmers have to rethink their pest and disease management strategies. While the *Allium* species are known to repel most insect pests, they host viruliferous thrips and provide IYSV inocula that may infect newly planted crops.

IYSV caused two types of foliar blight symptoms on *Allium* species both of which reduced crop photosynthetic area, thereby lowering crop yield, marketable and edible portions. The occurrence of two distinct symptoms could be due to the presence of different IYSV strains and variations in environmental conditions (Bulajic *et al*., 2009; Nischwitz *et al*., 2007). In addition, plant age, time of infection and differences in genetic makeup of cultivars also affect symptom expression (Bag *et al*., 2012). The occurrence of different symptoms could lead to misdiagnosis, especially if farmers and researchers rely solely on symptom expression for IYSV diagnosis (Krauthausen *et al*., 2012). This underscores the importance of having reliable IYSV detection and diagnostic methods. In samples lacking distinct symptoms, DAS-ELISA and RT-PCR followed by cloning and sequencing, detected IYSV. The consistent detection of IYSV by RT-PCR in this study demonstrates the value of this technique for IYSV diagnosis.

The study showed that Zimbabwean IYSV is mechanically transmissible. This confirms earlier studies by Kumar & Dhawan (2013) and Bag *et al*. (2015) who reported that some IYSV isolates are mechanically transmissible under greenhouse conditions. Kumar & Dhawan (2013) reported that the virus has 62.5% mechanical transmission efficiency, while Bag *et al*. (2015) reported a 30% rate of virus transmission. Because of IYSV's low mechanical transmission efficiency in most instances, most strain differentiation studies are based on differential responses on the indicator hosts *Nicotiana benthamiana* and *Datura stramonium* (Bag *et al.,* 2012).

Phylogenetic analysis revealed that all Zimbabwean IYSV isolates were closely related to each other. This may suggest that there has been little mutation of Zimbabwean isolates. It may also suggest that there may have been no multiple IYSV introductions into the country. The isolates were most closely related to the South African IYSV isolate with which they formed a sub-cluster. This indicates that Zimbabwean IYSV may have been introduced from South Africa or vice versa. Since IYSV is not seed transmitted, and long distance transport of viruliferous thrips vectors with the wind is very unlikely, IYSV is most likely to have been introduced by virus-infected plants or

viruliferous thrips on plants or cut flowers. A lot of trade in agricultural commodities occurs between Zimbabwe and South Africa.

The fact that Zimbabwean IYSV isolates were not all 100% identical to each other indicates a certain degree of IYSV diversity in the country. Several studies have pointed out that diversity occurs amongst various IYSV isolates (Nischwitz *et al*., 2007; Bag *et al*., 2012; Fuji *et al*., 2015; Gawande *et al*., 2015). Over time, new Zimbabwean IYSV strains may arise thereby complicating diagnosis through serology as no single serology kit can detect all isolates (Tomassoli *et al*., 2009). Knowledge on the nature and prevalence of isolates would aid in more effective screening of onion cultivars, breeding material and germplasm to identify and select for IYSV resistance that is more durable and effective across onion production regions (Bag *et al*., 2012).

In conclusion, the widespread occurrence of IYSV in Zimbabwe underscores the need for regular disease surveys to be undertaken so that new diseases can be detected early. This would enable early development and implementation of disease management strategies. Furthermore, farmer education about IYSD needs to be pursued for increased disease knowledge, awareness and control. This would ultimately lead to improved crop yield and quality.

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CHAPTER 5: NATURAL NON-*ALLIUM* HOSTS OF *IRIS YELLOW SPOT ORTHOTOSPOVIRUS* IN ZIMBABWE

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Abstract

Iris yellow spot orthotospovirus, IYSV, a tospovirus species that has recently been reported in Zimbabwe, causes serious yield losses in alliaceous and ornamental crops worldwide. A survey was conducted in five Zimbabwean districts to identify IYSV non-*Allium* weed hosts. Weed leaf samples of the dominant species in and around alliaceous fields were collected in RNA*later*® solution. Leaves that displayed mosaics, chlorosis and crinkling and had thrips-feeding damage symptoms were targeted for sampling. Samples were subjected to DAS-ELISA and RT-PCR followed by Sanger sequencing. IYSV was detected in *Amaranthus hybridus*, *Amaranthus spinosus* and *Eleusine indica* by both DAS-ELISA and RT-PCR. The weed-infecting IYSV isolates were closely related to each other and to Zimbabwean *Allium*-infecting isolates at the nucleotide and amino acid levels. The IYSV-infected weeds also supported *Thrips tabaci* feeding and reproduction. These weeds are important in the epidemiology of IYSV as they are natural non-alliaceous hosts that can act as epidemiological bridges in the seasonal carryover of the pathogen.

Keywords: *Allium*, dead-end, epidemiological bridge, tospovirus, weed.

5.1 Introduction

Iris yellow spot orthotospovirus (genus *Orthotospovirus*: family *Tospoviridae*) is an emerging pathogen of *Allium* species and some ornamental plants (Bag *et al*., 2015). It has been reported to be present in many countries worldwide, including Zimbabwe (Karavina *et al*., 2016). It is currently known to be vectored by *Thrips tabaci* and *Frankliniella fusca* (Srinivasan *et al*., 2012). Like most other tospoviruses, IYSV is neither seed nor transovarially transmitted (Kritzman *et al*., 2001; Robene-Soustrade *et al*., 2006).

IYSV is carried over between cropping seasons in infected onions transplants, volunteer onions and weeds (Hsu *et al*., 2011; Smith *et al*., 2011). Weeds that are susceptible to both the thrips vectors and the virus are important in IYSV epidemiology (Groves *et al*., 2002; Smith *et al*., 2011). Such weeds can support an early generation of thrips whose viruliferous adults disperse to infect newly planted alliaceous crops (Smith *et al*., 2011).

Currently, at least 20 weed species are known to be natural hosts of IYSV worldwide. Amongst the weed hosts are the annuals *Amaranthus retroflexus* (redroot pigweed), *Ambrosia* (ragweed), *Chenopodium album* (fat hen), *Linnaria canadensis* (blue toadflax), *Portulaca oleracea* (purslane), *Setaria viridis* (green foxtail), and *Tribulus terrestris* (puncture vine), the biennial *Arctium minus* (common burdock), and the perennials *Cichorium intybus* (chicory), *Lactuca serriora* (prickly lettuce), *Rumex crispus* (curly dock) and *Taraxacum officinale* (dandelion) (Hsu *et al*., 2011; CABI, 2016). Most studies on alternative IYSV hosts have been conducted in the USA. However, different geographical regions of the world have different weed flora. Thus, some weeds reported to be IYSV hosts in the USA may not be present elsewhere in the world. Given the worldwide distribution of IYSV, a study of the alternative hosts of the virus in other climatic conditions becomes critical. This study sought to determine the alternative hosts of IYSV in Zimbabwe. Knowledge on IYSV host range is important in understanding pathogen epidemiology and in the development of effective control measures against the IYSD.

5.2 Materials and Methods

5.2.1 Weed and thrips samples collection

Weed leaf samples were collected between July and October 2015 in Chegutu, Gutu, Harare West, Nyanga and Shamva districts of Zimbabwe (see Figure 2.1). A total of 280 samples representing dominant weed species in and around IYSV-infected alliaceous crop fields from nine families were collected. The sampled weeds displayed a wide range of symptoms that included chlorosis, mosaics, stunting and thrips-feeding damage, while others were symptomless. Collected samples were preserved in RNA*later*® solution and later refrigerated at -80^{o}C until analysis by DAS-ELISA and RT-PCR. Thrips inhabiting the weeds were collected and preserved in absolute ethanol. They were identified at The International Centre for Insect Physiology and Ecology (ICIPE), Kenya.

5.2.2 Serological detection

All samples were screened for IYSV by DAS-ELISA using a commercial kit supplied by Loewe Biochemica GmbH (Sauerlach, Germany) as described in Section 4.2.2. A weed species was considered IYSV-positive if at least one sample tested positive by DAS-ELISA.

5.2.3 Molecular characterization and Phylogenetic analysis

All samples were subjected to molecular characterization of IYSV as described in Section 4.2.5. Raw sequence data was checked for quality, edited and consensus sequences compiled using BioEdit Software. Partial N gene sequences of IYSV isolates generated in this study have been deposited in GenBank under the accession numbers highlighted in red on Figure 5.1.

The evolutionary history of the IYSV isolates generated in this study was inferred using the Maximum Likelihood method (Tamura, 1992). N gene sequences reported from elsewhere were retrieved from GenBank and, together with the Zimbabwean isolates, were aligned by ClustalW embedded in MEGA6 software (Tamura *et al*., 2013). The bootstrap consensus tree inferred 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Genetic distances between Zimbabwean IYSV isolates and those from elsewhere were calculated based on the

Tamura 3-parameter model (Tamura, 1992). Nucleotide sequence identity and similarity were computed using the SIAS program (www.imed.ucm.es/Tools/sias.html).

5.3 Results

5.3.1 Serological diagnosis

Of the 280 weed samples analysed, the species that were ELISA-positive are shown in Table 5.1. Weeds in the Asteraceae and Solanaceae families were negative for IYSV though some of them supported high populations of *Thrips tabaci*, displayed viral symptomatology characterized by leaf mottling and mosaics, and had visible thrips-feeding damage symptoms.

5.3.2 Molecular detection and Phylogenetic analysis

RT-PCR confirmed the presence of IYSV in *Amaranthus hybridus*, *A. spinosus* and *Eleusine indica* samples that had been positive by DAS-ELISA. In addition, all the samples that had been negative by DAS-ELISA were also negative by RT-PCR. The accession numbers of isolates that were generated in this study are KX192315 (*A. spinosus*), KX192321 (*E. indica*) and KX192325 (*A. hybridus*). Phylogenetic analysis placed the Zimbabwean IYSV weed isolates in the same group with IYSV isolates from other African countries, and were most closely related to the onion-infecting South African isolate (EF579801). The Zimbabwean isolates were 98.72-99.57% and 94.14-199% similar and identical to each other, respectively at nucleotide amino acid levels.

Table 5.1 Incidence of *Iris yellow spot orthotospovirus* on weeds collected in five districts of Zimbabwe

Family	Weed species	Number tested	Number positive	% positive
Amaranthaceae	*Amaranthus hybridus*	25	10	40.00
	Amaranthus spinosus	31	8	25.81
Asteraceae	*Bidens pilosa*	38	0	0
	Galinsoga parviflora	32	0	0
	Tagetes erecta	16	0	0
	Tagetes minuta	20	0	0
Commelinaceae	*Commelina benghalensis*	12	0	0
Convolvulaceae	*Ipomoea plebeia*	13	0	0
Oxalidaceae	*Oxalis latifolia*	14	0	0
Poaceae	*Eleusine indica*	25	6	24.00
	Digitaria spp	17	0	0
Solanaceae	*Nicandra physalodes*	15	0	0
	Physalis angulata	22	0	0

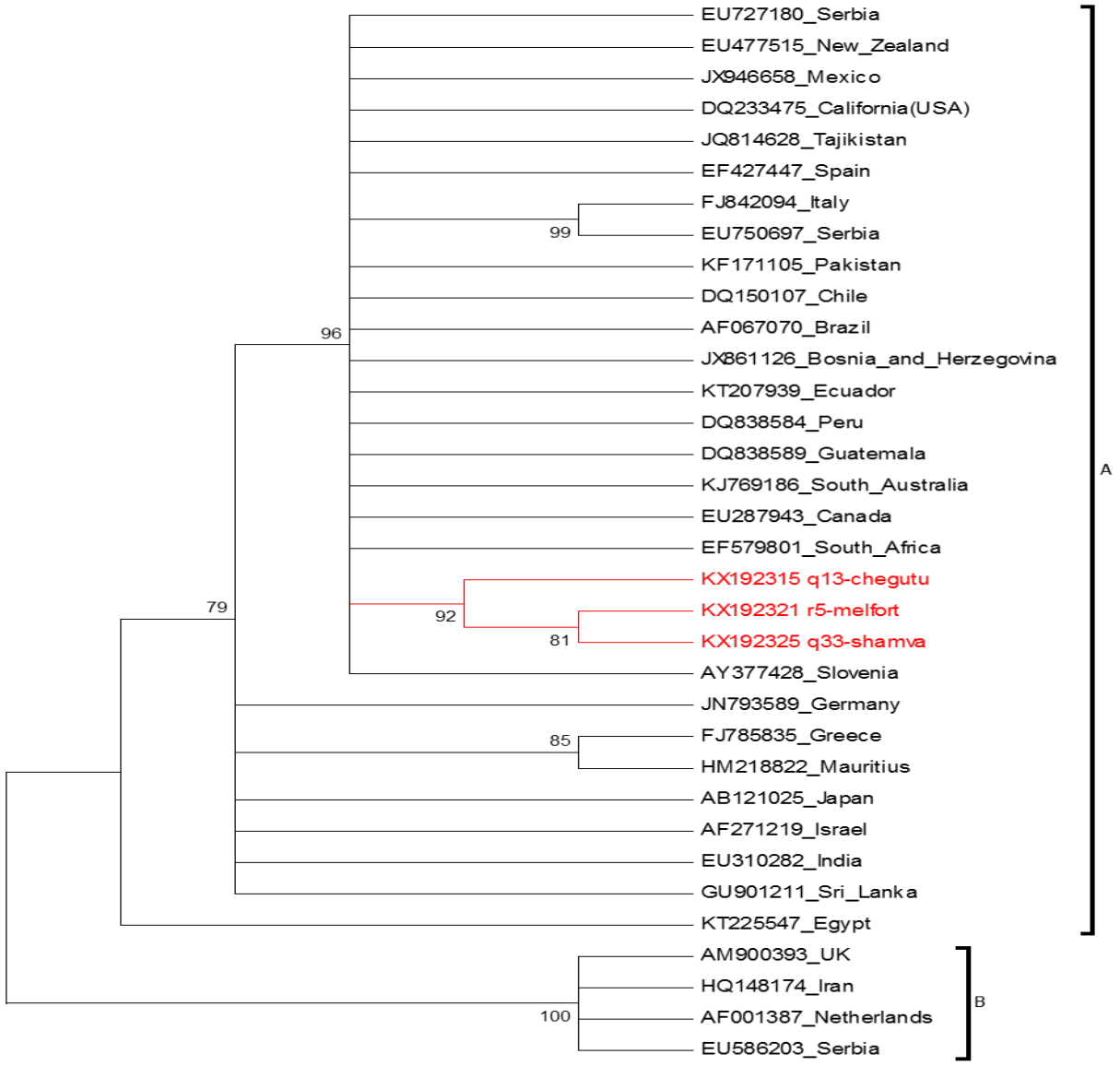

Figure 5.1. Phylogenetic analysis of the weed-infecting *Iris yellow spot orthotospovirus* isolates from Zimbabwe. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. Evolutionary analyses were conducted in MEGA6. Branches corresponding to partitions reproduced in less than 75% bootstrap replicates are collapsed.

5.4 Discussion

This study showed that the weeds *Amaranthus hybridus, A. spinosus* and *Eleusine indica* are naturally infected by IYSV in Zimbabwe. To the best of my knowledge, this is the first time that these weeds have been reported as IYSV natural hosts anywhere in the world. Previously, Gent *et al*. (2006) and Sampangi *et al*. (2007) reported *Amaranthus retroflexus* as a natural host of IYSV. *Eleusine indica* is one of two grasses that is naturally infected by IYSV, with the other grass being *Setaria viridis* (Evans *et al*., 2009). Results from this study adds to the body of knowledge on the non-alliaceous natural hosts of IYSV.

These weed hosts act as epidemiological bridges of IYSV. In Zimbabwe, alliaceous crops are normally grown in the cool season that commences in April/May. By the time the alliaceous crops are harvested in September or October, temperatures would have risen to levels that promote the growth of many summer weeds. Thrips migrate from the ageing alliaceous crops onto the weeds where they may feed and reproduce. If the thrips had acquired IYSV at the larval stage, they transmit the virus onto the weeds which could support virus replication. Such weeds are important in sustaining both the virus and vectors following the onion crop (Schwartz *et al*., 2014). The fact that both larvae and adult thrips were recovered from the IYSV-positive weeds indicated that the weeds supported vector reproduction and development. Such weeds ought to be controlled as part of IYSD management.

In studies by Smith *et al*. (2011), biennial and perennial weeds were found to be more important than annual weeds in IYSV overwintering in New York as the virus overwintered in perennating structures. In Zimbabwe, annuals are likely to play a more significant role in IYSV seasonal carryover due to the country's sub-tropical climate that favours all-year-round weed growth in cropped fields.

Several weeds, including *Bidens pilosa, Galinsoga parviflora, Nicandra physalodes, Tagetes erecta* and *T. minuta* supported *T. tabaci* multiplication and feeding but not IYSV. Such weeds, though important in Zimbabwe (Drummond, 1984), represent 'dead-ends' in IYSV epidemiology (Hsu et al., 2011). However, they still need to be controlled as they harbor thrips vectors which can then migrate onto infected alliaceous hosts, reproduce and acquire the virus. According to Smith *et al*. (2011), *T. tabaci* are likely to use a weed species as a host due to attraction by plant

volatiles, nutrition, relative absence of predators, and plant architecture favourable to thrips crypophilic and thigmotrophic behavior.

Both *A. hybridus* and *A. spinosus* (amaranths) are grown as traditional vegetables by some people in Zimbabwe. The production and consumption of amaranths is being promoted because of the nutritional and health benefits they confer (Maroyi, 2013). In an attempt to control pests and diseases, some farmers tend to intercrop the amaranths with alliaceous crops like garlic, onion and shallot. While this could control pests like aphids and leaf-eating caterpillars, it does not control *T. tabaci* and IYSV because both survive and reproduce on the the amaranths and alliaceous crops. Therefore, alternative pest and disease management strategies are needed to effectively manage IYSV and *T. tabaci* in such cropping systems.

Of the sampled weeds, only a low proportion were positive for IYSV. This could be due to the fact that IYSV is weakly systemic and unevenly distributed in plants (Kritzman *et al*., 2001; Gent *et al*., 2006; Smith *et al*., 2006; Fuji *et al*., 2015). Weed species in the Asteraceae and Solanaceae families have been reported as IYSV hosts elsewhere (Bag *et al*., 2015). Although no hosts were reported from these families in this study, there is need to continuously screen more weed samples in future. Host jumps are possible in future given the fact that tospoviruses like IYSV are generalist and opportunistic pathogens (Pappu *et al*., 2009).

Phylogenetically, the weed-infecting IYSV isolates were most closely related to the South African onion-infecting and the Zimbabwean *Allium*-infecting isolates that have been reported in the previous chapter. This shows that the same strain of IYSV is infecting both alliums and weeds in Zimbabwe. So, the same serological kit can be used to detect the virus in both the weeds and alliaceous crops.

In conclusion, this study suggests one strategy that might be implemented to reduce IYSV and thrips incidence in Zimbabwe. Weeds in and around alliaceous crop fields should be controlled so that they do not harbor the virus and the vector. This study opens avenues for future research on alternative hosts of IYSV in Zimbabwe. More weeds, including biennials and perennials, need to be screened for their possible role in this regard.

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CHAPTER 6: CHARACTERIZATION OF THREE FULL *IRIS YELLOW SPOT ORTHOTOSPOVIRUS* GENES OF A GARLIC-INFECTING ISOLATE FROM ZIMBABWE USING NEXT-GENERATION SEQUENCING

Abstract

Iris yellow spot orthotospovirus (IYSV) is an important pathogen of *Allium* species worldwide. It has a tripartite genome consisting of the large (L), medium (M) and small (S) RNA segments. Despite its worldwide distribution, very few complete gene and genome sequences are available in public databases. The aim of this study was to obtain full gene sequences of a garlic-infecting IYSV isolate by Next-generation sequencing (NGS) for the purposes of understanding pathogen evolution. Total RNA was extracted using the Quick-RNA MiniPrep Kit (Zymo Research, USA) and sent for NGS at The Agricultural Research Council (Pretoria, South Africa). Sequencing was done on the Illumina HiSeq using paired-end chemistry 2 x 125bp reads. The quality of raw reads was assessed using FastQC software before trimming with Trimmomatic version 0.36. The resultant paired-end sequences were used for both *de novo* and reference-based genome assembly. The consensus gene sequences generated from the two methods were analyzed in several bioinformatics softwares and subjected to phylogenetic analyses in MEGA6. The Garlic-Zimbabwe isolate's genome was consistent with other tospoviruses. Three full gene sequences i.e. nucleocapsid (N), nonstructural protein (NSs) and movement protein (NSm) were recovered from both *de novo* and reference-based mapping. The N gene was 822 nucleotides long while the NSs gene was 1332 nucleotides long. The NSm gene was 983 nucleotides long and coded for a protein with a molecular weight of 34.73 kDa. The N gene did not show any clustering patterns based on geographical locations and was most identical to an onion-infecting isolate from Serbia (Accession KT272878). The NSs and NSm genes clustered closely with homologous sequences of IYSV isolates that were retrieved from the databases. This study lays the foundation for complete genome studies of IYSV in Zimbabwe.

6.1 Introduction

Iris yellow spot orthotospovirus, IYSV, is an important emerging pathogen of *Allium spp* worldwide responsible for causing significant yield and quality losses in ornamental and food crops (Bag *et al*., 2015). IYSV was first isolated and characterized in iris (*Iris holandica*) in The Netherlands (Cortês *et al*., 1998) and has now been reported in over 40 countries worldwide (CABI, 2016). In Zimbabwe, IYSV was first identified in onions (*Allium cepa*) in 2014 (Karavina *et al*., 2016), and was subsequently reported in garlic (*A. sativum*), leeks (*A. ampeloprasum*) and shallots (*A. cepa* var. *aggregatum*) (Karavina and Gubba, 2017).

IYSV belongs to the genus *Orthotospovirus* in the family *Tospoviridae*. Like other tospoviruses, IYSV has quasi-spherical enveloped particles that are 80-120 nm in diameter (Pappu *et al*., 2009). It has a tripartite single-stranded RNA genome consisting of the large (L), medium (M) and small (S) segments. The L RNA segment is 8800 nucleotides long and encodes the RNA-dependent RNA polymerase (RdRp) in the viral complementary sense (Bag *et al*., 2010). The M RNA (~4800 nucleotides) is ambisense and has two open reading frames (ORFs). The viral sense strand of the M RNA encodes the non-structural movement (NSm) protein while the anti-viral sense strand encodes the Gn/Gc glycoproteins. The NSm protein is responsible for movement of virus particles during systemic infection while the two glycoproteins serve as virus attachment proteins (Bandla *et al*., 1998). The S RNA (~2900 nucleotides) is also ambisense and encodes the non-structural (NSs) protein in the viral sense strand and the nucleocapsid (N) protein in the antiviral sense strand. The NSs protein is involved in suppressing RNA silencing, while the N protein encapsidates the RNA segments (Bag *et al*., 2015). In addition to these genes, both M and S RNAs contain intergenic regions (IGRs) capable of forming stable hairpin structures (King *et al*., 2012). IYSV is currently known to be transmitted by two thrips species, *Thrips tabaci* and *Frankliniella fusca* in a persistent and propagative mode (Srinivasan *et al*., 2012).

IYSV identification and characterization is carried out by employing biological, serological, morphological and molecular techniques (Bag *et al*., 2015). Biological characterization, also known as host indexing, involves the inoculation of indicator plant species that produce typical symptoms. However, it normally takes several days for symptoms to develop on inoculated hosts

(Bag *et al*., 2012). Serological detection can lead to ambiguous results, especially for closely related viruses like IYSV and Tomato yellow ring virus. Also, antibodies to IYSV must be raised if serological detection is to be successful. Morphological identification requires the use of electron miscroscopes which are not affordable in most research and academic institutions. Being RNA viruses, reverse transcription-polymerase chain reaction (RT-PCR) can also be used for IYSV identification (Walsh *et al*., 2001). However, primers specific for IYSV must be available / must be designed.

Next-Generation Sequencing (NGS) provides an unbiased, quick, cost- and labour-effective characterization procedure for plant viruses (Kreuze *et al*., 2009). Two assembly methods namely; *de novo* assembly and reference-based mapping are used to recover the virus genome from the sequenced data. With NGS, full viral genomes can be recovered (Adams *et al*., 2009).

Several dozens of partial N gene sequences of IYSV are available in public databases like NCBI and EMBL, but few complete genome and gene sequences are available. This has negatively impacted both evolutionary and phylogenetic studies of IYSV. In this study, the characteristics of full-length N, NSs and NSm genes of a garlic-infecting IYSV isolate from Zimbabwe obtained using both reference-based mapping and *de novo* assembly methods are described.

6.2 Materials and Methods

6.2.1 Sources of materials used

The IYSV isolate was from garlic plants collected at a horticultural enterprise in Chegutu District (18º07ˈS, 30º09ˈE; 1159 m above sea level) during a survey for tospoviruses in 2015. Garlic plants displaying necrotic, irregularly-shaped and grey-to-bleached lesions typical of IYSV infection were targeted for sampling. Symptomatic leaves were collected in RNA*later*® solution (Thermo Fisher Scientific, USA) and transported to the University of KwaZulu-Natal (South Africa) for pathogen characterization.

6.2.2 Serological detection

All eight samples collected in RNA*later*® solution were analyzed with a commercial IYSV DAS-ELISA diagnostic kit (Loewe Biochemica GmbH, Germany) according to manufacturer's instructions as described in Section 4.2.2.

6.2.3 RNA extraction and RT-PCR

Total RNAs were extracted from the sampled leaves using the Quick RNA MiniPrep Kit (Zymo Research, USA) according to manufacturer's instructions. To ascertain the presence of IYSV in the samples, RT-PCR was performed following the procedure described in Section 4.2.5.

6.2.4 Sample preparation, sequencing and data analysis for Next-Generation Sequencing

One sample that had been positive for IYSV by RT-PCR was randomly selected and RNA extracted for NGS at the Agricultural Research Council's Biotechnology Platform (ARC-BTP) (Pretoria, South Africa). RNA quality and quantity were assessed for using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA samples were pre-treated with Ribo-Zero (NEB, UK) prior to library preparation.

NGS was performed on the Illumina HiSeq using paired-end chemistry 125 x 125 bp reads. Subsequent sample demultiplexing was done using the CASAVA pipeline software (Illumina, USA). Read lengths less than 25 nucleotides were trimmed and pair-end sequence libraries were generated. FastQC version 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess quality of the raw reads generated by NGS before and after trimming with the Trimmomatic v 0.36 with the following settings: (ILLUMINACLIP: TrueSeq3-PE-2.fa:2:30:9:1:true LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15:MINLEN:36) (Bolger *et al*., 2014). The pair-end sequences were subsequently used for both *de novo* assembly and reference-based mapping. *De novo* assembly was performed using the SPAdes Genome Assembler version 3.10.1 with the parameters: careful mode; only assembler; –k 21, 33, 55, 77, 99 (Bankevich *et al*., 2012), while referenced-based genome mapping was done using the BBMap Short Read Aligner version 37.28 (Bushnell, 2014) using IYSV genomes as references. All contigs generated

by the *de novo* assembly method were subjected to BLAST using ncbi-blast 2.6.0+ (www.ncbi.nih.gov).

6.2.5 IYSV sequence analysis and phylogeny

All contigs that matched IYSV genomes were selected and aligned using the Clustal W embedded in MEGA 6.06 software (Tamura *et al*., 2013) to generate the consensus sequences of the N, NSs and NSm genes. The open reading frames (ORFs) on the IYSV genes were identified using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder). The molecular weight (Mw) of the proteins was determined by using the online ExPASy bioinformatics tool (Gasteiger *et al*., 2005). Phylogenetic trees of the nucleotide sequences of the three IYSV ORFs were inferred by Maximum Likelihood method based on the best evolutionary models as determined by MEGA 6.06. The phylogenetic trees were rooted using the TSWV as an outgroup. The bootstrap analyses were conducted using 1000 replicates. Details of isolates used in the phylogenetic analysis are given in Table 6.3. Nucleotide and amino acid sequence compositions and sequence identities were calculated using SIAS program (www.imed.ucm.es/Tools/sias.html).

6.3 Results

6.3.1 Serology and RT-PCR analyses

Of the eight samples tested, six were positive for IYSV by both DAS-ELISA and RT-PCR.

6.3.2 RNA quality assessment and NGS data analysis

The concentration of the sample sent for NGS was 194.38 ng/µL, with an absorbance A_{260}/A_{280} ratio of 1.95. The size of the NGS data generated was 3.3 gigabytes and consisted of 6 921 806 raw reads. FastQC output indicated the presence of adapters in the reads. The average reads length after trimming was 119 bp (Table 6.1). A total of 107 102 contigs were generated by *de novo* analysis. Of these, 18 matched to the L (2), M (8) and S (8) RNA segments of the IYSV genome. From the reference-based mapping, a total of 671 reads were mapped to the IYSV genome (Table

6.1). Although the results obtained using both *de novo* assembly and reference-based mapping methods were consistent, none of the IYSV genome segments of the isolate in this study were fully recovered. However, three genes, the N and NSs found on the S RNA segment and the NSm present on the M RNA segment, were found to be complete after visual inspection and analysis on the ORF Finder. Consequently, these genes were considered for phylogenetic analyses. The nucleotide sequences of these genes were also deposited in GenBank under the accession numbers given in Table 6.2.

Table 6.1 Characteristics of the Next-Generation Sequencing data generated from total RNA infected by *Iris yellow spot orthotospovirus*

Characteristics	Statistics
Number of raw reads	6 921 806
Average read length	125 bp
Number of reads after trimming	5 758 205
Average reads length after trimming and adapter removal	119 bp
Number of contigs generated	107 102
Length of contigs	100-31303 bp
Contigs matching IYSV	18 (S: 2, M: 8, L: 8)
Number of reads mapped to L RNA	336
Number of reads mapped to M RNA	133
Number of reads mapped to S RNA	202

6.3.3 Sequence characteristics and phylogenetic analyses of the N, NSs and NSm genes from the IYSV Garlic-Zimbabwe (Gar-Zim) isolate

The N and NSs genes are found on the S RNA segment while the NSm gene is present on the 5'-end of the M RNA segment. The N gene of the IYSV Gar-Zim isolate was found to be 822 nt long and coded for a protein with a molecular weight of 30.46 kDa. The NSs and NSm proteins had molecular weights of 50.11 and 34.73 kDa, respectively (Table 6.2).

Table 6.2 Sequence characteristics of the N, NSs and NSm genes of the Gar-Zim isolate

Segment	ORF polarity	Accession Number	Protein coded	ORF length (nt)	Number of amino acids	Protein weight (kDa)
S	(-)	MF359019	N	822	273	30.46
	(+)	MF359021	NSs	1332	443	50.11
M	(+)	MF359020	NSm	983	311	34.73

The Gar-Zim isolate N gene had a sequence identity of 93.06% to the onion-infecting Serbian isolate (KT272878) at the nucleotide level, while at the protein level, it was most identical (95.25%) to the onion-infecting Sri Lankan isolate (GU901211). It shared the lowest nucleotide and protein sequence identity with the Iranian isolate (HQ148173). The NSs gene sequence of the Gar-Zim isolate had 91.66% identity to The Netherlands isolate (AF001387) at the nucleotide level. The NSm gene of the Zimbabwean isolate was most identical to the USA isolate (FJ361359) at both nucleotide and amino acid levels (Table 6.3).

Table 6.3 Percentage nucleotide (nt) and protein (aa) sequence identities between the IYSV N, NSs and NSm genes and other IYSV isolates

Accession No.	Isolate	N		NSs		NSm	
		Nt	aa	nt	aa	nt	aa
AF001387	-	41.36	17.14	91.66	82.02	-	-
AF067070	-	92.33	93.79	-	-	-	-
AF271219	-	91.11	92.7	-	-	-	-
KT272878	163-14	93.06	93.23	-	-	-	-
JQ973066	Washington	92.94	94.52	-	-	-	-
AB180919	SgOniD1	42.09	16.08	-	-	-	-
AM900393	-	41.22	17.62	-	-	-	-
AY345226	NSW-1	89.9	92.7	-	-	-	-
EU310295	IYSV-On-Vir	91.97	93.79	-	-	-	-
EU477515	New Zealand	92.7	93.23	-	-	-	-
EU586203	605-SRB	41.96	16.18	-	-	-	-
GU901211	-	92.57	95.25	-	-	-	-
HQ148173	5	41.05	15.82	-	-	-	-
KF171105	-	92.21	94.52	-	-	-	-
KT225547	IYSV-Egyptian	85.76	86.49	-	-	-	-
KJ868797	DOGR	-	-	91.51	83.59	-	-
FJ361359	-	-	-	-	-	97.11	99.67
AF213677	-	-	-	-	-	95.19	95.83
AF214014	-	-	-	-	-	89.63	94.55
KM035409	DOGR	-	-	-	-	87.07	89.42

Phylogenetic analysis of the N genes produced two distant groups (A and B; Figure 6.1). The N gene of the IYSV Gar-Zim isolate was in cluster A along with homologous sequences of isolates from Australia, Brazil, Egypt, India, Israel and Sri Lanka. Group B was composed of N sequences of isolates from Iran, Japan, Serbia, The Netherlands and The UK (Figure 6.1).

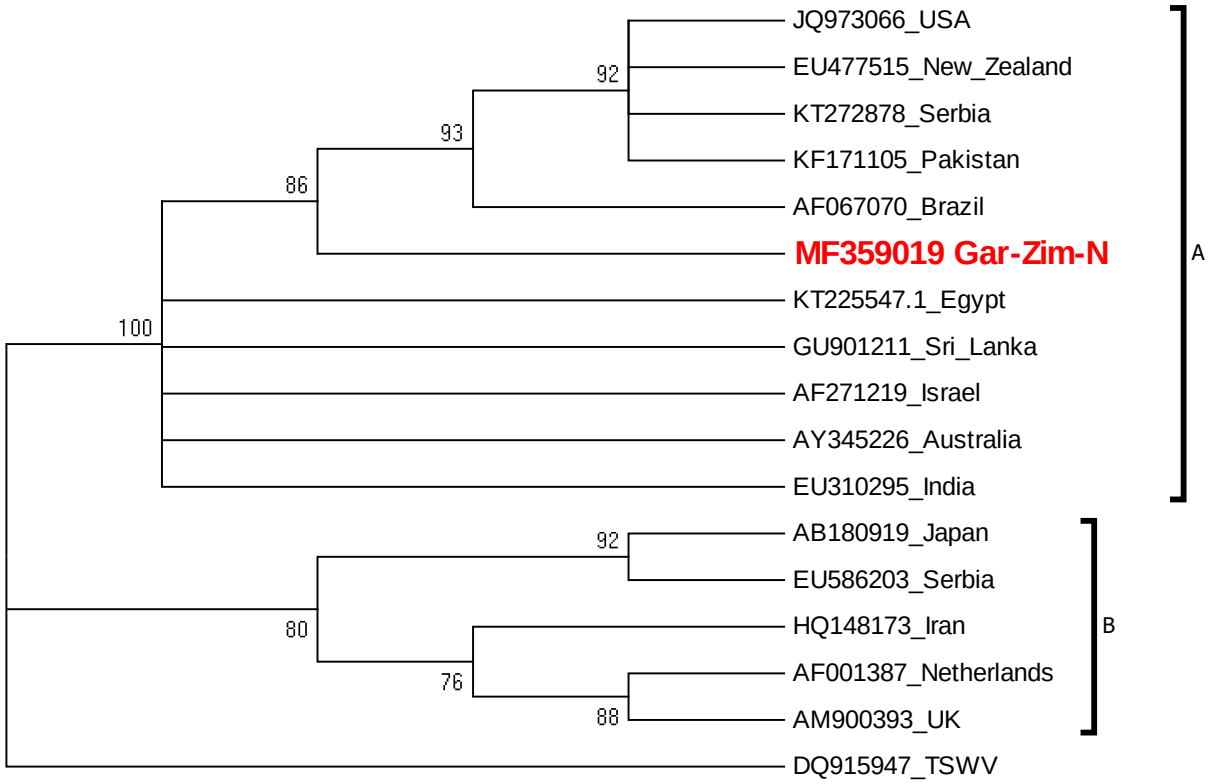
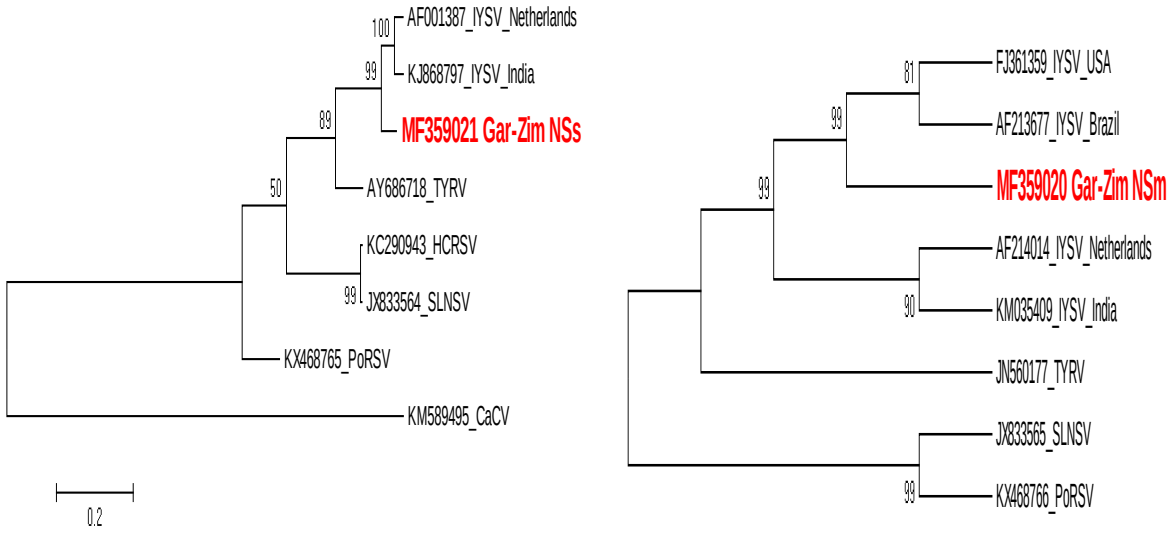


Figure 6.1 Phylogenetic analysis of the N gene of the Gar-Zim isolate by the Maximum Likelihood method based on the Tamura 3-parameter model.

Phylogenetically, the NSs gene of the IYSV Gar-Zim isolate clustered with the homologous sequences of isolates from India and The Netherlands (Figure 6.2A). As for the NSm gene of the Gar-Zim isolate, it also clustered with homologous sequences of IYSV isolates from Brazil (AF213677) and The USA (FJ361359) (Figure 6.2B).



A B

Figure 6.2 Molecular phylogenetic analyses of the NSs (A) and NSm (B) genes by Maximum Likelihood method based on the Tamura 3-parameter model.

6.4 Discussion

In this study, NGS was used to identify and characterize a garlic-infecting IYSV isolate from Zimbabwe. When compared to serology and RT-PCR, NGS rapidly identifies the IYSV from the sample and produces nucleic acid sequences for substantial parts of the viral genome. The use of NGS further confirmed the occurrence of IYSV in Zimbabwe that had been reported by Karavina *et al*. (2016). Knowledge of the presence of IYSV in Zimbabwe helps in epidemiological research towards developing management strategies against the pathogen.

In order to maximize the chances of detecting IYSV in the sequenced data, two different methods (*de novo* assembly and reference-based mapping) were employed. *De novo* assembly recreates the original genome sequence through overlapping reads while reference-based mapping requires a previously assembled genome to be used as a reference. A major advantage of *de novo* assembly over reference-based mapping is that it gives the virome of the host(s) studied. In some cases, other

viruses that were not meant for study are detected (Martin and Wang, 2011). In this study, *Garlic common latent virus*, *Garlic virus B*, *Garlic virus C* and *Shallot virus X* were detected (data not shown). Both reference-based sequencing and *de novo* assembly methods were used in this study for comparison purposes, and there were no significant differences in the IYSV genome recovered.

The fact that viruses other than IYSV were detected in samples sent for NGS shows that mixed and multiple infections are a common occurrence in nature. This compromises the reliance on symptomatology for IYSD diagnosis as symptoms expressed are a result of more than one pathogen.

A major advantage of NGS over other conventional specific approaches like ELISA and PCR is that the latter approaches require reagents designed exclusively to detect their viral target and any variation in the virus genome may cause the assay to fail. NGS is non-targeted and requires no prior knowledge of the target. Therefore, NGS is able to detect existing strains, new variants and even new strains (Adams *et al*., 2009).

The genomic organization of the Gar-Zim isolate is typical of tospoviruses (Pappu *et al*., 2009), with the S, M and L RNA segments recovered by both referenced-based mapping and *de novo* assembly. Though full IYSV genomes were not recovered in this study, NGS enabled the simultaneous recovery of the two full genes on the S RNA segment and the NSm gene on the M segment. With Sanger sequencing, it is normally a time-consuming and expensive process to get so many gene sequences. This study lays the foundation for future studies on the full genome of IYSV in Zimbabwe as only few nucleotides were missing from the recovered segments (data not shown).

IYSV, as already noted, is an important emerging pathogen of alliaceous crops worldwide. Despite its global importance, only a few full genome sequences have been characterized (Gawande *et al*., 2015). The majority of IYSV disease reports are based on partial N gene sequences. This greatly compromises studies to understand pathogen evolution and management. This is the first study in Africa where more than one full IYSV genes of the same isolate have been described. To date, only two other full IYSV N gene sequences (Accessions KT225547 and KC161369) from Africa have been deposited in public databases.

Phylogenetic analysis of the N gene showed no clustering patterns based on geographical location. This could suggest the possibilities of long-distance migration, recombination and reassortment events in IYSV. Such events are highly prevalent in tospoviruses (Zhang *et al*., 2016; Margaria *et al*., 2015). Since IYSV is a pathogen of some ornamental plants that are traded on the world market (CABI, 2016; Bag *et al*., 2015), it is possible that both the pathogen and its thrips vectors have been unintentionally distributed worldwide. Also, smuggling of live host plants across borders could also have contributed to pathogen's worldwide distribution. The S RNA segment is known to be substantially more prone to recombination than the M and L RNA segments (Gawande *et al*., 2015). For either recombination or reassortment to be verified in the Gar-Zim isolate, full genomes of the IYSV segments must be recovered and analyzed.

In conclusion, the characterization of the three IYSV genes from the Gar-Zim isolate lays the foundation for future studies on the full genome of this important pathogen. Knowledge of the full genome is critical in developing management strategies and understanding the evolutionary patterns of IYSV.

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CHAPTER 7: MARKER-ASSISTED IDENTIFICATION OF *TOMATO SPOTTED WILT ORTHOTOSPOVIRUS*-RESISTANT TOMATO (*Solanum lycopersicum* L.) GENOTYPES IN COMMERCIAL AND ACCESSION LINES

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Abstract

Marker-assisted selection has been widely applied in breeding for resistance to *Tomato spotted wilt orthotospovirus* (TSWV), a major constraint to tomato (*Solanum lycopersicum*) production worldwide. An experiment was carried out to screen Zimbabwean tomato germplasm for the *Sw-5b* gene allele using molecular markers. A total of 33 tomato genotypes consisting of 26 commercial and 7 accessions lines were grown for four weeks under greenhouse conditions. Genomic DNA of each tomato genotpye was separately extracted from tender leaves after being imprinted onto FTA cards and used to perform the polymerase chain reaction using gene-based (sw5b-f1/sw5b-r1) and SNP (sw5-f/sw5-r) markers. The resultant amplicons were excised, purified, cloned and sequenced in both directions. Consensus sequences were generated in BioEdit software. All tomato genotypes had the 662bp fragment with the sw5b-f1/sw5b-r1 primer pair, but 21 commercial cultivars and only 3 accession lines had the 541bp with the sw5f/sw5r primer pair. The study showed that most of the cultivars grown in Zimbabwe have the *Sw-5b* resistance gene. This could partly explain the low incidence and severity of TSWV disease in Zimbabwean tomatoes as local TSWV isolates probably have not yet been able to break the resistance conferred by the *Sw-5b* gene. The accessions carrying the *Sw-5b* allele can be advanced in TSWV resistance breeding programs. There is need to incorporate TSWV resistance information on tomato seed labels for the benefit of farmers as they decide on which cultivars to grow.

Keywords: breeding, disease management, molecular markers, *Sw-5b* gene.

7.1 Introduction

Marker-assisted selection (MAS) has been widely applied in breeding for resistance to *Tomato spotted wilt orthotospovirus* (TSWV), a pathogen that seriously affects tomato (*Solanum lycopersicum* L.) production worldwide (Gordillo *et al*., 2008; Shi *et al*., 2011). Yield losses attributed to TSWV infection can be as high as 100%, especially if susceptible cultivars are infected within 30 days post-transplanting (Rosello *et al*., 1996; Saidi and Warade, 2008). According to Cho *et al*. (1996), some Hawaiian farmers had to abandon tomato production due to serious tomato spotted wilt disease (TSWD) outbreak. The disease has become a major limiting factor to tomato production in Argentina, Brazil, Italy, Portugal and Spain (Gordillo *et al*., 2008). TSWV is now prevalent in tropical and subtropical environments, primarily due the spread of its principal vector *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) in international trade of cut flowers (Kirk and Terry, 2003). Early detection and management are critical to prevent TSWD epidemics.

TSWD management can be achieved by employing biological, cultural, chemical, host-plant resistance and physical methods. However, disease management by most of these methods has largely been ineffective due to several reasons. Biological control, though ecologically-friendly, is perceived to be slow-acting and more applicable to perennial than annual cropping systems. Insecticide-use for thrips vector control is largely ineffective because thrips quickly develop resistance to most available insecticides (Cloyd, 2009). Sometimes, thrips populations are suppressed but this does not prevent disease spread (Soler *et al*., 2003). The use of host resistance appears to be the best medium- to long-term TSWD control method (Garland *et al.,* 2005). Wild relatives of domesticated tomatoes have been screened for resistance to TSWV isolates (Stevens *et al*., 1994). So far, eight TSWV resistance genes have been identified namely: *Sw-1a, Sw-1b, Sw-2, Sw-3, Sw-4, Sw-5, Sw-6* and *Sw-7*. The *Sw-1a, Sw-1b, Sw-5, Sw-6* and *Sw-7* are dominant genes, while *Sw-2, Sw-3* and *Sw-4* are recessive (Saidi and Warade, 2008). The *Sw-5* gene, first identified in *Lycopersicon peruvianum* Mill. (Stevens *et al*., 1996), is the most widely deployed TSWV resistance gene in tomato breeding because of its durability to multiple tospoviruses, even across geographical locations (Rosello *et al*., 1998; Soler *et al*., 2003; Gordillo *et al*., 2008). This broad resistance is attributed to the presence of *Sw-5* alleles (*Sw-5a*, *Sw-5b, Sw-5c*, *Sw-5d* and *Sw-5e*) and homologs. The *Sw-5b* allele is responsible for conferring resistance to TSWV (Garland *et*

al., 2005). Plants carrying this allele can restrict systemic virus spread, showing only localized symptoms represented as tiny local lesions caused by a hypersensitive reaction (Dianese *et al*., 2010). MAS has been applied to identify TSWV-resistant tomato germplasm by utilizing markers linked to the *Sw-5* gene (Shi *et al*., 2011; Lee *et al*., 2015).

The available marker-assisted systems include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), sequence characterized amplified regions (SCAR), cleaved amplified polymorphic sequence (CAPS), Insertion-Deletion, and single nucleotide polymorphisms (SNPs) (Stevens *et al*., 1995; Chague *et al*., 1996; do Nasciamento *et al*., 2009; Dianese *et al*., 2010; Foolad and Panthee, 2012). CAPS and RFLP markers are based on the presence of SNPs or insertion/deletion (INDEL) mutations (Nasu *et al*., 2002). SNPs have also been used to develop SNP markers useful for MAS (Wu *et al*., 2008).

MAS eliminates the drawbacks associated with conventional breeding. With MAS, plants can be screened at any stage of development and it is also not affected by Genotype × Environment interactions (Foolad and Sharma, 2005). In addition, MAS enables the selection of a single resistance gene or the accumulation of several resistance genes in plants, thereby accelerating crop breeding (Soler *et al*., 2003; Arens *et al*., 2010). The use of gene-based markers is advantageous for increasing the accuracy of MAS compared to utilization of markers generated based on DNA polymorphisms located adjacent to the gene of interest (Salgotra *et al*., 2014). In this study, tomato germplasm lines sourced from Zimbabwe were screened for the *Sw-5b* allele using gene-based and SNP markers. This will form the basis of marker-assisted breeding for TSWV resistance in tomatoes.

7.2 Materials and Methods

7.2.1 Genetic material

Thirty three (33) tomato genotypes consisting of 26 commercial cultivars and 7 accessions were evaluated in this study. The 26 commercial cultivars consisted of three positive controls sourced from Sakata Seeds Southern Africa (Pty) Ltd (Johannesburg, South Africa) and 23 commercial genopytes sourced from Zimbabwean seed dealers. The accession lines were provided by the

Zimbabwe National Gene Bank. Both the accession lines and commercial cultivars were imported into South Africa under Permit Number 048717A issued by the Ministry of Agriculture's Plant Quarantine Services Department.

7.2.2 DNA extraction

Genomic DNA was extracted from tender leaves of four-week-old greenhouse-grown tomato plants. For each tomato genotype, plant sap was separately imprinted onto FTA® (Whatman International, USA) cards and air-dried at room temperature for two hours. DNA was extracted from the FTA cards as described below:

A Harris® punch was used to cut out four discs from a card. The discs were place in a 2 mL microcentrifuge tube and 500 µL Millipore water added. Each tube was pulse-vortexed for 30 s and discs transferred to a new microcentrifuge tube to which 50 µL Millipore water was added. The tube was pulse-vortexed for 5 s, up to three times. Thereafter, it was heated at 95ºC for 30 min, pulse vortexed three times for 5 s each time and then centrifuged at 14 800 rpm for 5 min. The discs were removed and the eluate used to perform polymerase chain reaction (PCR). Extracted genomic DNA quality and quantity were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA) prior to storage at -20ºC.

7.2.3 PCR amplification

PCR amplification was performed following standard PCR procedures. Each 20 µL PCR reaction mixture consisted of 3 µL total nucleic acid, 2 µL of each primer, 10 µL KAPA mix and 3 µL ddH2O. The primer pairs, sw5b-f1/sw5b-r1 and sw5-f/sw5-r, (Table 7.1) designed by Shi *et al*. (2011), were used in amplification. The thermal cycler was programmed as follows: initial denaturation at 95ºC for 2 min; followed by 35 cycles of 5s at 98ºC (denaturation step), 20s at appropriate annealing temperature (annealing step), and 15s at 72ºC (elongation step), and a final elongation step of 72ºC for 5 min. PCR products were separated by gel electrophoresis with 1.5% agarose gel in TAE buffer, stained with SYBR Safe DNA Gel Stain (Life Technologies, USA) and visualized with UV light on a GelVue UV Transilluminator.

7.2.4 Gel purification, sequencing and analysis

The expected amplicons were excised and purified using the QiaQuick Gel Extraction Kit (Qiagen, Germany) following manufacturer's instructions. The purified amplicons were either cloned into the TA cloning vector pCR2.1 and transformed into TOP10 competent cells (Life Technologies, USA) or directly sequenced at Inqaba Biotechnical Laboratories (Pretoria, South Africa). Consensus sequences were generated from the sequences through alignment with ClustalW in MEGA6 (Tamura *et al.*, 2013). The consensus sequences were blasted into MEGA6.

Table 7.1 Primers used to amplify the *Sw-5* gene

Primer name	Primer sequence	Tm (°C)	Amplicon (bp)
Sw5b-f1	5'-AACCACTAGGGGCAGTCCTT-3'	62.45	662
Sw5b-r1	5'-CTCACTATGTGGCTGCTCCA-3'	62.45	
Sw5-f	5'-CGGAACCTGTAACTTGACTG-3'	60.4	541
Sw5-r	5'-GAGCTCTCATCCATTTTCCG-3'	60.4	

7.3 Results and Discussion

7.3.1 Evaluation of the tomato genotypes for TSWV resistance using the *Sw-5b* gene-based and SNP markers

To determine whether Zimbabwean tomato genotypes are resistant to TSWV, the cultivars were screened by PCR with the primer pairs given in Table 7.1. These primers had previously been used by Shi et al. (2011) and Lee at al. (2015). PCR amplified 662 bp and 541 bp DNA fragments with sw5b-f1/sw5b-r1 and sw5-f/sw5-r primer pairs, respectively. The 662 bp fragment was produced in all tomato genotypes (Table 7.2). This indicated that this primer pair did not distinguish between the *Sw-5* alleles. With the sw5-f/sw5-r primer pair, only two commercial cultivars (Star 9065 and UC-82-B) and four accession lines (6370, 6373, 6374 and 6403) did not have the 541 bp fragment

(Table 2), indicating that these genotypes did not carry the *Sw-5b* allele for TSWV resistance. The control lines MFH7032, Mion and Disco were already described by the provider to carry the *Sw-5b* gene. Previous studies by Shi et al. (2011) and Lee et al. (2015) also screened both commercial cultivars and accessions for TSWV resistance. Only 21.4% of the commercial cultivars and 10% of the accessions screened by Shi et al. (2011) and 18.8% of the commercial cultivars screened by Lee et al. (2015) carried the *Sw-5b* gene allele. In this study, 91.3% of the commercial varieties and 42.9% of the accession lines sourced from Zimbabwe had the *Sw-5b* gene.

The fact that most tomato varieties grown in Zimbabwe have the *Sw-5b* gene could partly explain why there has not been TSWV epidemics in the country. The TSWV strains in the country probably have not yet been able to overcome the resistance gene in these varieties. However, the occurrence TSWV resistance-breaking strains in Australia, South Africa and Spain (Lopez et al., 2011; Gabor et al., 2012) highlights the need to regularly monitor the disease in this country. This is particularly so in the face of the major drivers of TSWV epidemics like climate change, global trade in plants and plant products and agricultural intensification and diversification affecting agriculture in Zimbabwe.

In the study by Lee et al. (2015), the cultivar 'Moneymaker' was employed as a TSWV-susceptible line. In this study, a cultivar with the same name was found to have the *Sw-5b* resistance gene. This indicates the fact that cultivar names vary from region to region. Therefore, the best way to ascertain any cultivar's disease resistance status is to carry out some evaluations. Molecular evaluations are particularly desirable because they are quick and independent of Genotype × Environment interactions.

The accession lines carrying the *Sw-5b* gene can be advanced for use in breeding for TSWV resistance. Those accessions without the *Sw-5b* gene can be evaluated for other horticultural traits like resistance to other diseases, yield potential, fruit size, shape and firmness which are important in tomato production and these can be pyramided into the TSWV-resistant accessions if needed (Robbins et al., 2010). For example, though accession 6370 does not carry the *Sw-5b* allele, it produces big fruits. On the other hand, accession 6401 has the *Sw-5b* gene but produces small fruits.

Table 7.2: Tomato genotypes screened for the *Sw-5b* gene using gene-based and SNP markers

No.	Cultivar/Accession	Source	Genotype per marker	
			Sw5b-f1/sw5b-r1	Sw5-f/Sw5-r
1	Floradade	ZGS	+	+
2	Red Khaki	ZGS	+	+
3	Rodade	ZGS	+	+
4	Rio Grande	ZGS	+	+
5	Heinz	ZGS	+	+
6	Star 9003	Prime Seeds	+	+
7	Star 9065	Prime Seeds	+	-
8	UC-82-B	Prime Seeds	+	-
9	Goldilocks	Prime Seeds	+	+
10	CAL-J	Prime Seeds	+	+
11	Rodade	Prime Seeds	+	+
12	Oxheart	Prime Seeds	+	+
13	Little Wonder	Prime Seeds	+	+
14	Gold Nugget	Prime Seeds	+	+
15	Opal	EWS	+	+
16	Rodade Plus	EWS	+	+
17	Tejas	EWS	+	+
18	Moneymaker	ZGS	+	+
19	Marglobe	NTS	+	+
20	Roma	NTS	+	+
21	Tengeru 97	Prime Seeds	+	+
22	Nemo Netta	Pedstock Investments	+	+
23	Daniela	Pedstock Investments	+	+
24	6366	ZNGB	+	+
25	6370	ZNGB	+	-
26	6373	ZNGB	+	-
27	6374	ZNGB	+	-
28	6401	ZNGB	+	+
29	6402	ZNGB	+	+
30	6403	ZNGB	+	-
31	MFH7032	Sakata Seeds	+	+
32	Mion	Sakata Seeds	+	+
33	Disco	Sakata Seeds	+	+

ZGB – Zimbabwe Garden Seeds; **ZNGB** – Zimbabwe National GenBank; **EWS** – East West Seeds; "**+**" – PCR fragment present; "**-**" – PCR fragment absent.

None of the tomato cultivars marketed in Zimbabwe have TSWV-resistance information displayed on their seed labels. However, information on resistance to *Verticillium*, *Fusarium*, *Alternaria alternata*, bacterial wilt and *Tobacco mosaic virus* is provided on the seed packs. Given the fact that TSWV infects tomatoes in Zimbabwe (Masuka et al., 1998), there are chances that serious disease outbreaks may occur in the country should TSWV resistance-breaking strains occur. Therefore, farmers should be provided with information on the TSWV resistance status of the commercially available tomato cultivars to enable them to decide accordingly when purchasing seed.

The study showed that MAS is a quick method that can be used to screen tomato genotypes for the *Sw-5b* gene. Tomato plant DNA was extracted from four-week-old seedlings and the *Sw-5b* gene detected by PCR. This greatly speeds up breeding, as tomato lines with the this gene can be quickly advanced through the breeding program without the need for phenotypic evaluation (Foolad and Sharma, 2005) which can take at least four months depending on the tomato cultivar being evaluated.

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CHAPTER 8: THESIS OVERVIEW

8.1 Introduction

This study was undertaken with the broad objective of increasing knowledge about tospoviruses in Zimbabwe. Prior to this study, only TSWV was known to be present in Zimbabwe. Now, IYSV is also known to be present. The information generated in this study will greatly contribute to the body of knowledge about tospoviruses both locally and internationally. Several first reports and full papers about tospoviruses in Zimbabwe have been published in peer-reviewed journals. This chapter highlights the main findings of the study, their implications and gaps for future research.

8.2 Knowledge and percetions of plant viral diseases by different stakeholders in Zimbabwe's agricultural sector: Implications for disease management

A survey undertaken at the start of this project highlighted how poorly understood and appreciated plant viral diseases are in Zimbabwe by the various stakeholders in the agricultural sector. The majority (72%) of the technical staff rated MSV as the most important virus, followed by TMV, CMV, ToMV and GRV. None of the respondents could name tospovirus species other than TSWV. A major contributory factor to the poor knowledge of plant viruses is inadequate training in plant virology that both agricultural graduates and farmers are exposed to. The training of plant virologists is expensive, requiring specialized equipment and consumables most of which are not available in most research and academic institutions in the country. Over 30% of farmers were self-taught to identify viral diseases, thus greatly increasing chances of misdiagnosis and control. The survey highlighted the need to train agricultural graduates in the discipline of plant virology so that they, in turn, train farmers about plant viral diseases.

8.3 Detection and characterization of *Tomato spotted wilt orthotospovirus* infecting field and green-house grown crops in Zimbabwe

TSWV was already known to be present in Zimbabwe. However, there were no records of how pathogen identification and characterization were done. In this study, protocols and procedures for TSWV detection are given. Tospovirus immunostrips were used for field detection of TSWV,

while FTA cards and RNA*later*® solution were used for archiving nucleic acid in collected samples. The use of FTA cards and RNAlater® solution during sampling is significant as these can replace the need for dry ice, snap-freezing of samples in liquid nitrogen, immediate processing after collection or refrigeration to preserve sample integrity. In a developing country like Zimbabwe, liquid nitrogen, dry ice and freezers are not readily available. Total nucleic acid extracted from the FTA cards and RNA*later*-stored samples was used for molecular detection of TSWV. The virus was detected in only 50% of the surveyed districts, mostly in the country's prime agricultural region. TSWV disease incidence and severity were both low in the surveyed areas. The virus was detected for the first time in *Cucurbita moschata, C. pepo, Cucumis sativus* and *Gypsophilla elegans*. In addition, a mixed infection of TSWV and PVY in *Capsicum annuum* was reported for the first time in Zimbabwe. Though the host range of TSWV is expanding, the virus was not detected in legumes and brassicas. All Zimbabwean TSWV isolates are highly similar and identical (≥ 93.99%) to each other at amino acid and nucleotide levels.

8.4 *Iris yellow spot orthotospovirus* in Zimbabwe: Incidence, severity and characterization of *Allium*-infecting isolates

IYSV was reported in Zimbabwe for the first time in this study. Zimbabwe is the seventh African country to report the presence of IYSV after Reunion Islands (Robene-Saustrede *et al*., 2006), South Africa (du Toit *et al*., 2007), Mauritius (Lobin *et al*., 2010), Egypt (Hafez *et al*., 2012), Kenya and Uganda (Birithia *et al*., 2011). The virus was detected in onion, garlic, leek and shallot crops in all districts surveyed. This showed that the virus has been present in the country for much longer than reported. High incidences (up to 78.3%) and severities (up to 3.7) were reported in some districts. Zimbabwean IYSV isolates were highly similar and identical to each other at both nucleotide and amino acid sequence levels. All of them clustered with the South African IYSV isolate, suggesting that IYSV may either have been introduced from South Africa or vice versa. Volunteer and intercrop alliaceous crops were observed to be important in the seasonal carry-over of the virus. Farmers do not know anything about the occurrence of this disease in the country. Therefore, they do not implement any control measures against IYSD.

8.5 Natural non-*Allium* hosts of *Iris yellow spot orthotospovirus* in Zimbabwe

Besides *Allium* species, IYSV was also detected in *Amaranthus hybridus, A. spinosus* and *Eleusine indica*. However, it was not detected in some important weeds like *Bidens pilosa, Commelina benghalensis, Nicandra physalodes* and *Tagetes erecta* and *T. minuta*. *Bidens pilosa* and *Nicandra physalodes* supported *Thrips tabaci* reproduction and survival. The weed-infecting IYSV isolates were closely related to the *Allium*-infecting isolates. The weed hosts identified in this study are important in IYSV epidemiology because as summer weeds, they provide a green bridge for the survival of the virus in the *Allium* species off-season in Zimbabwe. *E. indica* is one of only two grasses reported to be naturally infected by IYSV worldwide, while *A. hybridus* and *A. spinosus* are grown as vegetables, sometimes in intercrops with IYSV-infected *Allium* species.

8.6 Characterization of three full *Iris yellow spot orthotospovirus* genes of a garlic-infecting isolate from Zimbabwe using Next-generation sequencing

Three full genes of IYSV namely N, NSs and NSm, were obtained from a garlic-infecting isolate by NGS. Phylogenetic analyses of the N gene did not reveal a clustering pattern based on geographical location. Rather, the clustering pattern suggested possibilities of recombination, reassortment and long-distance migration events. The NSs and NSm genes clustered with homologous IYSV genes from other regions of the world. Though the full genome of IYSV was not recovered by NGS, this study lays the foundation for future studies on the pathogen.

8.7 Marker-assisted identification of *Tomato spotted wilt orthotospovirus*-resistant tomato (*Solanum lycopersicum* L.) genotypes in commercial and accession lines

The tomato is an important vegetable in Zimbabwe. While information on resistance to several diseases is displayed on the seed label, no such information is available for TSWV. To speed up tomato breeding programs, MAS using molecular markers can be applied. Tomato genotypes consisting of commercial cultivars (23) and accession lines (7) sourced from Zimbabwe, and TSWV-resistant cultivars (3) sourced from Sakata Seeds Southern Africa in South Africa, were screened using molecular markers. Over 91% of the commercial cultivars and 42.9% of the

accession lines had the *Sw-5b* gene allele. The accession lines with the *Sw-5b* gene could be used in future tomato breeding programs. As a recommendation, seed packs should indicate the TSWV-resistance status of tomato varieties grown in Zimbabwe in order to assist farmers with choosing cultivars to grow.

8.8 Way forward

Even though TSWV was reported much earlier than IYSV in Zimbabwe, the latter is now a more serious problem threatening alliaceous crop production. Given the high IYSV incidence and severity in the country, there is need to quantify losses the pathogen is causing. More weeds, including perennials, should be screened in order to broaden the host range of the virus in the country. Onion varieties that are being grown in Zimbabwe should also be evaluated for resistance to IYSV. Farmers also need to be educated about the disease so that they can start to implement control measures against it. The education can be done through extension outreach programs and mass media broadcasts.

A number of viruses other than tospoviruses were detected in samples that were collected during the surveys. These include *Bell pepper alphaendornavirus*, Brassica yellows virus, Garlic viruses B and C, *Garlic common latent virus*, *Moroccan watermelon mosaic virus*, *Pepper cryptic virus 1*, Pepo aphid-borne yellows virus, *Potato leafroll virus*, *Potato virus Y, Tomato mosaic virus*, *Turnip yellows virus*, *Shallot virus X* and *Zucchini shoestring virus*. This study opens avenues for future research into these viruses. It will be interesting to evaluate how widespread some of these viruses are and their effects on crops.

Because it was not possible to transport TSWV-infective plant material from Zimbabwe to South Africa, more studies need to be undertaken to phenotypically evaluate Zimbabwean tomato germplasm for their reaction to TSWV infection. This has to be done in Zimbabwe in order to overcome challenges associated with movement of pathogens of quarantine importance across international borders. Even though TSWV is currently not a serious problem in tomato production, there is need to continuously grow TSWV-resistant cultivars. The accession lines with the *Sw-5b* gene can be utilized as germplasm for future breeding programs.

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APPENDICES

QUESTIONNAIRE FOR TOSPOVIRUS SURVEY (Form 1)

To be completed by Technical staff (Extension staff, agronomists, researchers, academics etc)

The information collected in this survey is for academic purposes only. It will not be used for any commercial purposes.

DATE: …… NAME OF RESPONDENT (optional): ……

DISTRICT: …… WARD: ……

A. BACKGROUND INFORMATION

1. Position
   ◯ Research officer ◯ Extension officer ◯ Extension worker ◯ Agronomist
   ◯ Teacher/Lecturer ◯ Other

2. Employer:
   ◯ Government ◯ Private sector ◯ Non-governmental organization
   ◯ Other

3. Gender …… ◯ Male ◯ Female

4. AGE: …….. Years

5. Educational level: ◯ Certificate ◯ Diploma ◯BSc ◯ MSc ◯ PhD

6. MARITAL STATUS: ◯ Single ◯ Married ◯ Divorced ◯ Widowed

7. Agricultural experience post first qualification

   ◯ <2 years ◯ 2-5 years ◯ 6-10 years ◯ 11-19 years ◯ > 20 years

B. KNOWLEDGE OF PLANT DISEASES

Which three major diseases you deal with on these crop families in your area?

8. Solanaceae: ……..

9. Cucurbitaceae: ……

10. Leguminosae: ……..

11. Ornamentals: ……

12. Do you train farmers/people in disease identification and control? ◯Yes ◯No

If yes, briefly explain how you do it.

◯ Short courses ◯ Pamphlet distribution

◯Set demonstration plots ◯ Plant inspection

◯ Other(s)

Please specify: ……..

13. Do farmers appreciate the impact of plant diseases on crops? ◯ Yes ◯ No
    Explain your answer

    Yes, because:

    ◯ Regularly seek information on disease identity

    ◯Purchase chemicals

    ◯ Appreciate yield and financial losses

    ◯ Attend agricultural shows

    ◯ Set up on-farm demonstration plots

    ◯ Other(s)
    Please specify other(s): ……

    No because: ……

C. VIRUS DISEASES

14. When compared to other pests, how important are viruses as crop pests?

    ◯ Not important ◯ Fairly important ◯ Important ◯ Very important ◯ Don't know

15. What three major virus diseases do you know? Give the crop and the virus.
    ……
    ……
    ……

16. What can be done to improve awareness of virus diseases amongst farmers?

……
……
……

D. **KNOWLEDGE OF TOMATO SPOTTED WILT VIRUS OR TOSPOVIRUSES**.

17. Have you ever heard about tospoviruses (or tomato spotted wilt virus)?

◯ Yes ◯ No

If yes, please answer the following questions:

18. Where did you learn/hear about these viruses?

◯ University/college ◯ Print media ◯ Electronic media

◯ Others (please specify) ……..

19. Which crops do they attack? ……..

……

How do you rate these viruses as crop pests in Zimbabwe?

◯ Not important ◯ Fairly important ◯ Important ◯ Very important ◯ Not sure

20. How is the tomato spotted wilt virus transmitted?

◯ Aphids ◯ Red spider mites ◯ Thrips ◯ Whiteflies
◯ Other (Please specify) ……..

21. Give three ways in which they are controlled

……
……
……

22. What can be done to improve awareness of tospoviruses?

◯ Print/electronic media awareness campaigns ◯ Farmer training

◯ University extension ◯ Student training in colleges/universities/schools

◯ Workshops/short courses for research/extension staff ◯ Agricultural shows

◯ Other(s): ……..

Thank you for your participation!!

QUESTIONNAIRE FOR TOSPOVIRUS SURVEY (Form 2)

To be administered to farmer/farm manager

The information collected in this survey is for academic purposes only. It will not be used for any commercial purposes.

DATE: …… NAME OF FARM/FARMER: ……

DISTRICT: …… WARD: …… VILLAGE: ……..

PROVINCE: …… NATURAL REGION: …….. GPS: ……

A. BACKGROUND INFORMATION

23. GENDER …… ◯ Male ◯ Female

24. Age: …….

25. Educational level: ◯ None ◯ Primary ◯ Secondary ◯ Post-secondary

26. MARITAL STATUS: ◯ Single ◯ Married ◯ Divorced ◯ Widowed

27. Farming experience …….. (years)

28. Farm type: ◯ Communal ◯ A1 ◯ A2 ◯ Large scale commercial ◯ Plot/Nursery ◯ Other

B. CROPPING SYSTEMS

29. Which three major crops have you grown in the last three years?
……
……

30. How does farmer grow crops? ◯ Dryland ◯ Irrigation ◯ Both [Tick appropriate option]

C. KNOWLEDGE OF PLANT DISEASES

31. What three major diseases affected your crops?

……
……
……..

32. Who trained you to identify diseases?

◯ School/college ◯ AREX/Research Officer ◯ Agrochemical/Seed companies/seedling supplier ◯ Self-taught ◯ International expert ◯ Former landowner ◯ Colleagues ◯ Combination of above options

33. How do you manage diseases attacking your crops?

◯ Spray with chemicals ◯ Grow resistant varieties ◯ Destroy infected plants

◯ Crop rotation ◯ Disinfection of entry points ◯ Use of biological control ◯ integrated management

◯ No action

D. VIRUS DISEASES

34. When compared to other pathogens, how important are viruses as crop pests?

◯ Not important ◯ Fairly important ◯ Important ◯ Very important ◯ Don't know

35. Are you able to identify virus diseases attacking your crop? ◯ Yes ◯ No

If yes, explain how you do so.

……
……

36. What can be done to improve awareness of viruses as plant pathogens?

◯ AREX should train farmers ◯ AREX should be trained

◯ University extension and research ◯ Radio/TV/Newspaper bulletins/articles

◯ Color pictures of disease symptoms ◯ College/school training

◯ Field shows ◯ Combination of above options

37. Have you heard about tospoviruses or the tomato spotted wilt virus? ◯ Yes ◯ No

If yes, please answer the following questions:

38. Where did you learn/hear about these viruses?

◯ Arex Officer ◯ Print media ◯ Electronic media ◯ School/College ◯ Agrochemical/seed rep

◯ Other(s) ……..

39. Name any three crops they attack

……

40. How do you rate them as disease agents?

◯ Not important ◯ Fairly important ◯ Important ◯ Very important

41. What are the vectors for tospoviruses?

◯ Aphids ◯ Red spider mites ◯ Thrips ◯ Whiteflies ◯ Others(s) ……..

42. Give any four methods of tospovirus disease control

……
……
……
……

Thank you for participating in this exercise