Pre-empting Maize Lethal Necrosis Disease in South Africa: potyviruses of maize (*Zea mays*)

by

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Supervisor: Professor Gerhard Pietersen

Declaration

I, Susanna Elizabeth Schulze declare that the dissertation, which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SE Schulze	6 August 2018
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Summary

The devastating Maize Lethal Necrosis Disease (MLND) hampers the production of maize, especially in developing countries. It causes mottling, necrosis and chlorosis of host plants, which results in severely reduced growth and yield and in extreme cases plant death in *Zea mays* (maize). Maize is regarded as one of three main staple food crops worldwide and in sub-Saharan Africa and South America it provides nutrition for over 1.2 billion people. Therefore, this disease is a severe food security threat and needs to be managed where it is present or pre-empted where it is predicted to spread to.

MLND is caused by the co-infection of Maize chlorotic mottle virus (MCMV) and a cereal-infecting member of the Potyviridae family such as Sugarcane mosaic virus (SCMV), Johnsongrass mosaic virus (JGMV) or Maize dwarf mosaic virus (MDMV). MCMV is not present in South Africa, but was recently introduced into East Africa. The disease is predicted to disperse to neighbouring countries including South Africa (SA) in the near future. A number of potyviruses of maize have, however, been reported from SA, although the status, distribution and their diversity of these viruses has not been studied and a molecular tool to simultaneously detect these viruses has not yet been developed.

In this study the status of potyviruses on maize in SA was determined, the diversity of the major maize potyviruses including those from SA and Tanzania was investigated, and an assay to simultaneously detect four maize-infecting viruses that are likely to be involved in MLND was developed. The results from this study allow us to pre-empt the introduction of MLND into SA by enabling us to 1) gain insight into which potyviruses are most likely to be present in potential MLND infections in SA and predict which areas are likely to be most affected, 2) better understand the evolution and diversity of the most common potyvirus on South African maize, SCMV isolates, in Africa and produce evidence that the MLN viral complex (or epidemiology thereof) will be more similar in SA to that seen in Asian countries rather than that seen in other African countries, and 3) verify that rapid, accurate and cost effective disease diagnosis can be made using the tool developed in this study.

The conclusions of this study will contribute to future research regarding the viral components and epidemiology of MLND, especially in Africa. It also highlights some factors to consider in the development of genetically modified virus-resistant maize towards a durable control strategy to curb the impact of this disease. The maize industry, seed companies, quarantine services and research facilities working on MLN will benefit from the knowledge generated as well as the assay developed here. The study will also be useful in the subsequent selection of future research directions.

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List of acronyms

ARC-BTP	Agriculture Research Council-Biotechnology Platform
ASARECA	Association for Strengthening Agricultural Research in Eastern and Central Africa
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CABI	Centre for Agriculture and Bioscience International
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
CIMMYT	International Maize and Wheat Improvement Center
CLN	Corn Lethal Necrosis
CMMoV	Cactus mild mottle virus
CMV	Cucumber mosaic virus
CP	Coat protein
CoMV	Commelina mosaic virus
CTAB	cetyltrimethylammonium bromide
DRC	Democratic Republic of the Congo
DTT	dichlorodiphenyltrichloroethane
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPPO	European and Mediterranean Plant Protection Organization
FAO	Food and Agriculture Organization
FAOSTAT	Statistical databases and data-sets of the FAO
GARP	Genetic Algorithm for Rule-Set Prediction
GPS	Global Positioning System

HC-Pro	Helper Component-Proteinase
ICTV	International Committee on Taxonomy of Viruses
ISSCT	International Society of Sugar Cane Technologists
JGMV	Johnsongrass mosaic virus
kb	kilo base pairs
LAMP	loop-mediated isothermal amplification
MAFFT	Multiple Alignment using Fast Fourier Transform
MaYMV	Maize yellow mosaic virus
MCMV	Maize chlorotic mottle virus
MDMV	Maize dwarf mosaic virus
MEGA	Molecular Evolutionary Genetic Analysis
MLN	Maize Lethal Necrosis
MLND	Maize Lethal Necrosis Disease
M-MLV	Moloney-Murine Leukemia Virus
MMV	Maize mosaic virus
MRFV	Maize rayado fino virus
mRNA	messenger RNA
MSV	Maize streak virus
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NIb	Nuclear Inclusion body ‘b’
PCR	Polymerase Chain Reaction
PeMoV	Peanut mottle virus
PenMV	Pennisetum mosaic virus

PepMV	Pepino mosaic virus
PTGS	Post transcriptional gene silencing
PVP	polyvinyl pyrrolidone
PVY	Potato virus Y
q-RT-PCR	quantitative RT-PCR
RdRp	RNA dependent RNA polymerase
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SA	South Africa
SAWS	South African Weather Services
SCMV	Sugarcane mosaic virus
SCSMV	Sugarcane streak mosaic virus
SCYLV	Sugarcane yellow leaf virus
SDT	Sequence Demarcation Tool
SrMV	Sorghum mosaic virus
TBTV	Tobacco bushy top virus
TuMV	Turnip mosaic virus
UP	University of Pretoria
WSMV	Wheat streak mosaic virus
ZeMV	Zea mosaic virus

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Preface

Maize (*Zea mays* L.) plays a crucial role in ensuring food security as it is a staple-food crop for both people and livestock in many parts of the world, especially on the African continent. The production of this crop is threatened by the devastating viral disease known as Maize Lethal Necrosis Disease that is spreading throughout East Africa. The disease is usually caused by the co-infection by Maize chlorotic mottle virus – which is currently not present in South Africa – together with a cereal-infecting member of the Potyviridae family (most often Sugarcane mosaic virus) of which some members have been reported in South Africa. South Africa is the second largest producer of maize on the continent and due to the abundance of Maize Lethal Necrosis Disease-hosts in South Africa, the introduction of MCMV would be devastating to the local commercial and subsistence maize-growers and the economy as a whole. Due to the vital role that maize plays in feed, food and fuel production, it is imperative that we generate knowledge, scientific resources and references, as well as diagnostic tools in order to aid ongoing MLND research, contribute to the development of control strategies and to manage a future outbreak of this devastating disease, especially in South Africa. The aim of this study is to pre-empt the introduction of Maize Lethal Necrosis Disease in South Africa by determining the status of the potyviruses that are present on maize in South Africa, elucidating the diversity of the predominant potyvirus found in that survey, and by developing an assay that could simultaneously identify the viral components potentially present in Maize Lethal Necrosis Disease infections in future, should Maize chlorotic mottle virus enter the country.

Chapter 1: Relevant literature

1.1 Introduction

"...perhaps the worst enemy of the maize crops in recent times…" –Kiruwa *et al*. (2016)

Maize Lethal Necrosis Disease (MLND) is a serious threat to crop production as it can cause severe to complete yield loss of *Zea mays* L. *ssp. Mays* (Linnaeus, 1758) (maize) (Wangai *et al*., 2012a). It has been described as one of the most devastating foliar disease responsible for highest yield loss in maize and has lead to major economic losses, especially in areas where maize is the key staple food crop for consumption by humans and livestock (Kiruwa *et al*., 2016; Mahuku *et al*., 2015a). The symptoms induced by MLND include: chlorotic mottling, necrosis, stunting and hindered development that results in slowed physiological processes of the plant, leading to yield losses and ultimately plant death (Wangai *et al*., 2012a). The disease has been present on the African continent since 2011 and is anticipated to spread to South Africa (SA) in the near future from countries such as Kenya or Tanzania where it is already present, via Mozambique or Zimbabwe, through porous borders between these countries. It therefore poses a massive threat to food security in SA (Flett & Mashingaidze, 2016).

The genus *Zea* includes *Z. mays* and belongs to the family Gramineae (Poaceae) together with the other major cereal crops and their non-domesticated relatives (Harris & Hillman, 2014). Maize was first domesticated in the highlands of Mexico about 8000-10 000 years ago when a common grass called teosinte was developed into the comestible crop through strategic cross-breeding and selection of desirable qualities (Wilkes, 2014). Since its domestication, maize has primarily been produced for human and animal consumption, in addition to being used as a raw material for the manufacturing of various other products; for example, nearly 40% of maize in the USA is used to produce biofuel (Zhuang *et al*., 2013). The maize crop is also an essential model organism in the study of genome evolution and comparative genomics, epigenetics, plant physiology, the evolution of plant domestication, pest and pathogen resistance, and quantitative inheritance of traits (Strable & Scanlon, 2009).

Globally, over the last decade, maize-growing areas increased from 158 to 188 million km^2 in total. This area of the earth's surface was used to produce 714 million

metric tonnes in 2007 and increased to over one billion metric tonnes of maize in the 2016 season (FAOSTAT, 2018). Maize is regarded as the third most important cereal crop for food security following rice and wheat; and is the main staple food source of more than 1.2 billion people residing in sub-Saharan Africa and South America (Iken & Amusa, 2004). In Africa, small scale farmers constitute the majority of maize growers (Onasanya *et al*., 2009). These farmers produce over 38 million metric tonnes of grain annually (FAO, 2012) of which 85% is consumed by humans and the remainder is consumed by livestock (Shiferaw *et al*., 2011). In SA, around 12 million tonnes of maize is produced annually on average, grown on approximately 2.8 million km^2 of land (Fig. 1.1, years 2008-2014). Maize is cultivated commercially in seven of SA's nine provinces: Limpopo Province, KwaZulu-Natal, Free State, North-West Province, Northern Cape, Mpumalanga and Gauteng (Statistical databases and data-sets of the FAO (FAOSTAT, 2018). Maize production in SA relies greatly on rain and most cultivation takes place on dry land (Flett & Mashingaidze, 2016). It plays an important role in the economy in terms of exportation, which amounted to 2.4 million tonnes in the marketing year of 2017/18 (FAO, 2018).

Various abiotic and biotic factors limit the production of maize. Maize diseases caused by viruses cause variable but noteworthy losses for producers throughout the maize-growing regions of the world. Lapierre and Signoret (2004) listed more than 50 viruses that infect maize and at least twelve of these – distributed amongst eight families – cause major agronomic losses worldwide (Redinbaugh & Zambrano-Mendoza, 2014). These viruses cause diseases in their hosts which most often result in yield loss and, significantly for this study, MLND is possibly the most threatening of these yet to be reported (Kiruwa *et al*., 2016).

MLND is caused by the synergistic co-infection of maize with two viruses (Uyemoto *et al*., 1981; Goldberg & Brakke, 1987; Scheets *et al*., 1998). These viruses include one or more members of the Potyviridae family – such as Sugarcane mosaic virus (SCMV), Johnsongrass mosaic virus (JGMV) and Maize dwarf mosaic virus (MDMV), all belonging to the *Potyvirus* genus, or Wheat streak mosaic virus (WSMV) (genus: *Tritimovirus*) (Uyemoto *et al*., 1980; Goldberg & Brakke, 1987; Giolitti *et al*., 2005) – together with Maize chlorotic mottle virus (MCMV) (family: Tombusviridae; genus: *Machlomovirus*) (Scheets, 1998; Stewart *et al*., 2017; Uyemoto *et al*., 1981).

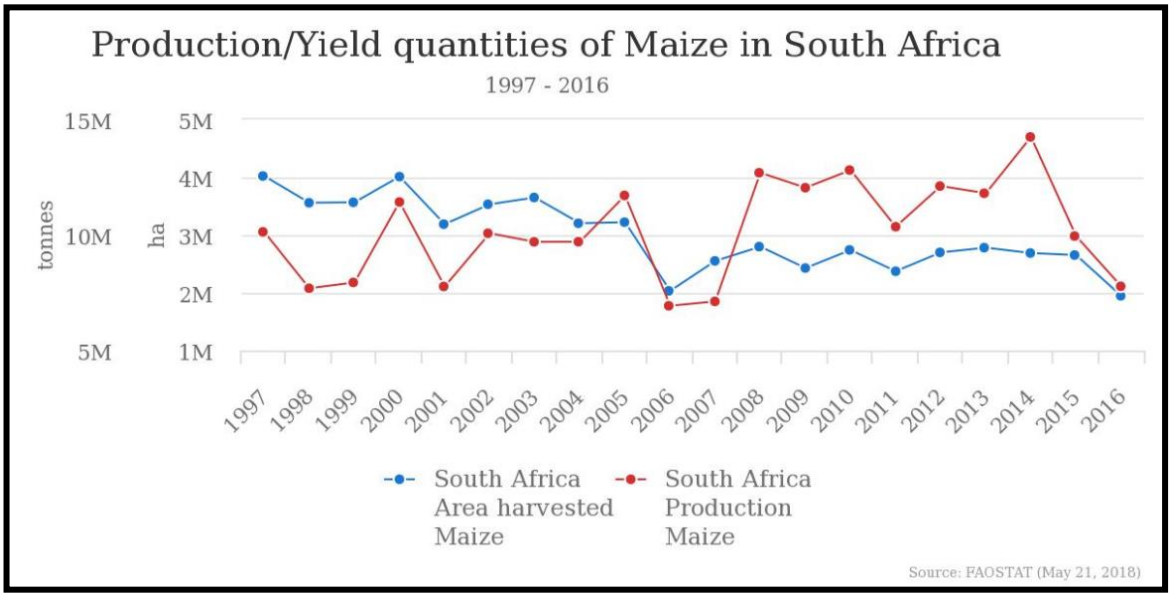

Figure 1.1 Line graph showing annual maize production yields and area harvested over the last two decades in South Africa. (FAOSTAT, 2018; accessed on: 21 May 2018; available online at: [http://www.fao.org/faostat/en/#data/QC/visualize]).

All growth stages of maize are typically susceptible to the disease and the host range is restricted to members of the grass (Poaceae) family including maize (Scheets, 2000), *Sorghum bicolor* (L.) Moench, 1794 (sorghum) (Huang *et al*., 2016), *Saccharum officinarum* (Linnaeus, 1753) (sugarcane) (Wang *et al*., 2014) and *Eleusine coracana* (L.) Gaertner, 1770 (finger millet) (Kusia *et al*., 2015), but various experimental hosts have been identified (Mahuku *et al*., 2015a).

In Africa the disease was first reported in Kenya (Wangai *et al*., 2012a) where its effects have been so devastating that smallholder farmers have experienced more than 80% crop losses in severely affected regions of the South Rift Valley region (Wangai *et al*., 2012b). MLND's "sudden" outbreak may be attributed to planting of susceptible hybrids, the widespread presence of SCMV that pre-disposed maize to the disease, the presence and rising population numbers of insect vectors and all-year growing of maize in many regions where the climate allows (Mahuku *et al*., 2015a). The disease is especially challenging to manage for various reasons including: lack of resources and knowledge of subsistence farmers to practice pathogen exclusion or disease management, no knowledge that resistant varieties exist and because the disease has a complicated development and dispersal cycle (De Groote *et al*., 2016). The contributing viruses can also have reservoir hosts

where they reside during the off-season, providing the inoculum source for the following growing-season (Roossinck, 2015). Various authors have discussed the particular vulnerability of subsistence farmers in rural areas to the impact of MLND attributable to the lack of financial resources to ensure virus-free crops and the implementation of back-to-back planting seasons due to two rain seasons, amongst other factors (De Groote *et al*., 2016; Fentahun *et al*., 2017).

While MLND is fairly new to Africa, it was initially described as Corn Lethal Necrosis Disease Kansas in the USA in 1974 where yield losses between 50% and 90% had been experienced (Niblett & Claflin, 1978). During the past seven years, MLND has spread to at least six new countries in eastern Africa largely due to poor phytosanitation and regulatory systems as well as porous borders (FAO, 2013). MLND has also been detected in South America (Castillo & Hebert, 1974) and Asia (Deng *et al*., 2014; Xie *et al*., 2011) and, to our knowledge, Australia and Europe are the only continents that remain MCMV-free therefore do not have MLND.

MCMV has not yet been reported in SA but two *Potyvirus* species known to co-operate in the MLND disease complex have been detected on various crops in many farming regions in SA. These viruses are SCMV (International Society of Sugar Cane Technologists (ISSCT), 1989; Handley *et al*., 1998) and MDMV (Knox *et al*., 1986). Maize, sorghum, sugarcane and various grasses are known host plants of these potyviruses and in SA, SCMV has been reported on sugarcane and maize (ISSCT, 1989) and MDMV on maize (von Wechmar *et al*., 1987). Despite being reported on maize nearly three decades ago, relatively little knowledge regarding the species, distribution and genetic diversity of these potyviruses in SA is available, especially those occurring on maize (Flett & Mashingaidze, 2016).

While MCMV is not known to be present in SA, ecological niche models constructed using mathematical algorithms to make predictions based on data of current disease epidemiology, showed that approximately 298 000km^2 of SA is a suitable habitat for MCMV (Isabirye & Rwomushana, 2016). Furthermore, Isabirye and Rwomushana (2016) predicted that MCMV by itself has great potential to become established in warm- and semi-arid regions, including the sub-tropics; climatic zones which are widespread in SA and further contributes to the prediction that MCMV could establish itself if introduced to this country. Thus, maize production in both

commercial and subsistence growing regions of SA are under threat and pre-emptive research is essential.

Gaining insights into the status of potyvirus species currently present on maize in SA and where they are distributed, will indicate which potyvirus species are likely to be present in potential Maize lethal necrosis (MLN) infections in SA. This will allow identification of areas to be monitored more carefully and will lay the crucial foundation for future research in SA on MLND. Determining the genetic diversity of the predominant potyvirus species in SA and how they are related to potyviruses from other countries will elucidate the evolution of these isolates, reveal the genetic diversity of the virus present within Africa and thus provide researchers with useful information for further studies on the disease epidemiology as well as disease management in terms of the development of genetically modified crops resistant to the viral components of MLND. Determining the prevalent maize-infecting potyvirus species on maize in SA is not only important to increase the available knowledge resources but also for the development of an improved diagnostic system for this disease.

The first crucial step in managing a plant disease is an accurate and rapid diagnosis of the causal agent(s) (Adams *et al*., 2013; Boonham *et al*., 2014). One such test is multiplex Polymerase Chain Reaction (PCR) which is a variant of the conventional PCR in which more than one target sequence is amplified using more than one primer pair set (Shen *et al*., 2010). Several monospecific PCR based methods have been reported for a number of maize viruses including for MCMV (Zhang *et al*., 2011; Wangai *et al*., 2012a; Mahuku *et al*., 2015a), Maize streak virus (MSV) (Briddon & Markham, 1995; Rybicki & Hughes, 1990), specific potyviruses (Jiang *et al*., 2002; Smith & Velde, 1994; Wangai *et al*., 2012a) and universal potyvirus-detecting PCR systems (Chen *et al*., 2001; Pappu *et al*., 1993; Zheng *et al*., 2010) but, to our knowledge, none have been developed or published that can simultaneously identify more than one maize virus in order to diagnose diseases including MLND. Developing a system such as a multiplex assay will reduce time and costs of disease diagnostics and is vital to pre-empt the disease in various regions and countries, will aid research facilities in studies of the viral complex epidemiology, aid ongoing monitoring at quarantine facilities, ensure cost-effective

quality control at seed companies and benefit plant pathogen diagnostic service providers, thereby allowing efficient management of the disease.

In order to achieve our aim of pre-empting MLND in SA three main objectives were identified and were addressed in three subsequent research chapters. These objectives were: 1) to determine the status and distribution of potyviruses on maize in SA 2) to determine the diversity of the predominant potyvirus detected on South African maize, and 3) to develop a multiplex RT-PCR diagnostic tool to detect the viruses that are expected to be the main role players in the disease complex of potential MLN infections on maize in SA in future.

1.2 Pathogens potentially present in the Maize Lethal Necrosis Disease-complex

A member of the Potyviridae family (genera: *Potyvirus* and *Tritimovirus*) in co-infection with MCMV has been the most common combination detected in MLN infections (Mahuku *et al*., 2015a; Uyemoto *et al*., 1981). As there may be other unidentified potential viral role players, it was deemed necessary to review a number of the viruses that are likely to contribute to this disease complex.

1.2.1 The potyviruses

The genus *Potyvirus* is one of eight belonging to the family Potyviridae, which is the largest plant virus family, and was comprised of nearly 150 species in 2008 (Gibbs *et al*., 2008) but has considerably more members now. The Potyviridae family was originally established as one of the plant virus groups with elongate particles in 1959 (Brandes & Wetter, 1959). The different genera within the family were initially distinguished largely based on which vector transmitted them, with those which are transmitted by aphids (superfamily: Aphidoidea) grouped in the *Potyvirus* genus (Agrios & Hadwiger, 2005). This genus is significant due to the virulence of its members against nearly all economically important crops over a wide geographical range (Ivanov *et al*., 2014).

Most potyviruses infect dicotyledons and to date, only seven grass-infecting species have been described and they are: SCMV, JGMV, MDMV, Pennisetum mosaic virus (PenMV), Sorghum mosaic virus (SrMV), Zea mosaic virus (ZeMV) and Cocksfoot

streak virus (CSV). The SCMV subgroup contains five of these viruses: SCMV, JGMV, MDMV, SrMV and ZeMV (Achon *et al*., 2012; Xu *et al*., 2008). Characteristic of a potyvirus infection, scroll- or pinwheel-shaped inclusion bodies can be observed in the cytoplasm using electron microscopy (Edwardson *et al.*, 1984). Potyviruses also cause of a wide array of symptoms on their host plants including mosaics, mottling, chlorosis, streaks, yellowing and discolouration of the leaves as well as stunting, reduced vigour and yield losses (Agrios & Hadwiger, 2005).

Potyviruses have flexuous filamentous virions, approximately 680 – 900 nm long and 12 nm in diameter. Their genomes are positive-sense single-stranded RNA of 9000 – 10 000 base pairs (bp) long (Ivanov *et al*., 2014). When potyviral RNA is encapsidated it is covalently linked to a genome-linked viral protein called VPg whose function has not yet been elicited but appears to be involved in stabilisation of the genome when disassembly occurs prior to viral translation (Ivanov & Mäkinen, 2012; Mäkinen & Hafrén, 2014). Potyvirus genomes encode 11 proteins and ten of these are derived from a single polyprotein precursor (Fig. 1.2 A) which is proteolytically cleaved at nine sequence- specific sites (Fig. 1.2 B) (Chung *et al*., 2008). These proteins include, from the N to C terminus of the polyprotein (represented by coloured blocks on Fig. 1.2 B), the trypsin-like serine proteinase responsible for symptomatology (P1), the Helper Component-Proteinase which functions in aphid-transmission, systemic movement and pathogenicity (HC-Pro), another protein involved in pathogenicity (P3), a 6K protein with unknown function (6K1), an RNA helicase protein involved in cell-to-cell movement (CI), the anchor protein of the replication complex to cellular membranes (6K2), Nuclear Inclusion body protein ‘a’ (NIa-pro) with dual functioning as a proteinase (whose cleavage sites are indicated by downward pointing arrows in Fig. 1.2 B) and that is involved in cellular localisation during the infection cycle, and also acts as the genome-linked VPg protein in some instances, the Nuclear Inclusion protein ‘b’ (NIb), which acts as the RNA dependent RNA polymerase (RdRp) and lastly, the capsid protein known as the Coat protein (CP) that forms the protective protein sheath around the virus RNA during packaging and is also involved in aphid transmissibility and virus movement (Chung *et al*., 2008; Ivanov *et al*., 2014; Raccah *et al.,* 2001). The 11th protein (“pretty interesting Potyviridae ORF”, PIPO) is produced from a shorter polyprotein which is translated when a +2 frame-shift occurs at the terminal 5’ end of the P3

gene within the potyvirus genome (Fig. 1.2 B) (Chung *et al*., 2008). The positive-sense genome can serve as either a template for genome replication or for translation of potyviral proteins. The nucleic acid is enclosed by a capsid protein, comprised of multiple units of an identical type of protein (Ivanov *et al*., 2014).

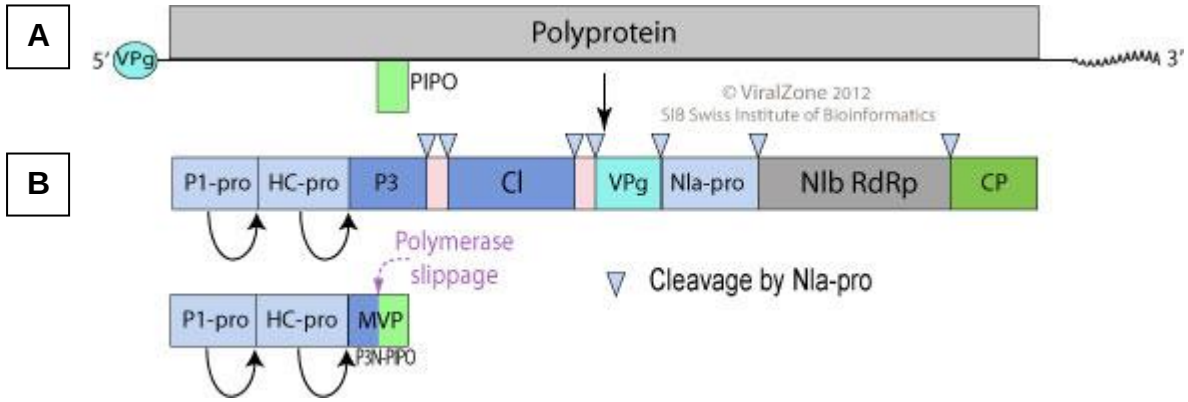

Figure 1.2 Organisation of typical potyvirus genome. **A.** Polyprotein precursor with VPg attached at N terminal end and Poly-A tail at C terminal end. **B.** Different potyviral protein products indicated by coloured blocks with names as abbreviations discussed in text. (Image courtesy SIB Swiss Institute of Bioinformatics; accessed on 20 May 2018; available online at: [https://viralzone.expasy.org/50?outline=all_by_species])

Potyvirus infections involve a complex and tightly coordinated set of molecular pathways which rely on the participation of both viral and host cell proteins (Beauchemin *et al*., 2007; Ivanov *et al*., 2014; Urcuqui-Inchima *et al*., 2001). Tasks such as replication, translation, translocation to neighbouring cells and encapsidation rely on allocating viral genomic material in balanced ratios at the correct time in the infection cycle (Mäkinen & Hafrén, 2014). Currently, it is believed that regulatory ribonucleoproteins (either their availability or competition for their active sites) play a role in executing this balance (Ivanov *et al*., 2014). The molecular methods underlying potyvirus infection are not yet fully understood and a sophisticated array of techniques would need to be combined to elucidate the links between the molecular pathways involved, the roles of host and viral proteins as well as the cellular location and timing of the processes involved (Ivanov *et al*., 2014; Roudet-Tavert *et al*., 2007). Such studies would allow for a greater understanding of potyviral spread within a plant and to new plants, and thus aid: 1) employing strategies to curb the spread of virus, 2) identify when to apply surveillance methods in order to keep

planting material virus free as well as 3) to detect the presence of new infections in the field.

1.2.1.1 *Potyvirus* species already present in South Africa and potentially present on maize

1.2.1.1.1 Sugarcane mosaic virus

Initially reported in *Saccharum* spp. nearly a century ago (Brandes, 1920), SCMV causes large-scale economic losses of sugarcane, sorghum and maize world-wide (Marie-Jeanne *et al*., 2011; Wu *et al*., 2012) and is the main potyvirus associated with MLN infections (Mahuku *et al*., 2015a). Other natural hosts are grasses in the following genera: *Panicum*, *Eleusine* and *Setaria* (L.) (Ford *et al*., 1972; Penrose, 1974; Rosenkranz, 2006; Tosic *et al*., 1972). The most economically important disease caused by potyviruses on sugarcane is known as Sugarcane mosaic disease and is caused by a complex of viruses that consists of four distinct potyviruses: SCMV, JGMV, SrMV and MDMV (Shukla *et al*., 1992).

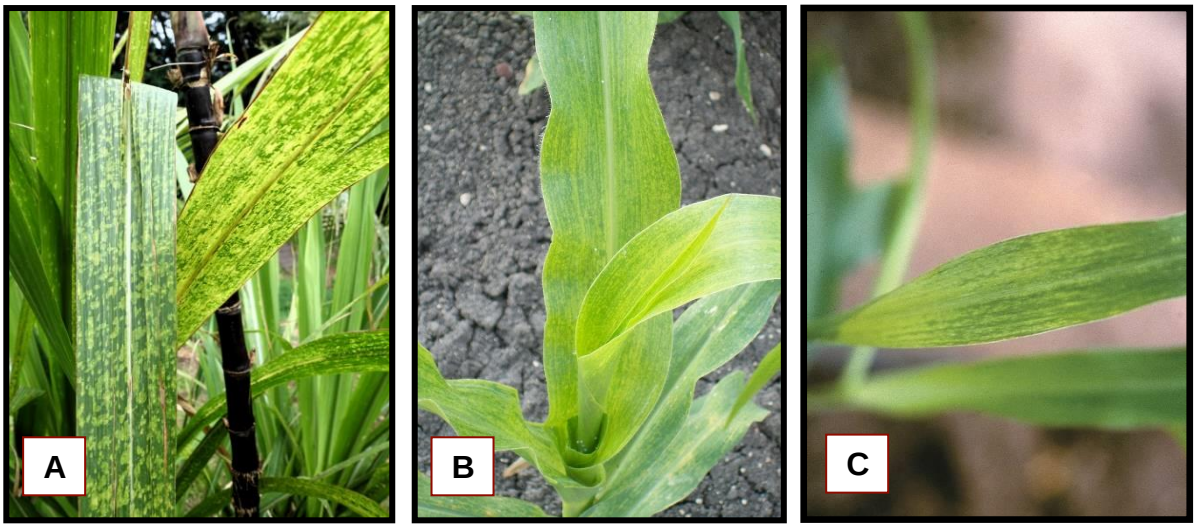

Figure 1.3 Typical symptoms caused by SCMV on maize. **A.** Mosaic on leaves. **B.** Mottling of leaves. **C.** Mottling on young leaf. (Images courtesy: A. Agdia Biofords, 2013; accessed on 20 May 2018; available online at: [http://www.agdia-biofords.com/en/product/sugarcane-mosaic-virus-2]. B & C. The International Maize and Wheat Improvement Center (CIMMYT); accessed on 21 May 2018; available online at: [http://mln.cimmyt.org/mln-field-guide/])

The symptoms caused on its hosts include mosaics (Fig. 1.3 A), mottling (Fig. 1.3 B and C) and stunting amongst others. In maize, stunting and chlorosis are induced and result in reduced grain and forage yield and early infected crops can be

completely barren (Fuchs & Grüntzig, 1995; Shukla *et al*., 1998). Due to the poor proofreading ability of the RdRp and fast multiplication, SCMV has a high mutation rate, which gives rise to numerous strains that occur and replicate in a complex quasispecies within a single plant (Elena & Sanjuán, 2005; Li *et al*., 2013; Xie *et al*., 2016). Consequently, SCMV has a broad genetic diversity amongst its species – between 70% and 99% sequence identities – and the isolates from different hosts and geographical origins have been seen to cluster together phylogenetically (Xie *et al*., 2016).

1.2.1.1.2 Maize dwarf mosaic virus

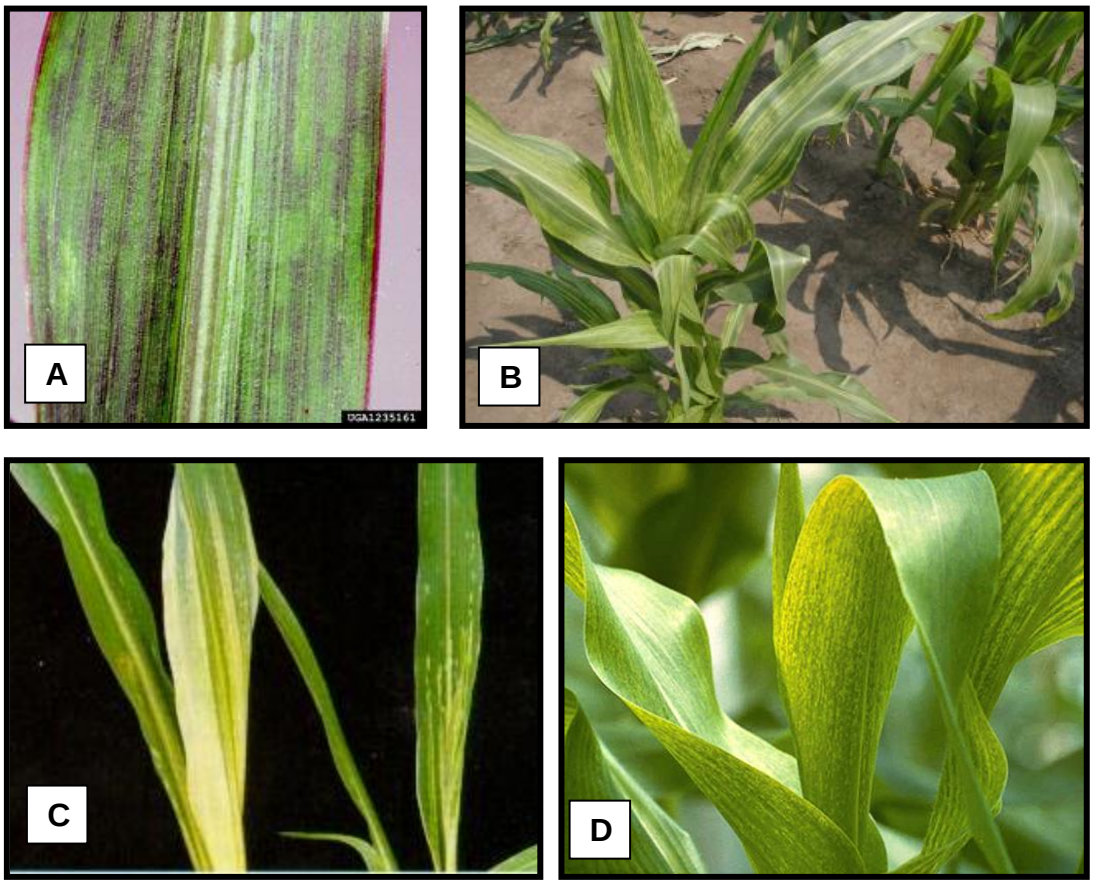

Figure 1.4 Typical symptoms and effects Maize dwarf mosaic virus infections on maize. **A.** Red streaks on leaves. **B.** Dwarfing of plants. **C.** Chlorotic streaks. **D.** Mosaic of leaves. (Images courtesy: A. University of Georgia website; accessed on: 18 May 2018; available online at:[https://www.forestryimages.org/ browse/detail.cfm?imgnum=1235161]. B. Iowa State University website; accessed on: 18 May 2018; available online at: [https://crops.extension.iastate.edu/maize-dwarf-mosaic]. C. Tsai & Falk; University of Minnesota website; accessed on: 18 May 2018; available online at: [https://ipmworld.umn.edu/tsai-maize-tropics]. D. Ryzhkov & Protsenko. Atlas of viral plant diseases. Moscow: Nauka, 1968. 136 pp.)

Janson *et al.* (1965) and Williams and Alexander (1965) first identified MDMV in maize. The virus occurs in temperate regions worldwide and is present in Asia and

the United States of America (USA), and specifically in African countries such as Zambia (Toler *et al*., 1989), Kenya (Thottappilly, 1993) and SA (Knox *et al.,* 1986). It is one of a complex of potyviruses that infect tropical grasses that were revealed to be isolates of four species; MDMV, SCMV, SrMV and JGMV by immunoblot and virion protein sequencing (McKern *et al*., 1991; Shukla & Teakle, 1989). The hosts of MDMV include maize, sorghum and, importantly, *S. halepense* (L.) Persoon, 1807 (Johnsongrass) which acts as a host plant and reservoir for the virus as well as its aphid vectors (Centre for Agriculture and Bioscience International (CABI), 2018).

MDMV causes dwarfing disease of maize (Fig. 1.4 B) and several strains of MDMV exist: A, C, D, E and F (Louie & Knoke, 1975). The symptoms caused by MDMV include chlorotic spotting leading to mottles, mosaics and fine streaks on young leaves (Fig. 1.4 C and D), dark red streaks (Fig. 1.4 A), yellowing of the leaves as the infection progresses resulting in suboptimal photosynthesis and stunted plants, a reduction in the number of ears produced, decelerated ear development which leads to grain loss (Fuchs & Grüntzig, 1995). Yield loss can range from 42% to 75% depending on whether the maize is planted early or late in the season, respectively, of the maize cultivar (CABI, 2018; Louie & Darrah, 1980).

1.2.1.2 Other Poaceae-infecting potyviruses potentially present in South Africa on maize

1.2.1.2.1 Johnsongrass mosaic virus

JGMV was first reported in Johnsongrass and maize by Taylor and Pares (1968) in Australia as Australian MDMV, but later named as a definitive member of the potyvirus group as JGMV by Shukla *et al*. (1987) and had not yet been detected in SA prior to our study. The virus causes mosaic and necrotic symptoms which lead to stunting and yield reduction of maize and *Zea mays* L. var. *rugosa* (sweet corn) as well as fodder, grain and weed sorghums*, Panicum miliaceum* (L.) (proso millet)*, Pennisetum glaucum* (L.) Robert Brown, 1818 (pearl millet) and wild grasses (McDaniel & Gordon, 1985; Teakle & Grylls, 1973).

1.2.1.2.2 Pennisetum mosaic virus

PenMV was most likely first reported as SCMV on *Pennisetum spp.* (L.) (R. Br.) in various earlier studies, possibly including those by Martins and Kitajima (1993) and Rishi *et al*. (1973) and also not yet reported in SA prior to our study. PenMV is a distinct potyvirus in the SCMV sub-group and is most closely related to MDMV, SCMV and SrMV (Deng *et al*., 2008). It is known to display relatively severe mosaic symptoms – compared to SCMV – on the leaves of its hosts (Fan *et al*., 2003; Fan *et al*., 2004). The virus infects indigenous Gramineae species such as *Panicum spp.* (L.)*, Setaria viridis* (L.) Palisot de Beauvois (green foxtail)*, Eleucine indica* (L.) Joseph Gaertner (Goose grass) and *Spodiopogon sibiricus* (Siberian frost grass); as well as commercially planted sorghum and maize (Deng *et al*., 2008). Phylogenetic analyses by Deng *et al*. (2008) of the complete genome and polyproteins indicated that PenMV is a distinct potyvirus within the SCMV subgroup and is closely related to MDMV, SrMV and SCMV.

1.2.1.2.3 Sorghum mosaic virus

First reported in sorghum by Abbott and Tippet (1966), SrMV was originally regarded as a strain of SCMV and therefore strains described as SCMV-I, SCMV-M, SCMV-H and SCMV-H SI are essentially strains of SrMV (Shukla *et al*., 1992). In 1997, Yang and Mirkov used RT-PCR-based Restriction fragment length polymorphism (RFLP) to differentiate SCMV and SrMV strains and confirmed this. The natural hosts of SrMV are sugarcane, where the predominant symptom seen is a mosaic, and sorghum, where mosaic and red-leaf are observed (Brunt *et al*., 2010).

1.2.1.2.4 Zea mosaic virus

ZeMV is a recently described maize-infecting virus in the *Potyvirus* genus, first reported in Israel (Seifers *et al.*, 2000). The virus causes necrosis in its hosts and was able to systemically infect its experimental host, Johnsongrass. Seifers *et al.* (2000) showed by nucleotide and amino acid analysis of different genome regions that ZeMV is a distinct potyvirus and not merely a different strain of the other members of the SCMV subgroup. The virus has not been extensively characterised as SCMV and MDMV.

1.2.1.2.5 Cocksfoot streak virus

CSV is also a recently described monocot-infecting potyvirus with a narrow host range, restricted to members of the Poaceae (Götz & Maiss, 2002). The complete genome sequence was determined by Götz and Maiss (2002). Phylogenetic analysis revealed that, of monocot infecting viruses it was compared to, CSV is most closely related to MDMV and JGMV and is also closely related to potyviruses that infect dicots (Götz & Maiss, 2002).

1.2.2 Wheat streak mosaic virus

WSMV, first identified by McKinney (1937), previously belonged to the *Rymovirus* genus and currently belongs to the *Tritimovirus* genus (Stenger *et al.*, 1998), which is one of the aforementioned eight genera in the Potyviridae family (Scheets, 1998). As with the potyviruses, WSMV has a single-stranded RNA genome, of approximately 8.5 kilo base pairs (kb) in size (Brakke & van Pelt; 1970). The tritimoviruses are mite-transmitted, with WSMV specifically transmitted by *Eriophyes tosichella* Keifer, 1969 (wheat-curl mite) (Slykhuis, 1953) and known to infect a number of species in the Poaceae family (Christian & Willis, 1993; Ellis *et al*., 2004).

1.2.3 Maize chlorotic mottle virus

MCMV is the only (and type) species of the genus *Machlomovirus* which belongs to the Tombusviridae family (Russo *et al*., 1994) as mentioned in the introduction. The virus has a 4.4 kb positive-sense single-stranded RNA genome encapsulated in a 30nm icosahedral-shaped virion (Nutter *et al*., 1989; Scheets, 2004; Wang *et al*., 2017). The genome of MCMV has been sequenced and studied by various groups (Adams *et al*., 2013; Mahuku *et al*., 2015a; Stenger & French, 2008; Wang *et al*., 2015; Wang *et al*., 2017). This unipartite genome encodes six overlapping open reading frames – five of which are essential for viral replication and cell-to-cell movement and one which translates into the Coat protein (Adams *et al*., 2014; Stenger & French, 2008).

Depending on plant genotype, environmental factors and the age of the infection, MCMV on its own causes a variety of disease symptoms in maize (Uyemoto *et al*.,

1981). These include mild necrosis (Fig. 1.5 A) or mottling (Fig. 1.5 B) to severe mottling (Fig. 1.5 C), chlorosis (Fig. 1.5 C), stunting, yellowing and mosaics of the leaves, partially filled ears, malformed or shortened ears or male inflorescences and premature plant death (Goldberg & Brakke, 1987; Uyemoto, 1983). Yield losses can be up to 15% in naturally occurring infections (Uyemoto *et al*., 1981).

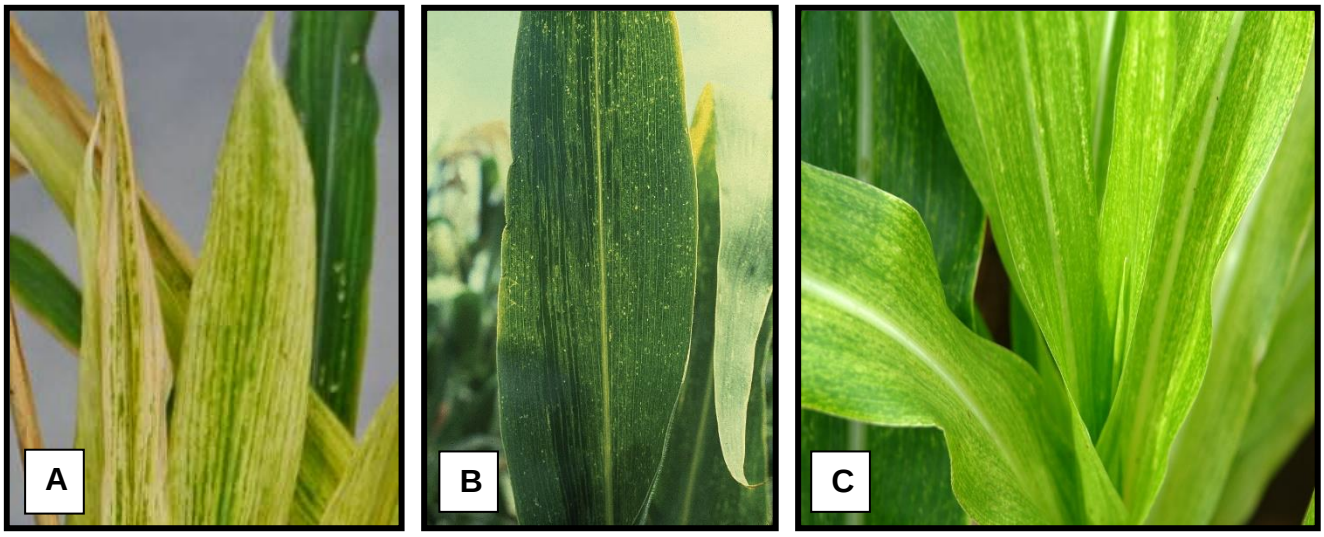

Figure 1.5 Typical symptoms caused by Maize chlorotic mottle virus on maize. A. Leaf necrosis. **B.** Mild mottle on leaves. **C.** Severe mottle and onset of chlorosis. (Images courtesy: A. Laikipia Rural Voices blog, 2015; accessed online; 21 May 2018; available online at:[http://laikipiaruralvoices.blogspot.com/2015/02/understanding-maize-lethal-necrosis.html]. B. CIMMYT; accessed on 21 May 2018; available online at: [http://mln.cimmyt.org/mln-field-guide/]. C. CIMMYT; accessed on 21 May 2018; available online at: [http://mln.cimmyt.org/mln-field-guide/])

The natural host range of MCMV seems to be restricted as it has only been identified in maize (Uyemoto *et al*., 1981), finger millet (Kusia *et al*., 2016) and sugarcane (Wang *et al*., 2014) but experimentally MCMV has been successfully transmitted to at least 19 grass species (Bockelman *et al*., 1982). Mahuku *et al*. (2015a) reported that sorghum, *Pennisetum purpureum* (L.) (R. Br.) (Napier grass), sugarcane and *Pennisetum clandestinum* (Kikuyu grass) (L.) (R. Br.) samples collected from the field in Uganda and Kenya tested positive for MCMV by ELISA (enzyme-linked immunosorbent assay) (Mahuku *et al*., 2015a) but were not verified using RT-PCR.

1.2.4 Maize streak virus

MSV, first named in 1925 (Storey), belongs to the genus *Mastrevirus*, in the family Geminiviridae (Briddon *et al*., 1994; Lazarowitz *et al*., 1987). It was first recorded in SA in 1901 and is widespread in sub-Saharan Africa as well as islands in the Indian

Ocean (Fuller, 1901; Thottappilly *et al*., 1993). MSV has been the subject of an extensive amount of research including its geographical distribution, transmission and vectors, characterisation and resistance breeding to it (Bosque-Pérez, 2000; Sheperd *et al*., 2010; Thottappilly *et al*., 1993). The various MSV strains are known to infect various plants, including sugarcane in SA (Van Antwerpen *et al.*, 2008), which could act as host plants, aiding in its proliferation (Rose, 1978; Rossel & Thottappilly, 1985). It is possible that MSV may play a role in MLND because other maize-infecting viruses that are not potyviruses such as Maize rayado fino virus (MRFV) and Maize mosaic virus (MMV) have also been reported to produce synergistic co-infections with MCMV (Nelson *et al*., 2011) and MSV is widely distributed in sub-Saharan Africa (Bonga & Cole, 1997; CABI & EPPO (European and Mediterranean Plant Protection Organization), 1997; Thottappilly *et al*., 1993).

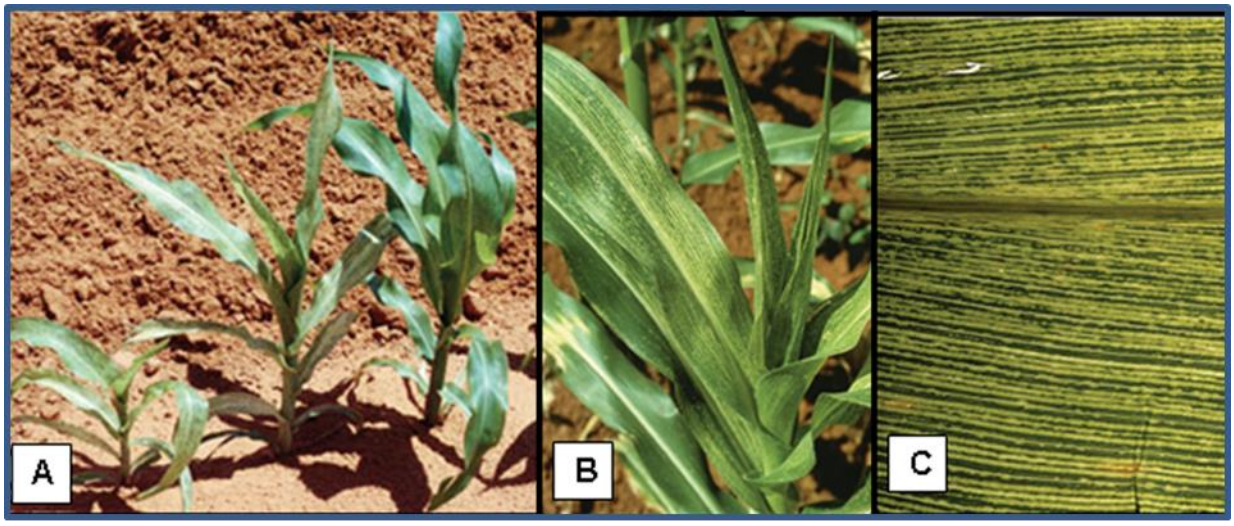

Figure 1.6 Typical symptoms caused by Maize streak virus on maize. **A.** Stunting and reduced vigour. **B.** Streaks on leaves. **C.** Onset of coalescence of chlorotic streaks. (Images courtesy: A. Flett, 2012; accessed on 24 May 2018; available online at: [http://www.grainsa.co.za/transmission-of-maize-streak-virus-from-grasses-to-maize]. B. Thomson, 2004; AgBioForum; accessed on 23 May 2018; available online at: [http://www.agbioforum.org/v7n12/v7n12a02-thomson.htm]. C. Djibnet, 2016; accessed on 23 May 2018; available online at: [http://www.djibnet.com/photo/s%C3%ADntoma/maize-streak-virus-on-maize-leaf-4927612620.html]).

The virus has a circular single-stranded DNA genome – approximately 2700 bp in length – and occurs as twinned or geminate icosahedral particles with 18 x 20 nm being the approximate measurement of each particle (Mullineaux *et al*., 1984; Zhang *et al*., 2001). MSV is vectored by several leafhopper species (family: Cicadellidae) and transmitted in a persistent manner (Storey; 1928; Webb, 1987). Symptoms vary depending on age and genotype of the plant (Pinner *et al*., 1988) but usually begin as sparse pale spots on the base of leaves, and eventually form parallel chlorotic

tallow of white streaks (Fig. 1.6 B and C), and even chlorosis of the whole lamina, which in turn affect the plant's photosynthesis and can lead to shortened internode spaces resulting in stunting (Fig. 1.6 A) and inability to produce completely set cobs or dieback of the plant (Martin & Sheperd, 2009; Martin *et al.*, 2008; Thottappilly *et al*., 1993).

1.3 Impact of Maize Lethal Necrosis Disease on its host

In order to understand the enormity of the impact of this devastating disease, it is important to study the effects caused as well as the extent of losses attributable to MLND.

1.3.1 Virus symptomatology and effects on plant physiology

The symptoms and severity observed due to MLND are influenced by variables that include: the genetic makeup of the plant, the environmental conditions which affect the vigour of the plant, the insect vector movement and populations as well as the growth stage of the plant upon infection (De Groote *et al*., 2016; Gowda *et al*., 2015). For example, if a maize plant is infected early in the cropping cycle, it could result in complete yield loss or dieback of the plant (Uyemoto *et al*., 1980; Wangai *et al.*, 2012a). Symptoms are inadequate to identify the causal agent of the disease due to factors causing similar virus-like symptoms such as nutrient deficiencies, herbicides, unfavourable environmental conditions and other pathogens or pests (Agrios & Hadwiger, 2005; Nelson *et al*., 2011). However, they can be useful for monitoring areas at risk for MLND, to putatively identify infected plants that should be rogued in order to prevent further spread within a field (Kiruwa *et al*., 2016) and when conducting community-based survey assessments to estimate the distribution and impact of a crop disease as was done by De Groote *et al*. (2016).

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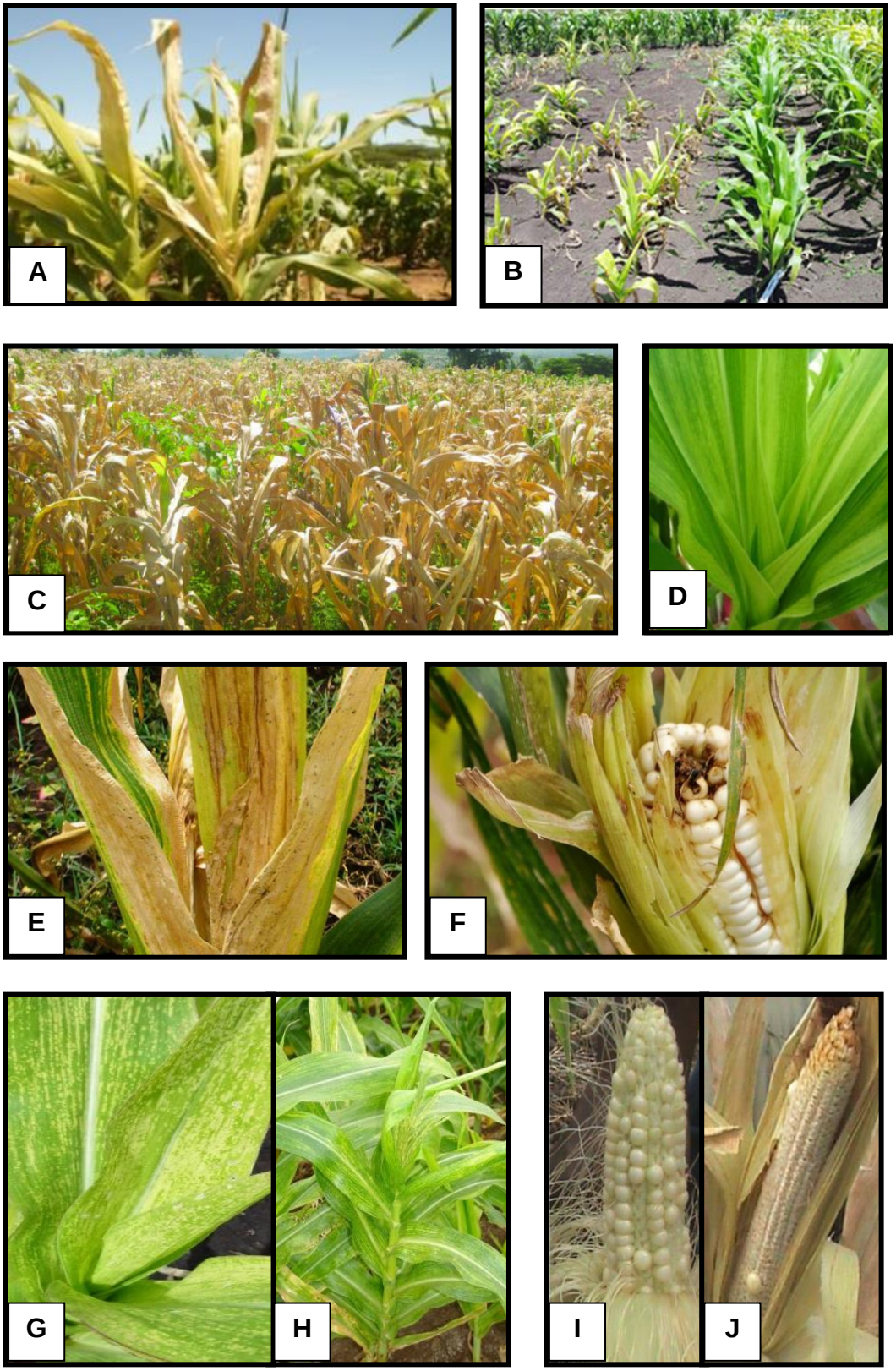

Figure 1.7 Disease symptoms of Maize Lethal Necrosis Disease. **A.** Yellowing and necrosis of leaves. **B.** Stunting of plants. **C.** Extreme necrosis of entire field of crops. **D.** Chlorosis of leaves. **E.** 'Dead heart' symptoms. **F.** Secondary fungal infection on cob. **G.** Chlorotic mottle/mosaic. **H.** Shortened internodes. **I.** Poor seed set. **J.** Severe lack of seed set. (Images courtesy: A, B, D, F, G-J. CIMMYT; accessed on 21 May 2018; available online at: [http://mln.cimmyt.org/mln-field-guide/]. C. The Organic Farmer; 2015; available online at: [http://theorganicfarmer.org/?q=Articles/maize-seed-can-transmit-maize-lethal-necrosis-disease-mln]. E. Wamboga-Mugirya; 2014; accessed on: 21 May 2018; available online at: [http://www.monitor.co.ug/Magazines/Farming/East-African-scientists-in-fight-to-combat-maize-lethal-necrosis/6898602342898-13jh3tf/index.html])

Separately, MCMV and the potyviruses cause some similar symptoms to when the two viruses are in co-infection, but most often when the viruses are in combination a diverse spectrum of virus-like symptoms can be seen (Fig. 1.7). The symptoms and subsequent effects caused by MLND on the host usually start with elongated narrow yellow streaks running parallel to the leaf veins (Fig. 1.7 H), which is the most common symptom observed (Nelson *et al*., 2011) resulting in insufficient photosynthesis taking place. These streaks can coalesce, giving rise to a chlorotic mottle (Fig. 1.7 G) starting at the base of young leaves and extending to the tips.

Chlorotic mottle symptoms are usually followed by further leaf chlorosis (Fig. 1.7 D) and then by leaf necrosis (Fig. 1.7 A and C) – usually starting at leaf margins, progressing to the mid-rib section, the eventual drying of the whole leaf and further reduction the total number of photosynthetic cells (Makone *et al*., 2014; Niblett & Claflin, 1978). Another common symptom referred to as 'dead heart' (Fig. 1.7 E) occurs when the leaves become necrotic in the centre of the whorl before expansion (Wangai *et al*., 2012a).

Other symptoms include shortened internode length (Fig. 1.7 H) leading to stunted plants (Fig. 1.7 B) and premature aging, failure to tassel and sterility in male plants (Uyemoto *et al*., 1981) which leads to small or malformed maize cobs that may fail to bear grains (Fig. 1.7 I and J) as well as secondary fungal infections (Fig. 1.7 F) which cause rotting of the maize cobs which results in severe yield losses or ultimately plant death (Kiruwa *et al*., 2016; Uyemoto *et al*., 1980, Uyemoto *et al*., 1981; Wangai *et al*., 2012a).

1.3.2 Extent of yield losses due to Maize Lethal Necrosis Disease

MLND threatens maize production in developing countries particularly (Mbega *et al.,* 2016) due to the reliance of rural communities on rain-fed, low-input crops such as maize, and it has been estimated that highly infected areas experience tremendous yield losses (Wangai *et al*., 2012b). By 2012, MLND had affected 26 000 km^2 of maize and by 2013, eight of the twenty maize-growing areas of Tanzania had already been reported to have MLND (Makumbi & Wangai, 2013). The exact figures for losses as a result of MLND is not available for many countries, but from the

information that is currently accessible it can be concluded that there is reason for major concern: in 2012, in Kenya, an estimated 22% of maize yield (0.5 million tonnes) was lost due to MLND, which amounted to US$ 52 million worth of losses (FAO, 2012).

A study by De Groote and colleagues (2016) showed that in certain areas in Kenya, farmers had experienced 86%-100% yield loss and up to half of all maize farmers in Western Kenya had MLND to some extent in their fields (De Groote *et al*., 2016). The FAO reported on a large volume of maize exported to Kenya from SA following the 2017/18 harvest year, due to reasons such as pests like *Spodoptera frugiperda* (fall armyworm) and insufficient long rains, but also possibly due to the effect that MLND has already had on production or that farmers were forced to rogue infected crops (FAO, 2018; Kimurto & Jeptanui, 2015). Niblett and Claflin (1978) reported 50%-90% losses in the USA. These losses are predicted to affect the livelihood of the communities that rely on this maize to feed their families and livestock and overall market prices in future.

1.4 Biology and epidemiology of Maize Lethal Necrosis Disease-causing viruses

Various factors contribute to the complexity of the MLND epidemiology and pose further challenges to disease management. Such factors include: the combination of various potential viral pathogens responsible for the establishment of the disease, the wide range of insects able to vectors these pathogens, the variety of mechanisms of both seasonal persistence and pathogen spread, the widespread planting of the natural hosts of these viral pathogens and its continuous spread across countries and continents.

1.4.1 Viral synergy

In terms of symptom severity, a synergistic – rather than additive – increase in symptom severity is observed during co-infection of the host by these two viruses (Fig. 1.7) as well as an increase in viral titer in some cases (Goldberg & Brakke, 1987; Hilker *et al*., 2017; Kiruwa *et al*., 2016; Mahuku *et al*., 2015a; Scheets, 1998). This synergistic increase in viral titer was shown for WSMV with MCMV, where

WSMV concentrations increased by the co-infection and the MCMV concentrations were 3-11 times higher than infection by only MCMV (Scheets, 1998). MCMV concentrations drastically increased when in combination with MDMV, of which the concentration remained the same (Goldberg & Brakke, 1987). In a study by Xie *et al.* (2016), SCMV allowed the multiplication of MCMV to a much higher titer than when MCMV infected a plant alone. The impact of this can be tremendous, for example, MCMV alone causes 10%-15% yield losses in natural infections (Uyemoto *et al*., 1981), but when in co-infection with a potyvirus can be as high as 90% recorded in the US (Uyemoto *et al*., 1980) and 30%-100% in Kenya (Wangai *et al*., 2012a).

The mechanism behind this in the case of MCMV co-infection with a potyvirus is thought to be the supply of HC-Pro movement proteins by the potyvirus, which facilitates cell-to-cell movement as well as long distance transport of both viruses (Syller, 2012) and also acts a strong suppressor of posttranscriptional gene silencing which serves as a defense mechanism against the virus, elicited by the plant (Kasschau *et al.,* 2003). However, in the case of MCMV co-infection with WSMV, Stenger *et al*. (2007) the synergism between WSMV and MCMV was shown to be independent of the WSMV HC-Pro protein, suggesting that WSMV uses a different gene to the HC-Pro to facilitate a similar post-transcriptional gene silencing (PTGS) to achieve synergism (Stenger *et al.*, 2007). MLND acts as a useful model to expand our understanding of synergistic interactions between viruses confined to a host range of monocots only (Scheets, 1998).

1.4.2 Vectors and transmission

Members of the *Potyvirus* genus are transmitted from infected to healthy plants by over 25 members of the Aphinidae family (Brault *et al*., 2010). Insect transmission is classified according to the length of time that the insect needs to feed before the virus can be acquired and the length of time that it takes for the insect to remain viruliferous amongst other factors (Bragard *et al*., 2013). Aphids transmit potyviruses in a non-circulative manner (previously referred to as non-persistent) (Brault *et al*., 2010). Aphids feed using a specialised mouthpart known as a stylet, which is extremely thin and flexible and allows feeding without damage to the cells (Tjallingii & Esch, 1993). Aphids first sample the cell content by probing epidermal layer of

cells, followed by feeding from the phloem, located deep under the epidermis, and while feeding, the aphid produces and excretes salivas containing different components including viruses (Miles *et al*., 1999). Aphids are able to acquire and inoculate viruses at any point in the feeding process, thereby making them suitable to vector many viral taxa with different tissue specificities (Brault *et al*., 2010).

Non-circulative viruses utilise either the 'capsid strategy' or the 'helper strategy' to mediate transmission by aphids, with potyviruses using the latter with the aid of their viral protein, the HC-Pro (Maia *et al*., 1996). The role of this protein in suppressor of gene silencing and viral movement within the plant was discussed above. Virus acquisition and transfer to a new plant can occur within a matter of seconds and aphids are no longer viruliferous a few minutes after acquisition (Brault *et al*., 2010). During vector transmission, much of the variation in a virus population is lost and only dominant strains are transmitted, which increases the probability of the virus success rate (Elena *et al.,* 2011). WSMV is transmitted persistently by mites (Wosula *et al*., 2016). The determinant of this transmissibility is also the WSMV HC-Pro protein (Stenger *et al*., 2005).

MCMV is thought to be transmitted non-circulatively by a number of insects (Cabanas *et al*., 2013; Jiang *et al*., 1992; Nelson *et al*., 2011). It has been observed that Chrysomelid beetles (*Diabrotica* spp.) (LeConte, 1868) transmit MCMV both under experimental conditions and in nature (Jensen *et al*., 1991; Nault *et al*., 1978). Beetles deposit a film of pre-digestive regurgitant material on leaf surfaces as they feed and introduce virus particles into the wounds created by them at the feeding sites (Fulton *et al*., 1987). Rootworms (*D. virgifera*) have been reported as MCMV vectors (Jiang *et al*., 1992; Nault *et al*., 1978) and so also thrips (*Frankliniella williamsi*) (Hood, 1915) from maize (Cabanas *et al*., 2013). Western flower thrips (*F. occidentalis*) (Pergande, 1895) specifically have also been implicated as vectors but this remains to be proven (Zhao *et al.*, 2014). In the case of both thrips and beetles, MCMV is transmitted following an acquisition period of three hours and all growth stages of the insects remain viruliferous for up to six days, but it was not evident if there was a latent period (Cabanas *et al*., 2013). Following surveys of Kenya, Uganda and the Democratic Republic of Congo (DRC), thrips were reported as widespread in East Africa, and several other species of stemborers and leafhoppers

are suspected based on their presence on maize in this survey (Moritz *et al*., 2013) but evidence of their involvement in MCMV spread is yet to be elucidated.

1.4.3 Seasonal persistence

As discussed earlier, in terms of seasonal persistence, MCMV and SCMV can survive in various alternate grass hosts during the maize-growing season and off-season with the aid of the various insect vectors (Kiruwa *et al*., 2016). Results of Bockleman *et al*. (1982) and Mahuku *et al*. (2015a) indicate the likelihood of an even wider range of MCMV reservoir hosts than currently thought. However, studies regarding the ability of insects to transmit MLND-causing viruses to the various alternate hosts remain to be conducted (Mahuku *et al*., 2014). Furthermore, in most areas in eastern Africa, maize, a primary host of MLND-causing viruses is cultivated all year round due to there being two rain seasons.. Soil transmissibility of the viruses, especially MCMV, is another major factor in seasonal persistence, potentially adding an additional element to the complexity of the MLND disease cycle (Nelson *et al*., 2011). Mahuku *et al.* (2015a) reported that when virus-free seed was planted in contaminated soil MCMV was detected using ELISA in 70% of the seedlings.

1.4.4 Mechanisms of dispersal

Insect-vector transmission (discussed in 1.2.1 and 1.2.3) and seed-transmission of MLND-causing viruses are considered the major mechanisms of regional dispersal, although these mechanisms remain to be clarified (Mahuku *et al*., 2015a). Seed transmissibility of SCMV in maize has been observed and, depending on maize genotype, can range from 0.4%-3.9% (Li *et al*., 2011). MDMV is mechanically transmissible and both MDMV and WSMV are seed-transmitted at 0.5%-2.5% and 0.1%, respectively (Hill *et al*., 1974; Mezzalama *et al*., 2005; Toler, 1985). MCMV is seed transmissible to a small – 0%-33% -- but epidemiologically significant extent because even a low rate of seed transmissibility can result in the introduction of pathogens in a new area (Jensen *et al*., 1991). Mahuku *et al.* (2015b) reported the presence of MCMV in up to 72% of the seed samples collected from a MCMV-infected plant, and MCMV was also detected in pooled samples of seeds, but does

not confirm that MCMV will be passed on to the progeny plants and this aspect remains to be studied. MLND-causing viruses are also dispersed by man via transportation, either illegally or legal importation of infected plant material, seed as well as soil containing infected plant debris (Nelson *et al*., 2011).

1.4.5 Host range of Maize Lethal Necrosis Disease-causing viruses when co-infected

The host ranges of the individual viral components of the MLND complex were discussed earlier (1.2.1 and 1.2.3, respectively) and for maize infecting potyviruses are generally narrow and restricted to members of the Poaceae family. Besides maize, MCMV has been reported to infect both sugarcane (Wang *et al*., 2014) and finger millet (Kusia *et al*., 2015) naturally as well as a number of experimental hosts – mostly grasses – reported by Bockleman *et al*. (1982).

Co-infections of MCMV with a potyvirus resulting in MLND are limited usually to maize as the main natural host and more recently reported finger millet (Kusia *et al.*, 2015). During a survey for alternate hosts of MCMV and SCMV conducted in 2014 in MLND hotspots in Kenya, leaves from finger millet with symptoms of viral infection were sampled and both SCMV and MCMV were confirmed by ELISA followed by RT-PCR in a number of these samples. Symptoms observed on finger millet leaves are chlorotic mottle and necrosis of the leaves (Kusia *et al*., 2015).

1.4.6 Geographical distribution

The disease was first detected in Peru in 1974 and first identified in the USA in the late 1970s where MCMV together with WSMV or MDMV caused the disease. It was formally described in Kansas and known as Corn lethal necrosis (Niblett & Claflin, 1978). Thereafter it was reported in Nebraska and Texas (Uyemoto *et al*., 1980) and in Hawaii (Jiang *et al*., 1992). Amongst the South American countries, MLND and consequently also MCMV have been reported in Peru (Castillo & Hebert, 1974; Loayza, 1977), Argentina (Teyssandier *et al*., 1983), Colombia (Morales *et al*., 1999) and more recently Ecuador (Quito-Avila *et al*., 2016). It has also been reported in Asia in countries including in China (Xie *et al*., 2011), Thailand (Sutabutra *et al*., 1982) and Taiwan (Deng *et al*., 2014). The African countries where MCMV has been

detected (and approximately when they were first detected) (indicated in red in Fig. 1.8) include: Kenya (September 2011; Wangai *et al*., 2012a), Tanzania (August 2012; Wangai *et al*., 2012a), Ethiopia (Mahuku *et al*., 2015b), Rwanda (February 2013; Adams *et al*., 2014), Uganda (October 2012; Kagoda *et al*., 2016), and the DRC (Adams *et al*., 2014; Lukanda *et al*., 2014) and suspected but not confirmed in Burundi and Southern Sudan (ASARECA (Association for Strengthening Agricultural Research in Eastern and Central Africa), 2016).

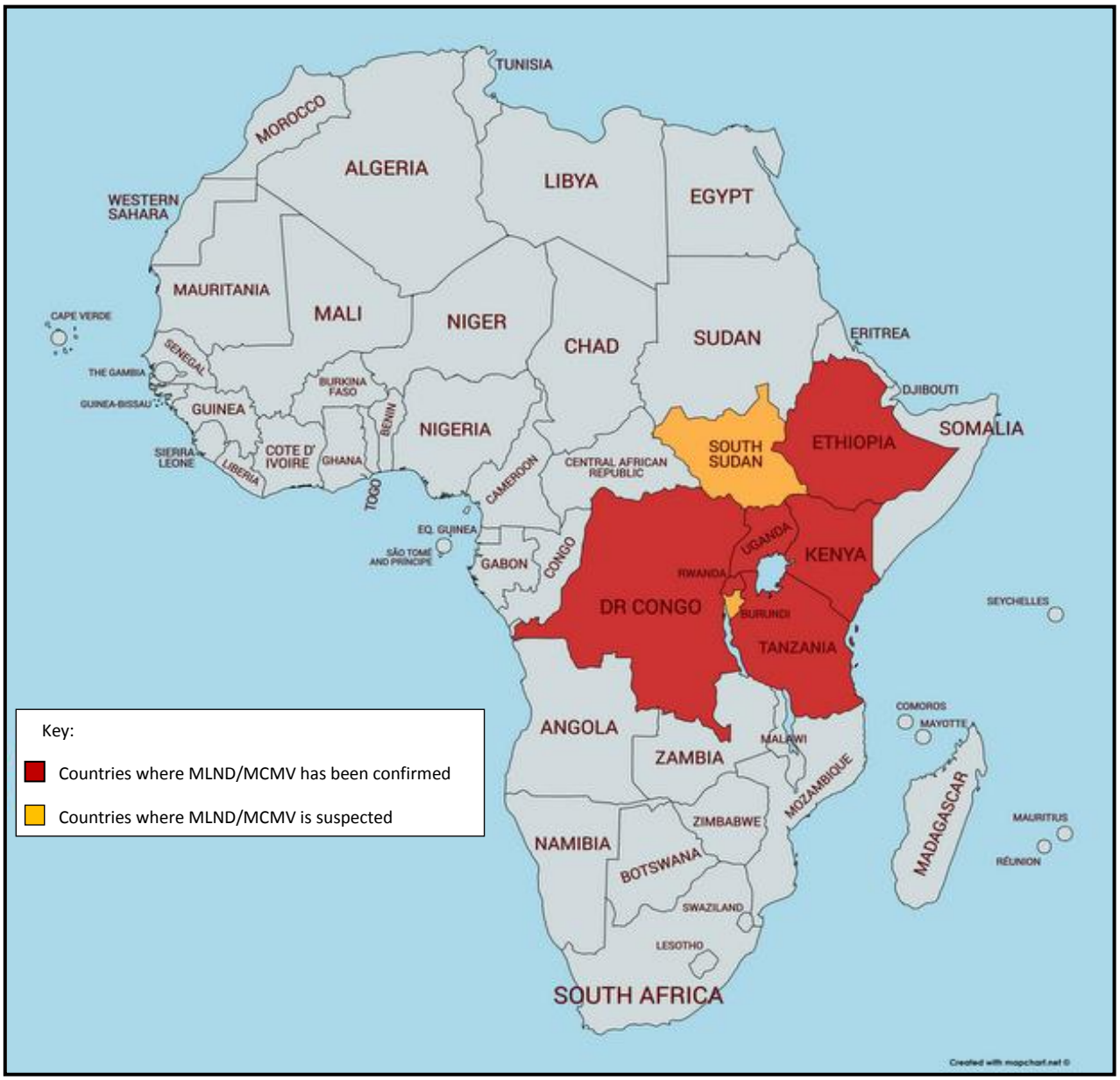

Figure 1.1 Map of Africa indicating countries where Maize Lethal Necrosis Disease/Maize chlorotic mottle virus has been reported: both confirmed (red) and suspected (orange). (Constructed using MapChart; accessed on 21 May 2018; available online: [https://mapchart.net/africa.html]).

1.5 Management and future prospects of MLND

A few successful MLND management attempts have been recorded in the literature including in Hawaii, where MCMV control was achieved by combining cultural practices, insecticides against vectors and virus tolerant plants (Nelson *et al*., 2011); and in the USA, where crop rotation aided in the reduction of MCMV incidence (Phillips *et al.*, 1982; Uyemoto, 1983). In East Africa, however, it is not yet clear what the ideal combination of vector management, host resistance and agronomic practices might entail as relatively little knowledge and resources are available and therefore such research is imperative. However, it is apparent that in the management of any disease it is important to direct efforts towards implementation of cultural practices in combination with molecular and field research. Such research needs to focus on all the aspects the disease: the viruses, the vectors, the host, and environment – by scientists (Agrios & Hadwiger, 2005; Redinbaugh & Zambrano-Mendoza, 2014) and collaboration with the local and national authorities.

1.5.1 Cultural practices-based management

Due to the fact that research takes a large amount of time, expertise and funding, cultural practices are the best place to start when attempting to manage a sudden and serious disease epidemic such as MLND in Africa. One of the biggest problems in management of MLND in East and Central Africa is that maize is grown continuously throughout the year and there are multiple cropping cycles in regions where the climate allows, causing build-up of contaminated plant debris and soil (De Groote *et al*., 2016). Implementing a closed maize-growing season and therefore avoidance of back-to-back planting seasons can reduce the load and carry-over from season to season: maize should not be planted in the short rain season, but rather in the main rain season to achieve an isolated planting season and thus aid in reducing disease persistence (Wangai *et al*., 2012b).

Infected plants need to be correctly identified and then rogued to eliminate the source of viruses and thereby avoid subsequent infections. Alternate host plants of the viruses can be removed so as to limit the presence of reservoir hosts (Phillips *et al.*, 1982; Uyemoto, 1983). Maize can be alternated with non-host crops such as potatoes, bananas, cassava, legumes, vegetables and onions in and effort to reduce

virus density (Makone *et al*., 2014). Farming implements also need to be cleaned thoroughly and plant debris and stumps from previous seasons should be removed from the soil (De Groote *et al*., 2016). Where possible, insecticides can be used to limit levels of virus vectors although this will not control migrating aphids (Agrios & Hadwiger, 2005).

Due to aphids' migratory abilities, lack of virus specificity, and mechanism of transfer the control of potyviruses is difficult (Ahlquist *et al*., 2003; Gibbs *et al*., 2008) but by planting maize early in the season to avoid key aphid population times, potyvirus infections can also be greatly reduced. However, because potyviruses such as SCMV are already relatively widespread compared to MCMV (Agrios & Hadwiger, 2005), and MCMV is implicated in almost all cases of MLND (Mahuku *et al*., 2015a), strategies should rather focus on avoiding and excluding MCMV infections specifically.

1.5.2 Research-based management

1.5.2.1 Focus on the viruses

1.5.2.1.1 Pathogen identification and disease diagnostics

According to Adams *et al*. (2013) and Kreuze *et al*. (2009), surveillance, early warning and fast implementation of disease management strategies, quick and accurate diagnostic tools are crucial for controlling the dispersal of pathogens. Visible symptoms are one of the definite signs that a plant is affected by a biotic or abiotic factor in the field. Diagnosing MLND based on symptoms alone is challenging and usually insufficient, as symptoms vary based on various factors, and herbicide damage, somatic mutations or nutrient deficiencies may resemble symptoms (Agrios & Hadwiger, 2005). Fortunately, different combinations of symptomatology, electron microscopy, serological tests and molecular techniques, depending on the available resources, allow for accurate detection and identification of the causal agent of viral diseases such as MLND (Kiruwa *et al*., 2016).

Since the 1960s, serological methods have formed part of the main diagnostic routine of plant viruses (Martin *et al*., 2000). Tests such as ELISA are efficient for

testing large numbers of samples while still retaining relatively high specificity (Boonham *et al*., 2014; Naidu *et al*., 2003). However, commercial ELISA kits may not always be useful in detecting SCMV or MCMV due to the lack of specificity of the antisera to all strains of these viruses (Adams *et al*., 2013). Therefore, variations of RT-PCR are used for the routine detection of MLND-causing viruses more often.

Before molecular techniques can be applied, total nucleic acid (RNA, in the case of potyviruses) is extracted and isolated from the plant material in question. High yields and relatively pure RNA can be extracted using cetyltrimethylammonium bromide (CTAB) such as the method used by White *et al*. (2008), which is less expensive than using commercial RNA extraction kits. Variants of molecular detection techniques include basic PCR, Reverse Transcription-PCR, nested PCR, real-time PCR, immunocapture PCR, loop-mediated isothermal amplification (LAMP) and multiplex-PCR and RT-PCR (Chen *et al*., 2017; Lopez *et al*., 2003; Webster *et al*., 2004). Detection of RNA viruses is achieved by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) or real-time RT-PCR, using the extracted viral RNA (amongst total extracted RNA from the plant material) as the template. RT-PCR allows rapid detection, and has the hypothetical sensitivity to detect a single target RNA strand, furthermore the primer binding is also highly specific and the test results are obtained relatively rapidly (Naidu *et al*., 2003). The PCR products can then be sequenced in order to identify specific species or strains of a virus (Webster *et al*., 2004).

For the genus *Potyvirus*, various pairs of degenerate primers have been used in RT-PCR reactions (Ha *et al*., 2008; Langeveld *et al*., 1991; Pappu *et al*., 1993; Zheng *et al*., 2010). Pappu *et al*. (1993) used the same sites (Fig. 1.9, blue arrows) as Langeveld *et al*. (1991) to develop their primers that were successful in detecting potyviruses and produced a PCR product approximately 700 bp in size. However, occasional problems arose, for example the reverse primer used by both Langeveld *et al*. (1991) and Pappu *et al*. (1993) contains a T-rich region – designed to bind to the poly-A tail at the 3’ end of the potyvirus genome – which: 1) caused occasional non-specific amplification in healthy tissue extracts because the oligo-dT primers can prime and amplify the polyadenylated mRNA (messenger RNA) of the plant RNA as well, as well as 2) occasional background virus fragments produced as mismatches occurred between the 3’ termini of the primers and the Poly-A tail and thus caused

amplification of a single product during PCR to be challenging (Pappu *et al*., 1993; Zheng *et al*., 2010) and therefore neds to be coupled with Sanger sequencing for accurate diagnosis.

More recently, a set of genus-specific primers, NIb3R and NIb2F that binds to two conserved sites within the NIb gene (RdRp) (Fig. 1.9, red arrows) have been developed for the aim of diagnosis of a potyvirus infection within a plant to the genus level. These sites flank a variable region, and the binding of the primers primes the amplification of this region, yielding a 350 bp fragment (Zheng *et al*., 2010). Zheng *et al.* (2010) showed that these primers allow the detection of all major groups belonging to the genus and that when analysed on an agarose gel, amplicons showed banding patterns that were simpler to interpret as fewer products were seen than with the primers used by Pappu *et al.* (1993) (Zheng *et al*., 2010). Furthermore, the cDNA (complementary DNA) that was synthesised had a consistent length. For these reasons, this primer pair was chosen for initial potyvirus detection in our study.

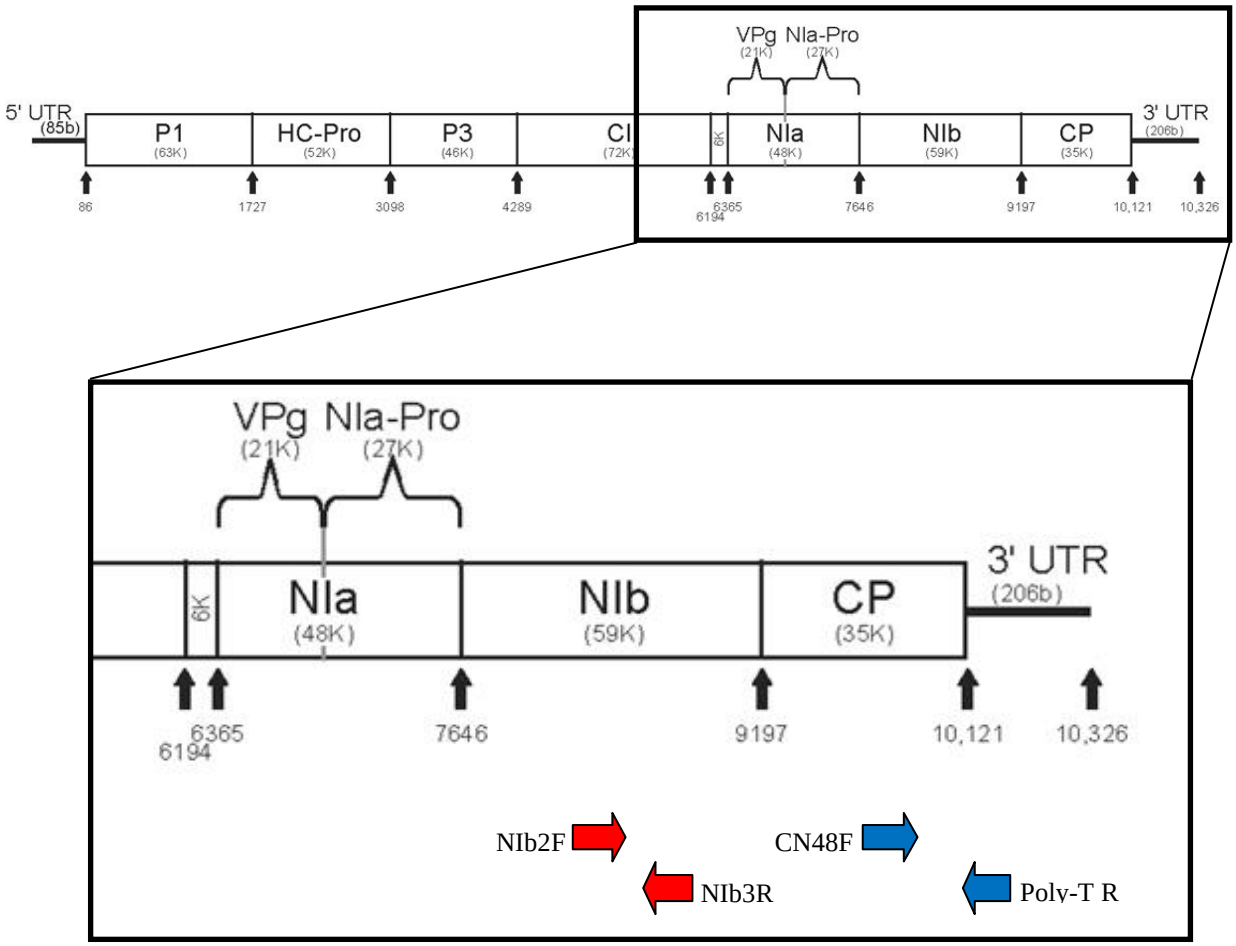

Figure 1.2 Schematic diagram of the binding sites of degenerate potyvirus primers developed by Pappu *et al*. (1993) (indicated by blue arrows) and Zheng *et al*. (2010) (indicated by red arrows) within the genome. (Image courtesy: Goncalves, 2010; APS; accessed on: 27 May 2018; available online at: [https://www.apsnet.org/edcenter/intropp/lessons/viruses/Pages/PapayaRingspotvirus.aspx]).

A potyvirus can be identified to the species level by direct Sanger sequencing of an amplicon to obtain its nucleotide sequence followed by comparison of the nucleotide sequence to previously obtained potyvirus genomes and DNA sequences on a database such as the NCBI (National Center for Biotechnology Information) Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Webster *et al*., 2004). Species demarcation depends on the species definition, which is currently proposed by the ICTV (International Committee on Taxonomy of Viruses) to be 85% nucleotide identity across the genome or 85% similarity in amino acid sequence over the Coat protein gene. The primers used by Zheng and colleagues, followed by subsequent Sanger sequencing, have been used to identify a novel potyvirus, Commelina mosaic virus (CoMV) on *Commelina spp.* (Zheng *et al.*, 2011), Peanut mottle virus (PeMoV) (Beikzadeh *et al*., 2015) and Bean yellow mosaic virus on three novel host plants in SA (Schulze *et al.*, 2017).

In maize seeds, MCMV is most often tested using real-time RT-PCR due its superior sensitivity compared to conventional RT-PCR and the low titer of virus usually present in seed (Adams *et al*., 2014; Adams *et al*., 2013). However, conventional RT-PCR has been routinely employed for the detection of MCMV and the potyviruses Sugarcane streak mosaic virus (SCSMV), Sugarcane yellow leaf virus (SCYLV) in sugarcane (Viswanathan *et al*., 2009; Xie *et al*., 2009) as well as the detection of SCMV in maize and sorghum (Rafael *et al*., 2013). However, no single test has been developed to our knowledge to test for MCMV and SCMV (or any other potyvirus) simultaneously.

1.5.2.1.2 Focus on the virus: surveys and diversity studies

In order to determine the geographical distribution of MCMV and SCMV, maize fields in Kenya, Uganda and Tanzania were surveyed from 2012 to 2014 (Mahuku *et al*., 2015a). In Tanzania, 60% of the nearly 2500 samples collected were positive for MCMV and only 28% were positive for SCMV. In Nigeria, pre-emptive surveys were conducted in 2014 to study SCMV and MLN-like diseases, showing that MSV was present in 66% of the fields but SCMV and MCMV were absent. Mahuku *et al*. (2015a) stated that it is necessary for countries in sub-Saharan Africa where MLND is not yet present to conduct similar surveys in order to identify other viruses or pathogens that may play a role in disease development as the information obtained

will be important when seed production and development strategies need to be established and containment protocols need to be drawn up (Mahuku *et al*. 2015a).

The genetic diversity of SCMV from various countries where MLND is present have been studied and have indicated that at least two groups of SCMV exist in Kenya of which one shares a common ancestor with Asian isolates (Mahuku *et al*. 2015a). Studies by Adams *et al*. (2014) and Adams *et al.* (2013) reported that East African MCMV is also genetically most similar to Asian isolates and implicate a shared origin. However, the epidemics in China and Africa occurred at a similar time and questions about the origin of East African MCMV remain and such an investigation may aid in the prevention of future outbreaks (Mahuku *et al*., 2015a). Some surveys or diversity studies on potyviruses have been conducted (Frenkel *et al*., 1991; Gemechu *et al*., 2006; Gonçalves *et al*., 2011; Götz *et al*., 1995; Kabululu *et al*., 2017; Stewart *et al*., 2014; Wang *et al.*, 2010; Xiao *et al*., 1993) but none have been conducted specifically on potyviruses from maize in Africa.

In 1998, Goodman and colleagues conducted a study to identify the SCMV strains present on sugarcane in SA to study their genetic diversity. Their study relied mainly on RT-PCR and Sanger sequencing for the diagnostics but sampling only took place in one province. The Coat protein region of four SCMV isolates from sugarcane were analysed, as most variation amongst strains resides in this region and enabled strains to be distinguished from one another (Goodman *et al*., 1998). Studies by Alegria *et al.* (2003) and Handley *et al.* (1998) also included 3 and 1 (respectively) SCMV isolates from SA in their SCMV diversity studies, but all isolates were isolated from sugarcane. However, in SA, knowledge about the maize-infecting potyvirus species, distribution and genetic diversity is limited and such surveys and studies need to be conducted (Flett & Mashingaidze, 2016).

1.5.2.2 Focus on the vectors

Some aforementioned studies report insects that transmit the MLND-causing viruses and identified potential insect vectors that require further analysis (Cabanas *et al*., 2013; Moritz *et al*., 2013; Nault *et al*., 1978; Nyasani *et al*., 2012; Zhao *et al*., 2014). Moritz *et al.* (2013) reported the use of diagnostic tools that can accurately identify

East African thrips. Knowing the identities of the vectors will aid in decisions regarding the insecticides to be used or developed and will be useful in predictive risk assessment studies. However, many other insects could potentially vector these viruses and much remains to be elucidated regarding: 1) the identities MCMV vectors, 2) their ecology, 3) the mechanisms behind MCMV transmission, 4) their role in dispersal of MLND-causing viruses, 5) their competence and efficiency as MCMV vectors, 6) robust diagnostic tools for other vectors will greatly aid the management of the disease (Mahuku *et al*., 2015a).

1.5.2.3 Focus on the host

Ultimately, disease resistant or tolerant maize lines would be the answer to control the dispersal of the causal agents of MLND on a molecular level as resistant varieties are a reliable, environmentally friendly and economical (over time) method to achieve disease free crops (Iqbal *et al*., 2017; Jones *et al*., 2018; Kumar *et al*., 2004; Manje *et al*., 2015; Marenya *et al*., 2018). Currently, ASARECA is working at producing resistant varieties of maize in eastern Africa (ASARECA, 2016). Mahuku *et al*. (2015a) reported on experiments in Kenya and Ohio that attempted to pinpoint sources of resistance or tolerance by studying maize seed germplasm. Studies in Kenya suggest that some maize lines are potentially resistant with some inbred lines (N211 and KS23-6) developing milder symptoms later in the study period. Other lines also showed delay in onset of symptoms, but at the end of the study period all lines tested positive for both the viruses (Mahuku *et al.*, 2015b). The tolerant lines identified in Mahuku *et al*. (2015a)'s study could potentially be developed into MLND-resistant hybrids in future (Mahuku *et al*., 2015b). Furthermore, genome-wide association studies have aided in identifying 1) certain gene regions that may be associated with MLND resistance, 2) single nucleotide polymorphisms that are associated with disease tolerance and 3) quantitative trait loci in the same regions that have been connected to virus resistance in the past (Redinbaugh & Zambrano-Mendoza, 2014). However, due to the high costs and time of this research, currently, other strategies of integrated disease management mentioned earlier as well as research regarding the pathogens and their vectors are imperative.

1.5.2.4 Focus on the environment

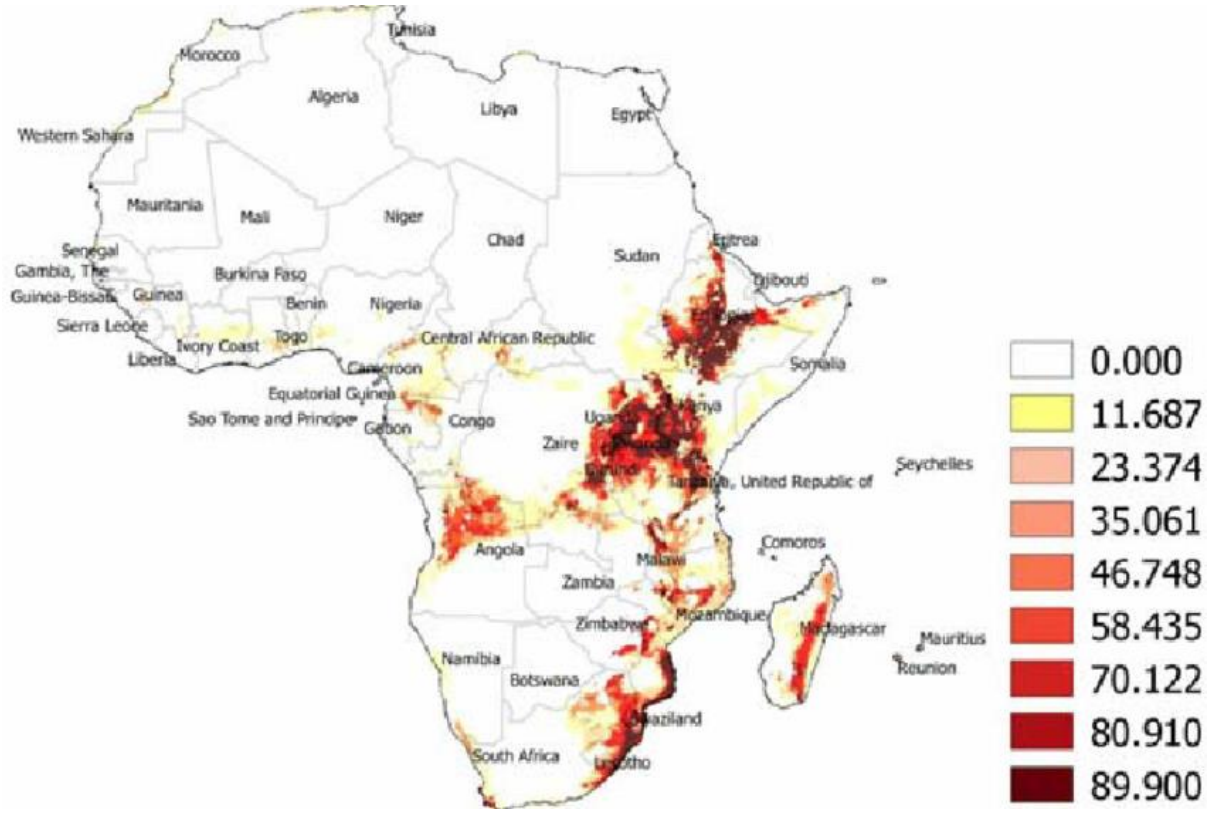

Figure 1.3 Map of Africa showing potential risk areas for MCMV spread and therefore also areas at risk for Maize Lethal Necrosis Disease outbreaks by 2050. Darkest colours indicate areas with the most suitable habitat for Maize Lethal Necrosis Disease to establish, and therefore also the areas at highest risk. (Image courtesy: Isabirye & Rwomushana, 2016).

Studying the current disease emergence and correlating that with the relevant environmental and landscape data allow researchers to predict and extrapolate the data to identify regions with climatic conditions that may favour MLND emergence. Thus, areas at risk of MLND outbreaks can be identified, which can lead to the establishment of successful response strategies in these priority regions (Lopez-Gardenas *et al*., 2005; Sarkar *et al*., 2010). As mentioned in the introduction, Isabirye and Rwomushana (2016) employed the Genetic Algorithm for Rule-Set Prediction (GARP) (Stockwell & Peters, 1999), which is a robust evolutionary computing application, in their ecological niche modelling in order to gain insight into the potential risk areas for MLND. It also makes future predictions by taking into account climatic data, current distribution data of MCMV and the climate change forecasts.

They predict that MCMV can manifest in a diverse range of climatic conditions including warm arid, semi-arid and sub-humid tropics and predicted that the highest risk areas (indicated by warmest colours on Fig. 1.10) in the following countries: Kenya, Tanzania, Uganda, Rwanda, Burundi, DRC, Angola and Ethiopia. Other noteworthy areas include: SA, Lesotho, Swaziland, Botswana and Mozambique. The current MLND risk for suitable MLND habitat in SA was estimated at 298 402km^2, and the prediction for 2020 increased to 337 870km^2, with a further slight increase to 342 082km^2 by 2050 (Isabirye & Rwomushana, 2016).

1.5.3 Authority-based management

Poverty and lack of resources is a big concern hindering the effective management of MLND as subsistence farmers cannot finance the application of pesticides to control the insect vectors of the viruses and cannot afford certified seed and often recycle seed too (De Groote *et al*., 2016). At a national level, governments can aim to supply certified virus-free seed as well as insecticides against insect vectors to subsistence farmers. Funds for research should be made available and surveillance should be encouraged (Makone *et al*., 2014). CIMMYT (International Maize and Wheat Improvement Center) and the National Maize Program have established a screening facility in Kenya in an effort to aid in effective pathogen identification. Furthermore, authorities can ensure MCMV exclusion from a specific area or country by ensuring strict quarantine when maize is imported. Quarantine services are provided by the FAO and are regarded as one of the most effective strategies of limiting the introduction of MCMV in new regions (Adams *et al*., 2014). Educating farmers through educational campaigns about the cultural practices discussed earlier will also aid in reducing virus infections and further spread as Makone *et al*. (2014) stated that various studies indicated that farmers lacked knowledge on MLND and its management.

1.6 Concluding Remarks

This review confirms that the threat that MLND poses to Africa's food security is far-reaching and is especially concerning to SA given that a significant part of SA's maize production area is a suitable habitat for MLND to manifest, should MCMV enter the country. It also highlighted that the incurable nature of viral diseases on

plants, lack of strict quarantine, distribution of infected plant material by mankind, as well as environmental abiotic and biotic factors such as climate and insect vectors, in both Africa and globally, contribute greatly to the proliferation of MLND and make it especially difficult to manage. The importance of the maize crop as a source of food, fodder, fuel and, in effect, livelihood in many parts of the world and especially in Africa, and its consequently particular economic significance, was also emphasised. Furthermore, it was underlined that ensuring SA's maize remains MLND free is imperative due to the important role that SA plays as a net exporter of maize and therefore also in food security for other countries and this endeavour can be aided by adequate knowledge resources for quarantine services and supported through research.

In a country where MCMV is not yet present, but where MLND is predicted to establish in future, research regarding the identities, diversity and optimal diagnostics of viruses currently present in that country form part of the foundation of imperative pre-emptive research. In SA, uncertainty remains regarding the potyvirus species present on maize, their distribution, the symptoms that they cause, their diversity and how they are related to the potyviruses present in other countries, among other aspects. A diagnostic tool for the simultaneous detection of the major MLND-causing viruses also remains to be developed. Thus, knowledge of the identities, distribution and genetic diversity of these pathogens in SA as well as an accurate, rapid and inexpensive diagnostic technique will aid in pre-empting MLND before MCMV enters SA and ultimately implementing management strategies, should MCMV enter the country.

In conclusion, this review clearly illustrated that the first steps in pre-empting the entry of MLND to SA, which are thus the aims of this study, are to: 1) determine the status and distribution of the potyviruses within SA, 2) elucidate the genetic diversity of South African potyviruses in a global context, 3) develop a multiplex RT-PCR system for the detection of the viruses predicted to be present in MLN infections, should MCMV enter the country. Achieving these aims will prepare SA, should MCMV enter the country, by contributing directly to the scientific resources and tools that are available to researchers and thereby pre-empting MLND in a country where the future of an exceptionally vital food-security crop is under threat.

References

Abbott EV and Tippett RL (1966) Strains of Sugarcane mosaic virus. *US Department of Agricultural Technology Bulletin* **1340**:25.

Achon MA, Larrañaga A & Alonso-Dueñas N (2012) The population genetics of Maize dwarf mosaic virus in Spain. *Archives of Virology* **157**: 2377-2382.

Adams IP, Harju VA, Hodges T, Hany U, Skelton A, Rai S, Deka MK, Smith J, Fox A & Uzayisenga B (2014) First report of Maize Lethal Necrosis Disease in Rwanda. *New Disease Reports* **29**: 22-22.

Adams IP, Miano D, Kinyua Z, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R & Hany U (2013) Use of next-generation sequencing for the identification and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus causing Maize Lethal Necrosis in Kenya. *Plant Pathology* **62**: 741-749.

Agrios GN & Hadwiger LA (2005) Diseases caused by Potyviridae. In: *Plant pathology*. Elsevier Academic Press: Amsterdam. 764-771.

Ahlquist P, Noueiry AO, Lee W, Kushner DB & Dye BT (2003) Host factors in positive-strand RNA virus genome replication. *Journal of Virology* **77**: 8181-8186.

Alegria OM, Royer M, Bousalem M, Chatenet M, Peterschmitt M, Girard J & Rott P (2003) Genetic diversity in the Coat protein coding region of eighty-six Sugarcane mosaic virus isolates from eight countries, particularly from Cameroon and Congo. *Archives of Virology* **148**: 357-372.

ASARECA (2016) NaCRRI, ASARECA Maize Lethal Necrosis Disease trials in Eastern Uganda. *ASARECA website*. Available online at: http://www.asareca .org/highlights/nacrri-asareca-mlnd-trials-eastern-uganda. [25 October 2016].

Beauchemin C, Boutet N & Laliberté J-F (2007) Visualization of the interaction between the precursors of VPg, the viral protein linked to the genome of Turnip mosaic virus, and the translation eukaryotic initiation factor iso 4E *in planta*. *Journal of Virology* **81**: 775-782.

Beikzadeh N, Hassani-Mehraban A & Peters D (2015) Molecular identification of an isolate of Peanut Mottle Virus (PeMoV) in Iran. *Journal of Agricultural Science and Technology* **17**: 765-776.

Beyene Y, Gowda M, Suresh LM, Mugo S, Olsen M, Oikeh SO, Juma C, Tarekegne A & Prasanna BM (2017) Genetic analysis of tropical maize inbred lines for resistance to Maize Lethal Necrosis Disease. *Euphytica: International Journal of Plant Breeding* **213**: 1-13.

Bockelman D, Claflin L & Uyemoto J (1982) Host range and seed-transmission studies of Maize chlorotic mottle virus in grasses and corn. *Plant Disease* **66**: 216-218.

Bonga J & Cole D (1997) Identification of viruses infecting maize in Zimbabwe. *African Plant Protection* **3**: 1-9.

Boonham N, Kreuze J, Winter S, van der Vlugt R, Bergervoet J, Tomlinson J & Mumford R (2014) Methods in virus diagnostics: from ELISA to next generation sequencing. *Virus Research* **186**: 20-31.

Bosque-Pérez NA (2000) Eight decades of Maize streak virus research. *Virus Research* **71**: 107-121.

Bragard C, Caciagli P, Lemaire O, Lopez-Moya J, MacFarlane S, Peters D, Susi P & Torrance L (2013) Status and prospects of plant virus control through interference with vector transmission. *Annual Review of Phytopathology* **51**: 177-201.

Braidwood L, Quito-Avila DF, Cabanas D, Bressan A, Wangai A & Baulcombe DC (2018) Maize chlorotic mottle virus exhibits low divergence between differentiated regional sub-populations. *Scientific Reports* **8**: 1173.

Brakke MK & Van Pelt N (1970) Properties of infectious ribonucleic acid from Wheat streak mosaic virus. *Virology* **42**: 699-706.

Brandes EW (1920) Mosaic disease of corn. *Journal of Agricultural Research* **19**: 517-521.

Brandes J & Wetter C (1959) Classification of elongated plant viruses on the basis of particle morphology. *Virology* **8**: 99-115.

Brault V, Uzest M, Monsion B, Jacquot E & Blanc S (2010) Aphids as transport devices for plant viruses. *Comptes Rendus Biologies* **333**: 524-538.

Briddon R & Markham P (1995) Use of PCR in the detection and characterization of geminiviruses. *European and Mediterranean Plant Protection Organization Bulletin* **25**: 315-320.

Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ, Watson L & Zurcher EJ (eds.) (2010) *Plant Viruses Online: Descriptions and Lists from the VIDE Database*. Available online at: http://biology.anu.edu.au/Groups/MES/vide. [15 January 2018].

Cabanas D, Watanabe S, Higashi C & Bressan A (2013) Dissecting the mode of Maize chlorotic mottle virus transmission (Tombusviridae: Machlomovirus) by *Frankliniella williamsi* (Thysanoptera: Thripidae). *Journal of Economic Entomology* **106**: 16-24.

CABI (2018) Maize dwarf mosaic virus Datasheet. Available online at: https://www.cabi.org/isc/datasheet/8157. [4 March 2018].

CABI & EPPO (1997) Maize streak monogeminivirus. *Distribution Maps of Plant Diseases*, 1st edition. CAB International: Wallingford, UK. Map 739.

Castillo J & Hebert T (1974) A new virus disease of maize in Peru. *Fitopatologia* **9**: 79-84.

Chen J & Adams M (2001) A universal PCR primer to detect members of the Potyviridae and its use to examine the taxonomic status of several members of the family. *Archives of Virology* **146**: 757-766.

Chen L, Jiao Z, Liu D, Liu X, Xia Z, Deng C, Zhou T & Fan Z (2017) One-step reverse transcription loop-mediated isothermal amplification for the detection of Maize chlorotic mottle virus in maize. *Journal of Virological Methods* **240**: 49-53.

Christian ML & Willis WG (1993) Survival of Wheat streak mosaic virus in grass hosts in Kansas for wheat harvest to fall wheat emergence. *Plant Disease*. **77**: 239-242.

Chung BYW, Miller WA, Atkins JF & Firth AE (2008) An overlapping essential gene in the Potyviridae. *Proceedings of the National Academy of Sciences* **105**: 5897-5902.

De Groote H, Oloo F, Tongruksawattana S & Das B (2016) Community-survey based assessment of the geographic distribution and impact of Maize Lethal Necrosis (MLN) Disease in Kenya. *Crop Protection* **82**: 30-35.

Deng T-C, Chou C-M, Chen C-T, Tsai C-H & Lin F-C (2014) First report of Maize chlorotic mottle virus on sweetcorn in Taiwan. *Plant Disease* **98**: 1748.

Deng C, Wang W, Wang Z, Jiang X, Cao Y, Zhou T, Wang F, Li H & Fan Z (2008) The genomic sequence and biological properties of Pennisetum mosaic virus, a novel monocot-infecting potyvirus. *Archives of Virology* **153**: 921-927.

Edwardson J, Christie R & Ko N (1984) Potyvirus cylindrical inclusions- Subdivision-IV. *Phytopathology* **74**: 1111-1114.

Elena SF, Bedhomme S, Carrasco P, Cuevas JM, de la Iglesia F, Lafforgue G, Lalić J, Pròsper À, Tromas N & Zwart MP (2011) The evolutionary genetics of emerging plant RNA viruses. *Molecular Plant-Microbe Interactions* **24**: 287-293.

Elena SF & Sanjuán R (2005) Adaptive value of high mutation rates of RNA viruses: separating causes from consequences. *Journal of Virology* **79**: 11555-11558.

Ellis M, Rebetzke G, Kelman W, Moore C & Hyles J (2004) Detection of Wheat streak mosaic virus in four pasture grass species in Australia. *Plant Pathology* **53**: 239.

Fan Z, Chen H, Cai S, Deng C, Wang W, Liang X & Li H (2003) Molecular characterization of a distinct potyvirus from whitegrass in China. *Archives of Virology* **148**: 1219-1224.

Fan Z, Wang W, Jiang X, Liang X, Wang F & Li H (2004) Natural infection of maize by Pennisetum mosaic virus in China. *Plant Pathology* **53**: 796.

FAO (2012) Sub-Regional Emergency Office for Eastern & Central Africa (REOA). Factsheet: Smallholders and family farmers. Available online at:

http://www.fao.org/fileadmin/templates/nr/sustainability_pathways/docs/Factsheet_Smallholders.pdf. [28 January 2018].

FAO (2013) Maize Lethal Necrosis Disease (MLND) - A snapshot. FAO Sub-Regional Emergency Office for Eastern & Central Africa (REOA). Available online at: http://www.fao.org/emergencies/resources/documents/resources-detail/en/c/179179/. [10 June 2017].

FAO (2018) Country briefs: South Africa. *FAO*, South Africa. Available online at: http://www.fao.org/giews/countrybrief/country.jsp?code=ZAF. [10 February 2018].

FAOSTAT (2018) *FAO*, South Africa. Available online at: http://www.fao.org/faostat/en/#data/QC/visualize. [9-21 May 2018].

Fentahun M, Feyissa T, Abraham A & Kwak HR (2017) Detection and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus associated with Maize Lethal Necrosis Disease in Ethiopia: an emerging threat to maize production in the region. *European Journal of Plant Pathology* **149**: 1011-1017.

Flett B & Mashingaidze K (2016) Maize Lethal Necrosis: Possible threat to local maize production. *Grain SA*. Available online at: http://www.grainsa.co.za/maize-lethal-necrosis:-possible-threat-to-local-maize-production. [23 October 2017].

Ford RE & Tosic M (1972) New hosts of Maize dwarf mosaic virus and Sugarcane mosaic virus and a comparative host range study of viruses infecting corn. *Journal of Phytopathology* **75**: 315-348.

Frenkel MJ, Jilka J, McKern NM, Strike PM, Clark Jr J, Shukla DD & Ward CW (1991) Unexpected sequence diversity in the amino-terminal ends of the Coat proteins of strains of Sugarcane mosaic virus. *Journal of General Virology* **72**: 237-242.

Fuchs E & Grüntzig M (1995) Influence of Sugarcane mosaic virus (SCMV) and Maize dwarf mosaic virus (MDMV) on the growth and yield of two maize varieties. *Journal of Plant Diseases and Protection* **102**: 44-50.

Fuller C (1901) First report of the government entomologist, Natal. 1899-1900. Available online at: http://archive.org/stream/reportgovernmen00agrigoog/reportgovernmen00agrigoog_djvu.txt. [10 January 2018].

Fulton J, Gergerich R & Scott H (1987) Beetle transmission of plant viruses. *Annual Review of Phytopathology* **25**: 111-123.

Gemechu AL, Chiemsombat P, Attathom S, Reanwarakorn K & Lersrutaiyotin R (2006) Cloning and sequence analysis of Coat protein gene for characterization of Sugarcane mosaic virus isolated from sugarcane and maize in Thailand. *Archives of Virology* **151**: 167-172.

Gibbs AJ, Ohshima K, Phillips MJ & Gibbs MJ (2008) The prehistory of potyviruses: their initial radiation was during the dawn of agriculture. *Public Library of Science One* **3**: e2523.

Giolitti F, Herrera M, Madariaga M & Lenardon S (2005) Detection of Maize dwarf mosaic virus (MDMV). *Maydica* **50**: 101-104.

Goldberg K-B & Brakke MK (1987) Concentration of Maize chlorotic mottle virus increased in mixed infections with Maize dwarf mosaic virus, strain B. *Phytopathology* **177**: 162-169.

Gonçalves MC, Galdeano DM, de G. Maia I & Chagas CMOL (2011) Genetic variability of Sugarcane mosaic virus causing Maize Mosaic in Brazil. *Pesquisa Agropecuária Brasileira* **46**: 362-369.

Goodman B, MacDonald D & Huckett B (1998) A survey of South African Sugarcane mosaic virus (SCMV) strains based on CP gene sequence analysis. *Proceedings of the South African Sugarcane Technologists' Association* **72**: 146-148.

Götz R, Huth W & Maiss E (1995) Molecular analyses of the Coat protein region of different viruses on Poaceae belonging to the Potyviridae. *Agronomie* **15**: 491-494.

Götz R & Maiss E (2002) The complete sequence of the genome of Cocksfoot streak virus (CSV), a grass infecting potyvirus. *Archives of Virology* **147**: 1573-1583.

Gowda M, Das B, Makumbi D, Babu R, Semagn K, Mahuku G, Olsen MS, Bright JM, Beyene Y & Prasanna BM (2015) Genome-wide association and genomic prediction of resistance to Maize Lethal Necrosis Disease in tropical maize germplasm. *Theoretical and Applied Genetics: International Journal of Plant Breeding Research* **128**: 1957-1968.

Ha C, Coombs S, Revill P, Harding RM, Vu M & Dale JL (2008) Design and application of two novel degenerate primer pairs for the detection and complete genomic characterization of potyviruses. *Archives of Virology* **153**: 25-36.

Handley J, Smith G, Dale J & Harding R (1998) Sequence diversity in the Coat protein coding region of twelve Sugarcane mosaic potyvirus isolates from Australia, USA and South Africa. *Archives of Virology* **143**: 1145-1153.

Harris DR & Hillman GC (2014) Maize: domestication, racial evolution, and spread. In: *Foraging and farming: the evolution of plant exploitation*. Routledge: London. 632-658.

Hilker FM, Allen LJS, Bokil VA*, et al.* (2017) Modeling virus co-infection to inform management of Maize Lethal Necrosis in Kenya. *Phytopathology* **107**: 1095-1108.

Hill J, Martinson C & Russell W (1974) Seed transmission of Maize dwarf mosaic and Wheat streak mosaic viruses in maize and response of inbred lines 1. *Crop Science* **14**: 232-235.

Huang J, Wen G, Li M, Sun C, Sun Y, Zhao M & He Y (2016) First report of Maize chlorotic mottle virus naturally infecting sorghum and coix seed in China. *Plant Disease* **100**: 1955-1955.

Iken J & Amusa N (2004) Maize research and production in Nigeria. *African Journal of Biotechnology* **3**: 302-307.

Iqbal MS, Jabbar B, Sharif MN, Ali Q, Husnain T & Nasir IA (2017) *In silico* MCMV silencing concludes potential host-derived miRNAs in maize. *Frontiers in Plant Science* **8**: 372.

Isabirye EB & Rwomushana I (2016) Current and future potential distribution of Maize chlorotic mottle virus and risk of Maize Lethal Necrosis Disease in Africa. *Journal of Crop Protection* **5**: 215-228.

ISSCT, 1989. Sugarcane diseases and their world distribution. In: *Diseases of Sugarcane.* Ricaud C, Egan BT, Gillaspie AG Jr, Hughes CG (eds.). Elsevier Academic Press: New York, USA. 341-376.

Ivanov KI, Eskelin K, Lõhmus A & Mäkinen K (2014) Molecular and cellular mechanisms underlying potyvirus infection. *Journal of General Virology* **95**: 1415-1429.

Ivanov KI & Mäkinen K (2012) Coat proteins, host factors and plant viral replication. *Current Opinion in Virology* **2**: 712-718.

Janson BF, Williams LE, Findley W, Dollinger E & Ellett C (1965) Maize dwarf mosaic: new corn virus disease in Ohio. *Plant Disease Reporter* **47**: 1107-1108.

Jensen S, Wysong D, Ball E & Higley P (1991) Seed transmission of Maize chlorotic mottle virus. *Plant Disease* **75**: 497-498.

Jiang J & Zhou X (2002) Maize Dwarf Mosaic Disease in different regions of China is caused by Sugarcane mosaic virus. *Archives of Virology* **147**: 2437-2443.

Jiang X, Meinke L, Wright R, Wilkinson D & Campbell J (1992) Maize chlorotic mottle virus in Hawaiian-grown maize: vector relations, host range and associated viruses. *Crop Protection* **11**: 248-254.

Jones MW, Penning BW, Jamann TM, Glaubitz JC, Romay C, Buckler ES & Redinbaugh MG (2018) Diverse chromosomal locations of quantitative trait loci for tolerance to Maize chlorotic mottle virus in five maize populations. *Phytopathology* **108(6)**: 748-758.

Kabululu MS, Ndunguru J, Ndakidemi P & Feyissa T (2017) Genetic diversity of maize accessions for Maize Lethal Necrosis Disease resistance. *Indian Journal of Agricultural Research* **51**: 17-24.

Kagoda F, Gidoi R & Isabirye BE (2016) Status of Maize Lethal Necrosis in eastern Uganda. *African Journal of Agricultural Research* **11**: 652-660.

Kasschau KD & Carrington JC (1998) A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell* **95**: 461-470.

Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA & Carrington JC (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. *Developmental Cell* **4**: 205-217.

Kimurto P & Jeptanui L (2015) Beware, lethal maize disease is still on prowl. *Daily Nation.* Available online at: https://www.nation.co.ke/business/seedsofgold/Beware-lethal-maize-disease-is-still-on-prowl/2301238-2757856-t4es9jz/index.html. [8 March 2018].

Kiruwa FH, Feyissa T & Ndakidemi PA (2016) Insights of Maize Lethal Necrotic Disease: a major constraint to maize production in East Africa. *African Journal of Microbiology Research* **10**: 271-279.

Knox A, Chauhan R & von Wechmar B (1986) *Mixed infections of Maize dwarf mosaic virus (MDMV) and Cucumber mosaic virus (CMV) in maize*. Technical Communication - Department of Agriculture and Water Supply, South Africa.

Kreuze JF, Perez A, Untiveros M, Quispe D, Fuentes S, Barker I & Simon R (2009) Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. *Virology* **388**: 1-7.

Kumar PP, Mohan S & Balasubramanian G (2004) Effect of whole-pea flour and a protein-rich fraction as repellents against stored-product insects. *Journal of Stored Products Research* **40**: 547-552.

Kusia E, Subramanian S, Nyasani J, Khamis F, Villinger J, Ateka E & Pappu H (2015) First report of Lethal Necrosis Disease associated with co-infection of finger millet with Maize chlorotic mottle virus and Sugarcane mosaic virus in Kenya. *Plant Disease* **99**: 899-900.

Langeveld SA, Dore J-M, Memelink J, Derks AF, van der Vlugt CI, Asjes CJ & Bol JF (1991) Identification of potyviruses using the Polymerase Chain Reaction with degenerate primers. *Journal of General Virology* **72**: 1531-1541.

Lapierre H & Signoret P-A (2004) Poaceae family. In: *Viruses and virus diseases of Poaceae (Gramineae)*. Editions Quae, Paris. 13-47.

Lazarowitz SG, Pinder AJ, Damsteegt VD & Rogers SG (1989) Maize streak virus genes essential for systemic spread and symptom development. *The European Molecular Biology Organization Journal* **8**: 1023-1032.

Li L, Wang X & Zhou G (2011) Effects of seed quality on the proportion of seed transmission for Sugarcane mosaic virus in maize. *Cereal Research Communications* **39**: 257-266.

Li Y, Liu R, Zhou T & Fan Z (2013) Genetic diversity and population structure of Sugarcane mosaic virus. *Virus Research* **171**: 242-246.

Loayza J (1977) Maize virus and virus-like diseases in Peru. *International Journal of Pest Management* **27(3)**: 363-369.

López MM, Bertolini E, Olmos A, Caruso P, Gorris MT, Llop P, Penyalver R & Cambra M (2003) Innovative tools for detection of plant pathogenic viruses and bacteria. *International Microbiology* **6**: 233-243.

López-Cardenas J, Bravo FCG, Schettino PMS, Solorzano JSG, Barba ERZ, Mendez JM, Nchez-Cordero VS, Peterson AT & Ramsey JM (2005) Fine-Scale predictions of distributions pf Chagas Disease vectors in the state of Guanajuato, Mexico. *Journal of Medical Entomology*. **42**(6): 1068-1081.

Louie R & Knoke JK (1975) Strains of Maize dwarf mosaic virus. *Plant Disease Reporter* **59**: 518-522

Louie R & Darrah L (1980) Disease resistance and yield loss to Sugarcane mosaic virus in East African-adapted maize. *Crop Science* **20**: 638-640.

Lukanda M, Owati A, Ogunsanya P, Valimunzigha K, Katsongo K, Ndemere H & Kumar PL (2014) First report of Maize chlorotic mottle virus infecting maize in the Democratic Republic of the Congo. *Plant Disease* **98**: 1448-1448.

Makone SM, Menge D & Basweti E (2014) Impact of Maize Lethal Necrosis Disease on maize yield: a case of Kisii, Kenya. *International Journal of Agricultural Extension* **2**: 211-218.

Mahuku G, Lockhart BE, Wanjala B, Jones MW, Kimunye JN, Stewart LR, Cassone BJ, Sevgan S, Nyasani JO & Kusia E (2015a) Maize Lethal Necrosis (MLN), an emerging threat to maize-based food security in sub-Saharan Africa. *Phytopathology* **105**: 956-965.

Mahuku G, Wangai A, Sadessa K, Teklewold A, Wegary D, Adams I, Smith J, Braidwood L, Feyissa B & Regassa B (2015b) First report of Maize chlorotic mottle virus and Maize Lethal Necrosis on maize in Ethiopia. *Plant Disease* **99**: 1870.

Maia IG, Haenni A-L & Bernardi F (1996) Potyviral HC-Pro: a multifunctional protein. *Journal of General Virology* **77**: 1335-1341.

Mäkinen K & Hafrén A (2014) Intracellular co-ordination of potyviral RNA functions in infection. *Frontiers in Plant Science* **5**: 110.

Makone SM, Menge D & Basweti E (2014) Impact of Maize Lethal Necrosis Disease on maize yield: a case of Kisii, Kenya. *International Journal of Agricultural Extension* **2**: 211-218.

Makumbi D & Wangai A (2013) Maize Lethal Necrosis (MLN) disease in Kenya and Tanzania: Facts and actions. Available online at: http://www.cimmyt.org/en/where-

we-work/africa/item/maize-lethal-necrosis-mln-disease-in-kenya-and-tanzania-facts-and-actions. [27 January 2018].

Manje G, Biswanath D, Dan M, Raman B, Kassa S, George M, Michael SO, Jumbo MB, Yoseph B & Boddupalli MP (2015) Genome-wide association and genomic prediction of resistance to Maize Lethal Necrosis Disease in tropical maize germplasm. *Theoretical and Applied Genetics* **128**: 1957-1968.

Marenya PP, Erenstein O, Prasanna B, Makumbi D, Jumbo M & Beyene Y (2018) Maize Lethal Necrosis Disease: evaluating agronomic and genetic control strategies for Ethiopia and Kenya. *Agricultural Systems* **162**: 220-228.

Marie-Jeanne V, Signoret PA, Hariri D & Doucet R (2011) First report of Sugarcane mosaic virus on maize in the centre region of France. *Plant Disease* **95**: 70.

Martins CR & Kitajima EW (1993) A unique virus isolated from elephant grass. *Plant Disease* **77**: 726-729

Martin DP & Shepherd DN (2009) The epidemiology, economic impact and control of Maize Streak Disease. *Food Security* **1**: 305-315.

Martin DP, Shepherd DN & Rybicki EP (2008) Maize streak virus. In: *Encyclopaedia of Virology*, 3rd edition. Mahy, BWJ & Van Regenmortel MHV (eds.). Elsevier Academic Press, Oxford. 263–272.

Martin RR, James D & Lévesque CA (2000) Impacts of molecular diagnostic technologies on plant disease management. *Annual Review of Phytopathology* **38**: 207-239.

Mbega E, Ndakidemi P, Mamiro D, Mushongi A, Kitenge K & Ndomba O (2016) Role of potyviruses in synergistic interaction leading to Maize Lethal Necrotic Disease on maize. *International Journal of Current Microbiology and Applied Sciences* **5**: 85-96.

McDaniel L & Gordon D (1985) Identification of a new strain of Maize dwarf mosaic virus. *Plant Disease* **69**: 602-607.

McKern N, Shukla D, Toler R, Jensen S, Tosic M, Ford R, Leon O & Ward C (1991) Confirmation that the Sugarcane mosaic virus subgroup consists of four distinct potyviruses by using peptide profiles of Coat proteins. *Phytopathology* **81**: 1025-1029.

McKinney H (1937) Mosaic diseases of wheat and related cereals. United States Department of Agriculture Circular No. 442. 1-23.

Mezzalama M, Gilchrist L & McNab A (2005) Seed health: rules and regulations for the safe movement of germplasm. CIMMYT: Mexico. 20.

Miles PW (1999) Aphid saliva. *Biological Reviews* **74**: 41-85.

Moritz G, Brandt S, Triapitsyn S & Subramanian S (2013) *Identification and information tools for pest thrips in East Africa.* QAAFI Biological Information Technology: Brisbane.

Mullineaux P, Donson J, Morris-Krsinich B, Boulton M & Davies J (1984) The nucleotide sequence of Maize streak virus DNA. *The European Molecular Biology Organization Journal* **3**: 3063-3068.

Naidu R & Hughes JdA (2003) Methods for the detection of plant virus diseases. In: *Plant Virology in sub-Saharan Africa*. Hughes JdA & Odu BO (eds.). Proceedings of a Conference Organized by IITA, International Institute of Tropical Agriculture, Nigeria. 233–253.

Nault L, Styer W, Coffey M, Gordon D, Negi L & Niblett C (1978) Transmission of Maize chlorotic mottle virus by Chrysomelid beetles. *Phytopathology* **68**: 1071-1074.

Nelson S, Brewbaker J & Hu J (2011) Maize chlorotic mottle. *Plant Disease* **79**: 1-6.

Niblett C & Claflin L (1978) Corn Lethal Necrosis-a new virus disease of corn in Kansas. *Plant Disease Reporter* **62**: 15-19.

Nutter R, Scheets K, Panganiban L & Lommel S (1989) The complete nucleotide sequence of the Maize chlorotic mottle virus genome. *Nucleic Acids Research* **17**: 3163-3177.

Nyasani J, Meyhöfer R, Subramanian S & Poehling HM (2012) Effect of intercrops on thrips species composition and population abundance on French beans in Kenya. *Entomologia Experimentalis et Applicata* **142**: 236-246.

Onasanya R, Aiyelari O, Onasanya A, Oikeh S, Nwilene F & Oyelakin O (2009) Growth and yield response of maize (*Zea mays* L.) to different rates of nitrogen and phosphorus fertilizers in southern Nigeria. *World Journal of Agricultural Sciences* **5**: 400-407.

Pappu S, Brand R, Pappu H, Rybicki E, Gough KH, Frenkel MJ & Niblett C (1993) A Polymerase Chain Reaction method adapted for selective amplification and cloning of 3' sequences of potyviral genomes: application to Dasheen mosaic virus. *Journal of Virological Methods* **41**: 9-20.

Penrose L (1974) Distribution of a Johnsongrass strain of Sugarcane mosaic virus in New South Wales and studies of the host range of the Johnsongrass and sugarcane strains. *Australian Journal of Agricultural Research* **25**: 99-104.

Phillips N, Uyemoto J & Wilson D (1982) Maize chlorotic mottle virus and crop rotation: effect of sorghum on virus incidence. *Plant Disease* **66**: 376-379.

Pinner M, Markham P, Markham R & Dekker L (1988) Characterization of Maize streak virus: description of strains; symptoms. *Plant pathology* **37**: 74-87.

Quito-Avila DF, Alvarez RA & Mendoza AA (2016) Occurrence of Maize Lethal Necrosis in Ecuador: a disease without boundaries? *European Journal of Plant Pathology* **146**: 705-710.

Raccah B, Huet H & Blanc S (2001) Potyviruses. In: *Virus-insect-plant Interactions.* Harris K, Duffus JE & Smith OP (eds.). Academic Press: San Diego. 181-206.

Rafael H, de Souza I, Barros B, Pinto M, Moreira R, Gonçalves I & Carvalho S (2013) Molecular detection of the Sugarcane mosaic virus causing Mosaic disease in maize and sorghum in Brazil. *Proceedings of Congresso Brasileiro de Genética*, 59. 94.

Redinbaugh MG & Zambrano JL (2014) Control of virus diseases in maize. *Control of Plant Virus Diseases: Seed-Propagated Crops* **90**: 391-429.

Rishi N, Okuda S, Arai K, Doi Y, Yora K & Bhargava K (1973) Mycoplasma-like bodies, possibly the cause of grassy shoot disease of sugarcane in India. *Japanese Journal of Phytopathology* **39**: 429-431.

Roossinck MJ (2015) Plants, viruses and the environment: ecology and mutualism. *Virology* **479**: 271-277.

Rose D (1978) Epidemiology of Maize Streak Disease. *Annual Review of Entomology* **23**: 259-282.

Rosenkranz E (1983) Susceptibility of representative native Mississippi grasses in six subfamilies to Maize dwarf mosaic virus strains A and B and Sugarcane mosaic virus strain B. *Phytopathology* **73**: 1314.

Rosenkranz E (2006) Grasses native or adventive to the United States as new hosts of Maize dwarf mosaic and Sugarcane mosaic viruses. *Phytopathology* **68**: 175-179.

Rossel H & Thottappilly G (1985) Virus diseases of important food crops in tropical Africa. *Virus diseases of important food crops in tropical Africa*. International Institute of Tropical Agriculture: Nigeria. 1-61.

Roudet-Tavert G, Michon T, Walter J, Delaunay T, Redondo E & Le Gall O (2007) Central domain of a potyvirus VPg is involved in the interaction with the host translation initiation factor eIF4E and the viral protein HcPro. *Journal of General Virology* **88**: 1029-1033.

Russo M, Burgyan J & Martelli GP (1994) Molecular biology of Tombusviridae. *Advances in Virus Research* **44**: 381-428.

Rybicki EP & Hughes FL (1990) Detection and typing of Maize streak virus and other distantly related geminiviruses of grasses by Polymerase Chain Reaction amplification of a conserved viral sequence. *Journal of General Virology* **71**: 2519-2526.

Sarkar S, Strutz SE, Frank, DM, Rivaldi CL & Sissel B (2010) Chagas Disease risk in Texas. *Public Library of Science Neglected Tropical Diseases*. **4**(10): e836. DOI: 10.1371/ journal.pntd.0000836.

Scheets K (1998) Maize chlorotic mottle machlomovirus and Wheat streak mosaic Rymovirus concentrations increase in the synergistic disease Corn Lethal Necrosis. *Virology* **242**: 28-38.

Scheets K (2000) Maize chlorotic mottle machlomovirus expresses its Coat protein from a 1.47-kb subgenomic RNA and makes a 0.34-kb subgenomic RNA. *Virology* **267**: 90-101.

Schulze A, Roberts R & Pietersen G (2017) First Report of the Detection of Bean yellow mosaic virus (BYMV) on *Tropaeolum majus*; *Hippeastrum spp*., and *Liatris spp.* in South Africa. *Plant Disease* **101**: 846.

Seifers D, Salomon R, Marie-Jeanne V, Alliot B, Signoret P, Haber S, Loboda A, Ens W, She Y-M & Standing K (2000) Characterization of a novel potyvirus isolated from maize in Israel. *Phytopathology* **90**: 505-513.

Shen Z, Qu W, Wang W, Lu Y, Wu Y, Li Z, Hang X, Wang X, Zhao D & Zhang C (2010) MPprimer: a program for reliable multiplex PCR primer design. *BMC Bioinformatics* **11**: 143.

Shepherd RJ (1965) Properties of a mosaic virus of Corn and Johnsongrass and its relation to the Sugarcane mosaic virus. *Phytopathology* **55**: 1250-1256.

Shepherd DN, Martin DP, Van der Walt E, Dent K, Varsani A & Rybicki EP (2010) Maize streak virus: an old and complex 'emerging' pathogen. *Molecular Plant Pathology* **11**: 1-12.

Shiferaw B, Prasanna BM, Hellin J & Bänziger M (2011) Crops that feed the world 6. Past successes and future challenges to the role played by maize in global food security. *Food Security* **3**: 307.

Shukla DD, Frenkel MJ, McKern NM, Ward CW, Jilka J, Tosic M & Ford R (1992) Present status of the sugarcane mosaic subgroup of potyviruses. *Potyvirus Taxonomy.* Springer: New York. 363-373.

Shukla DD, Gough K & Ward C (1987) Coat protein of potyviruses. *Archives of Virology* **96**: 59-74.

Shukla DD & Teakle D (1989) Johnsongrass mosaic virus. *Mycological Institute and the Association of Applied Biologists Descriptions of Plant Viruses*. Available online at: http://www.dpvweb.net/dpv/showadpv.php?dpvno=340.

Slykhuis J (1953) Wheat streak mosaic in Alberta and factors related to its spread. *Canadian Journal of Agricultural Science* **33**: 195-197.

Smith GR & Velde R (1994) Detection of Sugarcane mosaic virus and Fiji Disease virus in diseased sugarcane using the Polymerase Chain Reaction. *Plant Disease* **78**: 557-561.

Stenger DC & French R (2008) Complete nucleotide sequence of a Maize chlorotic mottle virus isolate from Nebraska. *Archives of Virology* **153**: 995-997.

Stenger DC, Hall JS, Choi I-R & French R (1998) Phylogenetic relationships within the family Potyviridae: Wheat streak mosaic virus and Brome streak mosaic virus are not members of the genus *Rymovirus*. *Phytopathology* **88**: 782-787.

Stenger DC, Hein GL, Gildow FE, Horken KM & French R (2005) Plant virus HC-Pro is a determinant of Eriophyid mite transmission. *Journal of Virology* **79**: 9054-9061.

Stenger DC, Young BA, Qu F, Morris TJ & French R (2007) Wheat streak mosaic virus lacking Helper Component-Proteinase is competent to produce disease synergism in double infections with Maize chlorotic mottle virus. *Phytopathology* **97**: 1213-1221.

Stewart LR, Redinbaugh MG, Massawe D, Willie K, Wijeratne S, Niblett CL, Kiggundu A & Asiimwe T (2017) Johnsongrass mosaic virus contributes to Maize Lethal Necrosis in East Africa. *Plant Disease* **101**: 1455-1462.

Stewart LR, Teplier R, Todd J, Jones M, Cassone B, Wijeratne S, Wijeratne A & Redinbaugh M (2014) Viruses in maize and Johnsongrass in southern Ohio. *Phytopathology* **104**: 1360-1369.

Stockwell D & Peters D (1999) The GARP modelling system: problems and solutions to automated spatial prediction. *International Journal of Geographical Information Science* **13**: 143-158.

Storey H (1925) The transmission of streak disease of maize by the leafhopper *Balclutha mbila* Naude. *Annals of Applied Biology* **12**: 422-439.

Storey H (1928) Transmission studies of Maize streak disease. *Annals of Applied Biology* **15**: 1-25.

Strable J & Scanlon MJ (2009) Maize (*Zea mays*): a model organism for basic and applied research in plant biology. *Cold Spring Harbor Protocols* **2009**. DOI: 10.1101/pdb.emo132.

Sutabutra T, Kornkamhaeng P, Sirithorn P & Kositratana W (1976) A mosaic virus disease of maize in Thailand. Ohio Agricultural Research and Development Center: Ohio. 78-82.

Syller J (2012) Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular plant pathology* **13**: 204-216.

Taylor R & Pares R (1968) The relationship between Sugarcane mosaic virus and mosaic viruses of maize and Johnsongrass in Australia. *Australian Journal of Agricultural Research* **19**: 767-773.

Teakle D & Grylls N (1973) Four strains of Sugarcane mosaic virus infecting cereals and other grasses in Australia. *Australian Journal of Agricultural Research* **24**: 465-477.

Teyssandier E, Nome S & Dal BE (1983) Maize virus diseases in Argentina. Ohio Agricultural Research and Development Center: Ohio. 40-44.

Thottappilly G, Bosque-Pérez NA & Rossel HW (1993) Viruses and virus diseases of maize in tropical Africa. *Plant Pathology* **42**: 494-509.

Tjallingii W & Esch TH (1993) Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiological Entomology* **18**: 317-328.

Toler RW (1985) Maize dwarf mosaic, the most important virus disease of sorghum. *Plant Disease* **69**: 1011-1015.

Toler RW, Bhola N, Roose D & Kaula G (1989) Occurrence of Maize dwarf mosaic virus on sorghum in Zambia. *Plant Disease* **73**.

Tosic M & Ford R (1972) Grasses differentiating Sugarcane mosaic and Maize dwarf mosaic viruses. *Phytopathology* **62**: 1466-1470.

Urcuqui-Inchima S, Haenni A-L & Bernardi F (2001) Potyvirus proteins: a wealth of functions. *Virus Research* **74**: 157-175.

Uyemoto JK (1983) Biology and control. *Plant Disease* **67**: 7.

Uyemoto JK, Bockelman D & Claflin L (1980) Severe outbreak of Corn Lethal Necrosis Disease in Kansas. *Plant Disease* **64**: 99-100.

Uyemoto JK, Claflin L, Wilson D & Raney R (1981) Maize chlorotic mottle and Maize dwarf mosaic viruses; effect of single and double inoculations on symptomatology and yield. *Plant Disease* **65**: 39-41.

Van Antwerpen T, McFarlane S, Buchanan G, Shepherd D, Martin D, Rybicki E & Varsani A (2008) First report of Maize streak virus field infection of sugarcane in South Africa. *Plant Disease* **92**: 982.

Viswanathan R, Karuppaiah R, Malathi P, Kumar VG & Chinnaraja C (2009) Diagnosis of Sugarcane yellow leaf virus in asymptomatic sugarcane by RT-PCR. *Sugar Tech* **11**: 368-372.

Von Wechmar M, Chauhan R, Heam S & Knox E (1987) Applications of immunoelectroblotting to differentiate between strains of Maize dwarf mosaic virus and Sugarcane mosaic virus occurring in South Africa. In: *CSFRI Symposium: (Research into Citrus and Subtropical Crops)* Abstract 59.

Wang J-G, Zheng H-Y, Chen H-R, Adams MJ & Chen J-P (2010) Molecular diversities of Sugarcane mosaic virus and Sorghum mosaic virus isolates from Yunnan Province, China. *Journal of Phytopathology* **158**: 427-432.

Wang Q, Zhang C, Wang C, Qian Y, Li Z, Hong J & Zhou X (2017) Further characterization of Maize chlorotic mottle virus and its synergistic interaction with Sugarcane mosaic virus in maize. *Scientific Reports* **7**: 39960.

Wang Q, Zhou X & Wu J (2014) First report of Maize chlorotic mottle virus infecting sugarcane (*Saccharum officinarum*). *Plant Disease* **98**: 572-572.

Wang C-Y, Zhang Q-F, Gao Y-Z, Zhou X-P, Ji G, Huang X-J, Hong J & Zhang C-X (2015) Insight into the three-dimensional structure of Maize chlorotic mottle virus revealed by Cryo-EM single particle analysis. *Virology* **485**: 171-178.

Wangai A, Redinbaugh M, Kinyua Z, Miano D, Leley P, Kasina M, Mahuku G, Scheets K & Jeffers D (2012a) First report of Maize chlorotic mottle virus and Maize Lethal Necrosis in Kenya. *Plant Disease* **96**: 1582.

Wangai A, Sinkinyi E, Ochieng J, Miyogo S, Karanja T, Odour H, Kimani E, Irungu J, Kinyua Z, Ngaruiya P, Ligeyo D & Kipkemboi S (2012b) Joint assessment report: Report on status of Maize Lethal Necrosis Disease and general maize performance. Ministry of Agriculture, Kenya. Available online at: http://www.fao.org//fileadmin/user_upload/drought/docs/Maize%20Lethal%Nectrotic%Disease%20Kenya_Joint%20Assessment%20Report%20(July%202012).pdf. [28 November 2017].

Webb M (1987) Species recognition in *Cicadulina* leafhoppers (Hemiptera: Cicadellidae), vectors of pathogens of Gramineae. *Bulletin of Entomological Research* **77**: 683-712.

Webster CG, Wylie SJ & Jones MG (2004) Diagnosis of plant viral pathogens. *Current Science* 1604-1607.

White EJ, Venter M, Hiten NF & Burger JT (2008) Modified Cetyltrimethylammonium Bromide method improves robustness and versatility: The benchmark for plant RNA extraction. *Biotechnology Journal* **3**: 1424-1428.

Wilkes G (2014) Maize: domestication, racial evolution, and spread. *Foraging and Farming: The Evolution of Plant Exploitation* **31**: 440.

Williams LE & Alexander L (1965) Maize dwarf mosaic, a new corn disease. *Phytopathology* **55**: 802-804.

Wosula E, McMechan AJ, Oliveira-Hofman C, Wegulo SN & Hein G (2016) Differential transmission of two isolates of Wheat streak mosaic virus by five Wheat curl mite populations. *Plant Disease* **100**: 154-158.

Wu L, Zu X, Wang S & Chen Y (2012) Sugarcane mosaic virus–long history but still a threat to industry. *Crop Protection* **42**: 74-78.

Xiao XW, Frenkel MJ, Teakle D, Ward CW & Shukla DD (1993) Sequence diversity in the surface-exposed amino-terminal region of the Coat proteins of seven strains of

Sugarcane mosaic virus correlates with their host range. *Archives of Virology* **132**: 399-408.

Xie L, Zhang J, Wang Q, Meng C, Hong J & Zhou X (2011) Characterization of Maize chlorotic mottle virus associated with Maize Lethal Necrosis Disease in China. *Journal of Phytopathology* **159**: 191-193.

Xie X, Chen W, Fu Q, Zhang P, An T, Cui A & An D (2016) Molecular variability and distribution of Sugarcane mosaic virus in Shanxi, China. *Public Library of Science One* **11**: e0151549. DOI: 10.1371/journal.pone.0151549.

Xie Y, Wang M, Xu D, Li R & Zhou G (2009) Simultaneous detection and identification of four sugarcane viruses by one-step RT-PCR. *Journal of Virological Methods* **162**: 64-68.

Xu D-L, Zhou G-H, Shen W-K & Deng H-H (2008) Genetic diversity of Sorghum mosaic virus infecting sugarcane. *Acta Agronomica Sinica* **11**: 011.

Yang Z & Mirkov T (1997) Sequence and relationships of Sugarcane mosaic and Sorghum mosaic virus strains and development of RT-PCR-based RFLPs for strain discrimination. *Phytopathology* **87**: 932-939.

Zhang W, Olson NH, Baker TS, Faulkner L, Agbandje-McKenna M, Boulton MI, Davies JW & McKenna R (2001) Structure of the Maize streak virus geminate particle. *Virology* **279**: 471-477.

Zhang Y, Zhao W, Li M, Chen H, Zhu S & Fan Z (2011) Real-time TaqMan RT-PCR for detection of Maize chlorotic mottle virus in maize seeds. *Journal of Virological Methods* **171**: 292-294.

Zhao M, Ho H, Wu Y, He Y & Li M (2014) Western flower thrips (*Frankliniella occidentalis*) transmits Maize chlorotic mottle virus. *Journal of Phytopathology* **162**: 532-536.

Zheng L, Rodoni B, Gibbs M & Gibbs AJ (2010) A novel pair of universal primers for the detection of potyviruses. *Plant Pathology* **59**: 211-220.

Zhuang Q, Qin Z & Chen M (2013) Biofuel, land and water: maize, switchgrass or *Miscanthus*? *Environmental Research Letters* **8**: 015020.

Chapter 2: Status and distribution of cereal potyviruses in cultivated maize and potential reservoir within grass species across South Africa

Abstract

The destructive disease of maize, Maize Lethal necrosis Disease (MLND), is causing major crop losses in East Africa. Parts of South Africa (SA) may be at risk for the disease and thus it is important to act pre-emptively. MLND usually establishes due to the co-infection by Maize chlorotic mottle virus (MCMV) and a member of the Potyviridae family. Although MCMV is predicted to enter SA in the future, it is not currently present in SA to our knowledge. Although, members of the Potyviridae family have been reported in SA, the status of these potyviruses on maize is unknown. This single season maize-virus survey was conducted in an attempt to determine the status and distribution of potyviruses in maize in SA and thereby generate information on the relative incidence and diversity of potyviruses within the maize growing regions of SA. This information will assist in 1) identifying the potyvirus species present in potential Maize Lethal Necrosis (MLN) infections, 2) identifying key areas where the disease will manifest or potentially flourish, 3) gaining insight into the possible risk that MLND will cause an epidemic in SA's maize production areas as well as 4) identifying possible alternate reservoir host plants for the disease-causing viruses, should MCMV enter the country. In total 650 plant (611 maize and 39 grass) samples with virus-like symptoms were collected at 104 of the 129 sites surveyed across maize-growing regions of seven provinces in SA. Symptoms observed ranged from mild mottles, chlorosis, red vein-banding, mild and severe streaks and stunting. Between one and ten symptomatic samples were collected per site. RT-PCR using universal potyvirus-detecting primers in conjunction with Sanger sequencing and a subsequent BLAST analysis identified 56 Sugarcane mosaic virus (SCMV), 11 Johnsongrass mosaic virus (JGMV) (two on maize and nine on grass) and three Pennisetum mosaic virus (PenMV) isolates. These identities were further supported by alignment with appropriate reference sequences in a phylogenetic analysis. The potyviruses collected were at 18 different sites, mostly in the north-eastern parts of the country: the Limpopo Province and Mpumalanga Lowveld region, and were also detected in the north-western parts of SA: in the North-West Province and in Gauteng. The survey results suggest that SCMV will be the predominant potyvirus in future MLN infections on maize in SA and that JGMV may also play a role. It supports previous climatic predictions and suggests that the risk areas where MLND may establish in future, should MCMV

enter the country and allow establishment of the disease, are likely to be areas in the north-eastern part of the country. The study also indicates that follow-up surveys of maize and additional alternate hosts need to be conducted over a few successive seasons and that the host range of South African JGMV needs to be investigated.

2.1 Introduction

Worldwide and especially in sub-Saharan Africa, maize (*Zea mays*) is regarded as a significant fodder and food crop which is crucial for food security (Mahuku *et al*., 2015). Maize Lethal Necrosis Disease (MLND) is a recently introduced, major threat to maize production in Africa as it causes severe yield losses and threatens food security and the livelihood of subsistence farmers (Wangai *et al*., 2012). The viral disease was first detected on the African continent in 2011 in Kenya, and during the past seven years, it has spread to at least six countries in eastern Africa including Tanzania, Uganda and Rwanda (Kiruwa *et al.*, 2016). According to predictions based on ecological niche models generated based on climatic data as well as the present distribution of MLND, Isabirye and Rwomushana (2016) projected that various African countries, including South Africa (SA), are at risk for MLND outbreaks in future. The projected area that provides a suitable habitat for MLND to establish in SA is just under 300 000km^2, according to results produced in this study (Isabirye & Rwomushana, 2016) and thus threatens SA's future maize production.

MLND is caused by Maize chlorotic mottle virus (MCMV) in combination with a virus from the Potyviridae family. These have included Sugarcane mosaic virus (SCMV), Maize dwarf mosaic virus (MDMV), Johnsongrass mosaic virus (JGMV) (Bockelman *et al*., 1982; Stewart *et al*., 2017), from the genus *Potyvirus*, or Wheat streak mosaic virus (WSMV) (genus: Tritimovirus) (Scheets, 1998). These MLND-causing viruses are mainly spread by insects, but also via distribution of infected seed (De Groote *et al.*, 2016). While MCMV has not yet been reported in SA, SCMV (International Society of Sugar Cane Technologists (ISSCT), 1989) and MDMV (Knox *et al*., 1986) are known to be present in SA.

These viruses have been detected using various techniques including enzyme-linked immunosorbent assay (ELISA) (Adams *et al*., 2013; Giolotti *et al*., 2005), real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) (Zhang *et al*., 2011; Adams *et al*., 2013) and conventional RT-PCR (Wang *et al*., 2014; Wangai *et al*., 2012; Xie *et al*., 2011; Zheng *et al*., 2010) with each technique having its advantages and drawbacks (Kiruwa *et al*., 2016). RT-PCR is used routinely for virus detection due to its speed, specificity and sensitivity (Naidu *et al*., 2003) and when in combination with Sanger sequencing, allows the researcher to putatively identify the

virus species and/or strain present by comparison to the available sequences in a database such as GenBank (Webster *et al*., 2004). RT-PCR using the universal potyvirus primer pair developed by Zheng *et al*. (2010), NIb3R and NIb2F, is an example of a widely used potyvirus-detection tool which can be coupled with Sanger sequencing for species identification. The primers bind to two regions within the RNA dependent RNA polymerase (RdRp) gene (which are conserved amongst *Potyvirus* species) and amplify a variable region between which allows for differentiation between virus species in the genus based on the resulting nucleotide sequence (Zheng *et al*., 2010).

In SA, there are general trends for when the maize planting season commences: in the eastern parts of the country (KwaZulu-Natal, Eastern Cape, eastern Free State, eastern and southern Mpumalanga) maize is planted in October, while in the northern, central and some western parts of the maize-growing region maize is planted from November to December. In the most western parts of SA's maize-growing region, maize is planted from January up until February (Food and Agriculture Organisation of the United Nations (FAO), 2018). SA generally produces eight to ten million tonnes of maize, which increased to a record harvest of about 17.5 million tonnes in the 2017 season from both commercial and non-commercial farmers. This was more than twice the amount that was harvested in the 2015/2016 season, due to various factors such as a 35% increase in initial plantings (motivated by higher prices) due to dry weather conditions and thus lower yields in the 2016 season. These differences in distribution of rainfall were seen on average rainfall maps of SA: in October 2016, the eastern parts of SA's maize-growing regions had received much more rain relative to the previous year in October 2015 at the commencement of the 2015/2016 season; and the same applied for the western parts receiving comparatively more rain in November through to February when the latest sowing occurs (FAO, 2018; South African Weather Services (SAWS), 2018). This phenomenon is the most important contributing factor in the extreme production increase seen during the 2016/2017 season and the record harvest obtained (FAO, 2018). SA is also a net exporter of maize to East Asia: Taiwan, South Korea and Japan; as well as Africa, and about 2.4 million tonnes were exported to other countries after the 2017 season (Statistical databases and data-sets of the FAO (FAOSTAT), 2018). It is of utmost importance that MLND introduction in SA is pre-

empted in terms of knowledge, resources, and references available to the maize industry for research, due to the significant role that maize plays in the economy and food security. This is possible because even though MCMV is not present in SA, potyviruses that are important in Maize Lethal Necrosis (MLN) infections such as SCMV, are present in SA (Goodman *et al*., 1998; ISSCT, 1989).

While comprehensive surveys for potyviruses of cereals are generally lacking, some surveys have been conducted that could serve as a benchmark for the current study in terms of sample size and surveyed area size. The incidence for WSMV in *Hordeum vulgare* (barley) and *Triticum aestivum* (wheat) was studied in the Czech Republic where relatively low overall incidence of virus was observed (6.4% of the total samples tested) but the viruses were widely distributed (Singh & Kundu, 2017). Several grass species previously implicated as hosts of WSMV and weeds of cereal crops were also identified. In their study, 876 samples in total were collected and tested (Singh & Kundu, 2017). In 2010, Gell *et al*. published a study where 65 isolates from different cultivars of grain maize varieties, sorghum (*Sorghum bicolor*), Johnsongrass (*S. halepense*) and sweet corn (*Z. mays* L. var. *rugosa*) were collected and used in a diversity study of MDMV in Hungary. Interesting findings of their study were that differences in symptom expression were seen in terms of the intensities of chlorosis as well as symptom patterns amongst the variety of host plants (Gell *et al.,* 2010). MDMV, SCMV and Maize chlorotic dwarf virus (MCMV) were surveyed for in 89 sweet corn and Johnsongrass samples collected in Ohio over two seasons and identified Johnsongrass as a reservoir host (Stewart, 2014).

In terms of cereal virus surveys in Africa, Stewart *et al*. (2017) reported that JGMV contributes to MLND. They conducted a survey of 14 sites in Uganda and 27 in Kenya and pooled the samples. The aim was to establish the role of JGMV in MLND and not to investigate geographic distribution as is the case with our study, and therefore sample pooling was a time-efficient, cost-effective alternative for them to conducting nucleic acid extractions on all individual samples. Pande *et al*. (2017) studied the Maize streak virus (MSV) strain-A population in order to determine Kenya's role in cross-continental spread of the virus. A total of 170 maize samples and 122 grass samples were collected from 119 farms across the four major maize growing regions of the country (Pande *et al*., 2017). A comprehensive survey such

as that of Pande *et al*. (2017) is necessary in SA in order to accurately reflect the status and distribution of the maize potyviruses.

Although sugarcane is not a cereal, it is a monocotyledon and host of many of the cereal potyviruses (including SCMV) that may be role players in MLN infections (Wang *et al*., 2014). In terms of SCMV status in SA, Goodman and colleagues (1998) conducted a study to identify the SCMV strains present only on sugarcane. Their study relied mainly on RT-PCR and Sanger sequencing where the Coat protein region of the SCMV samples were analysed to distinguish the strains from one another (Goodman *et al*., 1998). Although this study provided a good idea of the variation of SCMV strains extant on South African sugarcane, the status and distribution of SCMV or other potyviruses on and within maize remains unknown.

MLND is a new disease in Africa and thus it is critical to do thorough foundational research. This includes studying the status of the relevant viruses, as well as the risk factors for example where subsistence farming takes place, and where international borders could be a porthole for MCMV into the country, along with the climate associated with these risk areas. The aim of this study is to pre-empt the introduction of MLND in SA by determining the status and distribution of potyviruses on maize and selected grasses in SA. This will identify: 1) the potyviruses likely to be present in future MLN infections in SA, 2) where MLND is likely to proliferate, should MCMV enter SA, 3) identify what climatic or other conditions are likely to be associated with MLN infections in SA, as well as 4) identify potential alternate hosts for the disease-causing viruses. This will be achieved by accomplishing the following objectives: 1) maize and grass samples will be identified and collected in commercial and subsistence, as well as irrigation and non-irrigation maize growing regions in all major maize growing provinces at sampling points of which latitude and longitude coordinates will be recorded with a Global Positioning System (GPS) , 2) potyvirus-detecting primers will be used in RT-PCR to detect potyviruses, 3) these amplicons will be directly sequenced in order to identify the potyviruses present, 4) a phylogeny will be constructed with the appropriate reference sequences to establish the diversity of South African potyviruses and confirm the sequence results. It is hypothesised that SCMV and MDMV which have been detected in SA before will be present on South African maize and that potyviruses will be widespread.

2.2 Materials and methods

2.2.1 Sampling of plant material and identification of grasses

In total 129 fields were surveyed across seven provinces in SA between November, 2016 and June, 2017. Altogether 650 leaf samples were collected from 104 of these maize fields where virus-like symptoms were observed. The maize fields were surveyed for at least 40 person-minutes per field with multiple evaluators. Maize and occasionally grasses growing next to maize fields were examined for any virus-like symptoms such as stunting, mosaic, vein banding, mottling, streaks, yellowing and discolouration of leaves. The grasses were identified using the field guide *Gids tot grasse van Suid-Afrika* (Van Oudtshoorn, 1992). A maximum of ten samples were collected per field. Sampling was by convenience sampling and, generally, four persons spread out along the border(s) of the field closest to where the vehicle was stopped, and monitoring to a depth of up to 50m for 10min at least. At least three symptomatic leaves from different sections of the plant were collected. A lat/long coordinate within each field was recorded with a differential GPS and points were differentially processed relative to base stations. All samples were assigned unique accession numbers and where possible, images of symptoms were recorded. Samples were stored at 4˚C until RNA was extracted.

2.2.2 RNA extraction and virus isolates

Total RNA was extracted from 200mg fresh leaf material from all the leaves collected from a specific plant using the cetyltrimethylammonium bromide (CTAB) (2% CTAB, 1% polyvinyl pyrrolidone (PVP), 20mM ethylenediaminetetraacetic acid (EDTA), 100mM Tris-hydrochloric acid (HCl), 1.4M sodium chloride (NaCl)) extraction method described by White *et al*. (2008), modified by excluding spermidine from the CTAB buffer. RNA was re-suspended in molecular-grade nuclease-free water (Sigma; St. Louis, MO, USA) and stored at -80°C.

2.2.3 Reverse Transcription-Polymerase Chain Reaction

The universal potyvirus-detecting primers NIb3R and NIb2F (Zheng *et al*., 2010) were used in an RT-PCR assay to identify samples that contain potyviruses. RT-

PCR was done in two-step reactions using the Moloney-Murine Leukemia Virus (M-MLV) RT system (Promega; Madison, WI, USA) and MyTaq system (Bioline; Taunton, MA, USA), with modifications to the manufacturer's protocol. Total RNA (3µl) and 2µl R primer (10µM; 4µM in final reaction) were incubated together at 70°C for 5 min prior to the cDNA synthesis reaction in order to facilitate primer binding and removal of RNA secondary structures. First-strand cDNA synthesis was performed in a total reaction volume of 10.5µl, containing 0.25µl (25 U) M-MLV Reverse Transcriptase, 2.25µl 10x M-MLV-buffer (5mM Tris-HCl pH 8.3, 3mM magnesium chloride ($MgCl_2$), 75mM KCl, 10 mM dichlorodiphenyltrichloroethane (DTT) in final working solution), 1.7µl molecular grade water and 1.25µl dNTPs (10mM each dATP, dCTP, dGTP and dCTP; Promega). A master mix was prepared and 5.5µl was added to 5µl RNA/ primer mixture. The RT reaction was carried out at 42°C for 1h. For the amplification of the cDNA a master mixture was prepared which contained, per reaction: 5µl MyTaq reaction buffer (5mM dNTPs, 15mM $MgCl_2$) (Bioline; Taunton, MA, USA), 0.5µl (0.2µM) NIb2F and NIb3R primers each, 0.25µl (25U) (MyTaq DNA polymerase (Bioline; Taunton, MA, USA) and 15.75µl molecular-grade nuclease-free water (H_2O) (Sigma; St. Louis, MO, USA). Three µl cDNA was added to 22µl PCR master mixture. Amplification conditions were 95°C for 5min, followed by 40 cycles denaturation at 95°C (15s), annealing (30s at the specific melting temperature of the primer pair as seen in Table 1) and extension at 72°C (10s), followed by a final extension step of 72°C for 10min.

2.2.4 Purification of Polymerase Chain Reaction products and Sanger sequencing

RT-PCR products were subjected to a PCR clean-up using 5U ExoI (Fermentas, MD, USA) and 1U FastAP (Fermentas) at 37°C followed by 85°C, for 15min each, respectively. This was followed by direct sequencing in both directions on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems; Foster City, CA, USA) at the University of Pretoria (UP) sequencing facility. Reaction volumes of 10µl in total were used containing: 3µl template DNA, 0.75µl (2µM) NIbF2 or NIb3R primer, 1µl BigDye Terminator Ready Reaction Mix (Applied Biosystems), 2.25µl Sequencing Buffer (Applied Biosystems) and 3µl molecular-grade nuclease-free H_2O (Sigma). Reaction products were precipitated using 1µl Sodium Acetate (NaOAc; 3M), 1µl ethylenediaminetetraacetic acid (EDTA; 125mM), and 100% molecular-grade

ethanol (Merck; Darmstadt, Germany) and centrifugation at 13000rpm for 30min, followed by washing the pellet with 70% molecular-grade ethanol (Merck) before submission to the sequencing facility.

2.2.5 Sequence processing and alignment

Partial CP sequences of South African isolates were manually edited using Chromas LITE Sequence Alignment Editor (v2.1.1) (Technelysium Pty Ltd, Australia), followed by assembling forward and reverse sequences in BioEdit Sequence Alignment Editor (v7.0.9.0) (Hall, 1999; 2011). Subsequently, consensus sequences between the two were curated and these sequences were subjected to a NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Sequence Tool) analysis (available online from https://blast.ncbi.nlm.nih.gov/Blast.cgi) for verification of the identity of the sequences.

2.2.6 Multiple alignment and phylogenetic analysis

The sequences of the isolates together with SCMV reference sequences obtained from NCBI Genbank (available from https://www.ncbi.nlm.nih.gov/genbank/) were submitted to the MAFFT (Multiple Alignment using Fast Fourier Transform) online (v7.0) (Katoh *et al*., 2002; Katoh & Standley, 2013) (available online from https://mafft.cbrc.jp/alignment/server/index.html) in order to verify the orientation of the sequences and to perform a multiple sequence alignment using MAFFT default parameters. The alignment was exported to BioEdit (v7.0.9.0) (Hall, 1999; 2011) where the overhangs of the alignment were trimmed.

The evolutionary model with the best fit was determined using MEGA (v6) (Molecular Evolutionary Genetic Analysis) (Kumar *et al*., 2008). The model selected for the analysis was Tamura 3-parameter (T92) (Tamura & Nei, 1993), including rate variation among sites (+G). MEGA (v6) (Kumar *et al*., 2008) was used to construct maximum-likelihood phylogenies by analysing a 270 bp of the NIb protein region, taking into account all sites in the alignment including the gaps. To obtain confidence in the topologies and branching points in the analysis, a bootstrap analysis of a 1000 replicates was applied. All isolates from this study together with 30 reference sequences were used to construct Fig. 2.1. The reference sequences were selected

based on the BLAST analyses of the South African sequences and the most closely related SCMV's were selected this way. WSMV was used as the outgroup as it is the closest relative to the species in the *Potyvirus* genus.

2.2.7 Pairwise nucleotide analysis

The same dataset was used to conduct a pairwise nucleotide analysis using Sequence Demarcation Tool (SDT) software (v1.2) (available online from http://web.cbio.uct.ac.za/SDT) (Muhire *et al*., 2014) to calculate the nucleotide sequence identities between isolates, using a distribution plot that represents the pairwise nucleotide sequence identity percentages graphically.

2.2.8 Construction of maps

Google Earth Pro (v7.3) (available online at: https://www.google.com/earth/desktop/) was used to contruct maps to illustrate where all the sampling sites were, using the GPS data collected during the survey (2.2.1). Blue and black dotes were used to denote sites where virus-like symptoms were seen and thus samples collected or where no symptoms were seen and no samples collected. Different coloured dots (red, orange and green) were used to show where different viral species were detected, as per map key in Fig. 2.1 and Figs. 2.6-2.8.

2.3 Results

Virus-like symptoms were observed in 104 of the 129 maize fields (Fig. 2.1) and between one and ten symptomatic samples were collected at such sites, along with symptomatic grass species seen directly next to these fields when they were present (Appendix A, Table 1.1). The grass samples were identified as either *Sorghum halepense* (Johnsongrass) or *Panicum maximum* (Guineagrass) (Appendix A, Table 1.1). In 26 of the fields no virus-like symptoms were observed and no samples were collected. Relatively low numbers of virus-like symptoms were observed in regions of central and southern Mpumalanga (Highveld), the north-eastern Free State and north and north-western parts of KwaZulu-Natal and the eastern part of Northern Cape (Appendix A, Fig. 2.6 A-C). Virus-like symptoms were relatively abundant in the remaining regions, especially in irrigation areas such as Loskop, Vaalharts and the Crocodile River irrigation schemes (Appendix A, Fig 2.6 C and Fig. 2.7 A and B).

Of the 650 samples collected, 611 were collected from symptomatic maize while 39 were symptomatic uncultivated grass samples found growing in close proximity to symptomatic maize. Virus-like symptoms observed and sampled included mosaics, mottles, fine or broad streaks, red streaks, yellowing, blotches and concentric ringspots if different severities and variations (Fig. 2.2 & 2.3). Streaks on the leaves, which probably represent MSV symptoms (Figs. 2.2: F, G, H, M, N, O), were the most commonly observed symptom but we did not test routinely for this virus and only tested 15 samples with variations in intensity of the prominent streak symptoms suspected to be MSV and confirmed the presence thereof. Nevertheless such material was collected as to avoid pre-judging that such symptoms were caused by or may be in combination with potyviruses, as some of the potyviruses symptoms are not easily distinguished from MSV. Occasionally, these plants were stunted and appeared to have reduced vigour, but infected plants were mostly the same height as symptomless plants. However, the symptoms observed on plants collected near Trichardtsdal and Ofcolaco, and on the UP Experimental farm in Pretoria showed a range of symptoms other than streaks such as definite mottles, mosaics and yellowing (Figs. 2.3: C, M, N, O). Symptomatic grass samples putatively identified as *S. halepense* and *P. maximum* using a field guide displayed similar symptoms such as fine streaks and mottles, respectively, on the leaves (Figs. 2.4 & 2.5).

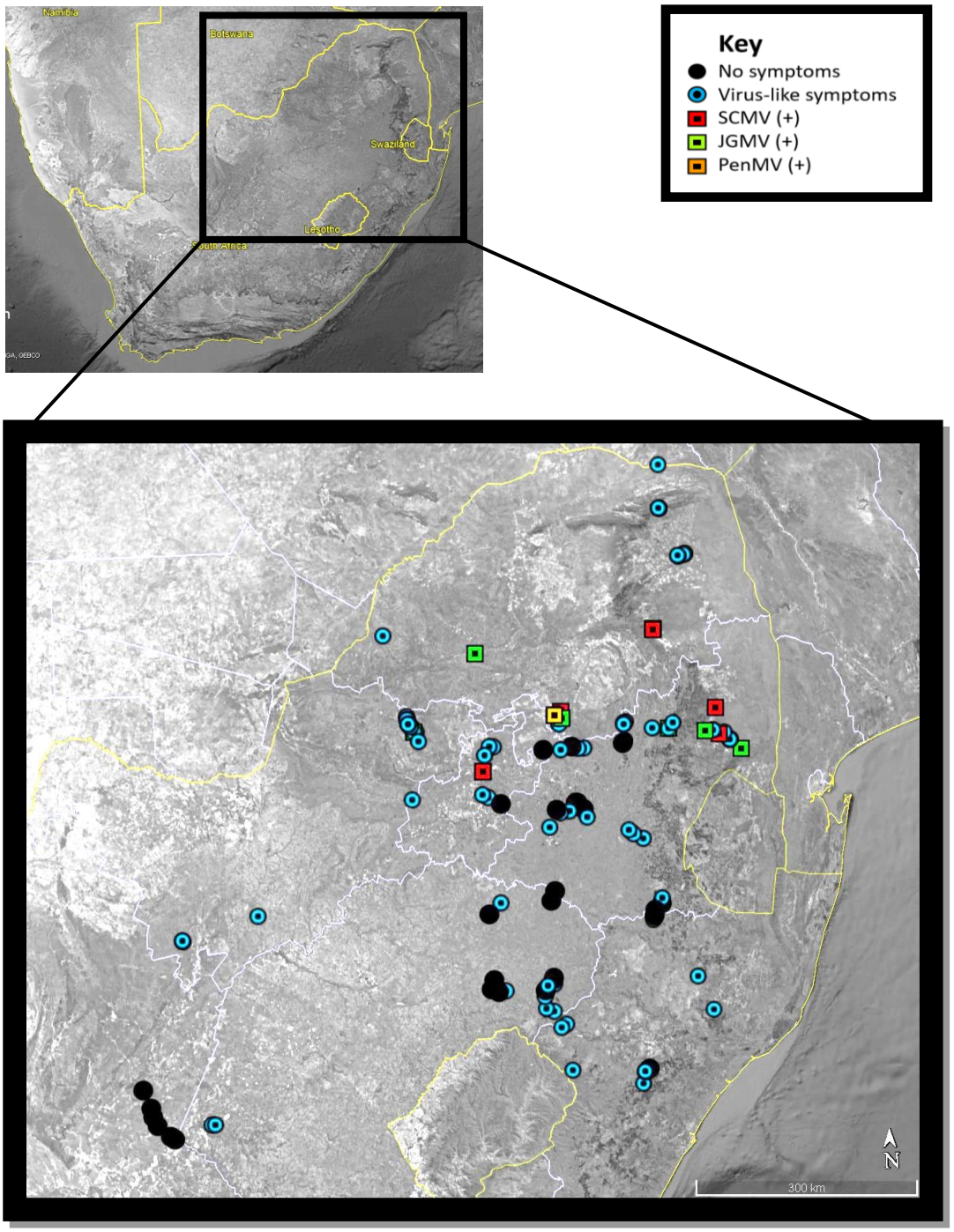

Figure 2.1 Map of South Africa showing sampling points across the provinces where the maize survey was conducted. Sites where no virus-like symptoms were observed (black dots), sites where virus-like symptoms were observed and sampled but tested negative for potyviruses (blue dots) as well as sites that tested positive for potyviruses (red, green and orange dots) are indicated.

Figure 2.2 A-O. Different degrees of severity and variations of virus-like streak and yellowing symptoms on maize.

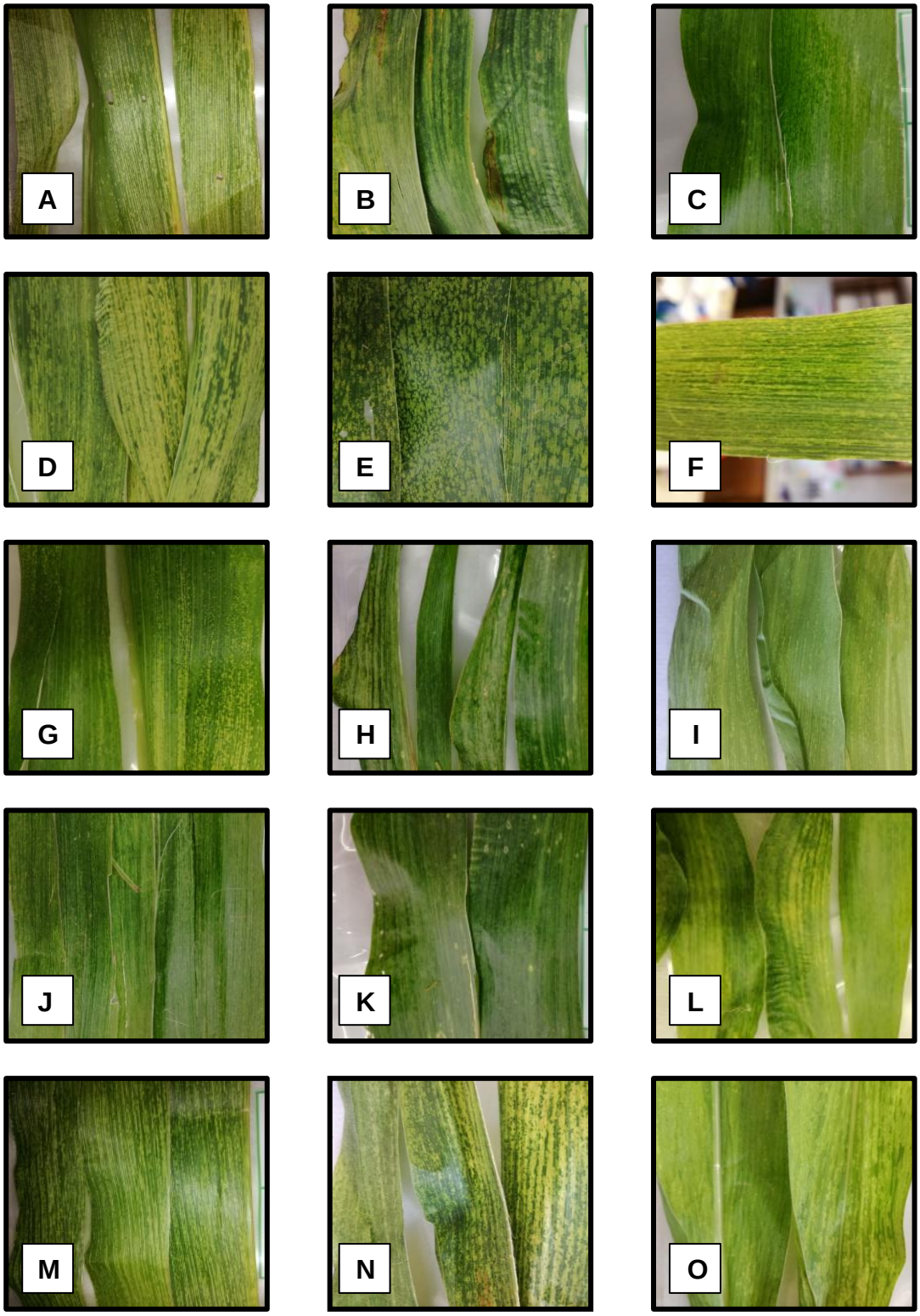

Figure 2.3 A-O. Different degrees of severity and variations of virus-like mottle and mosaic symptoms on maize.

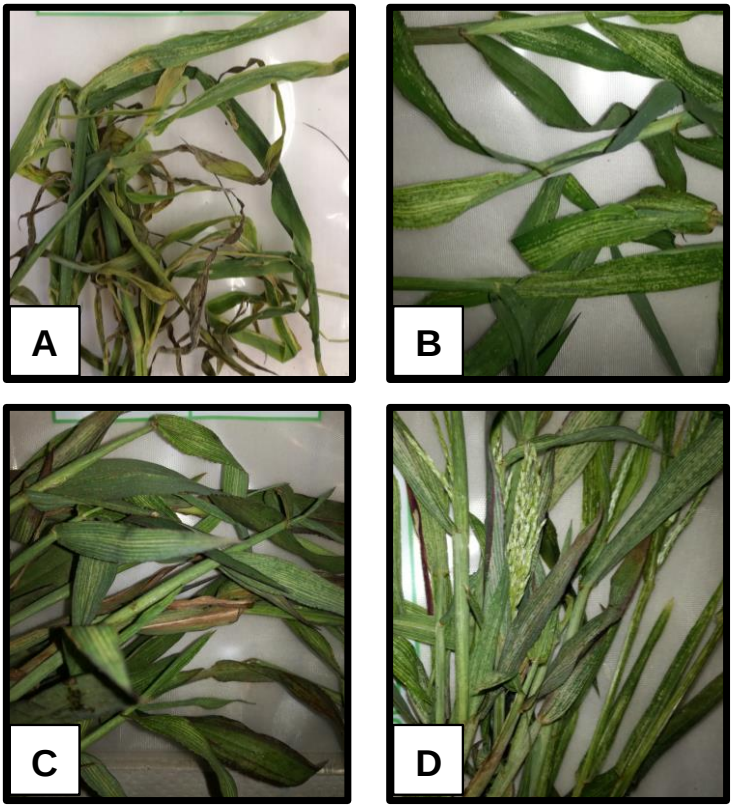

Figure. 2.4. A-D. Virus-like streak symptoms on (putatively) identified Johnsongrass.

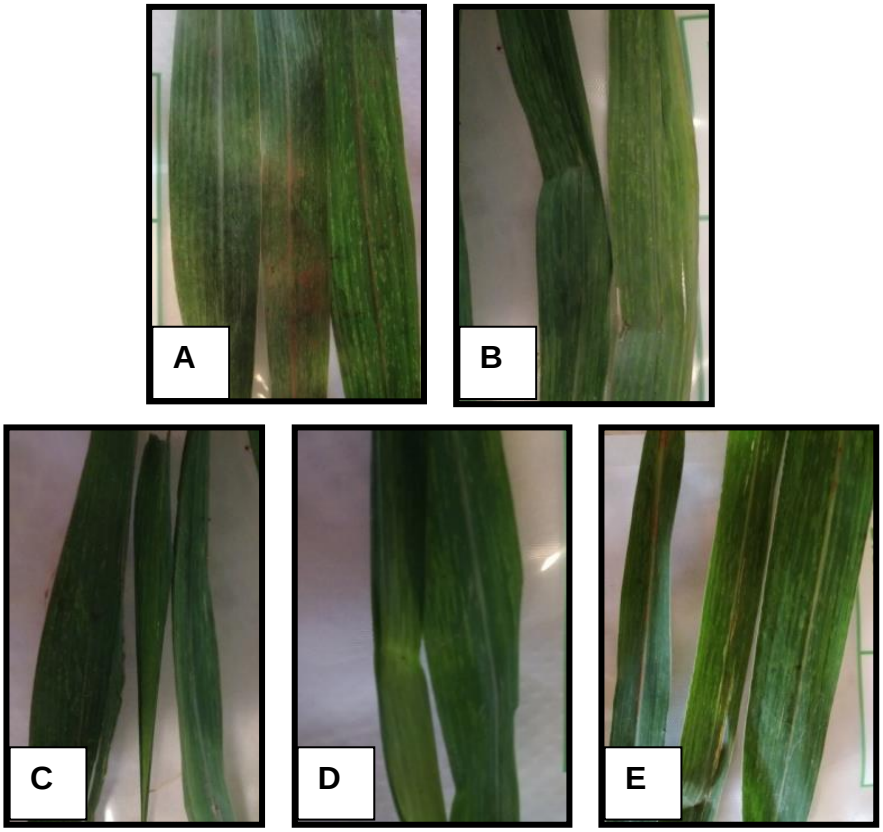

Figure 2.5 A-D. Virus-like fine mottle and streak symptoms on (putatively) identified Guineagrass.

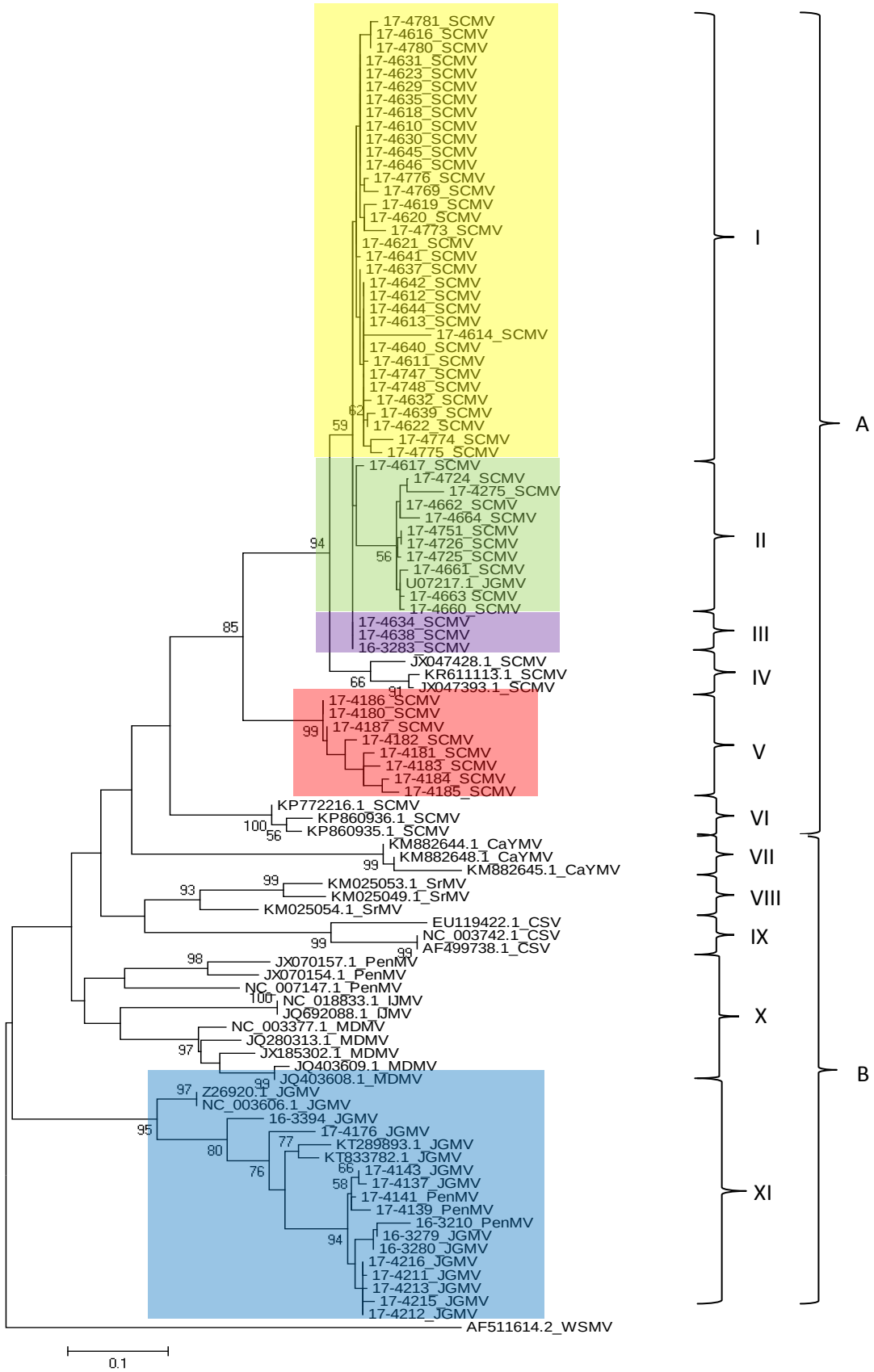

Figure 2.9 Maximum likelihood phylogenetic analysis reconstructed using partial NIb protein nucleotide sequences of 70 isolates potyviruses from this study, together with 31 reference isolates obtained from GenBank as reference sequences and a Wheat streak mosaic virus isolate as outgroup. The analysis was conducted using MEGA 6 with 1000 bootstrap replicates where the values are indicated as percentages and only scores above 50 are shown. Letters A and B indicate main groups and Roman numerals (I-XI) indicate sub-groups. Coloured boxes indicate groups containing South African isolates.

Of the 685 samples tested, 70 tested positive for the presence of potyviruses which corresponded to a roughly 10% relative prevalence of infection amongst the symptomatic samples collected. While there were more samples collected in commercial farming areas than in subsistence farming areas, the samples that tested positive in these two categories of farming relative to the total number of plants collected in each of these two categories were 10% and 9%, respectively. Samples collected at experimental farms were not included in these calculations. Based on the BLAST analyses of the Sanger sequences of amplicons of these isolates, a total of 56 maize samples putatively contained SCMV (7.88% of total samples collected) and two contained JGMV. Amongst the grass samples, nine samples contained JGMV and three contained Pennisetum mosaic virus (PenMV) (Appendix A, Table 1.1). The symptoms associated with these potyvirus-infections were not specific in pattern, colour or intensity and ranged from different degrees of severity of mottles and/or mosaics, as well as fine streaks and/or yellowing on maize, and fine streaks on Johnsongrass (Fig. 2.4) and fine mottle on Guineagrass (Fig. 2.5). Potyviruses were detected in four of the seven provinces of SA that have major maize-growing areas. These potyviruses were mostly detected in the north-eastern part of SA specifically, the Lowveld of Mpumalanga and also the Limpopo Province (Fig. 2.8), but were also detected although in lower levels in the north-western part of SA, specifically North-West Province and Gauteng (Fig. 2.7).

The phylogenetic analysis of all isolates obtained from this study with various monocot-infecting potyvirus sequences obtained from GenBank as reference sequences is presented in Fig. 2.9. Sub-groups in which South African isolates are located are shaded in different colours. WSMV was selected as the outgroup for the tree. The analysis revealed two major groups into which the isolates from our study grouped, the first (A), has six sub-groups (referred to as sub-groups I - VI) and contains all SCMV isolates isolated from maize, while the second main group, (B), is comprised five sub-groups that contain the other potyvirus species (referred to as sub-group VII-XI). The largest of the five sub-groups in Group A (sub-group I) comprises isolates of SCMV isolated from maize and collected mainly in the Limpopo Province, from sweet corn (shaded in yellow on Fig. 2.9). Other major clusters include isolates from Mpumalanga and the Limpopo Province (II, shaded in green), Groblersdal (III, shaded in purple), Chinese reference sequences (IV),

isolates from Gauteng (V, shaded in red) and Ethiopian reference sequences (VI). Isolates from sub-groups I - III are most closely related to Chinese isolates (III) and sub-groups I-V share a common ancestor with the Ethiopian isolates (VI). The partial NIb gene sequences of SCMV isolates in Group A, sub-group I shared the largest range of pairwise nucleotide identity (91.35%-100%), and isolates within sub-groups II and V have a higher degree of identity to sequences within their group (both ranged from 95.49%-100%). Between sub-groups I and II, the highest nucleotide identity was 95.86% and the lowest was 90.97%. The minimum nucleotide identity between group I and XI was 75.56%, while the maximum was 84.59%. The minimum nucleotide identity between groups II and XI was 74.43% and the maximum 80.45%. Putatively identified JGMV and PenMV isolates from this study, isolated from grasses, formed sub-group XI (shaded in blue) along with JGMV reference sequences, indicating that PenMV from this study was most closely related to JGMV references from Australia, and not other PenMV isolate. Relatively short terminal branches are seen in sub-groups I, II and III.

2.4 Discussion

This study reports on the status and distribution of potyviruses detected on South African maize and selected grass samples. The key findings of this study include: 1) potyviruses were detected in four out of seven provinces sampled in, 2) out of 129 sampling points, 18 sites were positive for potyviruses, of which all of these 18 sites were located in the northern parts of the country, 3) out of 685 samples, 56 tested positive for SCMV, 11 for JGMV, and three for PenMV, 4) SCMV was the most common potyvirus on maize, while JGMV was most common on grass, 5) potyvirus infections are more abundant in the north-eastern part of the country but were also detected in the north-western part of the country, 6) virus-like symptoms were observed at most of the sampling sites throughout the country and increased in intensity in irrigation areas, 7) few virus-like symptoms were observed in the Highveld (southern and central parts of Mpumalanga, northern part of KwaZulu-Natal and north-eastern part of the Free State) and in the eastern part of the Northern Cape near the border of the Free State, and 8) symptoms that were observed were mostly streaks on leaves and occasionally yellowing or mottle symptoms were seen.

Overall, the relative incidence of potyviruses was lower than expected. Potyvirus infections were anticipated to be more widespread due to the fact that 1) the host plants of these viruses are grown in large parts of SA (Flett & Mashingaidze, 2016) and 2) potyviruses are vectored by aphids, which are present in SA, that transport the virus and are often carried via wind currents (Zeyen *et al*., 1987). The 7.88% SCMV relative incidence observed in this study correlate with the SCMV relative incidence in previous studies. Luo *et al*. (2016) published a study where a viral survey of SrMV in co-infection with SCMV and Sugarcane streak mosaic virus (SCSMV) was conducted on 104 samples from China. Over 70% of samples contained SrMV, while 6.7% contained SCMV and SCSMV occurred in 3.7% of samples in mixed infections with either of the other two viruses. The infected samples were mostly collected from fields from an area near Trichardtsdal and Ofcolaco (Appendix A, Fig. 2.8 B; Appendix A, Table 1). This may indicate that there has been a recent introduction of SCMV either via infected seed which coincided with the presence of the aphids and appropriate climatic conditions for them to thrive, or that the virus was present on uncultivated grasses.

Notably, the maize samples collected from the Trichardtsdal and Ofcolaco area were sweet corn unlike the grain maize varieties representing the majority of other samples in this survey. The difference in planting time may play a role in the increased numbers of plants infected by SCMV in this area as these were generally winter plantings where irrigation took place. A similar phenomenon was observed in the large number of symptomatic plants were observed in irrigated areas such as Loskop, Groblersdal and Vaalharts irrigation schemes (Appendix A, Fig. 2.7). The large number of virus-infected plants in the sweet corn and the irrigation scheme areas may be due to the fact that irrigation allows farmers to plant before the rainy season commences. This increased succulence of such early-planted crops compared to the wild grasses adjacent to the fields that have not yet received rain, results in a large amount of pressure for potentially viruliferous insects to move over from dry natural vegetation to the young maize plants (Agrios & Hadwiger, 2005). Viruliferous insects are known to translocate to where green hosts are available, which includes irrigated areas (Duffus, 1971). In 2002, Azzam and Chancellor observed that in irrigated areas specifically, leafhoppers carrying two rice-infecting viruses move from Tungro, which is the primary inoculum source, to the rice plantation.

It was expected that positive samples collected in subsistence farming areas would exceed those collected in commercial farming areas where insecticides are regularly applied against potential vectors (Agrios & Hadwiger, 2005; De Groote *et al*., 2016; Mahuku *et al*., 2015), but the relative percentages were very similar for the two farming areas. Collecting equivalent numbers of samples from both subsistence and commercial farming areas in future surveys may clarify this result.

The results of the current study confirmed the hypothesis that SCMV will be present on South African maize but MDMV was not detected as expected. This may be because most modern maize cultivars are MDMV resistant due to a long history of studying genes that allow MDMV resistance and the subsequent breeding for such resistance. In SA there has most likely been an increase in planting MDMV-resistant varieties as MDMV has been a limiting factor on South African maize for a longer time compared to SCMV, which caused losses for the sugarcane industry at first (Goodman *et al*., 1998; Knox *et al*., 1986). This survey provides the first record of JGMV in SA which was detected in various different regions and on two different

hosts. JGMV was mainly detected on grass but in two instances was detected on maize. PenMV was also detected for the first time in SA and was also identified in grass. Only three samples were putatively identified as PenMV based on a BLAST analysis of the amplicon sequence. The infection of maize by JGMV indicates that these maize genotypes are susceptible and infection may occur if a JGMV source is in close proximity. The presence of JGMV is of concern as it was shown by Stewart *et al*. (2017) that JGMV plays a role in MLN infections in East Africa. JGMV is also able to infect sorghum and oats and cause significant yield losses in maize in Australia (Tosic *et al*., 1990) and thus has a number of potential hosts to proliferate in. Stewart *et al*. (2014)'s study did however show that JGMV mainly reside in Johnsongrass and sweet corn and not in grain maize varieties.

The beneficial weather conditions for the crop in 2017 was the main contributing factor to the high yields seen, and was largely influenced by the desired amount of rain at the appropriate time (FAO, 2018). It is not known whether these atypical weather conditions influenced the level of virus observed. Environmental factors can determine whether certain plant diseases develop or not and the two most important of these is moisture as well as temperature (Agrios & Hadwiger, 2005). These aspects have an effect on 1) the growth and development of the plant and therefore its susceptibility to diseases as well as 2) the interaction between the pathogen causing the disease and the plant. This results in differing levels expression of the disease, estimated in terms of symptom severity (Agrios & Hadwiger, 2005). The amount and distribution of rainfall in a particular area is often closely related to the occurrence of disease, and favourable rainfall conditions may determine whether a disease occurs at all within a season (Agrios & Hadwiger, 2005). Therefore, in a season where a record harvest was recorded due to favourable environmental conditions it can be expected that the presence of plenty of strong, large yield-bearing plants, the conditions for the development of infections by certain pathogens are likely to be abnormal. It is possible that this may not have been favourable for SCMV spread. It is also possible that fast initial plant growth rates may have decreased susceptibility of the plants. Furthermore, the fact that rain also fell on non-vegetation adjacent to the fields may also have contributed towards abnormal spread of the disease, possibly resulting in less pressure for viruliferous insects such as aphids (for potyviruses) and leafhoppers (for MSV) to move from infected wild

grasses to the maize fields. It is uncertain whether the SCMV from the 2016/2017 season represents higher or lower incidences of the disease than normal, and a multi-year survey is recommended for the future.

At the same time, it is also possible that the aphid vectors of potyviruses were not present, or at very reduced levels, during the 2016/2017 season during which we sampled. While initial introduction of a pathogen to an area is deemed as the most important driver of emerging infectious diseases (Anderson *et al*., 2004), the mere introduction of a pathogen will not be enough to cause emergence unless the natural vectors are present (Fereres, 2015). Various other authors have also discussed the presence of aphids as the determining factor of the risk and rapidity of spread of potyviruses such as SCMV once the virus has been introduced to a new area, thereby regulating where the virus can proliferate and become an epidemic (Claflin *et al.*, 2017; Jones, 2014). The limited number of symptomatic plants observed in certain areas such as northern part of the Free State (Vrede, Warden, Kestell, Bethlehem), southern part of Mpumalanga (Piet Retief, Ermelo, Secunda, Hendrina) and the eastern part of Northern Cape (Orania, Hopetown) (Appendix A, Fig. 2.6 A-C) are possibly due to the lack of insect vectors in these areas. This is perhaps due to the colder temperatures and frost experienced here in winter with lows reaching -4.1°C, -5°C (southern part of Mpumalanga) and -3.2°C (eastern part of the Northern Cape) during the coldest quarter of 2016 prior to the start of the 2016/2017 maize season (SAWS, personal communication, 6 April 2018). Insect vectors are not likely to survive or be able to overwinter in such low temperatures. The survival temperatures for spotted alfalfa aphids were shown to be between 6 and 32.5°C (Messenger, 1964). Low winter temperatures also could impede colonisation by invasive pests and insects from warmer areas and thus viruliferous insects might not be a threat to all adjacent areas where virus infections are not present (Bale & Hayward 2010). For example, the distribution of the leafhopper *Cicadulina bipunctata* (Melichar, 1904) was studied and it was found that a temperature below 5°C during winter made a noteworthy contribution to its dispersal. This is therefore an important aspect limiting the range expansion of these leafhoppers, and is equivalent to the temperatures experienced during the 2016 winter (Matsukura *et al*., 2016). Indirect chilling injury was the main cause of lethality of leafhoppers and as a result, the overwintering rates of the insects were very low (Matsukura *et al.,* 2014). While

these are completely different pathosystems, a similar effect may have been experienced with the MSV-leafhopper pathosystem due to comparatively low temperatures seen in the areas where very few/no virus symptoms were observed.

In contrast, in the Lowveld regions (Nelspruit, Malalane, White River and surrounding regions that were sampled) the lowest temperature recorded in 2016 winter was 4.3°C and average temperatures range from 21.4°C-27.9°C (SAWS, personal communication, 6 April 2018). These warmer temperatures and the lack of frost are likely to sustain aphid colonies or leafhopper populations in areas surrounding Nelspruit and Trichardtsdal, which is where plenty of virus-like symptoms were observed (Appendix A, Fig. 2.8 A & B). A study by Morgan *et al.* (2001) showed that the pea aphid, *Acyrthosiphon pisum* (Harris) shows the greatest population growth at temperatures between 11.9°C and 23.1°C. Insect vectors facilitate spread of viruses to new hosts and also the proliferation of a disease on the current host crop (Agrios & Hadwiger, 2005). In the current study, SCMV was found concentrated in the north-eastern parts of SA (Appendix A, Fig. 2.8) and were also detected in the north-western parts of SA (Appendix A, Fig. 2.7). This may be due to the climatic conditions and lack of frost that: 1) allow insect vectors to survive and proliferate to higher numbers, 2) allows maize to be grown all year round, especially on smallholder farmers' lands, causing a build-up of virus in the area and 3) allows reservoir hosts of viruses to flourish.

Variations in symptoms were observed in the maize which tested positive for SCMV and thus visual symptoms cannot be used to discriminate SCMV infections over infections by other viruses. SCMV can cause varying symptoms on different sugarcane genotypes, with the most distinct being interveinal chlorosis and mosaics of different shades of green (Holkar *et al.,* 2017) and differ based on host plant cultivar, growing conditions including temperature as well as the strain of SCMV involved (Luo *et al*., 2016). The mottle symptoms seen on the samples which tested positive for SCMV (Fig. 2.3 A-C, H, L-O) could be specific to SCMV infections on maize and may be useful to identify SCMV-infections in future. However, the presence of other viruses in mixed infections cannot be excluded. For example, Maize yellow mosaic virus (MaYMV) which does not cause streak symptoms (Gonçalves *et al*., 2017), but was detected in some of these samples (Welgemoed *et al*., unpublished). The lack of stunting seen in virus-infected plants in this study could

represent plants recently infected after plants had already reached full maturity (Agrios & Hadwiger, 2005). Furthermore, Poaceae-infecting viruses can also cause symptomless infections (Harris & Hillman, 2000). It would be worthwhile to screen the current study's samples for other monocot-infecting viruses to identify viruses present in these symptomatic samples. This is being pursued using next generation sequencing in a parallel study.

Although isolates grouped together in terms of geographic region where collected, some nucleotide similarity percentages between isolates from a particular region were lower than expected. Phylogenetic analysis revealed great similarity exists between isolates of SCMV on sugarcane from the same region (99.5%) (Goodman *et al.*, 1998). In the current study nucleotide percentage ranged between 91.35%-100% (sub-group I) or 95.49%-100% (sub-groups II and V) between isolates from the same region. The shared nucleotide percentages between isolates from different regions were lower than expected at approximately 95% between sub-groups I and II, and even lower between sub-groups II and V as this similarity was just below 85% while on sugarcane SCMV from two widely separated geographic locations was 98.5% (Goodman *et al.* 1998). These differences may be due to the relatively small sample size of their study (only two isolates from two regions). It may also be that these SCMV isolates were obtained from sugarcane which is clonally propagated (Butterfield *et al.,* 2002; Irvine & Benda, 1985), resulting in less strain variation if these SCMV isolates previously shared a source of planting material (Ramgareeb *et al*., 2010; Snyman *et al*., 2008). For members of the Potyviridae family, the species demarcation criteria for the NIb gene sequence of different potyviral species is below 76.6% nucleotide identity and below 89% amino acid identity (Adams *et al*., 2005). Although we do not have the whole NIb sequence, we could differentiate, based on the partial gene sequence, a number of putative JGMV and PenMV isolates in this study from the SCMV isolates from this study, as the minimum nucleotide similarities between certain sequences were 74.43%-75.56%. However, some isolates of putative JGMV and PenMV still shared 84.59%-80.45%, suggesting that they are genetically divergent but may be a less closely related strain of SCMV to the other isolates included in the current study. SCMV isolates from this study were identified by the initial BLAST analysis as those with a the minimum nucleotide similaritiies between these isolates and other SCMV isolates of 91.35%-95.86%, with a

maximum of 100% homology in this partial gene sequence. However, absolute proof requires that the entire NIb gene sequence must be determined for an accurate species demarcation based on polyprotein sequence nucleotide homology percentage comparison (Adams *et al*., 2005). The relatively short terminal branches seen in sub-groups I, II and III indicate isolates may have accumulated mutations over a relatively short period of time (Hosseini *et al*., 2017).

In addition to insect vectors, the inoculum source is also important in virus ecology and diversity (Roossinck, 2012). The alternate hosts of potyviruses in this study mainly harboured JGMV, and not SCMV, and were all panic grass. Interestingly JGMV were not detected on maize fields in proximity to the JGMV infections on panic grass. This may suggest that these strains of JGMV do not infect maize as shown by Stewart *et al.* (2014). The sample number of wild grasses in the current study was relatively small and a larger survey would need to be conducted to confirm these results and also to identify other species that may be reservoir hosts for SCMV – especially in the areas where SCMV was detected in high numbers. In the past, virus research was aimed at viruses on crops but more recent studies indicated that plant viruses are abundant on wild plants too (Roossinck, 2012) and therefore a whole range of wild grasses would need to be included in the survey.

Long distance dispersal of viruses can occur when their insect vectors travel and small insects specifically like cereal aphids are suspected to travel large distances by air currents (Agrios & Hadwiger, 2005). Zeyen *et al.* (1987) reported transport of MDMV via viruliferous aphids up to 1000km in 12h. This may put a larger region than only the neighbouring farms of the Ofcolaco and Trichardtsdal area (where SCMV was observed in high numbers) at risk for a SCMV outbreak in future. Furthermore, various studies have indicated that global warming can in time alter the distribution of species (Parmesan, 2006) and can change the climate in favour of aphids in regions that are currently unfavourable. It is therefore possible to extrapolate from such studies that increasing temperatures in the future may increase inhabitable regions for other insects and thus allow the spread of the viruses that they carry.

In critical evaluation of the experimental strategy used in this study, we consider the sample numbers tested is deemed sufficient to draw conclusions: samples surveyed compare favourably with other studies, 106 samples sampled in Benin (Afouda *et al*.,

2017) and 104 maize samples (Luo *et al.*, 2016) analysing co-infections between SrMV and SCMV or SCSMV. However, this study should be followed up by similar surveys over multiple seasons in order to confirm the current study's results. The season in which the survey was conducted is unusual in having a record harvest yield due to good rainfall at the appropriate stage, and may not reflect the general situation and status or diversity of potyviruses on maize in average years. Future studies regarding the risk and rapidity of SCMV spreading further on South African maize should address 1) the viruses present over multiple growing seasons, 2) investigating the host range of South African JGMV, 3) identifying additional alternate reservoir plants of these viruses, 4) the climatic conditions across all maize-growing regions in SA as well as 5) the distribution and ecology of the aphids.

In conclusion, this is the first comprehensive potyvirus survey of maize and selected alternate hosts conducted in SA. In this study, we report on a comprehensive single season survey conducted over the 2016/2017 growing season in SA in which the presence, identities, localities as well as potential reservoir hosts of potyviruses are identified. The most likely potyvirus to be associated with MLN infections in future in SA is SCMV as it is already present in a number of regions, and although JGMV was identified on a number of grass samples as well as two samples of maize. The ability of JGMV in SA to infect maize needs to be studied before predictions can be made regarding its role in MLND in SA. The current study also showed that symptoms can be cryptic due to the occurrence of a symptomless infection or multiple pathogens in one plant, and thus cannot identify a potyvirus infection and molecular techniques should be employed. In view of the potential introduction of MCMV to SA from neighbouring African countries, the large concentration of potyvirus infection in the two provinces in the north-eastern part of the country, closest to the border between SA and Zimbabwe, are reasons for concern as MLND is already present in Tanzania which borders (and may spread to) Zimbabwe. The current study, together with predictions in the study by Isabirye and Rwomushana (2016) suggests that the areas where 1) warmer minimum and average temperatures are experienced that allow the vectors to survive proliferate and 2) potyviruses were detected in high numbers during the current study, are some of the likely areas to be affected by MLND should MCMV enter SA. These areas seem to supply a habitat supportive of the aphids, alternate host plants and some of the MLND-causing pathogens: the potyviruses.

Based on the relatively low relative incidence of potyviruses on maize suggest that South African maize may not be particularly susceptible to potyvirus infections or that viruliferous vectors are not present in large sections of the maize-growing regions. This may indicate that MLND will most likely be containable and manageable should MCMV enter the country, at least for the first while. However, follow-up surveys and continuous surveillance should be carried out in future especially near the borders.

References

Adams IP, Miano D, Kinyua Z, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R & Hany U (2013) Use of Next Generation Sequencing for the identification and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus causing Maize Lethal Necrosis in Kenya. *Plant Pathology* **62**: 741-749.

Adams M, Antoniw J & Fauquet C (2005) Molecular criteria for genus and species discrimination within the family Potyviridae. *Archives of Virology* **150**: 459-479.

Afouda L, Kone D, Zinsou V, Dossou L, Kenyon L, Winter S & Knierim D (2017) Virus surveys of *Capsicum spp*. in the Republic of Benin reveal the prevalence of Pepper vein yellows virus and the identification of a previously uncharacterised *Polerovirus* species. *Archives of Virology* **162**: 1599-1607.

Agrios GN & Hadwiger LA (2005) Environmental effects on the development of infectious pant disease. *Plant pathology*. Elsevier Academic Press: Amsterdam. 251-257.

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR & Daszak P (2004) Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology and Evolution* **19**: 535-544.

Azzam O & Chancellor TC (2002) The biology, epidemiology, and management of Rice Tungro Disease in Asia. *Plant Disease* **86**: 88-100.

Bale J & Hayward S (2010) Insect overwintering in a changing climate. *Journal of Experimental Biology* **213**: 980-994.

Bockelman D, Claflin L & Uyemoto J (1982) Host range and seed-transmission studies of Maize chlorotic mottle virus in grasses and corn. *Plant Disease* **66**: 216-218.

Butterfield M, Irvine J, Garza MV & Mirkov T (2002) Inheritance and segregation of virus and herbicide resistance transgenes in sugarcane. *Theoretical and Applied Genetics* **104**: 797-803.

Claflin SB, Jones LE, Thaler JS & Power AG (2017) Crop-dominated landscapes have higher vector-borne plant virus prevalence. *Journal of Applied Ecology* **54**: 1190-1198.

De Groote H, Oloo F, Tongruksawattana S & Das B (2016) Community-survey based assessment of the geographic distribution and impact of Maize Lethal Necrosis (MLN) Disease in Kenya. *Crop Protection* **82**: 30-35.

Duffus J (1971) Role of weeds in the incidence of virus diseases. *Annual Review of Phytopathology* **9**: 319-340.

FAO (2018) Country briefs: South Africa. Available online at: http://www.fao.org/giews/countrybrief/country.jsp?code=ZAF. [10 February 2018].

FAOSTAT (2018) FAO website, South Africa. Available online at: http://www.fao.org/faostat/en/#data/QC/visualize. [9-21 May 2018].

Fereres A (2015) Insect vectors as drivers of plant virus emergence. *Current Opinion in Virology* 42-46.

Flett B & Mashingaidze K (2016) Maize Lethal Necrosis: Possible threat to local maize production. *Grain SA*. Available online at: http://www.grainsa.co.za/maize-lethal-necrosis:-possible-threat-to-local-maize-production. [23 October 2017].

Gell G, Balázs E & Petrik K (2010) Genetic diversity of Hungarian Maize dwarf mosaic virus isolates. *Virus Genes* **40**: 277-281.

Giolitti F, Herrera M, Madariaga M & Lenardon S (2005) Detection of Maize dwarf mosaic virus (MDMV). *Maydica* **50**: 101-104.

Gonçalves M, Godinho M, Alves-Freitas D, Varsani A & Ribeiro S (2017) First report of Maize yellow mosaic virus infecting maize in Brazil. *Plant Disease* **101**: 2156.

Goodman B, MacDonald D & Huckett B (1998) A survey of South African Sugarcane mosaic virus (SCMV) strains based on CP gene sequence analysis. In: *Proceedings of the South African Sugarcane Technologists' Association* **72**: 146-148.

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: *Nucleic Acids Symposium Series*. **41**: 95-98.

Hall TA (2011) BioEdit: an important software for molecular biology. *Green Earth Research Foundation Bulletin of Biosciences* **2**: 60-61.

Harris DR & Hillman GC (2014) Maize: domestication, racial evolution, and spread. *Foraging and farming: the evolution of plant exploitation*. Routledge: London. 632-658.

Holkar S, Kumar A, Meena M & Lal R (2017) Detection and partial molecular characterization of Sugarcane mosaic virus infecting sugarcane genotypes. *Journal of Environmental Biology* **38**: 409.

Hosseini H, Mehrvar M & Zaki AGM (2017) Comparative genetic diversity of potato virus Y populations based on Coat protein gene. *Acta Virologica* **61**: 161-174.

Irvine J & Benda G (1985) Sugarcane mosaic virus in plantlets regenerated from diseased leaf tissue. *Plant Cell, Tissue and Organ Culture* **5**: 101-106.

Isabirye EB & Rwomushana I (2016) Current and future potential distribution of Maize chlorotic mottle virus and risk of Maize Lethal Necrosis Disease in Africa. *Journal of Crop Protection* **5**: 215-228.

ISSCT (1989) Sugarcane diseases and their world distribution. Ricaud C, Egan BT, Gillaspie AG Jr, Hughes CG (eds.). In: *Diseases of Sugarcane*. Elsevier, New York. 341-376.

Jones R (2014) Plant virus ecology and epidemiology: historical perspectives, recent progress and future prospects. *Annals of Applied Biology* **164**: 320-347.

Katoh K, Misawa K, Kuma Ki & Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform. *Nucleic Acids Research* **30**: 3059-3066.

Katoh K & Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772-780.

Kiruwa FH, Feyissa T & Ndakidemi PA (2016) Insights of Maize Lethal Necrotic Disease: a major constraint to maize production in East Africa. *African Journal of Microbiology Research* **10**: 271-279.

Knox A, Chauhan R & von Wechmar B (1986) *Mixed infections of Maize dwarf mosaic virus (MDMV) and Cucumber mosaic virus (CMV) in maize*. Technical Communication - Department of Agriculture and Water Supply, South Africa.

Kumar S, Nei M, Dudley J & Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* **9**: 299-306.

Luo Q, Ahmad K, Fu HY, Wang JD, Chen RK & Gao SJ (2016) Genetic diversity and population structure of Sorghum mosaic virus infecting *Saccharum spp*. hybrids. *Annals of Applied Biology* **169**: 398-407.

Mahuku G, Lockhart BE, Wanjala B, Jones MW, Kimunye JN, Stewart LR, Cassone BJ, Sevgan S, Nyasani JO & Kusia E (2015) Maize Lethal Necrosis (MLN), an emerging threat to maize-based food security in sub-Saharan Africa. *Phytopathology* **105**: 956-965.

Matsukura K, Izumi Y, Kumashiro S & Matsumura M (2014) Cold tolerance of the Maize orange leafhopper, *Cicadulina bipunctata*. *Journal of Insect Physiology* **67**: 114-119.

Messenger P (1964) The influence of rhythmically fluctuating temperatures on the development and reproduction of the spotted alfalfa aphid, *Therioaphis maculata*. *Journal of Economic Entomology* **57**: 71-76.

Morgan D, Walters K & Aegerter J (2001) Effect of temperature and cultivar on Pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae) life history. *Bulletin of Entomological research* **91**: 47-52.

Muhire BM, Varsani A & Martin DP (2014) SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. *Public Library of Science One* **9**: e108277. DOI: 10.1371/journal.pone.0108277.

Naidu R & Hughes JA (2003) Methods for the detection of plant virus diseases. In: *Plant Virology in sub-Saharan Africa.* Proceedings of a conference organised by IITA, 4-8 June 2001. International Institute of Tropical Agriculture, Ibadan, Nigeria. 233-253.

Pande D, Madzokere E, Hartnady P, Kraberger S, Hadfield J, Rosario K, Jäschke A, Monjane AL, Owor BE & Dida MM (2017) The role of Kenya in the trans-African spread of Maize streak virus strain A. *Virus Research* **232**: 69-76.

Parmesan C (2006) Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology, Evolution, and Systematics* **37**: 637-669.

Ramgareeb S, Snyman S, Van Antwerpen T & Rutherford R (2010) Elimination of virus and rapid propagation of disease-free sugarcane (*Saccharum spp*. cultivar NCo376) using apical meristem culture. *Plant Cell, Tissue and Organ Culture* **100**: 175-181.

Roossinck MJ (2012) Plant virus metagenomics: biodiversity and ecology. *Annual Review of Genetics* **46**: 359-369.

Scheets K (1998) Maize chlorotic mottle machlomovirus and Wheat streak mosaic Rymovirus concentrations increase in the synergistic disease Corn Lethal Necrosis. *Virology* **242**: 28-38.

Singh K & Kundu JK (2017) Variations in Coat protein sequence of Wheat streak mosaic virus among crop and non-crop hosts. *Crop and Pasture Science* **68**: 328-336.

Snyman S, Baker C, Huckett B, McFarlane S, Van Antwerpen T, Berry S, Omarjee J, Rutherford R & Watt D (2008) South African Sugarcane Research Institute: embracing biotechnology for crop improvement research. *Sugar Tech* **10**: 1.

South African Weather Services (SAWS) website (2018) Historical rain maps of South Africa. Available online at: http://www.weathersa.co.za/climate/historical-rain-maps. [20 March 2018].

Stewart L, Teplier R, Todd J, Jones M, Cassone B, Wijeratne S, Wijeratne A & Redinbaugh M (2014) Viruses in maize and Johnsongrass in southern Ohio. *Phytopathology* **104**: 1360-1369.

Stewart LR, Redinbaugh MG, Massawe D, Willie K, Wijeratne S, Niblett CL, Kiggundu A & Asiimwe T (2017) Johnsongrass mosaic virus contributes to Maize Lethal Necrosis in East Africa. *Plant Disease* **101**: 1455-1462.

Tamura K & Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**: 512-526.

Tosic M & Ford R (1972) Grasses differentiating Sugarcane mosaic and Maize dwarf mosaic viruses. *Phytopathology* **62**: 1466-1470.

Van Oudtshoorn, F (1992) Spesiebeskrywings. *Gids tot grasse van Suid-Afrika.* Nasionale Boekdrukkery: Cape Town. 239, 245.

Wang Q, Zhou X & Wu J (2014) First report of Maize chlorotic mottle virus infecting sugarcane (*Saccharum officinarum*). *Plant Disease* **98**: 572.

Wangai A, Redinbaugh M, Kinyua Z, Miano D, Leley P, Kasina M, Mahuku G, Scheets K & Jeffers D (2012) First report of Maize chlorotic mottle virus and Maize Lethal Necrosis in Kenya. *Plant Disease* **96**: 1582.

Webster CG, Wylie SJ & Jones MG (2004) Diagnosis of plant viral pathogens. *Current Science* **86**: 1604-1607.

White EJ, Venter M, Hiten NF & Burger JT (2008) Modified Cetyltrimethylammonium Bromide method improves robustness and versatility: The benchmark for plant RNA extraction. *Biotechnology Journal* **3**: 1424-1428.

Xie L, Zhang J, Wang Q, Meng C, Hong J & Zhou X (2011) Characterization of Maize chlorotic mottle virus associated with Maize Lethal Necrosis Disease in China. *Journal of Phytopathology* **159**: 191-193.

Zeyen R, Stromberg E & Kuehnast E (1987) Long-range aphid transport hypothesis for Maize dwarf mosaic virus: history and distribution in Minnesota, USA. *Annals of Applied Biology* **111**: 325-336.

Zhang Y, Zhao W, Li M, Chen H, Zhu S & Fan Z (2011) Real-time TaqMan RT-PCR for detection of Maize chlorotic mottle virus in maize seeds. *Journal of Virological Methods* **171**: 292-294.

Zheng L, Rodoni B, Gibbs M & Gibbs AJ (2010) A novel pair of universal primers for the detection of potyviruses. *Plant Pathology* **59**: 211-220.

Chapter 3: Genetic diversity of two partial gene regions of Sugarcane mosaic virus isolates from *Zea mays* in South Africa and Tanzania

Abstract

Sugarcane mosaic virus (SCMV) is one of the main components of Maize Lethal Necrosis Disease (MLND) which is jeopardising food security across sub-Saharan Africa. While the other crucial pathogen for the disease, Maize chlorotic mottle virus (MCMV), is not present in South Africa (SA), SCMV does occur here. Tanzania is the geographically closest country to SA having MLND and it is a possible source of a future outbreak of the disease in SA. This study aims to elucidate the phylogenetic relationships of South African and Tanzanian SCMV isolates with those isolates from other countries, especially those on the African continent. SCMV infected samples from various regions in SA were selected and a partial region of the Nuclear Inclusion body gene and Coat protein gene were amplified from these during Reverse Transcription-Polymerase Chain Reaction (RT-PCR), followed by direct cycle sequencing of the amplicons. The resulting sequences, together with reference sequences from various countries (including Tanzania, for which Next Generation Sequencing (NGS) data was available) and host plants were analysed and used to construct phylogenies. The key results include that: 1) SCMV isolates from SA clusters in two clades based on the local geographical origin of the samples, 2) South African and Tanzanian isolates did not cluster together, 3) South African SCMV isolates are most closely related to Asian and European isolates, 4) Tanzanian isolates grouped with isolates from other East African countries, 5) clustering of isolates occurred based on host plant from which they were derived. This study pre-empts the possible outbreak of MLN in SA, and reveals the genetic diversity and evolution of SCMV, one of the two key viral components of this devastating disease of maize. The results suggest that the combination of SCMV in SA with MCMV may have biological differences to that of this combination in Tanzania or other East African countries. Our study aids ongoing research in Africa as a whole regarding: 1) the evolution and similarities of MLN-causing viruses in Africa and their epidemiology and 2) is critically important should genetically modified maize resistant to the viruses in the disease complex be developed in future.

3.1 Introduction

Maize lethal necrosis disease (MLND) causes severe yield loss of maize (*Zea mays*), Africa's major staple food and fodder crop (Adams *et al*., 2013; De Groote *et al*., 2016). It's possible, that rapid spread to neighbouring countries including South Africa (SA) in the near future is a looming threat (Flett & Mashingaidze, 2016). The disease is caused by the synergistic co-infection of a potyvirus such as Sugarcane mosaic virus (SCMV), in combination with Maize chlorotic mottle virus (MCMV) (Adams *et al.,* 2014; Goldberg & Brakke *et al*., 1987; Stewart *et al*., 2017). The virus was identified as the major potyvirus present on maize in SA (Chapter 2: 2.3) and has been identified as the most common potyvirus associated with Maize Lethal Necrosis (MLN) infections in eastern Africa (Mahuku *et al*., 2015). Due to its role in the formation of the MLND-complex, SCMV may predispose South African maize to this devastating disease.

The importance of studying SCMV-especially in areas that do not yet have MCMV or MLND-has been highlighted in the literature. Specifically, for SA, studying MLND-causing viruses from Tanzania is important as there is a strong possibility that MCMV from Tanzania may be the source of a future outbreak of MLND in SA due to Tanzania having the closest proximity to SA amongst the countries where MLND has already been identified (Flett & Mashingaidze, 2016; Wangai *et al*., 2012). The most likely route of MCMV entrance into SA is from Tanzania into Mozambique, in the sub-tropical north-east and northern parts of SA (Flett & Mashingaidze, 2016).

SCMV is a 3' polyadenylated, positive-sense single-stranded RNA virus with a protein (VPg) linked to the 5' terminal of the genome (Brunt, 1992). The 9.8 kilo base pairs (kb) genome is encapsulated by an elongated helix of Coat protein (CP)s that form a flexuous virion, 68-900nm in length and 11-15nm in width (Urcuqui-Inchima *et al*., 2001). The genome contains a long open reading frame (ORF) that is translated into a polyprotein and subsequently post- and/or co-translationally cleaved into 10 smaller proteins (Ivanov *et al*., 2014). An 11th protein, P3N-PIPO, is produced from a small open reading frame separately (Chung *et al*., 2008). Of greatest significance for this study are the potyviral Nuclear Inclusion body (NIb) as well as the CP whose genes are routinely used in virus identification (Ivanov *et al*., 2014).

The NIb protein is multifunctional as it works as the RNA-dependent RNA-polymerase (RdRp), covalently binds with the VPg protein to perform regulatory functions (Li *et al*., 1997; Urcuqui-Inchima *et al*., 2001) and for some potyviruses also forms crystalline particles in the nuclease of infected cells (Riedel *et al*., 1998). The NIb gene region contains variable sequences flanked by conserved regions to which diagnostic primers have been designed that can detect any species within the genus, while sequencing the amplicon produced allows the identification to the species level of the virus (Zheng *et al*., 2010).

Potyviral CPs are multifunctional, fulfilling significant roles of the virus life cycle and mediating virus-vector relationships and thus, this gene is probably a target of selection by both insect vectors and plant hosts (Urcuqui-Inchima *et al*., 2001). The gene encoding CP is well defined, unique in each viral type and includes a highly conserved region, which makes it ideal for analysing strain variation (Shukla *et al.*, 1987) and thus inferring phylogenetic relatedness amongst isolates. The study of Shukla *et al.* (1987) showed that CP gene sequence information is useful in potyvirus classification. In 1993, Pappu *et al*. established a universal degenerate pair of diagnostic primers which binds to and amplifies part of the 3'UTR and the CP regions. The forward primer was designed to bind to the conserved WCIEN and QMKAAA 'boxes' of the CP. The reverse primer to the poly-A tail at the 3' end of the genome and consists of a poly-T oligo tailed at its 3' end by either A, C or G in order to guarantee binding which the 3'UTR ends and the poly-A tail begins (Pappu *et al*., 1993).

A study by Green *et al.* (2018) showed that the nucleotides from 5,850-9,300 of the potyviral genome, where both the NIb and CP genes are located, has the most structural diversity and contains most of the novel recombinant segments suggesting that this area of the genome responds rapidly to new selection pressures. This means that sequence divergence and diversity can be best studied from genes in this region of the genome, reinforcing the validity of using these particular partial genes in our study.

In order to gain insight and understanding of the epidemiology and ecology of plant viruses, studies deciphering their genetic diversity are valuable (Aranda & Freitas-Astua, 2017; García-Arenal *et al*., 2001; Varsani *et al*., 2009). A number of studies

have been done on SCMV's biology and characterisation but systematic research is still lacking when compared to other potyviruses such as Potato virus Y (PVY) (Ogawa *et al*., 2008) and Turnip mosaic virus (TuMV) (Tomitaka & Ohshima, 2006). Li *et al*. (2013) conducted a study on the population structures and genetic diversity of SCMV isolated from both maize and sugarcane from various countries and showed certain trends that were coherent with previous studies on potyviruses. For example, SCMV generally clusters according to its host and also its geographical origin. A drawback of Li *et al*. (2013)'s study for our purposes is that their study focused on isolates from sugarcane and furthermore contained only one isolate from Africa. In studies by Alegria *et al*. (2003) and Li *et al.* (2013), SCMV isolates from different countries on the same continent, such as Egypt and SA (Alegria *et al*., 2003), and Germany and Spain (Li *et al*., 2013), are often genetically similar and thus, this was also hypothesised to be true for South African isolates and SCMV from other African countries.

Studies on the diversity of South African and Tanzanian SCMV are scarce, especially when considering SCMV isolates from maize. In fact, Kiruwa *et al*. (2016) stated that Tanzanian MLND-causing viruses, including Tanzanian SCMV, have not been studied extensively or characterised. SCMV diversity studies by Alegria *et al.* (2003), Goodman *et al*. (1998) and Handley *et al.* (1998) included South African isolates, but all isolates were isolated from sugarcane and included only a few from SA (3, 4 and 1, respectively). Thus, it is clear that there is a knowledge-gap regarding the diversity of SCMV isolated from maize in SA and Tanzania.

This study investigates genetic variation amongst African SCMV isolates in the countries where MLND is already prevalent, along with those from other countries worldwide. SCMV plays an integral role in MLN infections in Africa and is the predominant potyvirus present on South African maize, pre-disposing the food security crop to this lethal disease. Currently the diversity of South African and Tanzanian SCMV remains unexplored. The study of its diversity in these two countries will provide valuable information about how similar SCMV isolates from SA are to those from Tanzania, the closest country to SA where MLND is currently present. It will also provide insight into both the relationships of SCMV globally and the epidemiology of the MLND viral complex in Africa as well as the potential MLN infections in SA in future. It will also provide indispensable information regarding the

diversity of the SCMV population structure in Africa to aid the development of MLND resistant crops. The aim of this study was to determine the diversity of South African and Tanzanian SCMV isolates, their relationship with other SCMV isolates worldwide and thus, the origin of South African SCMV. This will be achieved by accomplishing the following objectives: 1) compare South African SCMV with those from Tanzania, the geographically closest country to SA where MLND is present, 2) investigate the genetic evolution of SCMV throughout Africa based on two partial gene sequences, and 3) obtain a holistic and current insight of the diversity of SCMV internationally using as many currently available as possible sequences..

3.2 Materials and Methods

3.2.1 RNA extraction

Purified RNA, prepared for the survey (Chapter 2: 2.2.1) was used except in instances where these RNA were depleted by previous tests. In that case, RNA was re-extracted using the method described by White *et al.* (2008) from 200mg of dried original leaf material with a slight modification: spermidine was omitted from the cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 1% polyvinyl pyrrolidone (PVP), 20mM ethylenediaminetetraacetic acid (EDTA), 100mM Tris-hydrochloric acid (HCl), 1.4M sodium chloride (NaCl)). Illumina HiSeq data for various samples collected from Tanzania where MLND is present was generated from RNA in order to monitor southward spread of the disease in Africa and was available for use in studying Tanzanian SCMV in this study.

3.2.2 Reverse Transcription-Polymerase Chain Reaction

Two pairs of universal potyvirus primers, 1) oligo dT and CN48F (Pappu *et al*., 1993) as well as, 2) NIb2F and NIb3R (Zheng *et al*., 2010) (Table 3.1), were utilised in RT-PCR. RT-PCR was done in two-step reactions using the Moloney-Murine Leukemia Virus (M-MLV) RT (Promega; Madison, WI, USA) and MyTaq (Bioline; Taunton, MA, USA) systems, with some alterations to the manufacturer's protocol. Total RNA (3µl) and 2µl R primer (4µM in final reaction) were incubated together at 70˚C for 5min. Subsequently, first-strand cDNA synthesis was completed by preparing a master mixture of 10mM dNTPs (dATP, dCTP, dGTP and dCTP; Promega), 25U M-MLV RT enzyme, M-MLV-buffer (5mM Tris-HCl pH 8.3, 3mM magnesium chloride ($MgCl_2$), 75mM potassium chloride (KCl), 10mM dichlorodiphenyltrichloroethane (DTT) in final working solution). A volume of 5.5µl master mixture and 5µl RNA/ primer mixture were mixed and the reactions were incubated at 42°C for 1h. For the amplification step, a master mix was prepared with the following per reaction: 0.2µM forward and reverse primers each, 25U MyTaq DNA polymerase (Bioline) and MyTaq reaction buffer (5mM dNTPs, 15mM $MgCl_2$) (Bioline). 22µl of the PCR master mix and 3µl cDNA were added together. cDNA amplification was carried out at the following conditions: 95°C for 5min; 40 cycles of 1) 95°C for 15s, 2) 42°C for 30s and 3) 72°C for 10s, and finally a 10min extension step at 72°C.

Table 3.1 Information on primers used in this study.

Primer name	Polarity	Author of publication	Primer sequence (5'-3')	Partial gene amplified	Expected product size (bp)
NIb2F	+	Zheng *et al.* (2010)	GTITGYGTIGAYGAYTTYAAYAA	NIb	350
NIb3R	-		TCIACIACIGTIGAIGGYTGNCC		
CN48F	+	Pappu *et al.* (1993)	TCGTGIATHGANAATGG	CP	700
Oligo-dT	-		$(T)_{21}V$		

3.2.3 Purification of Polymerase Chain Reaction products and Sanger sequencing

RT-PCR products were subjected to a PCR clean-up to remove 5'P and 3'OH overhangs using 5U ExoI (Fermentas, MD, USA) and 1U FastAP (Fermentas) at 37°C for 15min and subsequently 85°C for 15min. The purified DNA products were then used as a template in cycle sequencing reactions (Coenye *et al*., 1999). Reaction volumes of 10µl in total were used and contained: 3µl template DNA, 2.25µl Sequencing Buffer (Applied Biosystems; Foster City, CA, USA), 0.75µl (2µM) forward and reverse primer (Inqaba Biotec, SA), 3µl molecular-grade nuclease-free water (H_2O) (Sigma), 1µl BigDye Terminator Ready Reaction Mix (Applied Biosystems). Precipitation of the products was performed using the following per reaction: 1µl EDTA, 1µl sodium acetate (NaOAc), and 100% Molecular-grade ethanol (EtOH) (Merck; Darmstadt, Germany), followed by centrifugation at 13000rpm for 30min, and subsequently washing the pellet with 70% Molecular-grade Ethanol (Merck) before delivery to the sequencing facility. Direct sequencing in both directions was conducted at the University of Pretoria (UP) sequencing facility on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems).

3.2.4 Reference mapping to obtain Tanzanian Sugarcane mosaic virus consensus sequences

Next Generation Sequencing (NGS) data from MLND samples collected in Tanzania was generated at the time when this study started by the Agriculture Research Council-Biotechnology Platform (ARC-BTP), and the SCMV components could be obtained for the purpose of studying their genetic diversity. Illumina HiSeq data was

mapped to reference sequences in order to obtain SCMV partial genomes from the data. In order to identify and extract SCMV sequences from the Illumina data of the Tanzania MLND samples, reference mapping to various SCMV whole genomes were conducted. Processing and analyses of the Illumina HiSeq data was achieved using CLC Genomics Workbench (v6) (available online from: https://www.qiagen bioinformatics.com/). Following importation of data as paired end reads, adapter and quality trimming was achieved using the default program settings with the universal TruSeq adapter sequence (5' AATGATACGGCGACCACCGAGATCTACACTCTTT CCCTACACGACGCTCTTCCGATCT). The following sequences obtained from NCBI (National Center for Biotechnology Information) GenBank (available online from https://www.ncbi.nlm.nih.gov/genbank/) were utilised for reference mapping and are listed using their GenBank accession numbers: JX286706.1, KP860936.1, JX047394.1, GU474635.1. The default parameter of similarity/length fraction of 0.5/0.8 was used. The 'ignore' function was used, meaning that reads that were capable of multiple mappings are classified as unmapped. Consensus sequences were obtained and used in subsequent steps.

3.2.5 Sequence processing and alignment

Partial NIb and CP sequences of South African isolates were manually edited using Chromas LITE Sequence Alignment Editor (v2.1.1) (Technelysium Pty Ltd, Australia). Forward and reverse sequences were assembled in BioEdit Sequence Alignment Editor (v7.0.9.0) (Hall, 1999; 2011) followed by curating consensus sequences between the two. Sequences were subjected to a GenBank Basic Local Alignment Search Tool (BLAST) (available online from https://blast. ncbi.nlm.nih.gov/Blast.cgi) analysis for verification of the identity of the sequences. All curated sequences were submitted to NCBI GenBank to obtain accession numbers. All sequence names and accession numbers used in this study are listed in Table 3.2 and sequence data generated and analysed in this study is available on GenBank (available online from https://www.ncbi.nlm.nih.gov/genbank/).

3.2.6 Multiple alignment and phylogenetic analysis

The sequences of the isolates together with SCMV reference sequences were submitted to the MAFFT (Multiple Alignment using Fast Fourier Transform) online sequence alignment program (v7.0) (Katoh *et al*., 2002; Katoh & Standley, 2013) (available online from https://mafft.cbrc.jp/alignment/server/index.html) in order to verify the orientation of the sequences and to perform a multiple sequence alignment using MAFFT default parameters. The alignment was exported to BioEdit (v7.0.9.0) (Hall, 1999; 2011) where the overhangs of the alignment were trimmed.

Molecular Evolutionary Genetic Analysis (MEGA) software (v6) (Kumar *et al*., 2008) was used to construct maximum-likelihood phylogenies by analysing a 510 bp region of the partial CP gene or a 270 bp of the NIb protein region. A total of 17 SCMV isolates isolated from maize during the current study and 12 isolates from Illumina data from Tanzanian isolates, together with the same 19 reference sequences for the CP (Fig. 3.1) and NIb (Fig. 3.2) trees. These reference isolates were selected from all SCMV sequences available on Genbank, and chosen because they contained both CP and NIb genes.

A second, larger phylogeny was constructed for the CP to obtain better resolution, as more CP references than NIb sequences are available. This phylogeny (Fig. 3.3) was generated with 50 reference SCMV sequences from a variety of hosts as well as different countries. PVY (genus: *Potyvirus*, family: Potyviridae), the type strain for the genus *Potyvirus,* was selected as outgroup for the tree.

The evolutionary models with the best fit were determined and maximum likelihood analyses performed using MEGA (v6) (Kumar *et al*., 2008), taking into account all sites in the alignment including the gaps. The models selected for the analyses were Kimura 2-parameter (K2) (Fig. 3.1 and 3.3) and Tamura 3-parameter (T92) (Fig. 3.2) (Tamura & Nei, 1993), both including rate variation among sites (+G). A bootstrap analysis of a 1000 replicates was also implemented for the purpose of evaluating the confidence in the branching points.

3.2.7 Pairwise nucleotide analysis

Using the dataset generated in 3.2.6, the Sequence Demarcation Tool (SDT) software (v1.2) (available online from http://web.cbio.uct.ac.za/SDT) (Muhire *et al*., 2014) was used to calculate the nucleotide similarity percentages between isolates.

3.3 Results

Amplicons of the expected size were obtained when using both primer pairs. The multiple alignment and preparation of dendrograms using the NIb partial gene sequences (Fig. 3.1) revealed two main groups, group A, with eight sub-groups and group B, with one sub-group, most with high bootstrap values. SA isolates in sub-groups I and II are most closely related to Chinese isolates. The SA isolates in sub-groups I, II and VIII cluster separately and thus form novel clades on their own. Tanzanian isolates are distributed among two sub-groups, with those from sub-group VIII grouping with relatives from China, Thailand and Ecuador. However, the majority of Tanzanian isolates (sub-group IX), grouped among other East African isolates from Rwanda and Ethiopia.

The CP phylogeny (Fig. 3.2) contains three main groups of which the first, A, contains five sub-groups, the second group, B, three sub-groups, and the third group, C, one sub-group, most with high bootstrap values. This phylogeny confirms that South African isolates form distinct sub-groups and isolates in sub-group I group most closely to the same Chinese isolates as with the NIb gene (where sub-group I and II in Fig. 3.1 grouped closely to) but in this phylogeny they also group closely with the Spanish reference (which grouped with sub-group VIII in the NIb analysis, Fig. 3.1). The analysis showed that all the Tanzanian isolates cluster together in one sub-group in this phylogeny (as opposed to two separate groups in the NIb analysis), and grouped with Ethiopian and Rwandan isolates as their closest relatives again as seen with the NIb phylogeny. The CP phylogeny shows that reference sequences from Australia, Argentina and Iran isolated from sugarcane that had grouped with Tanzanian isolates based on the NIb partial gene sequence, now group on their own.

Concordance between both trees was observed with regards to the clustering of South African and Tanzanian isolates based on geographical origin. In both phylogenies the South African isolates are most closely related to two Chinese and a Spanish isolate. The two phylogenies constructed using different gene regions show strong statistical support at the upper nodes. The differences between the two phylogenies include: the relatively higher bootstrap support values for the South African clades (89% and 98%) revealed higher resolution in the NIb phylogeny (Fig. 3.1) than in the CP phylogeny (Fig. 3.2). The NIb phylogeny (Fig. 3.1) showed that

South African isolates from different regions in the same broader cluster (A) but grouping in three different sub-groups (I, II, VIII), and the CP phylogeny (Fig. 3.2) shows South African isolates in two different sub-groups (I and IV). As well as for the South African sub-groups positions and evolutionary distances between individual isolates differ somewhat between the two gene regions used for this analysis.

The more extensive CP analysis in Fig. 3.3 revealed multiple lineages into which the isolates from this study grouped and the 79 isolates in this phylogeny clustered in 3 main groups (A, B and C). These groups can be sub-divided into 17 sub-groups (I-XVII) relative to the reference sequences, but with lower bootstrap support values than those in the dendrograms (Figs. 3.1 & 3.2). Due to the fact that more international sequence data as reference sequences for the CP gene are available in GenBank, a more comprehensive study could be conducted which enables more accurate conclusions to be drawn regarding the relationships between isolates from different hosts and geographical areas. A strong correlation between South African and Tanzanian isolates with their respective geographical origins was observed as in Figs. 3.1 and 3.2 and verified in the phylogeny seen in Fig. 3.3; clearly geographical location had an influence on their evolution.

In Fig. 3.3, Group A (sub-group I-XIII) contains mostly maize from East Africa, and isolates from Asia and SA collected from Gauteng province, generally isolated from maize, Group B (sub-group XIV-XVI) contains mostly European, Limpopo Province/Mpumalanga isolates from SA as well as a few from China, and South America, also predominantly isolated from maize, Group C (Sub-group XVII) consisted of isolates from sugarcane from various countries. Isolates from SA grouped together in two main clusters (XIII and XVI) and Tanzanian isolates also mostly clustered together (I, II, V, VI). South African isolates in sub-group XIII are most closely related to Asian isolates (China, Philippines, Vietnam, Thailand). From this phylogeny, it is also clear that isolates in sub-group XVI cluster most closely with isolates from China and Argentina and are also closely related to isolates from Europe: Spain and Germany. Bootstrap support values for sub-groups I – VIII were below the 50% threshold and thus not shown (Fig. 3.3) and this lack of boostrap support results in less confidence in the topologies seen here. With dendrograms based on CP sequences (Figs. 3.2 and 3.3) relatively short terminal branches were seen as compared to terminal branch lengths with the NIb gene (Fig 3.1).

Investigating the pairwise nucleotide comparison provided support for the topologies and relationships seen in Fig. 3.3: SA isolates shared up to 99.54% nucleotide similarity with isolates from China and 96.51% with isolates from Spain, implying a more recent ancestor between these isolates than with any of the other examined isolates as the nucleotide divergence with other African isolates, such as maximums of 93.66% similarity with a Kenyan isolate and 93.63% with a Rwandan isolate and minimum similarities of 85.19% nucleotide similarity with other African isolates. Based on the pairwise nucleotide identity score, South African isolates shared 93.81%-99% and 97.45%-100% nucleotide similarity within sub-groups XIII and XVI, respectively, with 85.25%-93.10% similarity between the two groups. Isolates collected in Gauteng (sub-group XIII) and Limpopo Province/Mpumalanga (sub-group XVI) clustered in two discrete clusters, sharing 85.19%-93.66% and 86.66%-91.52% nucleotide similarity respectively, with the other African isolates from maize. These results show that there is the same amount of divergence between the two South African clades as there are between either of the two South African clades with most other African countries. South African isolates in sub-group XIII shared a relatively higher sequence similarity of 88.53%-97.5% with Tanzanian isolates, while South African SCMV in sub-group XVI shared 86.13%-92.68% with Tanzanian isolates. The Tanzanian isolates shared 98.78%-99.75% nucleotide similarity with each other. These are most closely related to, and share 98.29%-99.75% with, Rwandan, Kenyan and Ethiopian isolates with which they clustered in group A, sub-groups I-VIII. The lowest sequence similarity between two SCMV isolates was 78.52%, between the Argentinian isolate DQ973170.1 (sub-group XVI) and South African SCMV isolate 17-4181 (sub-group XIII).

Fig. 3.3 showed grouping of isolates based on host plant where lineage C contained only isolates from sugarcane and Groups A-B consisted of mostly maize isolates. The South African isolate previously obtained from sugarcane grouped in Group C with other sugarcane isolates and not with SCMV from South African maize in sub-groups XIII or XVI. It is also noteworthy to mention that isolates from other hosts such as *Digitaria velutina* (velvet fingergrass), *Eleusine coracana* (finger millet), *Vigna unguiculata* (cowpea), *Musa acuminata* (banana) and *Canna* spp. (canna lily) were situated amongst the maize sequences.

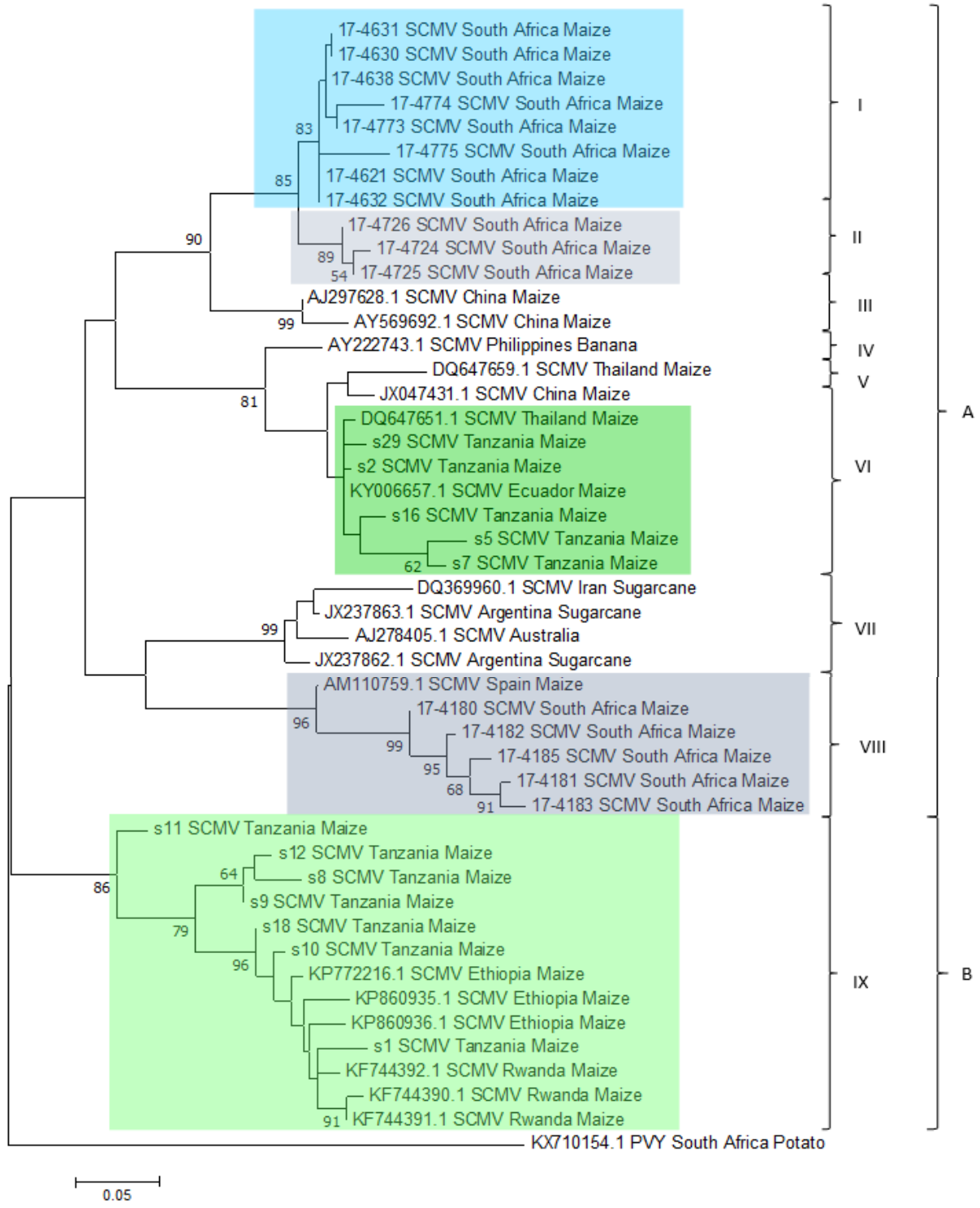

Figure 3.1 Maximum likelihood phylogenetic analysis reconstructed using partial Nuclear Inclusion body protein nucleotide sequences of 17 isolates Sugarcane mosaic virus from the current study, 12 isolates from Tanzania, together with 18 Sugarcane mosaic virus isolates obtained from GenBank as reference sequences and a Potato virus Y isolate as outgroup. The analysis was conducted using MEGA 6 with 1000 bootstrap replicates shown as percentages where the values are indicated as percentages and only scores above 50 are shown. Letters A and B indicate broad groups and Roman numerals (I-IX) indicate sub-groups. Sub-groups shaded in blues contain South African isolates, while sub-groups shaded in green contain Tanzanian isolates.

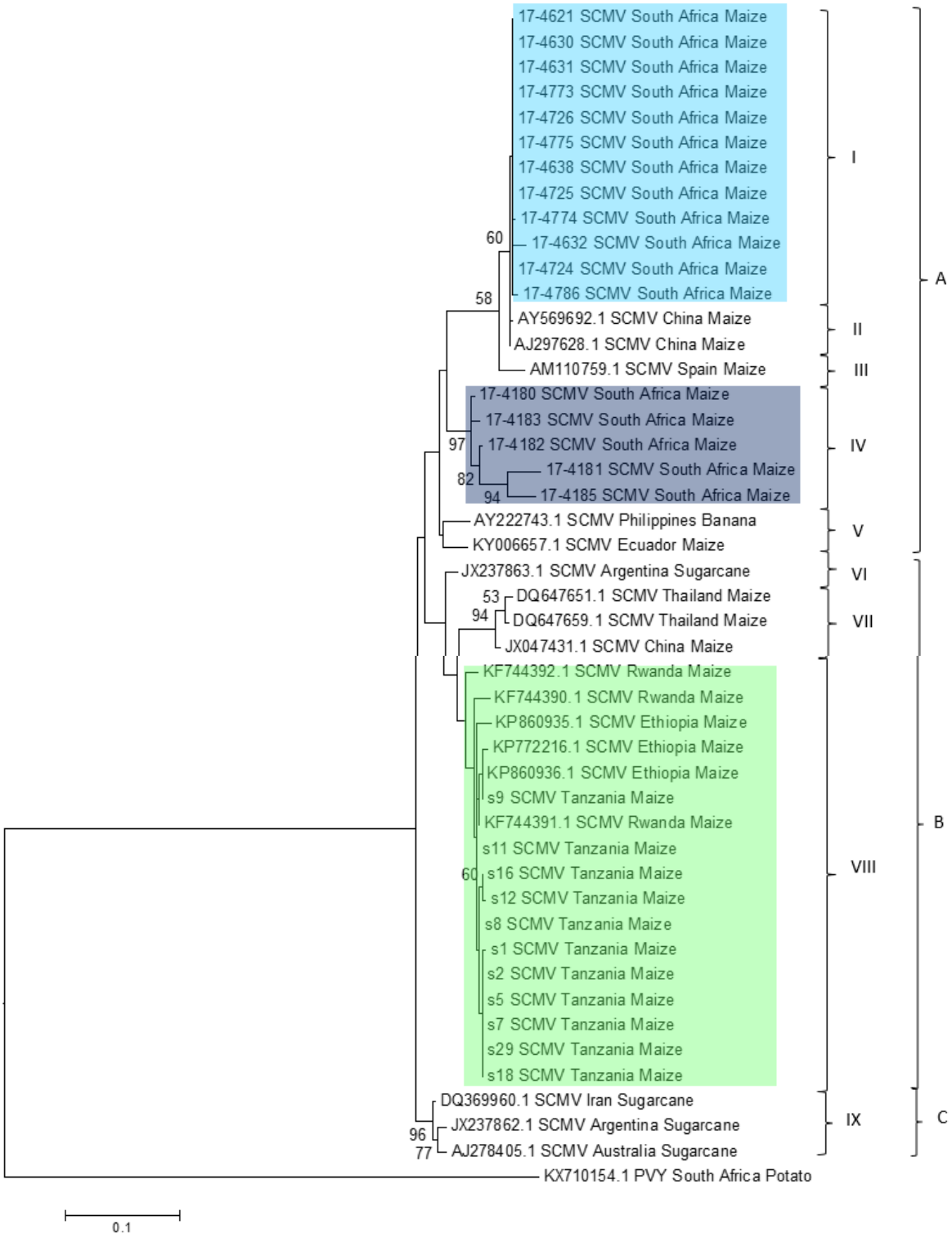

Figure 3.2 Maximum likelihood phylogenetic analysis constucted using partial Coat protein nucleotide sequences of 17 isolates Sugarcane mosaic virus from this study and 12 isolates from Tanzania. These were analysed in comparison with 18 Sugarcane mosaic virus reference sequences and a Potato virus Y isolate was used as outgroup. The phylogeny was generated using MEGA 6 with 1000 bootstrap replicates (only values of 50 or higher are shown) shown as percentages. Letters A-C indicate broad groups and Roman numerals (I-IX) indicate sub-groups. Sub-groups shaded in blues contain South African isolates and sub-groups shaded in green contain Tanzanian isolates.

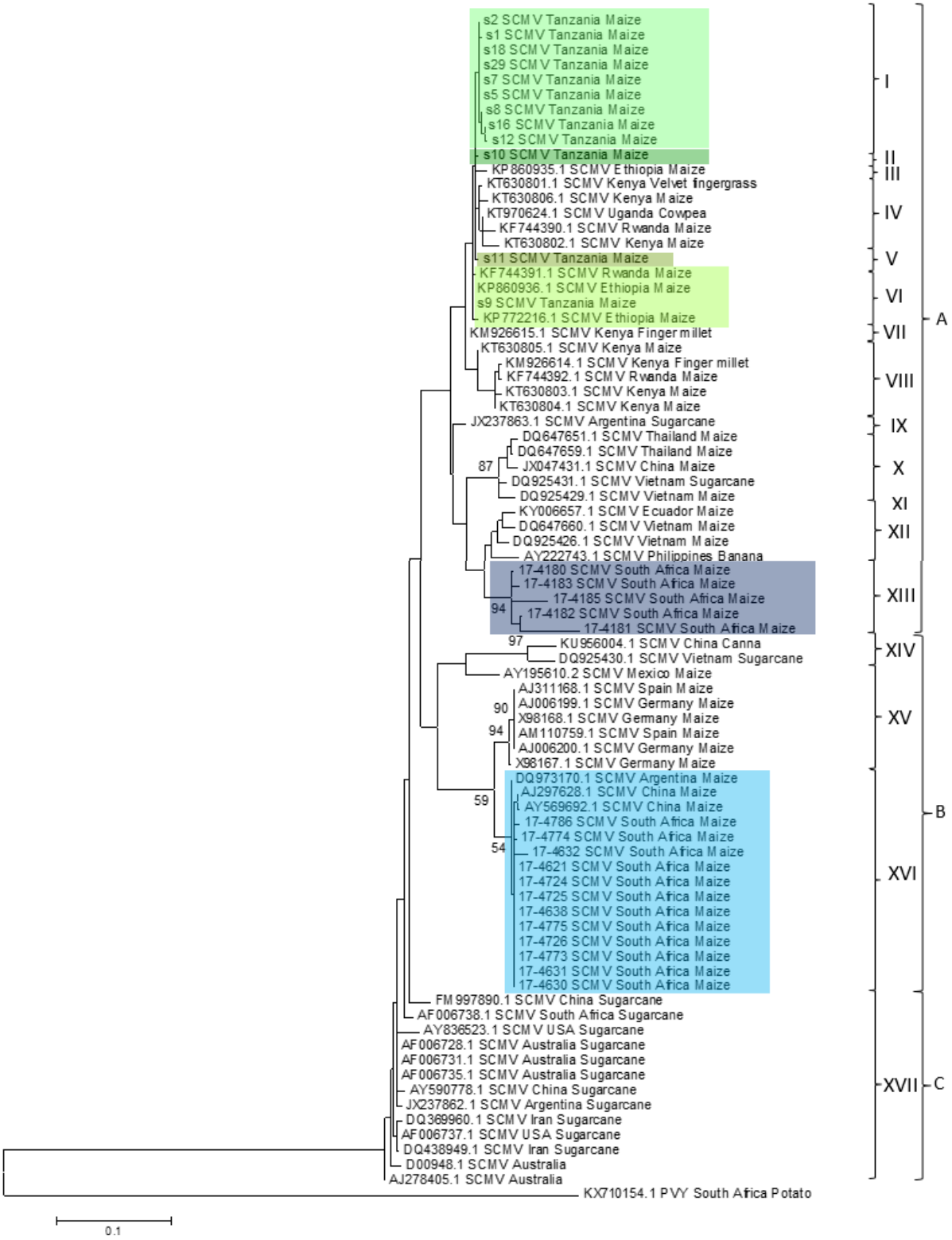

Figure 3.3 Maximum likelihood phylogenetic analysis of partial 510 bp Coat protein region of 17 Sugarcane mosaic virus isolates isolated from maize from this study and 12 isolates from Tanzanian isolates, together with the same 50 reference sequences. Potato virus Y was used as outgroup and phylogeny was constructed using MEGA 6. Bootstrap values following 1000 replicates are presented as percentages and only values above 50 are shown. Letters A-C indicate broad groups and Roman numerals (I-XVII) indicate sub-groups. Sub-groups shaded in blues contain South African isolates, while sub-groups shaded in green contain Tanzanian isolates.

Table 3.2 Table showing information on isolates used in the diversity study phylogenies.

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GenBank accession number	Accession number in this study	Description in Figs. 3.1, 3.2 and 3.3	Country of origin	Partial gene sequence
MH491463	17-4180	17-4180	South Africa	NIb
MH491462	17-4181	17-4181	South Africa	NIb
MH491461	17-4182	17-4182	South Africa	NIb
MH491460	17-4183	17-4183	South Africa	NIb
MH491459	17-4185	17-4185	South Africa	NIb
MH491451	17-4630	17-4630	South Africa	NIb
MH491450	17-4631	17-4631	South Africa	NIb
MH491449	17-4632	17-4632	South Africa	NIb
MH491448	17-4638	17-4638	South Africa	NIb
MH491458	17-4724	17-4724	South Africa	NIb
MH491457	17-4725	17-4725	South Africa	NIb
MH491456	17-4726	17-4726	South Africa	NIb
MH491455	17-4773	17-4773	South Africa	NIb
MH491454	17-4774	17-4774	South Africa	NIb
MH491434	17-4180	17-4180	South Africa	CP
MH491430	17-4181	17-4181	South Africa	CP
MH491431	17-4182	17-4182	South Africa	CP
MH491432	17-4183	17-4183	South Africa	CP
MH491433	17-4185	17-4185	South Africa	CP
MH491446	17-4630	17-4630	South Africa	CP
MH491445	17-4631	17-4631	South Africa	CP
MH491444	17-4632	17-4632	South Africa	CP
MH491443	17-4638	17-4638	South Africa	CP
MH491442	17-4724	17-4724	South Africa	CP
MH491441	17-4725	17-4725	South Africa	CP
MH491440	17-4726	17-4726	South Africa	CP
MH491439	17-4773	17-4773	South Africa	CP
MH491438	17-4774	17-4774	South Africa	CP
MH491464	16-0053	s1	Tanzania	NIb and CP
MH491465	16-0054	s2	Tanzania	NIb and CP
MH491466	16-0057	s5	Tanzania	NIb and CP
MH491467	16-0059	s7	Tanzania	NIb and CP
MH491470	16-0060	s8	Tanzania	NIb and CP
MH491471	16-0061	s9	Tanzania	NIb and CP
MH491475	16-0062	s10	Tanzania	NIb and CP
MH491473	16-0063	s11	Tanzania	NIb and CP
MH491472	16-0064	s12	Tanzania	NIb and CP
MH491469	16-0068	s16	Tanzania	NIb and CP
MH491476	16-0070	s18	Tanzania	NIb and CP
MH491468	16-0081	s29	Tanzania	NIb and CP

3.4 Discussion

This study reports on the diversity of South African and Tanzanian SCMV isolates, their relationships with SCMV isolates from other countries worldwide and the potential origin of SCMV detected in SA. The study clearly showed two separate clades of South African isolates with different geographical origins, that group with Asian or European relatives, while Tanzanian isolates generally belong to one clade and group with other East African isolates. Generally, isolates grouped mainly based on geographic origin and according to host plant from which they were isolated. Despite some differences in topologies seen, the two gene-trees were congruent in their display of closest relatives of the isolates from the two countries' SCMV isolates that were investigated. The CP gene region was found to allow better resolution of the phylogenies based on a larger number of available references sequences for this particular gene region.

All three phylogenies and the pairwise distance calculations were in agreement with South African isolates clustering together and forming discrete clades with other South African SCMV isolates from the same geographical origin. There is also a high degree of variability amongst the South African SCMV clusters. The association of the isolates included in the current study with their country/region of origin was similar to the findings of studies in other countries where the same correlation was observed (Achon *et al*., 2012; Alegria *et al*., 2003; Fentahun *et al.*, 2017; Li *et al*., 2013; Xiao *et al*., 1993). Alegria *et al*. (2003) observed that clusters of SCMV isolates from countries including Cameroon, Australia and the United States of America (USA) grouped based on their geographical origins and a study by Li *et al*. (2013), revealed that Asia- and Europe-originating SCMV isolates also clustered relative to their respective geographical origins. Similarly, Fentahun *et al*. (2017) showed that SCMV of maize from Ethiopia formed a distinct phylogenetic group, as with the Tanzanian and South African isolates in this study. This outcome was observed for other maize-infecting members of the Potyviridae family: Li *et al*. (2013) showed SCMV population genotypes correlated with geographical origin as well as the host plant from which they were obtained. RNA viruses evolve at an especially fast rate, a phenomenon caused by the high error rates of their RdRp enzymes, which is accelerated by molecular interactions with host-produced proteins such as

in Post-Transcriptional Gene Silencing (PTGS) (García-Arenal *et al*., 2001). Clustering of the current study's isolates based on geographic origin could indirectly be attributed to the evolution of these viruses on a broad range of alternate hosts as well as insect vectors present within a geographically isolated area, giving rise to genetic variation, resulting in evolution of the species and therefore phylogenetic divergence based on the vegetation in that region (Alexander, 2014; Power *et al*., 2000). Viruses usually have broad taxonomic host ranges and when a host is absent from an area, viruses and their vectors can persist in other types of vegetation including wild hosts (Alexander, 2014; Cooper & Jones, 2006; Elena, 2014; Wisler & Norris, 2005). Alexander (2014) discussed the plant virus-dynamics across the agro-ecological interface and highlighted the importance of the virus-host plant diversity in: 1) exchange of genes between crops and wild plants via seeds and pollen and 2) diversity of arthropods and microbes and their movement across the interface. This may also explain why SCMV isolated from hosts such as Finger millet and Velvet fingergrass grouped amongst the maize isolates.

For species within the Potyviridae family the species demarcation criteria is <78% nucleotide identity and <79.6% amino acid identity within the CP sequence (Adams *et al*., 2005), and although we only obtained two partial gene sequences in the current study, it appears that all isolates from this study can be considered to be SCMV species as 78.52% was the lowest sequence similarity between any two SCMV isolates. The phylogenies and nucleotide similarity calculations confirmed that South African isolates were not most closely related to SCMV from other African countries (as they shared minimum nucleotide similarities of 85.19%-86.66%, and maximum nucleotide similarities of 91.52%-93.66%) but that the two South African lineages are most closely related to a number of isolates from either Asia (China, Vietnam, Thailand and Philippines, sharing up to 99.54% nucleotide similarity), or Europe (Germany and Spain, sharing up to 96.51% nucleotide similarity). This indicates long-distance, inter-continental, rather than intra-continental, dispersal of SCMV to SA, from Asia and/or Europe (Achon *et al*., 2012; Gell *et al*., 2010). Differences in SCMV nucleotide identities amongst regions in SA forming such discrete clusters containing only South African isolates may be attributed to their proliferation over time on slightly different host crops. For example, changes in sugarcane cultivar have been linked to differences in sequences seen amongst

SCMV (Grisham & Pan, 2007; Koike & Gillaspie, 1989) and this may also hold true for changes in maize cultivar as the SCMV host in this study: most of the Limpopo Province samples (Fig 3.3, XVI) were *Z. mays* L. var. *rugosa* (sweet corn) but the Gauteng isolates (Fig 3.3, XIII) were isolated from grain maize varieties. The appearance of new genetic types on different hosts and the evolution of positive strand RNA viruses is usually driven by accumulation of mutations, recombination, and reassortment (Balasubramanian & Selvarajan, 2014; Green *et al*., 2018), caused by different selective pressures acting on the virus by factors associated with- or the specific host crop (Hajizadeh *et al*., 2017; He *et al*., 2015). The large genetic diversity of SCMV provides opportunity for overcoming resistance genes and adapting to new hosts, which leads to better fitness of a specific strain in a particular host and environment (Green *et al*., 2018).

Tanzanian SCMV isolates also shared high levels of similarity with each other (98.78%-99.75%) and clustered amongst isolates from other East African countries (with which they also shared high nucleotide similarities of 98.29%-99.75%). These isolates formed a less divergent, more homogenous group and these lineages are genetically more fluid than the discrete and isolated clusters of South African isolates discussed above (Aritua *et al*., 2008). This observation also suggests that the Tanzanian and other East African isolates share a common ancestor and are closely related (Pierce, 2012). All three dendrograms are congruent in showing that Tanzanian isolates grouped with Ethiopian and Rwandan isolates which implies a common ancestor to SCMV from these countries (Li *et al*., 1988; Pierce, 2012) and suggests possible dispersal from one country to the other as Rwanda and Tanzania are neighbouring countries. In Fig 3.3, a Kenyan isolate also grouped with the East African isolates and thus probably also shares a common ancestor as Kenya and Tanzania are also neighbours. Had a sequence of the NIb gene region of Kenyan SCMV isolate been available, the inclusion of this sequence in the dendrogram in Fig 3.1 may have provided further support of the above mentioned hypothesis. The apparent genetic divergence of East Africa's SCMV from SCMV isolates from other continents suggests evolution, possibly through recombination (Padhi & Ramu, 2011). East African isolates probably diverged from SCMV from other continents due to variations in the insect vector species that differ on the African continent, alternate host plants in these countries or the genotype of their respective host crops on which

they proliferated in East Africa that cause host adaptive selection (Alexander, 2014; Hajizadeh *et al*., 2017; Power *et al*., 2000).

The incongruency of Tanzanian isolates that all clustered together with regards their partial NIb gene sequence but formed different clusters with the CP gene sequence when compared to the same set of reference isolates can be due to different selection pressure acted upon these two genes. As the CP gene of potyviruses is a target of selection by host plants and vectors due to its roles in virus life cycle and virus-vector interaction (Li *et al*., 2013), it is known to have a lower diversity in most virus species (Tairo *et al*., 2005) when compared to cognate NIb genes. In a study by Parameswari *et al*. (2013) the nucleotide similarity percentage of various genes (including NIb and CP) of Sugarcane streak mosaic virus (SCSMV) from India with those from other countries were compared and a lower identity percentage amongst NIb genes than CP genes was observed, suggesting that the NIb gene is more variable, but separate phylogenies for the two genes were not constructed in their study. Thus, a higher degree of variability of NIb genes compared to the CP may have caused the differences in topologies and Tanzanian isolates clustering seen amongst Figs. 3.1 and 3.2. The CP gene of RNA viruses is under a great amount of purifying selection due to its central role in survival within the plant host and the insect vectors, and vector-borne RNA viruses had a higher level of genetic diversity than those that are not vector-transmitted (Chare & Holmes, 2004).

These differences in topologies of Tanzanian clusters between the two gene-trees correlate with the results of studies by Min *et al.* (2006) and Chenault and Melcher (1994), who also generated dissimilarities in dendrograms constructed using different genes. Chenault and Melcher (1994) concluded the relationships of isolates in their study were obscured due to recombination events in the evolution of the virus isolates. Recombination was not investigated in the current study as the genome fragments that we used were too short (Hajizadeh *et al*., 2017), but based on the discussion above, we can conclude that recombination events may be the cause of sequence divergence resulting in the disagreement seen between Tanzanian SCMV gene-trees in the current study. Thus, sequencing and comparison of nucleotides and amino acid sequence of the entire CP gene would provide more conclusive evidence to support this hypothesis. The low bootstrap values seen for some of the

upper nodes especially in Fig 3.3 may likely be due to recombination events such as those delineated by Hosseini *et al*. (2017) and Ohshima *et al.* (2018).

Various hypotheses could explain the clustering of samples based on host plant for the South African and Tanzanian maize isolates and the South African SCMV from sugarcane grouping in terms of host plant and not country of origin. Besides the observation that the host plays an integral role in diversification of viruses by causing host adaptive selection as mentioned previously (García-Arenal, 2001; Hajizadeh *et al*., 2017), differences in the proliferation mechanisms of the two hosts and thus also the likely methods of virus transmission may drive host-associated diversity for SCMV. Sugarcane is vegetatively propagated, hence the diversity of virus populations is kept largely constant and the same virus genotype can be transported within the host plant over a long distance, particularly between neighbouring countries (Singh *et al*., 2005). In maize, seed transmission (that keep the genetic diversity constant) does occur but is relatively low (Li *et al*., 2013) and new infections are primarily caused by feeding of viruliferous aphids that may travel great distances by means of air currents, giving rise to a more diverse virus population (Fereres, 2015; Zeyen *et al*., 1987) and thus spatial diversification has previously been recorded (Li *et al*., 2013). Xie *et al*. (2016) studied the distribution and molecular variability of SCMV in China and observed that SCMV isolates from the study could be divided into two divergent evolutionary groups based on their host plant species. They concluded that the host is one of the most powerful selection pressures on SCMV because it presents different conditions, causing the virus to adapt to the new environment (Xie *et al*., 2016). Xie *et al*. (2016) also observed that two evolutionary factors: recombination as well as negative selection were taking place in SCMV and were attributed to the different host species that provide different circumstances which require adaptation and steer evolution. These two factors seem to play a significant role in determining the genetic structure of SCMV populations and direct the development of more virulent strains (Xie *et al*., 2016). This explains why the molecular diversity of SCMV in maize in the field is increasing, and is significant because of the threat SCMV poses even to maize cultivars with resistance genes to SCMV and this host resistance can easily be overcome due to this accumulating molecular variability. In regions and countries where multiple potential hosts are

present and often grow in close proximity of one another, this is especially significant.

In a study by Fentahun *et al*. (2017), a similar trend was observed where the Ethiopian SCMV isolates from their study clustered with isolates from Kenya and other isolates from Ethiopia. In their study, the Ethiopian isolates showed the highest nucleotide similarities with isolates from African countries such as Kenya, Rwanda and other Ethiopian isolates with a common host: maize. This was confirmed by the findings of Mahuku *et al*. (2015) and Wangai *et al*. (2012). The occurrence of isolates from other hosts amongst those from maize in the dendrogram suggests a common ancestor and that the original introduction to the new host was made from a maize SCMV reservoir and subsequently come into with alternate hosts or with under various selective pressures evolved to extend their host range to infect new hosts (Cooper & Jones, 2006; García-Arenal, 2001).

An additional hypothesis arises for both clustering based-on-geographical origin or based-on-host-plant: vector-associated selection (He *et al*., 2015). Geographical location or the plant host may play a role in the vectors present or linked to the virus infection, and can indirectly influence the structure of the population. Previous studies have reported that genetic differentiation of plant viruses can be as a result of their vectors (García-Arenal *et al*., 2001; Gutiérrez *et al*., 2013; He *et al*., 2015). A study by Salvador *et al*. (2008) showed two important factors that contribute to this hypothesis: 1) predominant strains in a population are most efficiently transmitted by aphids and 2) the population size of a species of aphid could influence the virus strain diversity. Moreover, the Helper Component-Proteinase (HC-Pro) gene, and not the CP or NIb genes, is the determinant for virus-host interactions, suggesting its role in host-switching (Elena *et al*., 2011) and thus, by future analysis of the HC-Pro gene, a different representation of the relationships between isolates may be obtained which could indicate the involvement of the host in genetic diversity of SCMV. However, while using different protein-encoding gene regions to construct a species tree do provide an indication of the evolution genes, a subjective view of the evolution of the isolates will be obtained because different genes are under different selective pressures (García-Arenal, 2001) and therefore analysing whole genome sequences will provide a more holistic perspective.

A drawback of this study is that partial gene regions were used due to the product generated by selected primers used for initial detection. Therefore, by using whole genome information and constructing a species tree a more accurate assessment of the phylogenetic structure of the SCMV populations in SA and Tanzania could be conducted. Testing isolates obtained from both maize and sugarcane from the same country may show whether host plant or geography was a stronger determinant of the clustering seen within a single phylogeny.

This study reports the first diversity study of South African and Tanzanian SCMV isolated from maize as a host. The current study's investigations show that SCMV has a large genetic diversity, and a population structure based on both geography and the host plant. South African SCMV isolates share a common ancestor with Asian and European isolates and there are distinct lineages of SCMV present in SA that are confined to disparate geographic regions. In the phylogenies these discrete lineages of South African SCMV showed clustering SCMV from the same geographical regions. This may be due to; geographical isolation resulting in differences in natural vegetation, alternate hosts available for viruses and the aphid species present, as well as the differences in maize cultivars planted. The population structure of Tanzanian SCMV seems to be slightly more fluid and less discrete, or the genes selected to construct the phylogenies, may have played a role in the resulting relationships observed. Worldwide, spatial diversification was observed for maize SCMV isolates but not sugarcane SCMV isolates, which mostly clustered together regardless of geography but based on host, which may be related to the means of propagation utilised by the two hosts, the selection pressures due to the host such as different conditions, or the vector-associated selection caused by the two different hosts. The extent of the genetic diversification among SCMV host populations was moderate, but strong among geographical populations. The pathogens in South African are genetically less similar to those from other African countries and strain variation is present amongst South African SCMV. Thus, although many factors contribute to disease outbreak, the nature and severity of an MLND outbreak in SA may have a different epidemiology, biology, or severity, than those in the African countries where the disease is present. The current study's findings also extend current knowledge of SCMV genetic diversity in Africa, showing their common ancestors and the large SCMV diversity present on the continent. This

may be useful for clarifying facets of the SCMV evolution and variation amongst strains and contributes to the available knowledge resources for further MLND-related studies and predictions. However, probably the most significant impact of this study is the elucidation of the large diversity of SCMV present in Africa which would have to be considered during the potential development of genetically modified resistant crops. On the basis of the analyses described above, there is a risk of incomplete resistance if CP-mediated virus resistant transgenic plants are developed, due to the presence of variants of SCMV. Together with ongoing surveillance and additional studies, the current study could aid in predicting the risk of SCMV and MLND epidemics in countries such as SA, in future.

References

Achon MA, Larrañaga A & Alonso-Dueñas N (2012) The population genetics of Maize dwarf mosaic virus in Spain. *Archives of Virology:* **157**: 2377-2382.

Adams M, Antoniw J & Fauquet C (2005) Molecular criteria for genus and species discrimination within the family Potyviridae. *Archives of Virology* **150**: 459-479.

Adams IP, Miano D, Kinyua Z, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R & Hany U (2013) Use of Next Generation Sequencing for the identification and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus causing Maize Lethal Necrosis in Kenya. *Plant Pathology* **62**: 741-749.

Adams IP, Harju VA, Hodges T, Hany U, Skelton A, Rai S, Deka MK, Smith J, Fox A & Uzayisenga B (2014) First report of Maize Lethal Necrosis Disease in Rwanda. *New Disease Reports* **29**: 22.

Alegria OM, Royer M, Bousalem M, Chatenet M, Peterschmitt M, Girard J & Rott P (2003) Genetic diversity in the Coat protein coding region of eighty-six Sugarcane mosaic virus isolates from eight countries, particularly from Cameroon and Congo. *Archives of Virology* **148**: 357-372.

Alexander H, Mauck KE, Whitfield A, Garrett K & Malmstrom C (2014) Plant-virus interactions and the agro-ecological interface. *European Journal of Plant Pathology* **138**: 529-547.

Aritua V, Barg E, Adipala E, Gibson RW & Vetten HJ (2008) Further evidence for limited genetic diversity among East African isolates of Sweet potato chlorotic stunt virus. *Journal of Phytopathology* **156**: 181-189.

Aranda M & Freitas-Astúa J (2017) Ecology and diversity of plant viruses, and epidemiology of plant virus-induced diseases. *Annals of Applied Biology* **171**: 1-4.

Balasubramanian V & Selvarajan R (2014) Genetic diversity and recombination analysis in the Coat protein gene of Banana bract mosaic virus. *Virus Genes* **48**: 509-517.

Brunt A (1992) The general properties of potyviruses. In: *Potyvirus Taxonomy.* Springer: New York. 3-16.

Chare ER & Holmes EC (2004) Selection pressures in the capsid genes of plant RNA viruses reflect mode of transmission. *The Journal of General Virology* **85**: 3149-3157.

Chenault KD & Melcher U (1994) Phylogenetic relationships reveal recombination among isolates of Cauliflower mosaic virus. *Journal of Molecular Evolution* **39**: 496-505.

Chung BY-W, Miller WA, Atkins JF & Firth AE (2008) An overlapping essential gene in the Potyviridae. *Proceedings of the National Academy of Sciences* **105**: 5897-5902.

Cooper I & Jones RA (2006) Wild Plants and Viruses: Under-Investigated Ecosystems. *Advances in Virus Research* **67**: 1-47.

De Groote H, Oloo F, Tongruksawattana S & Das B (2016) Community-survey based assessment of the geographic distribution and impact of Maize Lethal Necrosis (MLN) Disease in Kenya. *Crop Protection* **82**: 30-35.

Elena SF, Bedhomme S, Carrasco P, Cuevas JM, de la Iglesia F, Lafforgue G, Lalić J, Pròsper À, Tromas N & Zwart MP (2011) The evolutionary genetics of emerging plant RNA viruses. *Molecular Plant-Microbe Interactions* **24**: 287-293.

Elena SF, Fraile A & García-Arenal F (2014) Evolution and emergence of plant viruses. *Advances in Virus Research* **88**: 161-191.

Fentahun M, Feyissa T, Abraham A & Kwak HR (2017) Detection and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus associated with Maize Lethal Necrosis Disease in Ethiopia: an emerging threat to maize production in the region. *European Journal of Plant Pathology* **149**: 1011-1017.

Fereres A & Raccah B (2015) Plant virus transmission by insects. *Essential for Life Science*. DOI: 10.1002/9780470015902.a0000760.pub3.

Flett B & Mashingaidze K (2016) Maize Lethal Necrosis: Possible threat to local maize production. *Grain SA*. Available online at: http://www.grainsa.co.za/maize-lethal-necrosis:-possible-threat-to-local-maize-production. [23 October 2017].

García-Arenal F, Fraile A & Malpica JM (2001) Variability and genetic structure of plant virus populations. *Annual Review of Phytopathology* **39**: 157-186.

Gell G, Balázs E & Petrik K (2010) Genetic diversity of Hungarian Maize dwarf mosaic virus isolates. *Virus Genes* **40**: 277-281.

Goldberg K-B & Brakke MK (1987) Concentration of Maize chlorotic mottle virus increased in mixed infections with Maize dwarf mosaic virus, strain B. *Phytopathology* **177**: 162-169.

Goodman B, MacDonald D & Huckett B (1998) A survey of South African Sugarcane mosaic virus (SCMV) strains based on CP gene sequence analysis. In *Proceedings of the South African Sugarcane Technologists' Association* **72**: 146-148.

Green KJ, Brown CJ & Karasev AV (2018) Genetic diversity of Potato virus Y (PVY): sequence analyses reveal ten novel PVY recombinant structures. *Archives of Virology* **163**: 23-32.

Grisham M & Pan Y-B (2007) A genetic shift in the virus strains that cause mosaic in Louisiana sugarcane. *Plant Disease* **91**: 453-458.

Gutiérrez S, Michalakis Y, Munster M & Blanc S (2013) Plant feeding by insect vectors can affect life cycle, population genetics and evolution of plant viruses. *Functional Ecology* **27**: 610-622.

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic Acids Symposium Series*. **41**: 95-98.

Hall TA (2011) BioEdit: an important software for molecular biology. *Green Earth Research Foundation Bulletin of Biosciences* **2**: 60-61.

Hajizadeh M, Bahrampour H & Abdollahzadeh J (2017) Genetic diversity and population structure of Watermelon mosaic virus. *Journal of Plant Diseases and Protection* **124**: 601-610.

Handley J, Smith G, Dale J & Harding R (1998) Sequence diversity in the Coat protein coding region of twelve Sugarcane mosaic potyvirus isolates from Australia, USA and South Africa. *Archives of Virology* **143**: 1145-1153.

He WB, Li J & Liu SS (2015) Differential profiles of direct and indirect modification of vector feeding behaviour by a plant virus. *Scientific Reports* **5**: 7682.

Hosseini H, Mehrvar M & Zaki AGM (2017) Comparative genetic diversity of potato virus Y populations based on Coat protein gene. *Acta Virologica* **61**: 161-174.

Ivanov K, Eskelin K, Lõhmus A & Mäkinen K (2014) Molecular and cellular mechanisms underlying potyvirus infection. *Journal of General Virology* **95**: 1415-1429.

Katoh K, Misawa K, Kuma Ki & Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform. *Nucleic Acids Research* **30**: 3059-3066.

Katoh K & Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772-780.

Kiruwa FH, Feyissa T & Ndakidemi PA (2016) Insights of Maize Lethal Necrotic Disease: a major constraint to maize production in East Africa. *African Journal of Microbiology Research* **10**: 271-279.

Koike H & Gillaspie AG (1989) Mosaic. In: *Diseases of Sugarcane.* Ricaud C, Egan BT, Gillaspie AG & Hughes CG (eds.). Elsevier Academic Press: Amsterdam. 301–322.

Kumar PP, Mohan S & Balasubramanian G (2004) Effect of whole-pea flour and a protein-rich fraction as repellents against stored-product insects. *Journal of Stored Products Research* **40**: 547-552.

Li W-H, Tanimura M & Sharp PM (1988) Rates and dates of divergence between AIDS virus nucleotide sequences. *Molecular biology and evolution* **5**: 313-330.

Li XH, Valdez P, Olvera RE & Carrington JC (1997) Functions of the Tobacco etch virus RNA polymerase (NIb): subcellular transport and protein-protein interaction with VPg/proteinase (NIa). *Journal of Virology* **71**: 1598-1607.

Li Y, Liu R, Zhou T & Fan Z (2013) Genetic diversity and population structure of Sugarcane mosaic virus. *Virus Research* **171**: 242-246.

Mahuku G, Lockhart BE, Wanjala B, Jones MW, Kimunye JN, Stewart LR, Cassone BJ, Sevgan S, Nyasani JO & Kusia E (2015) Maize Lethal Necrosis (MLN), an emerging threat to maize-based food security in sub-Saharan Africa. *Phytopathology* **105**: 956-965.

Min B, Chung B, Kim M, Ha J, Lee B & Ryu K (2006) Cactus mild mottle virus is a new cactus-infecting tobamovirus. *Archives of Virology* **151**: 13-21.

Muhire BM, Varsani A & Martin DP (2014) SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. *Public Library of Science One* **9**: e108277. DOI: 10.1371/journal.pone.0108277.

Ogawa T, Tomitaka Y, Nakagawa A & Ohshima K (2008) Genetic structure of a population of Potato virus Y inducing potato tuber necrotic ringspot disease in Japan; comparison with North American and European populations. *Virus Research* **131**: 199-212.

Ohshima K, Mitoma S & Gibbs AJ (2018) The genetic diversity of narcissus viruses related to turnip mosaic virus blur arbitrary boundaries used to discriminate potyvirus species. *Public Library of Science One* **13**: e0190511. DOI: 10.1371/journal.pone.0190511.

Padhi A & Ramu K (2011) Genomic evidence of intraspecific recombination in Sugarcane mosaic virus. *Virus genes* **42**: 282-285.

Pappu S, Brand R, Pappu H, Rybicki E, Gough KH, Frenkel MJ & Niblett C (1993) A Polymerase Chain Reaction method adapted for selective amplification and cloning of 3' sequences of potyviral genomes: application to Dasheen mosaic virus. *Journal of Virological Methods* **41**: 9-20.

Parameswari B, Bagyalakshmi K, Viswanathan R & Chinnaraja C (2013) Molecular characterization of Indian sugarcane streak mosaic virus isolate. *Virus Genes* **46**: 186-189.

Pierce BA (2012) Evolutionary genetics. In: *Genetics: A conceptual approach*. Macmillan: New York. 697-718.

Power AG (2000) Insect transmission of plant viruses: a constraint on virus variability. *Current opinion in Plant Biology* **3**: 336-340.

Riedel D, Lesemann D-E & Maiss E (1998) Ultrastructural localization of nonstructural and Coat proteins of 19 potyviruses using antisera to bacterially expressed proteins of Plum pox potyvirus. *Archives of Virology* **143**: 2133-2158.

Salvador B, Delgadillo M, Sáenz P, García J & Simón-Mateo C (2008) Identification of Plum pox virus pathogenicity determinants in herbaceous and woody hosts. *Molecular Plant-Microbe Interactions* **21**: 20-29.

Shukla DD, Gough K & Ward C (1987) Coat protein of potyviruses. *Archives of Virology* **96**: 59-74.

Singh N, Somai B & Pillay D (2005) In vitro screening of sugarcane to evaluate smut susceptibility. *Plant cell, tissue and organ culture* **80**: 259-266.

Tamura K & Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**: 512-526.

Tomitaka Y & Ohshima K (2006) A phylogeographical study of the Turnip mosaic virus population in East Asia reveals an 'emergent' lineage in Japan. *Molecular Ecology* **15**: 4437-4457.

Urcuqui-Inchima S, Haenni A-L & Bernardi F (2001) Potyvirus proteins: a wealth of functions. *Virus Research* **74**: 157-175.

Varsani A, Monjane AL, Donaldson L, Oluwafemi S, Zinga I, Komba EK, Plakoutene D, Mandakombo N, Mboukoulida J & Semballa S (2009) Comparative analysis of Panicum streak virus and Maize streak virus diversity, recombination patterns and phylogeography. *Virology Journal* **6**: 194.

Wangai A, Redinbaugh M, Kinyua Z, Miano D, Leley P, Kasina M, Mahuku G, Scheets K & Jeffers D (2012) First report of Maize chlorotic mottle virus and Maize Lethal Necrosis in Kenya. *Plant Disease* **96**: 1582.

White EJ, Venter M, Hiten NF & Burger JT (2008) Modified Cetyltrimethylammonium Bromide method improves robustness and versatility: The benchmark for plant RNA extraction. *Biotechnology Journal* **3**: 1424-1428.

Wisler GC & Norris RF (2005) Interactions between weeds and cultivated plants as related to management of plant pathogens. *Weed Science* **53**: 914-917.

Xiao XW, Frenkel MJ, Teakle D, Ward CW & Shukla DD (1993) Sequence diversity in the surface-exposed amino-terminal region of the Coat proteins of seven strains of Sugarcane mosaic virus correlates with their host range. *Archives of Virology* **132**: 399-408.

Xie X, Chen W, Fu Q, Zhang P, An T, Cui A & An D (2016) Molecular variability and distribution of Sugarcane mosaic virus in Shanxi, China. *Public Library of Science One* **11**: e0151549. DOI: 10.1371/journal.pone.0151549.

Zeyen R, Stromberg E & Kuehnast E (1987) Long-range aphid transport hypothesis for Maize dwarf mosaic virus: history and distribution in Minnesota, USA. *Annals of Applied Biology* **111**: 325-336.

Zheng L, Rodoni B, Gibbs M & Gibbs AJ (2010) A novel pair of universal primers for the detection of potyviruses. *Plant Pathology* **59**: 211-220.

Chapter 4: A multiplex Reverse-Transcription Polymerase Chain Reaction tool for the simultaneous detection of four maize-infecting viruses

Abstract

In order to prevent the entry of the devastating disease of maize, Maize Lethal Necrosis Disease (MLND), to regions or countries where it is not present such as South Africa (SA), testing for the pathogens present in this disease complex must be routinely done when importing maize, distributing seed and in general surveillance. Conducting diagnostic tests that are accurate and sensitive as well as cost-, time- and labour efficient manner are a priority. The aim of this study was to develop a multiplex Reverse Transcription-Polymerase Chain Reaction (RT-PCR) system that identifies four maize-infecting viruses, Sugarcane mosaic virus (SCMV), Maize chlorotic mottle virus (MCMV), Johnsongrass mosaic virus (JGMV) and Maize Streak virus (MSV), predicted to contribute to the disease complex, should an outbreak in SA occur. This was achieved by meticulous primer design followed by optimisation of the concentrations of the four sets of specific primers using available enzyme systems. The primer sets did not outcompete or interact with one another and based on amplicon size differences could differente the individual viruses present. The assay could correctly diagnose four two-way and three three-way combinations of the viruses. Within the limits of viruses available to us, cross-reactivity with other closely related viruses was tested, but none was observed. The assay is predicted to identify low concentrations of virus based on detection limits determined in our study and will thus be useful to quarantine facilities, seed producers and researchers carrying out surveillance for MLND-causing viruses.

4.1 Introduction

In 2011, Maize Lethal Necrosis Disease (MLND) was reported and identified for the first time on the African continent in Kenya (Wangai *et al*., 2012). The disease subsequently spread to other East African countries such as the Democratic Republic of the Congo (DRC) (Adams *et al*., 2014), Uganda (Kagoda *et al*., 2016) and Rwanda (Adams *et al*., 2013). The rapidity of its dispersal, its severe effects on maize yield, its seed-transmissibility, the general incurable nature of virus-caused diseases, as well as the widespread planting of MLND-susceptible hosts (maize (*Zea mays*) and sorghum (*Sorghum bicolor*)) in Africa, has lead to great concern about dispersal to other regions and countries as well as its impact on food security (Kiruwa *et al*., 2016).

Maize chlorotic mottle virus (MCMV; genus: *Machlomovirus*, family: Tombusviridae) was identified as the causal agent of the disease, either on its own or, more commonly, in co-infection with a member of the Potyviridae family (Uyemoto *et al*., 1980). The dual infection of MCMV and a potyvirus results in a more severe disease than the single infection of MCMV (Niblett & Clafin, 1978). Potyviruses identified in Maize Lethal Necrosis (MLN) infections include Sugarcane mosaic virus (SCMV) (Bockelman *et al*., 1982) or Johnsongrass mosaic virus (JGMV) (Stewart *et al.,* 2017) but the combination of MCMV with SCMV is by far the most widely reported (Mahuku *et al*., 2015; Uyemoto *et al*., 1981).

Polymerase Chain Reaction (PCR) is a versatile diagnostic technique capable of detecting small amounts of the target nucleic acid accurately and rapidly (Boonham *et al*., 2014). Currently, the most reliable method for the detection of the causal-agents of MLND is Reverse Transcription PCR (RT-PCR) and real-time TaqMan RT-PCR. While serological methods are more amenable to large scale testing, they have lower sensitivity, and are difficult and time consuming due to the production of antisera, which also may have cross-reactivity of antisera (Schaad *et al*., 2002). Although PCR-based detection methods are desirable, they are costly and laborious. To curb this limitation, ‘multiplex’ PCR or RT-PCR assays that simultaneously allow the detection of multiple targets using different primer pairs in the same reaction are ideal (Boonham *et al*., 2014). Multiplex RT-PCR saves time and reagent costs when compared to monospecific RT-PCR where multiple PCR tests are conducted

independently for each target (Bertolini *et al*., 2001) and are thus regularly applied for diagnostics in environmental, agricultural and clinical microbiology. This effectively overcomes the typical disadvantages associated with monospecific RT-PCR tests (Elnifro *et al*., 2000). To the best of our knowledge, MLND diagnosis is generally done by performing separate PCR tests to detect MCMV and the potyviruses, SCMV or JGMV. Clearly, detection of the MLND-causing viruses can however be performed more efficiently with the use of multiplex RT-PCR, especially when many samples are tested routinely (Li *et al*., 2012; Nassuth *et al*., 2000).

Multiplex PCR has been utilised to identify specific target regions in different lines of genetically modified maize, canola and soybean (James *et al*., 2003) but no multiplex RT-PCR systems have been published to detect maize viruses specifically. Other monocot crops for which multiplex systems have been developed include sorghum (Srinivas, 2013); sugarcane (Viswanathan *et al*., 2010) and wheat (Deb & Anderson, 2008; Tao *et al*., 2012; Zhang *et al*., 2017) and detect some of these crops' major viruses. Some of these viruses detected in these assays are able to infect maize but these assays are not suitable for our purposes as they do not detect the desired combination of viruses. In instances where a disease is caused by a co-infection of pathogens, such as with MLND, multiplex PCR has proved to be particularly useful (Opiyo *et al*., 2010).

Successful multiplex PCR reactions rely on the design of an optimum primer set combination (Nassuth *et al.*, 2000). Primer design for a conventional monospecific PCR routinely takes into account the ideal primer size (usually 18-30 base pairs (bp)), the melting temperature (Tm) of the primers in the set being similar, differing by 3°C or less, and between 58°C and 65°C, the GC content of primers being between 40% and 60%, the amplicon product being 100-500 bp in size and that for each primer the Gibbs free energy of the last five nucleotides at the 3' end should be more than or equal to that of -9 kcal/mol (Rozen & Skaletsky, 2000; Shen *et al*., 2010).

In addition to the normal requirements for primer design for conventional PCR, for multiplex PCR primer design various other factors must be considered such as synchronised annealing temperatures, competition between primers in amplifying

their targets, primer interactions that could result in dimer formation (Wei *et al*., 2008), and stringent target specificities. Furthermore, there is a need for different sized products (Nassuth *et al*., 2000; Wei *et al*., 2008) as the multiple targets in a single reaction need to be differentiated from each other by electrophoresis. Therefore, the GC compositions and lengths of the primers must be similar and target sequences that differ significantly should be chosen. Concentrations of multiplex primers to be used together need to be determined empirically and optimised to achieve a balanced product synthesis (Wei *et al*., 2008).

Due to the integral role of MCMV in MLN infections, its significance as quarantine pathogen in South Africa (SA) and many other maize-growing countries, and its consequent imperative accurate detection and identification, it will be included in the assay. Relatively little is known about the potyvirus species that could be present in MLND co-infections in Africa and possibly in future in SA, and thus it was decided against designing degenerate primers for the potyviral component of the MLND. Rather, it was decided to design primers against the potyviruses that were detected from the South African maize potyvirus survey (Chapter 2) in order to aid with identification of the specific potyvirus species present in a co-infection for routine diagnostics and quarantine testing, and thereby contribute to the understanding of MLND epidemiology. SCMV is the most common potyvirus detected in MLN infections (Wangai *et al*., 2012) and was by far the most prominent potyvirus detected in SA. The second most frequently detected potyvirus in the survey across SA was JGMV. In 2017, Stewart *et al.* reported that JGMV was associated with MLN infections of maize in East Africa in Tanzania, Uganda, Rwanda and Kenya. It was therefore decided to include the latter two potyviruses in this assay due to the risk of their possible future role in MLND in other countries and possibly SA. MSV has been widespread in SA for at least two decades (Centre for Agriculture and Bioscience International (CABI), & European and Mediterranean Plant Protection Organization (EPPO), 1997) and MSV symptoms were observed across most maize-growing regions in SA during the survey. The possibility of a co-infection of Maize streak virus (MSV) and other MLND components may thus arise here (Flett & Mashingaidze, 2016).

Since MLN infections in East Africa occur as the result of multiple viruses, the availability of a test that can simultaneously identify multiple viruses that potentially contribute to a MLND viral complex and thereby diagnose the disease had not been available to researchers. Such a test will enable rapid, accurate, cost-effective, less laborious detection and identification of four potentially significant MLND-contributing viruses in Africa. It has been highlighted that the use of this test will advance epidemiological studies of the MLND complex and diagnostics and therefore disease management at MLND-research facilities, aid quarantine facilities in effective and low-cost disease monitoring which will allow early detection as well as benefit the seed-producing industry to supply virus-free planting material to ultimately facilitate the control and management of this damaging disease of one of the world's most invaluable staple food crops. Therefore the aim of this study is to develop a multiplex assay that is capable of simultaneously detecting the two viral components most likely to occur in the MLND complex, as well as two others that may be present or contribute to the disease. The aim will be achieved by addressing the following objectives: 1) design a primer set in which all four primer pairs are compatible with each other, 2) determine the appropriate concentration of each primer pair, 3) test the ability of the assay to detect all four viruses simultaneously, 4) test the primer set in various combinations of simulated co-infections, 5) determine the sensitivity of the primer set, 6) evaluate the efficiency of the assay on selected field samples.

4.2 Materials and methods

4.2.1 Plant material, nucleic acid extraction and virus isolates

Maize plants with single infections of SCMV and JGMV were identified during a survey of potyviruses in SA (Chapter 2: 2.2.5) while plants with single infections of MCMV, as well as mixed infections of SCMV and MCMV, were obtained under permit from a MLND-infected region in Tanzania. Total nucleic acids were extracted from fresh leaf material (200mg) using a cetyltrimethylammonium bromide (CTAB) (2% CTAB, 1% polyvinyl pyrrolidone (PVP), 20mM ethylenediaminetetraacetic acid (EDTA), 100mM Tris-hydrochloric acid (HCl), 1.4M sodium chloride (NaCl)) extraction method (White *et al*. 2008) modified by omitting spermidine from the CTAB buffer. The nucleic acids were re-suspended in molecular-grade nuclease-free water (H_2O) (Sigma; St. Louis, MO, USA) and stored at -80°C. The original method reports the isolation of total RNA. However, the presence of MSV DNA in these extracts was confirmed via PCR in various samples. In this assay MSV RNA will be added before conducting complementary DNA (cDNA) synthesis of the RNA viruses.

4.2.2 Design of virus-specific primers

MCMV-, SCMV-, JGMV- and MSV-specific primers (Table 4.1) were designed based on published sequences of African isolates of these viruses on NCBI GenBank Database (Accession numbers: KP772217.1, KT833782.1, U84578.1, Y00514.1). MPprimer (Shen *et al*., 2010) based on Primer3 software (Rozen & Skaletsky, 2000) and primer specificity evaluation program MFEprimer (Qu *et al*., 2008) was used to design appropriate primer sets. The parameters applied were: Tm range of 57 – 63°C, the primer length of 18 bp to 27 bp, GC content between 40% and 60%. Potential interactions among primers were analysed using PriDimerCheck (Marshall, 2004). The characteristics of the individual primers were analysed to evaluate their use in multiplex PCR. Individual primers were subjected to a NCBI (National Center for Biotechnology Information) GenBank Primer-BLAST (Basic Local Alignment Sequence Tool) analysis (available online from https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to determine complementarity to any other sequences available on the database as well as to confirm the specificity to target sequences. After evaluation and selection of primer sets with the highest

quality scores provided by MPprimer (Shen *et al*., 2010), primers were synthesised by Inqaba Biotec (Pretoria, SA).

4.2.3 Establishment of each virus individually to evaluate specificity of primers

4.2.3.1 Uniplex Reverse Transcription

Primer pairs for the detection of each virus were tested in uniplex RT-PCR on total RNA extracts containing the individual respective templates. RT was done in a two-step reaction using Moloney-Murine Leukemia Virus (M-MLV) Reverse Transcriptase system (Promega; Madison, WI, USA), according to the manufacturer's specifications. For cDNA synthesis 2µl RNA extract containing SCMV, MCMV, JGMV or MSV (confirmed by previous tests) and 2.5µl of a given virus-specific MP1-R primer (4µM in final reaction) were incubated at 70°C for 5 min. This is followed by the addition of 7µl of RNA/primer mixture to 5.5µl of a RT master mix containing the following per reaction: 2.25µl 10x M-MLV-buffer (5mM Tris-HCl pH 8.3, 3mM magnesium chloride ($MgCl_2$), 75mM KCl, 10 mM dichlorodiphenyltrichloroethane (DTT) in final working solution), 0.125µl M-MLV Reverse Transcriptase enzyme, 1.875µl molecular grade nuclease-free H_2O (Sigma) and 1.25µl 10mM dNTPs (10mM each dATP, dCTP, dGTP and dCTP; Promega) and incubation at 42°C for 60min.

4.2.3.2 Polymerase Chain Reaction

The MyTaq system (Bioline; Taunton, MA, USA) was used to facilitate amplification of the cDNA, a total reaction volume of 25µl was used and a master mix was prepared that contained the following per reaction: 5µl MyTaq reaction buffer (5mM dNTPs, 15mM $MgCl_2$) (Bioline), 0.5µl (0.2µM) each of a given MP1-F and MP1-R primer set, 0.25µl (25 U) MyTaq DNA polymerase (Bioline) and 15.75µl molecular-grade nuclease-free H_2O (Sigma). A volume of 3µl cDNA was added to 22µl PCR mixture followed by the PCR reaction. Cycle conditions for PCR were 95°C for 15s, 35 cycles of 95°C for 15s, 60°C for 10s and 70°C for 15s, followed by a final extension step of 72°C for 5min.

4.2.3.3 Evaluation using gel electrophoresis

The product size as well as primer specificity and efficiency were evaluated by agarose gel electrophoresis on a 1%-2% Seakem agarose gel (Lonza; Basel, Switzerland) at 70-85V for 45-85min, depending on the experiment and the resolution desired. Ethidium bromide (EtBr; 0.6µg/mL) (Thermo Fisher Scientific; Waltham, MA, USA) was used for visualisation.

4.2.3.4 Verification of primer specificity via Sanger sequencing of Reverse Transcription-Polymerase Chain Reaction products

RT-PCR products were subjected to a PCR clean-up using ExoI (5U) and FastAP (1U) (Thermo Fisher Scientific) by incubating at 37°C followed by 85°C, for 15min each, respectively. The clean-up was followed by cycle sequencing and sequencing precipitation prior to submission to the University of Pretoria (UP) sequencing facility for direct Sanger sequencing on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems; Foster City, CA, USA). For the cycle sequencing, reaction volumes of 10µl in total were used that contained: 3µl template DNA, 0.75µl (2µM) virus-specific forward or reverse primer, 1µl BigDye Terminator Ready Reaction Mix (Applied Biosystems), 2.25µl Sequencing Buffer (Applied Biosystems) and 3µl molecular-grade nuclease-free H_2O (Sigma) per sample. Reaction products were precipitated using 1µl Sodium Acetate (NaOAc), 1µl EDTA and 100% Molecular-grade ethanol (Merck; Darmstadt, Germany) and centrifugation at 13000rpm for 30 minutes, followed by washing the pellet with 70% Molecular-grade Ethanol (EtOH) (Merck) before submission to the sequencing facility. This was followed by a NCBI BLAST analysis (available online from https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis for identification of sequenced products.

4.2.4 Determining the primer concentrations for use in multiplex Polymerase Chain Reaction

4.2.4.1 Standardisation of template

DNA amplicon was generated in RT-PCR reactions with the separate virus-specific primers from the total RNA extracted from maize. The concentrations of these were evaluated using Qubit (Thermo Fisher Scientific). To optimise the multiplex PCR

system, amplicons of all four viruses (MCMV (origin: Tanzania), SCMV, JGMV and MSV (origin: SA)) were diluted to a concentration of 50fg/µl in healthy maize nucleic acid and mixed together in equal volumes. Amplicon was used for optimisation instead of RNA as it is not possible to standardise RNA concentration of the individual viruses within a total RNA extraction. While the concentration of viral cDNA can be determined and standardised, there is a risk of breakdown of the cDNA if required in multiple reactions. Amplicons in contrast can be standardised and stored at 4°C to avoid multiple occurrences of thawing.

4.2.4.2 Determining optimal primer concentrations using Polymerase Chain Reaction

PCR was conducted as described in 4.2.3.2 except that instead of 3µl cDNA, 2µl amplicon mix of the four viruses (0.5µl (50fg/µl) each) was used. Initially, MCMV primers were used in 10µM (0.2µM in final reaction volume) with an equimolar concentration of SCMV primers but a strong bias for MCMV template was observed. The lower detection limit of the MCMV primers relative to 10µM SCMV primers was established and the primer concentration at which equal band intensities for both viruses were seen was established by running a MCMV primer concentration gradient in which five different concentrations of MCMV primers, ranging from 10µm to 2µM and descending with increments of 2µM, in conjunction with 10µM SCMV primers were used in the reaction with amplicon of equal concentrations of the four virus templates. The concentration at which the two products had equal band intensities was chosen as the optimal concentration of MCMV primer. The same approach was used using different concentrations of MSV and JGMV primers (together with the established concentrations for SCMV and MCMV primers). The concentration at which MSV or JGMV showed an equally bright band as the SCMV and MCMV products was selected as the ideal concentration for MSV or JGMV primers.

4.2.5 Multiplex Polymerase Chain Reaction

A volume of 2µl of the amplicon mix (0.5µl of each virus) was added to 23µl of PCR master mix which consisted of 4µl multiplex primer mix (consisting of: 10µM F and R SCMV-MP1 primers (0.2µM in final reaction), 4µM F and R MCMV-MP1 (0.08µM in

final reaction), 8µM F and R JGMV-MP1 primers (0.32µM in final reaction) and, 6µM F and R MSV-MP1 primers (0.24µM in final reaction)), 5µl MyTaq reaction buffer (5mM dNTPs, 15mM $MgCl_2$) (Bioline), 0.25µl (25U) (MyTaq DNA polymerase (Bioline) and 13.75µl molecular-grade nuclease-free H_2O (Sigma), following PCR cycling as described in 4.2.3.2.

4.2.6 Establishment of internal RNA/DNA control Polymerase Chain Reaction

Primers designed and developed by Van den Berg (2004) (Actin1F: 5'-ACCGAAGCCCCTCTTAACCC-3'; Actin2R: 5'-GTATGGCTGACACCATCACC-3') were ordered from Inqaba Biotec. RT-PCR was conducted in a second reaction, as in 4.2.3.1 and 4.2.3.2 with an annealing temperature of 42°C in the PCR step.

4.2.7 Gel extraction and sequence analysis

Gel electrophoresis was performed at 70V for 85 minutes, on a 2% Seakem agarose gel (Lonza) with EtBr staining (0.6µg/mL) (Thermo Fisher Scientific). After electrophoresis bands of all different sizes were excised and purified using the Nucleospin Gel and PCR Clean-Up kit (Macherey-Nagel; Düren, Germany). To verify the identity of the products obtained in the multiplex assay, amplicons were sequenced via direct sequencing in both directions on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems) at the UP sequencing facility using the preparation method described in 4.2.3.4.

4.2.8 Sensitivity and specificity testing of uniplex and multiplex Polymerase Chain Reaction systems

To compare the relative sensitivity of the multiplex RT-PCR system, the DNA amplicons were serially diluted from 50fg/µl (as standardised in step 2.4.1) 5-fold ($10^{-0.5}$ to $10^{-2.5}$) to obtain samples of 15.811fg/µl; 5fg/µl; 1.581fg/µl; 0.5fg/µl and 0.158fg/µl, respectively. All four virus templates were tested together using the primer pairs from this study. This was done in uniplex (all four primer pairs separately), as well as in multiplex.

To test the system's specificity, all samples available that contained cereal potyviruses related to SCMV were subjected to the multiplex assay. These included

Sorghum mosaic virus (SrMV) and Maize dwarf mosaic virus (MDMV). No isolates of viruses closely related to MCMV or MSV were available and therefore this specificity could not be tested.

4.2.9 Detection of viruses from mixed RNAs from naturally infected samples

To validate the assay, the nucleic acid samples from maize or grass in SA were selected that contain SCMV, JGMV and MSV, as well as RNA from Tanzania that contains MCMV. For each individual virus, 2µl each of nucleic acid samples containing SCMV, MCMV, JGMV and/or MSV was used. Where one or more of the viruses were left out the volume of 2µl was replaced by 2µl healthy maize nucleic acid. The samples were incubated with multiplex R primer mix containing: 2.5µl 10x SCMV-MP1-R primer (10µM), 2.5µl 10x MCMV-MP1-R primer (10µM), 2.5µl 10x JGMV-MP1-R primer (10µM) and 2.5µl 10x MSV-MP1-R primer (10µM). cDNA was synthesised using the steps and reagents described in 4.2.3.1 and 4.2.3.2. For the PCR step, 4µl cDNA was used as template. Thereafter the mixtures were subjected to PCR using the multiplex PCR primer mix and protocol described in 4.2.5, but using 11.75µl of water to maintain the total PCR reaction volume of 25µl.

4.2.10 Testing the system on field samples

To test the system on samples from the field, 16 selected nucleic acid samples from the survey (Chapter 2: 2.2.1) that tested positive for potyviruses were used because this study was primarily focussed on viruses from the *Potyvirus* genus. Two samples for which nucleic acid was available from Tanzania which contained MCMV and SCMV were also subjected to the established multiplex RT-PCR system as described in 4.2.9.

4.3 Results

4.3.1 Plant material, nucleic acid extraction and virus isolates

The extraction method produced good quality RNA. The nucleic acid protocol used here extracted both RNA and DNA although it was originally developed for the extraction of RNA. As no DNAse step was included, MSV could be amplified in PCR from various samples extracted using this protocol. DNA was also detected when using the internal control primers.

4.3.2 Design of virus-specific primers

A combination of primer pairs with similar Tm, appropriate length, negligible primer dimerization capacity and differentiable product sizes were selected with their individual properties summarised in Table 4.1.

Table 4.1 Primers used in the uniplex and multiplex Reverse Transcription-Polymerase Chain Reaction and Polymerase Chain Reaction system to detect four maize-infecting viruses.

Virus	Polarity	Primer name	Primer sequence 5'-3'	Tm	Length	GC (%)	Product size (nt)	Amplified partial gene
MCMV	+	MP1-MCMV-F	CAGGGCCAAAGCGCACCATGTA	60.0	22	47.2	460	Polymerase gene (p112K protein)
	-	MP1-MCMV-R	CGGTTGTGAATCTGCCACCGCA	60.2	22			
SCMV	+	MP1-SCMV-F	GTTGCAAAATGTCCCGGCCAGC	59.7	22	40.4	280	NIb gene (RNA-dependent-RNA-polymerase)
	-	MP1-SCMV-R	ACGTGCATTGTTGCATTCCCACG	59.2	23			
JGMV	+	MP1-JGMV-F	TTGCAGCAGGAGTGGCGATTGG	60	22	45.3	680	VPg-NIa gene
	-	MP1-JGMV-R	GTTGGCAACCCCATGGATCGCT	59.7	22			
MSV	+	MP1-MSV-F	AGGGCTGGAAGCAAGGCCGATA	60	22	58.6	360	Replicase gene
	-	MP1-MSV-R	ATGACACAGCTCCCGGCTCACT	59.9	22			

4.3.3 Establishment of each virus individually to evaluate specificity of primers

Virus-specific primer systems produced single bands of the expected size, 280 and 460 bp for SCMV and MCMV, respectively. In the case of the MSV primers a single band of the expected size was produced, but occasionally additional one or two extra bands, 490 and 310 bp in size occured. The JGMV specific primers produced a band of expected size but occasionally a band of 110 bp was also produced.

4.3.4 Determining the primer concentrations for use in multiplex Polymerase Chain Reaction

A balanced amplification with similar fluorescent intensity of bands (Fig. 4.2) was obtained when the primer concentrations were adjusted to 10µM SCMV-MP1: 4µM MCMV-MP1 : 6µM MSV primers : 8µM JGMV primers, and the expected amplicon sizes of 280 bp, 460 bp, 360 bp and 710 bp respectively, were obtained.

4.3.5 Multiplex Polymerase Chain Reaction

All four multiplex primers were used together successfully in the detection of their four target viruses (Fig 4.2). The various bands of expected sizes were clearly visible and easily differentiated from each other when electrophoresis was performed for the recommended amount of time and voltage. However, in some reactions extra bands were produced, identified as MSV and JGMV amplicons. When the selected concentrations of each of the four primers were used to detect equal concentrations of virus template, slightly lighter bands were observed for MSV as compared to the other three viruses (Fig. 4.2).

4.3.6 Establishment of internal RNA/DNA control primers

Two clear bands were produced of sizes 180 and 270 bp, corresponding to cDNA and genomic DNA respectively (Fig. 4.5), along with other faint bands of different sizes. The extracts were not treated with DNase and DNA viruses had been amplified from these extracts previously.

4.3.7 Gel extraction and sequence analysis

Following cycle sequencing of the individual gel purified PCR products and a GenBank BLAST analysis it was observed that the primers successfully detected their specific targets.

4.3.8 Sensitivity and specificity testing of uniplex and multiplex Polymerase Chain Reaction systems

In the 5-fold dilution series (Fig. 4.3), the primers could detect SCMV and MCMV templates in uniplex, as well as multiplex, at the lowest concentration/highest dilution ($10^{-2.5}$) but JGMV could only be detected clearly in multiplex to a concentration of 0.5fg/µl in both uniplex (Fig. 4.3 C, lane 4) and multiplex (Fig. 4.3 E, lane 4). MSV could only be detected clearly until a concentration 0.5fg/µl in uniplex but in multiplex could be detected clearly up until a concentration of 0.158fg/µl.

4.3.9 Detection of viruses from mixed RNAs from naturally infected samples

The multiplex assay confirmed the results of the conventional RT-PCR tests conducted in initial identification of the viruses. It also successfully detected different combinations of the four viruses within the simulated mixed-infection samples tested (Fig. 4.4 A & B). In some cases, a higher concentration of amplicon, as evidenced through brighter bands, were observed for some viruses than others within the sample for example Fig. 4.4 A in lane 4, MCMV band was much brighter compared to SCMV band and lanes 8 and 9, JGMV band much brighter than other bands.

4.3.10 Testing the system on field samples

When testing potyvirus-positive nucleic acid, the assay detected the potyvirus previously detected using NIb primers and the assay was able to amplify SCMV from Tanzanian samples effectively. Sixteen of the samples from the field tested using the system showed single infections of SCMV. One grass sample tested positive for both MSV and JGMV, and one sample from Tanzania tested positive for SCMV and MCMV.

4.4 Discussion

In this study we report the development of a multiplex assay for the simultaneous detection of four maize-infecting viruses that could potentially contribute to the MLND complex should MCMV enter SA. The key findings of this study can be summarised as follows: 1) all four viruses could be detected simultaneously in multiplex RT-PCR, 2) the six two- and three three-way combinations of the four viruses could be detected simultaneously, 3) products of the expected and identifiable size were obtained, 4) relatively low concentrations of their targets were amplified in uniplex and multiplex, 5) the multiplex primers detected the targeted viruses efficiently from both amplicon as well as RNA, and 6) no cross-reactivity took place between the virus-specific primers and other closely related viruses for which isolates were available.

Zhang *et al.* (2017) also described the use of RNA extracts to amplify four wheat viruses: three RNA viruses and one DNA virus, deeming it sufficient to extract only RNA instead of both RNA and DNA, as the mRNA of the DNA virus was reverse transcribed and then amplified, making the method less time consuming and cheaper than for example the multiplex system developed by Tao *et al*. (2012) for the detection of wheat-infecting viruses and phytoplasma (Zhang *et al*., 2017). In our study, MSV may have been detected from transcribed RNA, but could also be detected from DNA present within the extracts (as no DNase treatment is conducted on the nucleic acid extracts). To differentiate this, a sample containing MSV nucleic acid could be treated with DNase. However, it was not critical to determine this for the assay developed in this study.

In terms of primer concentrations, Zhang *et al*. (2017) discussed the importance of adapting the primer concentration of each primer pair. In a study by Liu *et al.* (2014), observed that the detection efficiency of Tobacco bushy top virus (TBTV) primers varied when changing primer concentrations. A general trend is that lower primer concentrations are used to amplify shorter sequences and higher concentrations for longer sequences Zhang *et al*. (2017) also held true in our assays for MSV and MCMV (360 bp and 460 bp, respectively) in which a lower primer concentration was used, and JGMV (680 bp), for which a higher primer concentration was used.

The relatively low concentrations of target amplicon that can be detected is ideal for routine quarantine testing when plants are often tested with new or symptomless infections and where signs of disease are not observable or viral titres are low (Li *et al*., 2012; Prendeville *et al*., 2012; Roossinck *et al*., 2010). Ideally, RNA should have been used when establishing the detection limit but the extraction protocol used was for total RNA and the concentration of virus could not be measured without using quantitative RT-PCR (q-RT-PCR) for each of the four viruses. Therefore, amplicon, for which concentration could be accurately measured, was used in the dilution series in order to test the combinations of primer concentrations and establish the detection limit. Had probes and primers be available, q-RT-PCR could have been used to quantify viral RNA within a sample in order to compare the detection limit. However, as the system was successful when mixed RNA samples were tested in RT-PCR it was apparent that determining the ideal primer concentration combinations using amplicon was feasible. The multiplex PCR system could detect South African as well as Tanzanian SCMV isolates efficiently regardless of the fact that SCMV strains vary amongst regions and countries (Alegria *et al.*, 2003; Gao *et al*., 2011; Li *et al*., 2012) and may thus be a useful tool to use in other African countries where surveillance for MLND-causing viruses is conducted. Bands corresponding to primer dimers were the same intensity in multiplex as they were in uniplex PCR. Therefore, negligible or no reaction inhibition was caused by primer dimers. Hot start Taq polymerase was therefore not needed as in previous studies (Du *et al*. 2006; Roy *et al*., 2005) thus making this method cheaper than such multiplex PCR systems.

In terms of both JGMV and MSV, the detection limit in uniplex was higher than in multiplex at equivalent template concentration. The reason for this reduction in sensitivity may be that in the multiplex assay, various templates are present and the mixture of primers competes for all four viruses instead of only one, making the detection limit higher (Roy *et al*., 2005; Uga & Tsuda, 2005). However, in this study the sensitivity of the multiplex and uniplex were the same for both SCMV and MCMV detection as observed by Li *et al*. (2012) on sweet potato viruses.

The extra bands observed occasionally in the amplification of MSV and JGMV using the primers in uniplex and in multiplex cannot be attributed to multiple component genomes that provide multiple binding sites for the primers as in the case of Banana

bunchy top virus (Burns *et al*., 1994) as both viruses have single-component genomes (Ivanov *et al*., 2014; Sheperd *et al*., 2010). It is possible that more than one binding site of one or both of the MSV and JGMV primers exist within their respective virus genomes and that either the forward or reverse primer binding to similar sequences in different virus isolates (Deb & Anderson, 2008). A primer which is capable of binding at more than one site is not necessarily completely complementary to the binding site and may have fewer complementary sites and more mismatches (Primrose & Twyman, 2009), resulting in the binding at that site occurring less often and therefore less of that product being made, and thus fainter bands are observed for these products.

Not all three MSV bands are seen in each reaction and this may be due to primer associated bias which is one of the most important factors where a population of viruses is present (Ihrmark *et al*., 2012). When virus titer is low and less template is available, primer-associated bias results in the amplicons which are biased against not being formed in high enough concentration to be visible as PCR products on the gel.

In a study by Nassuth *et al*. (2000), a similar phenomenon was observed with multiple bands produced from a primer pair in a multiplex PCR. In an effort to reduce the background noise of these extra bands, the amounts of KCl in the RT-PCR mixture were altered as K^+ and NH_4^+ concentrations were reported to affect the non-specific hybridisation and amplification efficiency for multiplex PCR (Henegariu *et al*., 1997). Alterations like this were not possible in this study due to the premixed MyTaq system that was used, but the use of different enzyme systems and kits could be explored where KCl concentrations could be adjusted to improve the assay further. The extra bands obtained in non-specific amplification in this study do not seem to affect the ability of the system to detect the four viruses and were therefore not taken into consideration further. Multiple bands can however, lead to false negatives (Robertson *et al.* 1991) and cognisance of this is required.

A regularly occurring problem in multiplex RT-PCR systems is the unbalanced amplification of some viruses compared to others (Wei *et al*., 2008). This was observed in the case of the 460 bp MCMV fragment (Fig. 1C) in spite of the relative concentrations of the primer pairs in the multiplex set being systematically

determined. Different concentrations of the four primer pairs were used to produce similar intensities of amplicons within the same reaction. The highest concentration primer was required for SCMV (10µM), while the lowest for MCMV (4µM).

In the experiment (4.2.9) where the nucleic acid concentration was not standardised beforehand due to the use of total RNA in a simulation of a naturally occurring co-infection, it was seen that certain viruses had brighter bands than others. For example, in the case in Fig. 4, lane 1, a bright band was observed for JGMV and in lane 4 a bright band for MCMV was produced. Very faint but visible bands were produced for MSV and SCMV compared to the bright luminescence of JGMV product in lane 8. It is possible that this apparent variation in virus titer is due to differences in the concentration and quality of RNA which can only be tested by q-RT-PCR as discussed above, but may be because of varying titers of the viruses in different plants. This could be due to age, health, genotype or species of the host (Pagán *et al*., 2012), age of infection (García-Arenál & Fraile, 2013), species and therefore nature of the virus, co-infections with other pathogens (Syller, 2012; Zhang *et al*., 2001) and various environmental parameters (Schenck & Lehrer, 2000), which result in naturally varying concentrations of viruses in infected plants (Alexander *et al*., 2014). It could also be due to inhibitors in the extraction method (Bertolini *et al*. 2001) Furthermore, experimentally mixed amplicons may not correctly simulate the equilibriums between co-infecting pathogens depending on how old or recent the newest pathogen introduction in a natural infection (Vidalakis *et al.*, 2004). When testing selected potyvirus-positive samples collected in the survey, the assay confirmed the presence of the viruses previously identified within the samples. More cases of co-infections may be identified in future.

A drawback of the system is that the 490 bp size band sometimes produced in reaction to the presence of MSV is only 30 bp larger than the MCMV band and could therefore lead to a mis-diagnosis of the presence of MCMV if care is not taken. However, in Fig. 4, lanes 1, 2 and 8 contain the 490 bp MSV band and lanes 4-7 and 9-10 contain the 460 bp MCMV band, and careful observation the bands of similar sizes are still differentiable. It is thus advised to perform gel electrophoresis as described in the methodology for best resolution and differentiation between bands that are close together in size.

A pair of primers for the detection of plant messenger RNA (mRNA) developed by Van den Berg (2004), was included in the assay as an internal control for RNA integrity and to ensure false negatives are avoided (Nassuth *et al*., 2000). In a study by Menzel *et al*. (2002) and Thompson *et al.* (2003), a plant mRNA specific internal control was included to avoid obtaining false-negative results in the RT-PCR and serves as an indicator of the success of the RNA extraction as well as the RT-PCR. This primer pair is especially useful because it differentiates between RNA and DNA by producing different sized bands. The larger bands represent genomic DNA (gDNA) present in the RNA extracts due to introns present in the banana Actin gene sequence against which they were designed, and the smaller fragment which is produced in the presence of reverse transcription, represents mRNA which is a shorter fragment following splicing of introns and exploiting the splicing of introns to design internal controls is similar to the strategy of previous authors such as Nassuth *et al.* (2000) and Thompson *et al.* (2003). The internal control primers developed by Van den Berg (2004), can be included in the assay in a second parallel reaction. The additional faint bands seen can be attributed to non-specific amplification from gDNA. Alternatively, to save costs, only samples that tested negative for all four viruses in the multiplex assay can then be subjected to RT-PCR using the internal RNA control primer pair. The protocol described here was initially designed for the purpose of total RNA extractions, and although a DNA virus could be detected from these nucleic acid preparations, future studies should investigate using an extraction protocol specific to total nucleic acids to use prior to RT-PCR.

Further research could test the primers using varying annealing temperatures in the PCR step or testing the assay using various PCR enzyme/buffer systems to alleviate some of the non-target amplicons observed. A hot-start polymerase increases DNA targets at low concentrations in the sample, as well as preventing mis-primed products and primer dimers from forming (Roy *et al*., 2005). This may alleviate some of the extra bands seen in our multiplex system. Samples from other African countries should be tested to assess the specificity of primers various quasispecies of the virus and to detect recombinants. Potyviruses are known to have high recombination rates (Nie & Singh, 2002) and SCMV is known to have high genetic variability (Xie *et al*., 2016). Various RNA extraction methods should be tested to shorten the process. The assay should also be applied on a larger scale to evaluate

the combinations and rates of co-infections that occur naturally between these four viruses.

Low titer of MCMV in seed remains a challenge (Zhang *et al*., 2011) and may affect the detection ability of the multiplex PCR in this study and therefore it is recommended not to use this method directly on seed. When in co-infection, the distribution and titer of some viruses can be affected as one virus can either act synergistically or antagonistically with another (Goldberg & Brakke, 1987; Mbega *et al*., 2016; Scheets, 1998), resulting in a lower detection capability using PCR of the virus which is in lowest titer. Vidalakis *et al.* (2004) highlighted that experimentally simulated co-infections may be different from natural infections because in naturally occurring co-infections, pathogens may establish equilibriums amongst themselves, altering their concentrations (Vidalakis *et al.*, 2004), therefore the method here requires testing with naturally infected plant samples.

To conclude, the multiplex RT-PCR system developed in this study is rapid, accurate, cost-effective, less laborious and effective in the simultaneous detection of three RNA viruses and one DNA virus from three different genera which – especially when in co-infection in a single plant – are predicted to be associated with MLND infections in SA, using a single PCR reaction. This assay is unique in the combination of pathogens tested for as well as the purpose for which it was developed. The system developed in this study is attractive for several reasons: 1) the method can greatly reduce the cost, labour and time involved when large scale diagnostics are conducted and many more samples can be tested in a given amount of time, 2) diagnostic laboratories that are still using ELISA to detect these four viruses due to its low cost as compared to conventional RT-PCR can now employ a cheaper system to achieve PCR-level sensitivity for the detection of these viruses, 3) four important maize-infecting viruses can now be detected within one day if extracted RNA is already available, 4) on a practical level, this tool will be useful for quarantine laboratories in both SA and other African countries where samples are tested on a large scale and where resources are limited as well as for seed-producers to ensure the supply virus-free seed. Furthermore, the method described here offers the researcher options to alter their experimental design should only SCMV and MCMV need to be tested for. This innovative diagnostic tool will significantly contribute to the surveillance, early detection, containment and

management of this devastating maize disease that the African continent is faced with at present.

References

Adams IP, Harju VA, Hodges T, Hany U, Skelton A, Rai S, Deka MK, Smith J, Fox A & Uzayisenga B (2014) First report of Maize Lethal Necrosis Disease in Rwanda. *New Disease Reports* **29**: 22.

Adams IP, Miano D, Kinyua Z, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R & Hany U (2013) Use of Next-Generation Sequencing for the identification and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus causing Maize Lethal Necrosis in Kenya. *Plant Pathology* **62**: 741-749.

Alegria OM, Royer M, Bousalem M, Chatenet M, Peterschmitt M, Girard J & Rott P (2003) Genetic diversity in the Coat protein coding region of eighty-six Sugarcane mosaic virus isolates from eight countries, particularly from Cameroon and Congo. *Archives of Virology* **148**: 357-372.

Alexander H, Mauck KE, Whitfield A, Garrett K & Malmstrom C (2014) Plant-virus interactions and the agro-ecological interface. *European Journal of Plant Pathology* **138**: 529-547.

Bertolini E, Olmos A, Martínez MC, Gorris MaT & Cambra M (2001) Single-step multiplex RT-PCR for simultaneous and colourimetric detection of six RNA viruses in olive trees. *Journal of Virological Methods* **96**: 33-41.

Bockelman D, Claflin L & Uyemoto J (1982) Host range and seed-transmission studies of Maize chlorotic mottle virus in grasses and corn. *Plant Disease* **66**: 216-218.

Boonham N, Kreuze J, Winter S, van der Vlugt R, Bergervoet J, Tomlinson J & Mumford R (2014) Methods in virus diagnostics: from ELISA to next generation sequencing. *Virus Research* **186**: 20-31.

Burns TM, Harding RM, Dale JL (1994) Evidence that Banana bunchy top virus has a multi component genome. *Archives of Virology* **137**: 371-380.

CABI; EPPO (1997) Maize streak monogeminivirus. *Distribution Maps of Plant Diseases*, 1st edition. CAB International: Wallingford, UK. Map 739.

Deb M & Anderson JM (2008) Development of a multiplexed PCR detection method for Barley and Cereal yellow dwarf viruses, Wheat spindle streak virus, Wheat streak mosaic virus and Soil-borne wheat mosaic virus. *Journal of Virological Methods* **148**: 17-24.

Du Z, Chen J & Hiruki C (2006) Optimization and application of a multiplex RT-PCR system for simultaneous detection of five potato viruses using 18S rRNA as an internal control. *Plant Disease* **90**: 185-189.

Elnifro EM, Ashshi AM, Cooper RJ & Klapper PE (2000) Multiplex PCR: optimization and application in diagnostic virology. *Clinical Microbiology Reviews* **13**: 559-570.

Flett B & Mashingaidze K (2016) Maize Lethal Necrosis: Possible threat to local maize production. *Grain SA*. Available online at: http://www.grainsa.co.za/maize-lethal-necrosis:-possible-threat-to-local-maize-production. [23 October 2017].

Gao B, Cui X-W, Li X-D, Zhang C-Q & Miao H-Q (2011) Complete genomic sequence analysis of a highly virulent isolate revealed a novel strain of Sugarcane mosaic virus. *Virus Genes* **43**: 390.

García-Arenal F & Fraile A (2013) Trade-offs in host range evolution of plant viruses. *Plant Pathology* **62**: 2-9.

Goldberg K-B & Brakke MK (1987) Concentration of Maize chlorotic mottle virus increased in mixed infections with Maize dwarf mosaic virus, strain B. *Phytopathology* **177**: 162-169.

Henegariu O, Heerema N, Dlouhy S, Vance G & Vogt P (1997) Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques* **23**: 504-511.

Ihrmark K, Bödeker I, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J, Brandström-Durling M & Clemmensen KE (2012) New primers to amplify the fungal ITS2 region–evaluation by 454-sequencing of artificial and natural communities. *Federation of European Microbiological Societies Microbiology Ecology* **82**: 666-677.

Ivanov K, Eskelin K, Lõhmus A & Mäkinen K (2014) Molecular and cellular mechanisms underlying potyvirus infection. *Journal of General Virology* **95**: 1415-1429.

James D, Schmidt A-M, Wall E, Green M & Masri S (2003) Reliable detection and identification of genetically modified maize, soybean, and canola by multiplex PCR analysis. *Journal of Agricultural and Food Chemistry* **51**: 5829-5834.

Kagoda F, Gidoi R & Isabirye BE (2016) Status of Maize Lethal Necrosis in eastern Uganda. *African Journal of Agricultural Research* **11**: 652-660.

Kiruwa FH, Feyissa T & Ndakidemi PA (2016) Insights of Maize Lethal Necrotic Disease: a major constraint to maize production in East Africa. *African Journal of Microbiology Research* **10**: 271-279.

Li R, Gao S, Hernandez AG, Wechter WP, Fei Z & Ling K-S (2012) Deep sequencing of small RNAs in tomato for virus and viroid identification and strain differentiation. *Public Library of Science One* **7**: e37127. DOI: 10.1371/journal.pone.0037127.

Liu F, Tan G, Li X, Chen H, Li R & Li F (2014) Simultaneous detection of four causal agents of Tobacco bushy top disease by a multiplex one-step RT-PCR. *Journal of Virological Methods* **205**: 99-103.

Mahuku G, Lockhart BE, Wanjala B, Jones MW, Kimunye JN, Stewart LR, Cassone BJ, Sevgan S, Nyasani JO & Kusia E (2015b) Maize Lethal Necrosis (MLN), an

emerging threat to maize-based food security in sub-Saharan Africa. *Phytopathology* **105**: 956-965.

Marshall OJ (2004) PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* **20**: 2471-2472.

Mbega E, Ndakidemi P, Mamiro D, Mushongi A, Kitenge K & Ndomba O (2016) Role of potyviruses in synergistic interaction leading to Maize Lethal Necrotic Disease on maize. *International Journal of Current Microbiology and Applied Sciences* **5**: 85-96.

Menzel W, Jelkmann W & Maiss E (2002) Detection of four apple viruses by multiplex RT-PCR assays with co-amplification of plant mRNA as internal control. *Journal of Virological Methods* **99**: 81-92.

Nassuth A, Pollari E, Helmeczy K, Stewart S & Kofalvi SA (2000) Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. *Journal of Virological Methods* **90**: 37-49.

Niblett C & Claflin L (1978) Corn Lethal Necrosis-a new virus disease of corn in Kansas. *Plant Disease Reporter* **62**: 15-19.

Nie X & Singh RP (2002) A new approach for the simultaneous differentiation of biological and geographical strains of Potato virus Y by uniplex and multiplex RT-PCR. *Journal of Virological Methods* **104**: 41-54.

Opiyo S, Ateka E, Owuor P, Manguro L & Miano D (2010) Development of a multiplex PCR technique for simultaneous detection of Sweet potato feathery mottle virus and Sweet potato chlorotic stunt virus. *Journal of Plant Pathology* **92**: 363-366.

Pagán I, González-Jara P, Moreno-Letelier A, Rodelo-Urrego M, Fraile A, Piñero D & García-Arenal F (2012) Effect of biodiversity changes in disease risk: exploring disease emergence in a plant-virus system. *Public Library of Science Pathogens* **8**: e1002796. DOI: 10.1371/journal.ppat.1002796.

Prendeville HR, Ye X, Jack Morris T & Pilson D (2012) Virus infections in wild plant populations are both frequent and often unapparent. *American Journal of Botany* **99**: 1033-1042.

Primrose SB & Twyman R (2009) Basic techiques. In: *Principles of Genome Analysis and Genomics*. John Wiley & Sons: Hoboken. 26-29.

Qu W, Shen Z, Zhao D, Yang Y & Zhang C (2008) MFEprimer: multiple factor evaluation of the specificity of PCR primers. *Bioinformatics* **25**: 276-278.

Robertson NL, French R & Gray SM (1991) Use of group-specific primers and the Polymerase Chain Reaction for the detection and identification of luteoviruses. *Journal of General Virology* **72**: 1473-1477.

Roossinck MJ (2010) Lifestyles of plant viruses. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**: 1899-1905.

Roy A, Fayad A, Barthe G & Brlansky R (2005) A multiplex Polymerase Chain Reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. *Journal of Virological Methods* **129**: 47-55.

Rozen S & Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols.* Humana Press: Totowa. 365-386.

Schaad NW & Frederick RD (2002) Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology* **24**: 250-258.

Scheets K (1998) Maize chlorotic mottle machlomovirus and Wheat streak mosaic Rymovirus concentrations increase in the synergistic disease Corn Lethal Necrosis. *Virology* **242**: 28-38.

Schenck S & Lehrer A (2000) Factors affecting the transmission and spread of Sugarcane yellow leaf virus. *Plant Disease* **84**: 1085-1088.

Shen Z, Qu W, Wang W, Lu Y, Wu Y, Li Z, Hang X, Wang X, Zhao D & Zhang C (2010) MPprimer: a program for reliable multiplex PCR primer design. *BMC Bioinformatics* **11**: 143.

Shepherd DN, Martin DP, Van der Walt E, Dent K, Varsani A & Rybicki EP (2010) Maize streak virus: an old and complex 'emerging' pathogen. *Molecular Plant Pathology* **11**: 1-12.

Srinivas K, Reddy MS, Hema M & Sreenivasulu P (2013) Multiplex RT-PCR for parallel detection of three RNA viruses associated with stripe and mosaic diseases of sorghum in India. *European Journal of Plant Pathology* **137**: 211-215.

Stewart LR, Redinbaugh MG, Massawe D, Willie K, Wijeratne S, Niblett CL, Kiggundu A & Asiimwe T (2017) Johnsongrass mosaic virus contributes to Maize Lethal Necrosis in East Africa. *Plant Disease* **101**: 1455-1462.

Syller J (2012) Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular Plant Pathology* **13**: 204-216.

Tao Y, Man J & Wu Y (2012) Development of a multiplex Polymerase Chain Reaction for simultaneous detection of wheat viruses and a phytoplasma in China. *Archives of Virology: Official Journal of the Virology Division of the International Union of Microbiological Societies* **157**: 1261-1267.

Thompson JR, Wetzel S, Klerks M, Vašková D, Schoen C, Špak J & Jelkmann W (2003) Multiplex RT-PCR detection of four aphid-borne strawberry viruses in *Fragaria spp*. in combination with a plant mRNA specific internal control. *Journal of Virological Methods* **111**: 85-93.

Uga H & Tsuda S (2005) A one-step Reverse Transcription-Polymerase Chain Reaction system for the simultaneous detection and identification of multiple tospovirus infections. *Phytopathology* **95**: 166-171.

Uyemoto J, Bockelman D & Claflin L (1980) Severe outbreak of Corn Lethal Necrosis Disease in Kansas. *Plant Disease* **64**: 99-100.

Uyemoto J, Claflin L, Wilson D & Raney R (1981) Maize chlorotic mottle and Maize dwarf mosaic viruses; effect of single and double inoculations on symptomatology and yield. *Plant Disease* **65**: 39-41.

Van den Berg N, Crampton BG, Hein I, Birch PR, Berger DK (2004) High-throughput screening of suppression subtractive hybridization cDNA libraries using DNA microarray analysis. *Biotechniques* **37**: 818-824.

Vidalakis G, Garnsey S, Bash J, Greer G & Gumpf D (2004) Efficacy of bioindexing for graft-transmissible citrus pathogens in mixed infections. *Plant Disease* **88**: 1328-1334.

Viswanathan R, Karuppaiah R & Balamuralikrishnan M (2010) Detection of three major RNA viruses infecting sugarcane by multiplex Reverse Transcription–Polymerase Chain Reaction (multiplex-RT-PCR). *Australasian Plant Pathology* **39**: 79-84.

Wangai A, Redinbaugh M, Kinyua Z, Miano D, Leley P, Kasina M, Mahuku G, Scheets K & Jeffers D (2012) First report of Maize chlorotic mottle virus and Maize Lethal Necrosis in Kenya. *Plant Disease* **96**: 1582-1582.

Wei T, Lu G & Clover G (2008) Novel approaches to mitigate primer interaction and eliminate inhibitors in multiplex PCR, demonstrated using an assay for detection of three strawberry viruses. *Journal of Virological Methods* **151**: 132-139.

White EJ, Venter M, Hiten NF & Burger JT (2008) Modified Cetyltrimethylammonium Bromide method improves robustness and versatility: The benchmark for plant RNA extraction. *Biotechnology Journal* **3**: 1424-1428.

Xie X, Chen W, Fu Q, Zhang P, An T, Cui A & An D (2016) Molecular variability and distribution of Sugarcane mosaic virus in Shanxi, China. *Public Library of Science One* **11**: e0151549. DOI: 10.1371/journal.pone.0151549.

Zhang P, Liu Y, Liu W, Massart S & Wang X (2017) Simultaneous detection of Wheat dwarf virus, Northern cereal mosaic virus, Barley yellow striate mosaic virus and Rice black-streaked dwarf virus in wheat by multiplex RT-PCR. *Journal of Virological Methods* **249**: 170-174.

Zhang Y, Zhao W, Li M, Chen H, Zhu S & Fan Z (2011) Real-time TaqMan RT-PCR for detection of Maize chlorotic mottle virus in maize seeds. *Journal of Virological Methods* **171**: 292-294.

Zhang W, Olson NH, Baker TS, Faulkner L, Agbandje-McKenna M, Boulton MI, Davies JW & McKenna R (2001) Structure of the Maize streak virus geminate particle. *Virology* **279**: 471-477.

Chapter 5: Concluding Remarks

The global importance of maize, especially in Africa, becomes apparent in its role as staple food for people, fodder for animals and its potential in fuel production. Continuous growth in world population and the need for alternate forms of fuel requires the limitation of factors that cause losses to maize production such as Maize lethal necrosis disease (MLND). The biology of this viral disease, its severity and extent of impact and the subsequent need for pre-emptive research in SA has been highlighted thoroughly in the previous chapters. The aim of this study was to pre-empt a future MLND outbreak in South Africa (SA) by 1) conducting a survey to determine the status of the MLND co-causing potyviral components present on maize in SA, 2) elucidating the diversity and relationships of the predominant potyvirus detected on maize in SA (Sugarcane mosaic virus; SCMV) with members of the same species from other countries worldwide, and 3) developing a tool that can be used to detect the viruses potentially present in the disease complex in SA.

In the first research chapter, the potyvirus survey is reported on, where a total of 650 plant samples (611 maize and 39 grass) with virus-like symptoms were collected at 104 of the 129 sites surveyed across maize-growing regions in seven provinces in SA. Symptoms observed ranged from mild mottles, yellowing, red vein-banding, mild and severe streaks and stunting and between one and ten samples were collected per site. A universal potyvirus-diagnostic primer pair in RT-PCR followed by direct Sanger sequencing and consequent BLAST analysis, 56 SCMV, 11 Johnsongrass mosaic virus (JGMV) (two on maize and nine on grass) and three Pennisetum mosaic virus (PenMV) cases were putatively identified. JGMV and PenMV are new reports for SA. The potyviruses were collected at 18 different sites, mostly in the north-eastern parts of the country: Limpopo Province and Mpumalanga Lowveld region, and were also detected in the north-western parts of SA: in the North-West Province and in Gauteng. The initial BLAST identification of the species was supported by a phylogenetic analysis as clustering with the appropriate reference sequences was observed. The 2016/2017 maize-growing season was exceptional in terms of the desired amount of rain at the ideal time, which resulted in record harvests, and this phenomenon may have played a role in the distribution and dispersal of virus infections on maize and the virus vectors, the observable symptoms that were present, as well as the range of potyvirus species detected in our study. For these reasons, it is recommended that such a survey be repeated in

the maize-growing regions of SA over multiple seasons. The survey results suggested that the risk areas where MLND may manifest in future, should MCMV enter the country, may be regions in the Limpopo Province and Mpumalanga as this is where higher relative concentrations of potyviruses were detected. The survey results also suggest that South African maize may not be particularly susceptible to potyvirus infections or that viruliferous vectors are not present in most parts of the country, which means that MLND will most likely be containable and manageable should MCMV enter the country, at least for the first while, taking into account that continuous surveillance should be carried out, especially near SA's borders. Determining the status, distribution and relative incidence of potyviruses provide a good idea of which potyviruses may be involved in MLND complexes in future and support previous predictions of where in SA MLND outbreaks may occur in future.

In the second research chapter, two partial-gene sequences of the RNA dependent RNA polymerase (RdRp) and Coat protein (CP) were used to conduct separate phylogenetic analyses. It was discovered that South African isolates of SCMV formed distinct clades based on their geographical origin. SCMV isolates from SA are most closely related to SCMV from Asia and Europe and not to other isolates from Africa as was initially hypothesised, which suggests a common ancestor with and a possible Asian or European source of SCMV introduced into SA. SCMV isolates from Tanzania were also studied (as Tanzania is the geographically closest country to SA where MLND is already present and is a possible source of MCMV in SA in future) and it was found that Tanzanian SCMV isolates are closely related to SCMV from other African countries but more distantly related to SCMV from SA. Isolates clustered mostly based on their geographical origin and and/or their host plant. The knowledge made available by the diversity study tentatively suggest that there may be differences in interactions between pathogens in the disease complex during a potential future MLND outbreak in SA compared to the outbreaks seen in other African countries. This was suggested because South African SCMV is genetically different to SCMV found in the rest of Africa which could have an impact on future co-infections with MCMV or the disease epidemiology of future MLND infections on maize in SA. It was seen that the partial CP gene region was the more ideal region of the two to use in a phylogenetic analysis as more reference sequences were available on GenBank for this region of the genome, thus allowing a

more complete view to be obtained. Future studies could expand on this work by including larger or additional gene regions, or the whole genomes to obtain a more holistic view of the diversity across the genome by following a phylogenomics approach. Studying the diversity of the predominant potyvirus species, SCMV, indicates the genetic variation of the SCMV which occurs in SA and therefore increase the available knowledge resources for designing alternate diagnostic tools or the development of virus-resistant crops, for example.

The third research chapter describes the multiplex RT-PCR system which could accurately and simultaneously identify four maize-infecting viruses: SCMV, MCMV, JGMV and MSV by means of four sets of specific primers that did not outcompete or interact with one another. The findings from the first research chapter were used to determine which viruses to include in the assay and thus the two potyvirus species detected most often in the survey were selected: SCMV and JGMV and Maize streak virus (MSV) is known to be widespread in SA and thought to contribute to MLN outbreaks in SA in future, and was therefore also included. The assay is predicted to identify low concentrations of virus and will thus be useful to for routine testing. Its cost, time and labour efficiency will allow larger numbers of samples to be screened, contributing to a better success rate of detecting infected plant material if samples are taken from a large batch. This assay could efficiently detect SCMV from Tanzania too, and can thus be used in other countries too, where surveillance is conducted. Some optimisation work remains to be done regarding multiple bands produced by two of the primer sets and the system should be used to test naturally occurring co-infections of these viruses of which not many were collected during our survey. The diagnostic tool will allow for accurate and sensitive detection that is less time-consuming, more cost-efficient and less labour-intensive and will thus greatly aid quarantine facilities performing routine diagnostics, researchers conducting surveys for viruses and also seed producers to ensure virus-free seed as a much greater number of tests will be done for the same current cost.

This study is unique and novel as it is the first work done to pre-empt MLN in SA. It reports the first comprehensive survey for virus-like symptoms in South African maize-growing regions in order to identify potyvirus species, the first diversity study of SCMV collected from South African maize and also reports the first use of multiplex RT-PCR to identify the four maize-infecting viruses: SCMV, MCMV, JGMV

and MSV, simultaneously. The success of the survey and diversity study is largely owed to the combination of: 1) the thorough survey for potential potyvirus-infections on maize grown in a variety of maize-growing regions where the climate, vectors and cultivars differ, 2) the use of two reliable RT-PCR/Sanger sequencing detection and identification methods developed by previous authors, and 3) the extensive resources available when developing the diagnostic assay such as range of positive controls, available MCMV RNA obtained under permit, and the good quality primer design software. The thorough survey allowed the screening of large numbers of samples from a range of locations, increasing the chances of detecting the potyviruses. The use of the two RT-PCR systems lead to the interesting observations made in the diversity study regarding the relatedness of South African SCMV isolates to those from other countries and also allowed the identification of two potyviruses that are novel to SA, one (JGMV) which has been identified in Maize lethal necrosis (MLN) infections in other parts of Africa which was thus, due to its presence in SA and role in MLND in East Africa, included in the multiplex assay.

Although all three aims of this study were achieved and a number of novel and useful observations were made, a great deal of work remains to be conducted in future studies. This includes: surveys over multiple seasons, which may also yield different results in subsequent diversity studies if potyviruses are identified in more regions or different species of potyviruses are identified, using additional gene regions or whole genome sequences for subsequent diversity studies, as well as certain optimisations and improvements for the multiplex assay. Future pre-emptive studies in SA can be directed at determining the diversity of insect populations around maize fields, studying their status, ecology and distributions and identifying alternate hosts of these potyviruses. Our study contributes greatly towards establishing the potyvirus species present in SA and their diversity, which provides insights into their identities, their distributions and genetic diversity, and towards developing a multiplex assay for identification of the potential MLND-causing viruses in future in SA. The findings from this study will be critically important in the containment of MLND in SA in the foreseeable future and have formed the foundation of MLND pre-emptive research in SA and thus aids pre-empting future MLND outbreaks in the country.

Appendices

Appendix A

Table 1.1 Results of survey for potyviruses on maize samples collected in South Africa

Field number	Latitude	Longitude	Type of farming	Accession number	Plant host	Description of symptoms	Presence of potyvirus	BLAST identity
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3300	Maize	Streaks	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3301	Maize	Streaks	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3302	Maize	Streaks	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3303	Maize	Streaks	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3304	Maize	Faint mottle	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3305	Maize	Streaks	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3306	Maize	Streaks	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3307	Maize	Streaks	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3308	Maize	Faint mottle	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3309	Maize	Streaks	Negative	n/a
1	25.4137665 063978°S	27.4702413 438913°E	Commercial	16-3310	Maize	Streaks	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3311	Sweet corn	Streaks (very prominent)	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3312	Sweet corn	Streaks	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3313	Sweet corn	Streaks	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3314	Sweet corn	Streaks	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3315	Sweet corn	Streaks	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3316	Sweet corn	Streaks	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3317	Sweet corn	Streaks	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3318	Sweet corn	Streaks (very prominent)	Negative	n/a
2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3319	Sweet corn	Streaks	Negative	n/a

2	25.3117325 290929°S	27.4444342 482201°E	Commercial	16-3320	Sweet corn	Streaks	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3321	Maize	Streaks	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3322	Maize	Streaks	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3323	Maize	Streaks	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3324	Maize	Streaks	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3325	Maize	Streaks	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3326	Maize	Streaks	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3327	Maize	Streaks & stunted	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3328	Maize	Streaks & stunted	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3329	Maize	Streaks & stunted	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3330	Maize	Streaks & stunted	Negative	n/a
3	25.1419742 208171°S	27.3302371 802152°E	Commercial	16-3331	Maize	Streaks & stunted	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3332	Maize	Streaks	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3333	Maize	Streaks	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3334	Maize	Streaks	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3335	Maize	Streaks	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3336	Maize	Streaks	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3337	Maize	Streaks	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3338	Maize	Streaks	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3339	Maize	Streaks	Negative	n/a
4	25.1736690 812038°S	27.3325216 589170°E	Commercial	16-3340	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3341	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3342	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3343	Maize	Streaks	Negative	n/a

5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3344	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3345	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3346	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3347	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3348	Maize	Streaks & stunted	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3349	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3350	Maize	Streaks (very broad)	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3351	Maize	Streaks	Negative	n/a
5	25.1740903 651013°S	27.3402414 836959°E	Commercial	16-3352	Maize	Streaks	Negative	n/a
6	25.2232712 157916°S	27.3510346 714934°E	Commercial	16-3353	Maize	Streaks	Negative	n/a
6	25.2232712 157916°S	27.3510346 714934°E	Commercial	16-3354	Maize	Streaks	Negative	n/a
7	25.2240379 674072°S	27.3446156 410799°E	Commercial	16-3355	Maize	Streaks	Negative	n/a
7	25.2240379 674072°S	27.3446156 410799°E	Commercial	16-3356	Maize	Streaks	Negative	n/a
7	25.2240379 674072°S	27.3446156 410799°E	Commercial	16-3357	Maize	Streaks	Negative	n/a
7	25.2240379 674072°S	27.3446156 410799°E	Commercial	16-3358	Maize	Streaks	Negative	n/a
7	25.2240379 674072°S	27.3446156 410799°E	Commercial	16-3359	Maize	Streaks	Negative	n/a
7	25.2240379 674072°S	27.3446156 410799°E	Commercial	16-3360	Maize	Streaks	Negative	n/a
8	25.2521991 641405°S	27.3753386 146646°E	Commercial	16-3361	Maize	Mosaic	Negative	n/a
8	25.2521991 641405°S	27.3753386 146646°E	Commercial	16-3362	Maize	Streaks	Negative	n/a
8	25.2521991 641405°S	27.3753386 146646°E	Commercial	16-3363	Maize	Streaks	Negative	n/a
8	25.2521991 641405°S	27.3753386 146646°E	Commercial	16-3364	Maize	Streaks	Negative	n/a
8	25.2521991 641405°S	27.3753386 146646°E	Commercial	16-3365	Maize	Streaks	Negative	n/a
8	25.2521991 641405°S	27.3753386 146646°E	Commercial	16-3366	Grass *(Panicum maximum)*	Streaks	Negative	n/a
8	25.2521991 641405°S	27.3753386 146646°E	Commercial	16-3367	Maize	Mosaic	Negative	n/a

8	25.2521991641405°S	27.3753386146646°E	Commercial	16-3368	Grass *(P. maximum)*	Streaks	Negative	n/a
8	25.2521991641405°S	27.3753386146646°E	Commercial	16-3369	Grass *(P. maximum)*	Streaks	Negative	n/a
8	25.2521991641405°S	27.3753386146646°E	Commercial	16-3370	Maize	Streaks	Negative	n/a
8	25.2521991641405°S	27.3753386146646°E	Commercial	16-3371	Maize	Streaks	Negative	n/a
8	25.2521991641405°S	27.3753386146646°E	Commercial	16-3372	Maize	Streaks	Negative	n/a
8	25.2521991641405°S	27.3753386146646°E	Commercial	16-3373	Maize	Streaks	Negative	n/a
8	25.2521991641405°S	27.3753386146646°E	Commercial	16-3374	Maize	Faint streaks	Negative	n/a
8	25.2521991641405°S	27.3753386146646°E	Commercial	16-3375	Maize	Faint mottle	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3376	Maize	Streaks	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3377	Maize	Streaks	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3378	Maize	Mosaic	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3379	Maize	Mosaic	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3380	Maize	Streaks	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3381	Maize	Streaks & stunted	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3382	Maize	Faint streaks & stunted	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3383	Maize	Streaks & stunted	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3384	Maize	Streaks & stunted	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3385	Maize	Streaks	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3386	Maize	Streaks	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3387	Maize	Streaks	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3388	Maize	Streaks	Negative	n/a
9	25.2626995292734°S	27.3927891322315°E	Commercial	16-3389	Maize	Streaks	Negative	n/a
10	25.3119549347904°S	27.4154948668337°E	Commercial	16-3390	Maize	Streaks	Negative	n/a
10	25.3119549347904°S	27.4154948668337°E	Commercial	16-3391	Maize	Streaks	Negative	n/a

10	25.3119549 347904°S	27.4154948 668337°E	Commercial	16-3392	Maize	Streaks	Negative	n/a
10	25.3119549 347904°S	27.4154948 668337°E	Commercial	16-3393	Maize	Streaks	Negative	n/a
10	25.3119549 347904°S	27.4154948 668337°E	Commercial	16-3394	Grass *(P. maximum)*	Streaks	Positive	Johnsongrass mosaic virus
10	25.3119549 347904°S	27.4154948 668337°E	Commercial	16-3395	Maize	Streaks	Negative	n/a
10	25.3119549 347904°S	27.4154948 668337°E	Commercial	16-3396	Maize	Streaks	Negative	n/a
10	25.3119549 347904°S	27.4154948 668337°E	Commercial	16-3397	Maize	Streaks	Negative	n/a
10	25.3119549 347904°S	27.4154948 668337°E	Commercial	16-3398	Maize	Streaks	Negative	n/a
10	25.3119549 347904°S	27.4154948 668337°E	Commercial	16-3399	Maize	Streaks	Negative	n/a
11	25.1319667 343225°S	29.1550514 880134°E	Commercial	16-3200	Maize	Streaks	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3201	Maize	Streaks	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3202	Maize	Streaks	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3203	Grass *(P. maximum)*	Faint mottle	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3204	Grass *(P. maximum)*	Faint mottle	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3205	Grass *(P. maximum)*	Streaks (very prominent)	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3206	Grass *(P. maximum)*	Faint mottle	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3207	Grass *(P. maximum)*	Streaks (very prominent)	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3208	Maize	Streaks	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3209	Grass *(P. maximum)*	Faint mottle	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3210	Grass *(P. maximum)*	Streaks	Positive	Pennisetum mosaic virus
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3211	Maize	Streaks	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3212	Maize	Streaks	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3213	Maize	Streaks	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3214	Maize	Streaks	Negative	n/a
12	25.1319674 678417°S	29.1550534 722612°E	Commercial	16-3215	Maize	Streak/mottle	Negative	n/a

13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3216	Grass *(P. maximum)*	Streaks (very prominent)	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3217	Maize	Streaks	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3218	Maize	Streaks	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3219	Maize	Streaks	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3220	Maize	Stipple on small area	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3221	Maize	Stipple on small area	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3222	Maize	Streaks	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3223	Maize	Streaks	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3224	Maize	Faint mottle	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3225	Maize	Streaks	Negative	n/a
13	25.1315007 598549°S	29.1556043 908753°E	Commercial	16-3226	Maize	Streaks	Negative	n/a
14	25.1156123 514060°S	29.1916253 958553°E	Commercial	16-3227	Maize	Streaks/possib le chimera	Negative	n/a
14	25.1156123 514060°S	29.1916253 958553°E	Commercial	16-3228	Maize	Asymptomatic	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3229	Maize	Faint mottle	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3230	Maize	Streaks	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3231	Maize	Streaks	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3232	Maize	Streaks	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3233	Maize	Streaks	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3234	Maize	Streaks	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3235	Maize	Streaks	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3236	Maize	Streaks	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3237	Maize	Streaks	Negative	n/a
15	25.2301350 013821°S	29.2100513 063339°E	Commercial	16-3238	Maize	Streaks	Negative	n/a
16	25.2106356 454978°S	29.2141021 051689°E	Commercial	16-3239	Maize	Streaks	Negative	n/a

16	25.2106356 454978°S	29.2141021 051689°E	Commercial	16-3240	Maize	Streaks	Negative*	n/a
16	25.2106356 454978°S	29.2141021 051689°E	Commercial	16-3241	Maize	Streaks	Negative	n/a
16	25.2106356 454978°S	29.2141021 051689°E	Commercial	16-3242	Maize	Streaks	Negative	n/a
16	25.2106356 454978°S	29.2141021 051689°E	Commercial	16-3243	Maize	Streaks	Negative	n/a
16	25.2106356 454978°S	29.2141021 051689°E	Commercial	16-3244	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3245	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3246	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3247	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3248	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3249	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3250	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3251	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3252	Maize	Stunted, light green/yellow streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3253	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3254	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3255	Maize	Streaks	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3256	Maize	Stunted, yellow plant	Negative	n/a
17	25.1819484 638977°S	29.2434831 721693°E	Commercial	16-3257	Maize	Severe stunting	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3258	Maize	Streaks	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3259	Maize	Streaks	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3260	Maize	Streaks	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3261	Maize	Streaks	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3262	Maize	Streaks	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3263	Maize	Streaks	Negative	n/a

18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3264	Maize	Streaks	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3265	Maize	Streaks	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3266	Maize	Streaks	Negative	n/a
18	25.1730297 569325°S	29.2448991 359731°E	Commercial	16-3267	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3268	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3269	Maize	Severe stunting	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3270	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3271	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3272	Maize	Streaks	Negative*	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3273	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3274	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3275	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3276	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3277	Maize	Streaks	Negative	n/a
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3278	Grass *(P. maximum)*	Streaks	Positive	Johnsongrass mosaic virus
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3279	Grass *(P. maximum)*	Faint mottle	Positive	Johnsongrass mosaic virus
19	25.1636458 254495°S	29.2457153 318993°E	Commercial	16-3280	Grass *(P. maximum)*	Faint mottle	Positive	Johnsongrass mosaic virus
20	25.0900601 114654°S	29.2336582 721028°E	Commercial	16-3281	Maize	Streaks	Negative	n/a
20	25.0900601 114654°S	29.2336582 721028°E	Commercial	16-3282	Maize	Streaks	Negative	n/a
20	25.0900601 114654°S	29.2336582 721028°E	Commercial	16-3283	Maize	Faint mottle	Positive	Sugarcane mosaic virus
20	25.0900601 114654°S	29.2336582 721028°E	Commercial	16-3284	Maize	Streaks	Negative	n/a
20	25.0900601 114654°S	29.2336582 721028°E	Commercial	16-3285	Maize	Streaks	Negative	n/a
20	25.0900601 114654°S	29.2336582 721028°E	Commercial	16-3286	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3287	Maize	Streaks	Negative	n/a

21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3288	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3289	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3290	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3291	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3292	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3293	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3294	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3295	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3296	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3297	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3298	Maize	Streaks	Negative	n/a
21	25.1208654 352388°S	29.1835706 292195°E	Commercial	16-3299	Maize	Streaks	Negative	n/a
22	25.5723354 209751°S	28.2817555 857201°E	Commercial	17-4000	Maize	Broad streak	Negative	n/a
22	25.5723354 209751°S	28.2817555 857201°E	Commercial	17-4001	Maize	Streaks	Negative	n/a
23	26.0038092 602530°S	28.2532240 746335°E	Commercial	17-4002	Maize	Streaks	Negative	n/a
23	26.0038092 602530°S	28.2532240 746335°E	Commercial	17-4003	Maize	Streaks	Negative	n/a
23	26.0038092 602530°S	28.2532240 746335°E	Commercial	17-4004	Maize	Streaks	Negative	n/a
24	26.0354374 096402°S	28.3245555 046226°E	Commercial	17-4005	Maize	Streaks	Negative	n/a
24	26.0354374 096402°S	28.3245555 046226°E	Commercial	17-4006	Maize	Streaks	Negative	n/a
24	26.0354374 096402°S	28.3245555 046226°E	Commercial	17-4007	Maize	Streaks	Negative	n/a
25	26.1150631 117912°S	28.4728539 849756°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
26	26.3643659 927117°S	29.0904598 461803°E	Commercial	17-4008	Maize	Streaks	Negative	n/a
26	26.3643659 927117°S	29.0904598 461803°E	Commercial	17-4009	Maize	Streaks	Negative	n/a
26	26.3643659 927117°S	29.0904598 461803°E	Commercial	17-4010	Maize	Streaks	Negative	n/a

26	26.3643659 927117°S	29.0904598 461803°E	Commercial	17-4011	Maize	Streaks	Negative	n/a
27	27.0748336 675158°S	29.1531001 518678°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
28	27.1829764 497714°S	29.1056843 077741°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
29	27.1921794 514980°S	28.4755703 769104°E	Commercial	17-4012	Maize	Spots, stunted	Negative	n/a
29	27.1921794 514980°S	28.4755703 769104°E	Commercial	17-4013	Maize	Streaks	Negative	n/a
29	27.1921794 514980°S	28.4755703 769104°E	Commercial	17-4014	Maize	Streaks	Negative	n/a
30	27.3259861 746848°S	28.3231473 100914°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
31	28.0504418 909090°S	28.3855004 163306°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
32	28.1526747 750079°S	28.3521848 573105°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
33	28.1844758 094904°S	28.4448430 915549°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
34	28.1625929 266199°S	28.5425036 413859°E	Commercial	17-4015	Maize	Streaks	Negative	n/a
34	28.1625929 266199°S	28.5425036 413859°E	Commercial	17-4016	Maize	Unevenly distributed yellow streaks	Negative	n/a
35	28.1722263 507367°S	29.0418135 253370°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
36	28.1106424 905035°S	29.0759010 299801°E	Commercial	17-4018	Maize	Streaks	Negative	n/a
37	28.0318848 968708°S	29.1453786 586300°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
38	28.0546256 272624°S	29.1609973 272396°E	Commercial	17-4019	Maize	Dwarfed, fine streaks	Negative	n/a
39	28.0923751 536629°S	29.1602259 758637°E	Commercial	17-4017	Maize	Streaks	Negative	n/a
40	28.2323830 012002°S	29.0421874 235372°E	Commercial	17-4020	Maize	Streaks	Negative	n/a
40	28.2323830 012002°S	29.0421874 235372°E	Commercial	17-4021	Grass *(Sorghum halepense)*	Streaks	Negative	n/a
41	28.3644375 728519°S	29.0600867 762648°E	Commercial	17-4022	Maize	Fine mottle	Negative	n/a
41	28.3644375 728519°S	29.0600867 762648°E	Commercial	17-4023	Maize	Yellow blotches	Negative	n/a
42	28.3959428 654104°S	29.1609414 418635°E	Commercial	17-4024	Maize	Yellow blotches	Negative	n/a
43	28.5755072 235991°S	29.2601580 882116°E	Commercial	17-4445	Maize	Streaks	Negative	n/a
44	28.5402840	29.3220751	Commercial	17-4025	Maize	Streaks	Negative	n/a

	884281°S	977709°E						
45	29.2146246 072963°S	30.3458628 565877°E	Commercial	17-4026	Maize	Streaks	Negative	n/a
45	29.2146246 072963°S	30.3458628 565877°E	Commercial	17-4027	Maize	Streaks	Negative	n/a
46	29.0750789 523456°S	30.3635486 793348°E	Commercial	17-4028	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4029	Maize	Small faint ringspots	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4030	Maize	Yellow blotches	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4031	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4032	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4033	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4034	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4035	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4036	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4037	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4038	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4039	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4040	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4041	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4042	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4043	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4044	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4045	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4046	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4047	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4048	Maize	Streaks	Negative	n/a
47	29.0822471	30.3716813	Commercial	17-4049	Maize	Streaks	Negative	n/a

	052362°S	033499°E						
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4050	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4051	Maize	Streaks	Negative	n/a
47	29.0822471 052362°S	30.3716813 033499°E	Commercial	17-4052	Maize	Streaks	Negative	n/a
48	29.0542756 469149°S	30.4142369 215343°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4053	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4054	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4055	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4056	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4057	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4058	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4059	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4060	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4061	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4062	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4063	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4064	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4065	Maize	Streaks	Negative	n/a
49	28.3852029 203555°S	31.2500780 024524°E	Commercial	17-4066	Maize	Streaks	Negative	n/a
50	28.0122808 417952°S	31.0259531 122487°E	Commercial	17-4067	Maize	Streaks	Negative	n/a
50	28.0122808 417952°S	31.0259531 122487°E	Commercial	17-4068	Maize	Streaks	Negative	n/a
50	28.0122808 417952°S	31.0259531 122487°E	Commercial	17-4069	Maize	Streaks	Negative	n/a
51	27.3827625 903682°S	30.4259887 277638°E	Commercial	17-4070	Maize	Streaks	Negative	n/a
51	27.3827625 903682°S	30.4259887 277638°E	Commercial	17-4071	Maize	Streaks	Negative	n/a
51	27.3827625	30.4259887	Commercial	17-4072	Maize	Streaks	Negative	n/a

	903682°S	277638°E						
51	27.3827625 903682°S	30.4259887 277638°E	Commercial	17-4073	Maize	Streaks	Negative	n/a
51	27.3827625 903682°S	30.4259887 277638°E	Commercial	17-4074	Maize	Streaks	Negative	n/a
51	27.3827625 903682°S	30.4259887 277638°E	Commercial	17-4075	Maize	Streaks	Negative	n/a
52	27.3518181 198854°S	30.4320350 201510°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
53	27.2828158 296351°S	30.4400258 130250°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
54	27.2131432 212045°S	30.5227361 960673°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
55	27.1425994 399968°S	30.5346350 735023°E	Commercial	17-4076	Maize	Streaks	Negative	n/a
55	27.1425994 399968°S	30.5346350 735023°E	Commercial	17-4077	Maize	Streaks	Negative	n/a
56	26.4855396 193252°S	30.2801602 589976°E	Commercial	17-4078	Maize	Streaks	Negative	n/a
56	26.4855396 193252°S	30.2801602 589976°E	Commercial	17-4079	Maize	Streaks	Negative	n/a
56	26.4855396 193252°S	30.2801602 589976°E	Commercial	17-4080	Maize	Streaks	Negative	n/a
56	26.4855396 193252°S	30.2801602 589976°E	Commercial	17-4081	Maize	Streaks	Negative	n/a
57	26.4351332 985270°S	30.1543261 620546°E	Commercial	17-4082	Maize	Streaks	Negative	n/a
57	26.4351332 985270°S	30.1543261 620546°E	Commercial	17-4083	Maize	Streaks	Negative	n/a
57	26.4351332 985270°S	30.1543261 620546°E	Commercial	17-4084	Maize	Streaks	Negative	n/a
57	26.4351332 985270°S	30.1543261 620546°E	Commercial	17-4085	Maize	Streaks	Negative	n/a
58	26.3912947 893386°S	30.0943277 292592°E	Commercial	17-4086	Maize	Streaks	Negative	n/a
58	26.3912947 893386°S	30.0943277 292592°E	Commercial	17-4087	Maize	Streaks	Negative	n/a
59	26.2519310 678366°S	29.5637087 016917°E	Commercial	17-4088	Maize	Streaks	Negative	n/a
60	26.1714094 340191°S	29.5124154 777650°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
61	26.1030534 454030°S	29.4321502 153219°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
62	26.1012906 154363°S	29.4125320 431388°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
63	26.1857068 939742°S	29.3401414 964990°E	Commercial	17-4089	Maize	Streaks	Negative	n/a
63	26.1857068	29.3401414	Commercial	17-4090	Maize	Streaks	Negative	n/a

	939742°S	964990°E						
63	26.1857068 939742°S	29.3401414 964990°E	Commercial	17-4091	Maize	Streaks	Negative	n/a
63	26.1857068 939742°S	29.3401414 964990°E	Commercial	17-4092	Maize	Streaks	Negative	n/a
63	26.1857068 939742°S	29.3401414 964990°E	Commercial	17-4093	Maize	Streaks	Negative	n/a
64	26.2054363 914959°S	29.2251936 948351°E	Commercial	17-4094	Maize	Streaks	Negative	n/a
64	26.2054363 914959°S	29.2251936 948351°E	Commercial	17-4095	Maize	Streaks	Negative	n/a
64	26.2054363 914959°S	29.2251936 948351°E	Commercial	17-4096	Maize	Streaks	Negative	n/a
65	26.1800124 404910°S	29.1713420 666247°E	Commercial	Negative field	Maize	Streaks	Negative	n/a
66	25.470695° S	31.534114° E	Subsistence	17-4215	Grass *(P. maximum)*	Mottle	Positive	Johnsongrass mosaic virus
66	25.470695° S	31.534114° E	Subsistence	17-4216	Grass *(P. maximum)*	Streaks	Positive	Johnsongrass mosaic virus
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4200	Maize	Red streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4201	Maize	Red streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4202	Maize	Red streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4203	Maize	Slight mottle	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4204	Maize	Red streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4205	Maize	Broad yellow streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4206	Maize	Broad yellow streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4207	Maize	Broad yellow streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4208	Maize	Streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4209	Grass *(S. halepense)*	Streaks	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4210	Grass *(S. halepense)*	Mottle	Negative	n/a
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4211	Grass *(P. maximum)*	Streaks	Positive	Johnsongrass mosaic virus
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4212	Grass *(P. maximum)*	Mottle	Positive	Johnsongrass mosaic virus
67	25.2623241 077435°S	30.5945539 277852°E	Commercial	17-4213	Grass *(P. maximum)*	Streaks	Positive	Johnsongrass mosaic virus
67	25.2623241	30.5945539	Commercial	17-4214	Grass *(S.*	Streaks	Negative	n/a

	077435°S	277852°E			*halepense)*			
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4219	Maize	Blotchy	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4220	Maize	Broad yellow streaks	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4221	Maize	Slight mottle	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4222	Maize	Slight mottle	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4223	Maize	Slight mottle	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4224	Maize	Concentric ringspots	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4225	Maize	Slight mottle	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4226	Maize	Slight mottle	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4227	Maize	Slight mottle	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4228	Maize	Broad yellow streaks	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4229	Maize	Streaks	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4230	Maize	Streaks	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4231	Maize	Streaks	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4232	Maize	Broad yellow streaks	Negative	n/a
68	25.2906823 075932°S	31.3103556 855110°E	Subsistence	17-4233	Maize	Slight mottle	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4250	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4251	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4252	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4253	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4254	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4255	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4256	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4257	Maize	Streaks	Negative	n/a
69	25.3705597	31.3842311	Commercial	17-4258	Maize	Large blocks of	Negative	n/a

	985882°S	666214°E				discolouration		
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4259	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4260	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4261	Maize	Yellowing and streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4262	Maize	Streaks	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4263	Maize	Mosaic	Negative	n/a
69	25.3705597 985882°S	31.3842311 666214°E	Commercial	17-4264	Maize	Streaks	Negative	n/a
70	25.3706729 181850°S	31.3855922 745397°E	Commercial	17-4265	Maize	Streaks	Negative	n/a
70	25.3706729 181850°S	31.3855922 745397°E	Commercial	17-4266	Maize	Yellow discolouration	Negative	n/a
71	25.3702261 925923°S	31.3923802 601829°E	Commercial	17-4267	Maize	Streaks	Negative	n/a
71	25.3702261 925923°S	31.3923802 601829°E	Commercial	17-4268	Maize	Slight mottle	Negative	n/a
71	25.3702261 925923°S	31.3923802 601829°E	Commercial	17-4269	Maize	Mosaic	Negative	n/a
72	25.3126748 933508°S	31.2451131 481093°E	Commercial	17-4270	Maize	Slight mottle	Negative	n/a
72	25.3126748 933508°S	31.2451131 481093°E	Commercial	17-4271	Maize	Slight yellowing	Negative	n/a
72	25.3126748 933508°S	31.2451131 481093°E	Commercial	17-4272	Maize	Slight mottle	Negative	n/a
72	25.3126748 933508°S	31.2451131 481093°E	Commercial	17-4273	Maize	Slight mottle	Negative	n/a
72	25.3126748 933508°S	31.2451131 481093°E	Commercial	17-4274	Maize	Slight mottle	Negative	n/a
72	25.3126748 933508°S	31.2451131 481093°E	Commercial	17-4275	Maize	Slight mottle	Positive	Sugarcane mosaic virus
73	25.2615199 984338°S	30.3858787 485332°E	Commercial	17-4131	Maize	Red streaks	Negative	n/a
73	25.2615199 984338°S	30.3858787 485332°E	Commercial	17-4132	Maize	Large blocks of discolouration	Negative	n/a
73	25.2615199 984338°S	30.3858787 485332°E	Commercial	17-4133	Maize	Concentric ringspots	Negative	n/a
73	25.2615199 984338°S	30.3858787 485332°E	Commercial	17-4134	Maize	Streaks	Negative	n/a
73	25.2615199 984338°S	30.3858787 485332°E	Commercial	17-4135	Maize	Broad yellow streaks	Negative	n/a
73	25.2615199 984338°S	30.3858787 485332°E	Commercial	17-4136	Maize	Slight mottle	Negative	n/a

74	25.2810618 201118°S	31.0644775 066508°E	Commercial	17-4137	Grass *(P. maximum)*	Slight mottle	Positive	Johnsongrass mosaic virus
74	25.2810618 201118°S	31.0644775 066508°E	Commercial	17-4138	Grass *(P. maximum)*	Streaks	Negative	n/a
74	25.2810618 201118°S	31.0644775 066508°E	Commercial	17-4139	Grass *(P. maximum)*	Slight mottle	Positive	Pennisetum mosaic virus
74	25.2810618 201118°S	31.0644775 066508°E	Commercial	17-4140	Grass *(P. maximum)*	Slight mottle	Negative	n/a
74	25.2810618 201118°S	31.0644775 066508°E	Commercial	17-4141	Grass *(P. maximum)*	Streaks	Positive	Pennisetum mosaic virus
74	25.2810618 201118°S	31.0644775 066508°E	Commercial	17-4142	Grass *(P. maximum)*	Streaks	Negative	n/a
74	25.2810618 201118°S	31.0644775 066508°E	Commercial	17-4143	Grass *(P. maximum)*	Slight mottle	Positive	Johnsongrass mosaic virus
74	25.2810618 201118°S	31.0644775 066508°E	Commercial	17-4144	Grass *(P. maximum)*	Streaks	Negative	n/a
75	25.4749677 278396°S	28.3412519 763910°E	Commercial	17-4276	Maize	Broad yellow streaks	Negative	n/a
75	25.4749677 278396°S	28.3412519 763910°E	Commercial	17-4277	Maize	Streaks	Negative	n/a
75	25.4749677 278396°S	28.3412519 763910°E	Commercial	17-4278	Maize	Streaks	Negative	n/a
76	25.4857990 918738°S	29.3624231 291156°E	Commercial	Negative field	n/a	n/a	n/a	n/a
77	25.4908149 723755°S	29.5202831 937142°E	Commercial	17-4279	Maize	Fine mottle	Negative	n/a
77	25.4908149 723755°S	29.5202831 937142°E	Commercial	17-4280	Grass *(S. Halepense)*	Yellowing	Negative	n/a
78	25.4056973 733243°S	30.0317704 078531°E	Commercial	17-4281	Maize	Fine mottle	Negative	n/a
79	25.4046416 833693°S	30.0325506 958067°E	Commercial	17-4282	Maize	Fine mottle	Negative	n/a
80	25.4429758 058181°S	30.0044547 145798°E	Commercial	Negative field	n/a	n/a	n/a	n/a
81	25.4953579 558371°S	29.4503419 371636°E	Commercial	17-4283	Maize	Yellow blotches/mosaic	Negative	n/a
82	25.5114868 820945°S	29.2305386 827497°E	Commercial	17-4284	Maize	Streaks/yellowing	Negative	n/a
82	25.5114868 820945°S	29.2305386 827497°E	Commercial	17-4285	Maize	Streaks	Negative	n/a
82	25.5114868 820945°S	29.2305386 827497°E	Commercial	17-4286	Maize	Streaks	Negative	n/a
82	25.5114868 820945°S	29.2305386 827497°E	Commercial	17-4287	Maize	Streaks	Negative	n/a
82	25.5114868 820945°S	29.2305386 827497°E	Commercial	17-4288	Maize	Streaks	Negative	n/a
82	25.5114868 820945°S	29.2305386 827497°E	Commercial	17-4289	Maize	Streaks	Negative	n/a

82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4290	Maize	Streaks/yellowing	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4291	Maize	Streaks	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4292	Maize	Streaks/yellowing	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4293	Maize	Streaks	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4294	Maize	Streaks/yellowing	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4295	Maize	Streaks	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4296	Maize	Streaks	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4297	Maize	Streaks	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4298	Maize	Streaks/yellowing	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4299	Maize	Streaks	Negative	n/a
82	25.5114868820945°S	29.2305386827497°E	Commercial	17-4300	Maize	Streaks	Negative	n/a
83	25.5212530179874°S	29.0055707867237°E	Commercial	Negative field	n/a	n/a	n/a	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4234	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4235	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4236	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4237	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4238	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4239	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4240	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4241	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4242	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4243	Maize	Streaks	Negative	n/a
84	25.5212533845301°S	29.0055708218769°E	Commercial	17-4244	Maize	Streaks	Negative	n/a
85	25.4800395327890°S	28.3916177751771°E	Commercial	17-4245	Maize	Streaks	Negative	n/a

85	25.4800395 327890°S	28.3916177 751771°E	Commercial	17-4246	Maize	Streaks	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4101	Maize	Streaks	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4102	Maize	Streaks	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4103	Maize	Streaks	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4104	Maize	Streaks	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4105	Maize	Streaks	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4106	Maize	Streaks	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4107	Maize	Slight mottle	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4108	Maize	Streaks	Negative	n/a
86	26.0515958 383308°S	27.3822853 812131°E	Commercial	17-4109	Maize	Broad yellow streaks	Negative	n/a
87	27.0137471 009883°S	26.0845136 721953°E	Commercial	17-4111	Maize	Streaks	Negative	n/a
87	27.0137471 009883°S	26.0845136 721953°E	Commercial	17-4112	Maize	Streaks	Negative	n/a
87	27.0137471 009883°S	26.0845136 721953°E	Commercial	17-4113	Maize	Streaks	Negative	n/a
87	27.0137471 009883°S	26.0845136 721953°E	Commercial	17-4110	Maize	Broad yellow streaks	Negative	n/a
88	27.2840307 280126°S	25.4503593 066503°E	Commercial	17-4114	Maize	Slight mottle	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4116	Maize	Streaks	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4117	Maize	Red streaks	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4118	Maize	Streaks	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4119	Maize	Streaks	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4120	Maize	Streaks	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4121	Maize	Red streaks	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4122	Maize	Streaks	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4123	Maize	Streaks	Negative	n/a
89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4124	Maize	Streaks	Negative	n/a

89	27.5353575 424051°S	24.5101221 624787°E	Commercial	17-4115	Maize	Streaks	Negative	n/a
90	27.5239325 857121°S	24.5047954 360003°E	Commercial	17-4126	Maize	Streaks	Negative	n/a
90	27.5239325 857121°S	24.5047954 360003°E	Commercial	17-4127	Maize	Streaks	Negative	n/a
90	27.5239325 857121°S	24.5047954 360003°E	Commercial	17-4128	Maize	Streaks	Negative	n/a
90	27.5239325 857121°S	24.5047954 360003°E	Commercial	17-4125	Maize	Streaks	Negative	n/a
91	29.695366° S	24.251805° E	Commercial	Negative field	n/a	n/a	n/a	n/a
92	29.712066° S	24.280644° E	Commercial	Negative field	n/a	n/a	n/a	n/a
93	29.727570° S	24.305363° E	Commercial	Negative field	n/a	n/a	n/a	n/a
94	29.469657° S	24.038557° E	Commercial	Negative field	n/a	n/a	n/a	n/a
95	29.37157°S	24.019331° E	Commercial	Negative field	n/a	n/a	n/a	n/a
96	29.167917° S	23.928693° E	Commercial	Negative field	n/a	n/a	n/a	n/a
97	29.570039° S	24.090742° E	Commercial	Negative field	n/a	n/a	n/a	n/a
98	29.570039° S	24.833006° E	Commercial	17-4151	Maize	Streaks	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4152	Grass *(P. maximum)*	Streaks	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4153	Maize	Streaks on leaves, red midrib	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4154	Grass *(P. maximum)*	Streaks	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4155	Maize	Streaks	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4156	Maize	Streaks on leaves, red midrib	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4157	Grass *(P. maximum)*	Streaks	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4158	Grass *(P. maximum)*	Streaks	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4159	Maize	Streaks	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4160	Maize	Streaks	Negative	n/a
98	29.570039° S	24.833006° E	Commercial	17-4161	Maize	Streaks	Negative	n/a
98	29.570039°	24.833006°	Commercial	17-4162	Grass *(P.*	Streaks	Negative	n/a

	S	E			*maximum)*			
99	29.570039° S	24.78906°E	Commercial	17-4164	Maize	Streaks and yellowing	Negative	n/a
99	29.570039° S	24.78906°E	Commercial	17-4165	Maize	Streaks	Negative	n/a
99	29.570039° S	24.78906°E	Commercial	17-4166	Maize	Streaks	Negative	n/a
99	29.570039° S	24.78906°E	Commercial	17-4167	Maize	Streaks	Negative	n/a
99	29.570039° S	24.78906°E	Commercial	17-4168	Maize	Streaks	Negative	n/a
99	29.570039° S	24.78906°E	Commercial	17-4169	Maize	Streaks	Negative	n/a
99	29.570039° S	24.78906°E	Commercial	17-4163	Maize	Streaks	Negative	n/a
100	22.3050°S	30.4910°E	Subsistence	17-4679	Maize	Streaks/Blotches	Negative	n/a
100	22.3050°S	30.4910°E	Subsistence	17-4680	Maize	Streaks/Blotches	Negative	n/a
100	22.3050°S	30.4910°E	Subsistence	17-4689	Maize	Streaks/Blotches	Negative	n/a
100	22.3050°S	30.4910°E	Subsistence	17-4690	Maize	Streaks/Blotches	Negative	n/a
100	22.3050°S	30.4910°E	Subsistence	17-4691	Maize	Streaks/Blotches	Negative	n/a
100	22.3050°S	30.4910°E	Subsistence	17-4692	Maize	Streaks/Blotches	Negative	n/a
100	22.3050°S	30.4910°E	Subsistence	17-4693	Maize	Streaks/Blotches	Negative	n/a
100	22.3050°S	30.4910°E	Subsistence	17-4710	Maize	Streaks/Blotches	Negative	n/a
101	23.3387°S	30.7288°E	Subsistence	17-4732	Maize	Streaks/Blotches	Negative	n/a
101	23.3387°S	30.7288°E	Subsistence	17-4733	Maize	Streaks/Blotches	Negative	n/a
101	23.3387°S	30.7288°E	Subsistence	17-4734	Maize	Streaks/Blotches	Negative	n/a
101	23.3387°S	30.7288°E	Subsistence	17-4735	Maize	Streaks/Blotches	Negative	n/a
101	23.3387°S	30.7288°E	Subsistence	17-4736	Maize	Streaks/Blotches	Negative	n/a
101	23.3387°S	30.7288°E	Subsistence	17-4737	Maize	Streaks/Blotches	Negative	n/a
101	23.3387°S	30.7288°E	Subsistence	17-4738	Maize	Streaks/Blotches	Negative	n/a
102	22.7995°S	30.4925°E	Subsistence	17-4671	Maize	Streaks/Blotches	Negative	n/a
102	22.7995°S	30.4925°E	Subsistence	17-4675	Maize	Streaks/Blotch	Negative	n/a

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102	22.7995°S	30.4925°E	Subsistence	17-4676	Maize	Streaks/Blotches	Negative	n/a
102	22.7995°S	30.4925°E	Subsistence	17-4677	Grass *(P. maximum)*	Streaks/Blotches	Negative	n/a
102	22.7995°S	30.4925°E	Subsistence	17-4678	Maize	Streaks/Blotches	Negative	n/a
103	22.8032°S	30.4956°E	Subsistence	17-4694	Maize	Streaks/Blotches	Negative	n/a
103	22.8032°S	30.4956°E	Subsistence	17-4695	Maize	Streaks/Blotches	Negative	n/a
103	22.8032°S	30.4956°E	Subsistence	17-4696	Maize	Streaks/Blotches	Negative	n/a
103	22.8032°S	30.4956°E	Subsistence	17-4709	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4742	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4743	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4744	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4745	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4746	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4747	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4748	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4749	Maize	Streaks/Blotches	Negative	n/a
104	25.2712°S	31.1649°E	Subsistence	17-4750	Maize	Streaks/Blotches	Negative	n/a
105	22.3050°S	30.4910°E	Subsistence	17-4681	Maize	Streaks/Blotches	Negative	n/a
105	22.3050°S	30.4910°E	Subsistence	17-4682	Maize	Streaks/Blotches	Negative	n/a
105	22.3050°S	30.4910°E	Subsistence	17-4683	Maize	Streaks/Blotches	Negative	n/a
105	22.3050°S	30.4910°E	Subsistence	17-4684	Maize	Streaks/Blotches	Negative	n/a
105	22.3050°S	30.4910°E	Subsistence	17-4685	Maize	Streaks/Blotches	Negative	n/a
105	22.3050°S	30.4910°E	Subsistence	17-4699	Maize	Streaks/Blotches	Negative	n/a
106	23.3264°S	30.7193°E	Subsistence	17-4727	Maize	Streaks/Blotches	Negative	n/a
106	23.3264°S	30.7193°E	Subsistence	17-4728	Maize	Streaks/Blotch	Negative	n/a

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106	23.3264°S	30.7193°E	Subsistence	17-4757	Maize	Streaks/Blotches	Negative	n/a
106	23.3264°S	30.7193°E	Subsistence	17-4758	Maize	Streaks/Blotches	Negative	n/a
106	23.3264°S	30.7193°E	Subsistence	17-4759	Maize	Streaks/Blotches	Negative	n/a
107	25.2716°S	31.1649°E	Subsistence	17-4729	Maize	Streaks/Blotches	Negative	n/a
107	25.2716°S	31.1649°E	Subsistence	17-4730	Maize	Streaks/Blotches	Negative	n/a
107	25.2716°S	31.1649°E	Subsistence	17-4731	Maize	Streaks/Blotches	Negative	n/a
107	25.2716°S	31.1649°E	Subsistence	17-4739	Maize	Streaks/Blotches	Negative	n/a
107	25.2716°S	31.1649°E	Subsistence	17-4740	Maize	Streaks/Blotches	Negative	n/a
107	25.2716°S	31.1649°E	Subsistence	17-4741	Maize	Streaks/Blotches	Negative	n/a
108	25.2280°S	31.1628°E	Subsistence	17-4673	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4711	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4712	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4713	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4718	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4719	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4720	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4721	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4755	Maize	Streaks/Blotches	Negative	n/a
109	23.3150°S	30.7919°E	Subsistence	17-4756	Maize	Streaks/Blotches	Negative	n/a
110	22.7987°S	30.4842°E	Subsistence	17-4672	Maize	Streaks/Blotches	Negative	n/a
110	22.7987°S	30.4842°E	Subsistence	17-4674	Maize	Streaks/Blotches	Negative	n/a
110	22.7987°S	30.4842°E	Subsistence	17-4686	Maize	Streaks/Blotches	Negative	n/a
110	22.7987°S	30.4842°E	Subsistence	17-4687	Maize	Streaks/Blotches	Negative	n/a
110	22.7987°S	30.4842°E	Subsistence	17-4688	Maize	Streaks/Blotch	Negative	n/a

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111	23.3050°S	30.8089°E	Subsistence	17-4697	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4698	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4700	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4701	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4702	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4703	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4704	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4705	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4722	Maize	Streaks/Blotches	Negative	n/a
111	23.3050°S	30.8089°E	Subsistence	17-4723	Maize	Streaks/Blotches	Negative	n/a
112	23.3050°S	30.8103°E	Subsistence	17-4706	Maize	Streaks/Blotches	Negative	n/a
112	23.3050°S	30.8103°E	Subsistence	17-4707	Maize	Streaks/Blotches	Negative	n/a
112	23.3050°S	30.8103°E	Subsistence	17-4708	Maize	Streaks/Blotches	Negative	n/a
113	25.2249°S	30.0228°E	Subsistence	17-4666	Maize	Streaks/Blotches	Negative	n/a
114	25.1980°S	30.6484°E	Subsistence	17-4667	Maize	Streaks/Blotches	Negative	n/a
115	25.2121°S	30.0225°E	Subsistence	17-4668	Maize	Streaks/Blotches	Negative	n/a
115	25.2121°S	30.0225°E	Subsistence	17-4669	Maize	Streaks/Blotches	Negative	n/a
116	25.0233°S	31.2250°E	Subsistence	17-4716	Maize	Streaks/Blotches	Positive	
117	25.1972°S	30.0465°E	Subsistence	17-4670	Maize	Streaks/Blotches	Negative	n/a
118	25.2280°S	31.1628°E	Subsistence	17-4660	Maize	Streaks/Blotches	Positive	Sugarcane mosaic virus
118	25.2280°S	31.1628°E	Subsistence	17-4661	Maize	Streaks/Blotches	Positive	Sugarcane mosaic virus
118	25.2280°S	31.1628°E	Subsistence	17-4662	Maize	Streaks/Blotches	Positive	Sugarcane mosaic virus
118	25.2280°S	31.1628°E	Subsistence	17-4663	Maize	Streaks/Blotches	Positive	Sugarcane mosaic virus
118	25.2280°S	31.1628°E	Subsistence	17-4664	Maize	Streaks/Blotch	Positive	Sugarcane mosaic virus

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118	25.2280°S	31.1628°E	Subsistence	17-4665	Maize	Streaks/Blotches	Negative	n/a
118	25.2280°S	31.1628°E	Subsistence	17-4753	Maize	Streaks/Blotches	Negative	n/a
118	25.2280°S	31.1628°E	Subsistence	17-4754	Maize	Streaks/Blotches	Negative	n/a
119	25.0209°S	31.1958°E	Subsistence	17-4714	Maize	Streaks/Blotches	Negative	n/a
119	25.0209°S	31.1958°E	Subsistence	17-4715	Maize	Streaks/Blotches	Negative	n/a
119	25.0209°S	31.1958°E	Subsistence	17-4717	Maize	Streaks/Blotches	Negative	n/a
119	25.0209°S	31.1958°E	Subsistence	17-4724	Maize	Streaks/Blotches	Positive	Sugarcane mosaic virus
119	25.0209°S	31.1958°E	Subsistence	17-4725	Maize	Streaks/Blotches	Positive	Sugarcane mosaic virus
119	25.0209°S	31.1958°E	Subsistence	17-4726	Maize	Streaks/Blotches	Positive	Sugarcane mosaic virus
119	25.0209°S	31.1958°E	Subsistence	17-4751	Maize	Streaks/Blotches	Positive	Sugarcane mosaic virus
119	25.0209°S	31.1958°E	Subsistence	17-4752	Maize	Streaks/Blotches	Negative	n/a
120	24.2502°S	27.0604°E	Commercial	17-4600	Maize	Streaks	Negative	n/a
120	24.2502°S	27.0604°E	Commercial	17-4601	Maize	Streaks	Negative	n/a
120	24.2502°S	27.0604°E	Commercial	17-4602	Maize	Streaks	Negative	n/a
120	24.2502°S	27.0604°E	Commercial	17-4603	Maize	Streaks	Negative	n/a
121	24.1625°S	30.3981°E	Commercial	17-4610	Sweet corn	Blotchy mottle	Positive	Sugarcane mosaic virus
121	24.1625°S	30.3981°E	Commercial	17-4611	Sweet corn	Blotchy mottle	Positive	Sugarcane mosaic virus
121	24.1625°S	30.3981°E	Commercial	17-4612	Sweet corn	Blotchy mottle	Positive	Sugarcane mosaic virus
121	24.1625°S	30.3981°E	Commercial	17-4613	Sweet corn	Broad streak/mottle	Positive	Sugarcane mosaic virus
121	24.1625°S	30.3981°E	Commercial	17-4614	Sweet corn	Streaks	Positive	Sugarcane mosaic virus
121	24.1625°S	30.3981°E	Commercial	17-4615	Sweet corn	Mottle	Negative	n/a
121	24.1625°S	30.3981°E	Commercial	17-4616	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
121	24.1625°S	30.3981°E	Commercial	17-4617	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
121	24.1625°S	30.3981°E	Commercial	17-4618	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
122	24.1603°S	30.3982°E	Commercial	17-4619	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
122	24.1603°S	30.3982°E	Commercial	17-4620	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
122	24.1603°S	30.3982°E	Commercial	17-4621	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
122	24.1603°S	30.3982°E	Commercial	17-4622	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
122	24.1603°S	30.3982°E	Commercial	17-4623	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
122	24.1603°S	30.3982°E	Commercial	17-4624	Sweet corn	Mottle	Positive	Sugarcane mosaic virus

122	24.1603°S	30.3982°E	Commercial	17-4625	Sweet corn	Mottle	Negative	n/a
122	24.1603°S	30.3982°E	Commercial	17-4626	Sweet corn	Mottle	Negative	n/a
122	24.1603°S	30.3982°E	Commercial	17-4627	Sweet corn	Mottle	Negative	n/a
122	24.1603°S	30.3982°E	Commercial	17-4629	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
123	24.157308° S	30.397082° E	Commercial	17-4628	Sweet corn	Mottle	Negative	n/a
123	24.157308° S	30.397082° E	Commercial	17-4630	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
123	24.157308° S	30.397082° E	Commercial	17-4631	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
123	24.157308° S	30.397082° E	Commercial	17-4632	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
123	24.157308° S	30.397082° E	Commercial	17-4633	Sweet corn	Mottle	Negative	n/a
123	24.157308° S	30.397082° E	Commercial	17-4634	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
123	24.157308° S	30.397082° E	Commercial	17-4635	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
123	24.157308° S	30.397082° E	Commercial	17-4636	Sweet corn	Mottle/streak	Negative	n/a
123	24.157308° S	30.397082° E	Commercial	17-4637	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
123	24.157308° S	30.397082° E	Commercial	17-4638	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4639	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4640	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4641	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4642	Sweet corn	Mottle/streak	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4643	Sweet corn	Mottle	Negative	n/a
124	24.161067° S	30.403777° E	Commercial	17-4644	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4645	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4646	Sweet corn	Mottle/streak	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4647	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
124	24.161067° S	30.403777° E	Commercial	17-4648	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
125	24.1000°S	30.3833°E	Commercial	17-4649	Maize	Streaks	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4650	Maize	Streaks	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4651	Maize	None	Negative	n/a

125	24.1000°S	30.3833°E	Commercial	17-4652	Maize	None	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4653	Maize	Streaks	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4654	Maize	Streaks	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4655	Maize	Streaks	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4656	Maize	Streak/blotchy mosaic	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4657	Maize	Fine streak	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4658	Maize	Fine streak/bottle	Negative	n/a
125	24.1000°S	30.3833°E	Commercial	17-4659	Maize	Streak/blotchy mosaic	Negative	n/a
126	29.0553°S	29.4134°E	Commercial	17-4782	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4760	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4761	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4762	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4763	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4764	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4765	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4766	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4767	Maize	Faint mottle	Positive	Johnsongrass mosaic virus
127	24.453421° S	28.181062° E	Commercial	17-4768	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4769	Maize	Faint mottle	Positive	Sugarcane mosaic virus
127	24.453421° S	28.181062° E	Commercial	17-4770	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4771	Maize	Faint mottle	Negative	n/a
127	24.453421° S	28.181062° E	Commercial	17-4772	Maize	Faint mottle	Negative	n/a
128	29.0809°S	30.3739°E	Commercial	17-4783	Maize	Faint mottle	Negative	n/a
129	24.168233° S	30.397082° E	Commercial	17-4773	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
129	24.168233° S	30.397082° E	Commercial	17-4774	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
129	24.168233° S	30.397082° E	Commercial	17-4775	Sweet corn	Mottle	Positive	Sugarcane mosaic virus
129	24.168233° S	30.397082° E	Commercial	17-4776	Sweet corn	Streaks	Positive	Sugarcane mosaic virus

129	24.168233° S	30.397082° E	Commercial	17-4777	Sweet corn	Streaks	Negative	n/a
129	24.168233° S	30.397082° E	Commercial	17-4778	Sweet corn	Streaks	Negative	n/a
129	24.168233° S	30.397082° E	Commercial	17-4779	Sweet corn	Blotchy	Negative	n/a
129	24.168233° S	30.397082° E	Commercial	17-4780	Sweet corn	Fine mottle	Positive	Sugarcane mosaic virus
129	24.168233° S	30.397082° E	Commercial	17-4781	Sweet corn	Fine mottle	Positive	Sugarcane mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4170	Maize	Streaks	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4171	Maize	Streaks	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4172	Maize	Streaks/Yellowing	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4173	Maize	Streaks	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4174	Maize	Streaks	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4175	Maize	Blotchy streaks	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4176	Maize	Streak	Positive	Johnsongrass mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4177	Maize	Streaks	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4178	Maize	Concentric ringspots	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4179	Maize	Faint mottle	Negative	n/a
130	25.750349° S	28.260126° E	Experimental farm	17-4180	Maize	Blotchy	Positive	Sugarcane mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4181	Maize	Blotchy	Positive	Sugarcane mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4182	Maize	Blotchy	Positive	Sugarcane mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4183	Maize	Blotchy	Positive	Sugarcane mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4184	Maize	Mottle	Positive	Sugarcane mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4185	Maize	Blotchy	Positive	Sugarcane mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4186	Maize	Concentric ringspots	Positive	Sugarcane mosaic virus
130	25.750349° S	28.260126° E	Experimental farm	17-4187	Maize	Concentric ringspots	Positive	Sugarcane mosaic virus

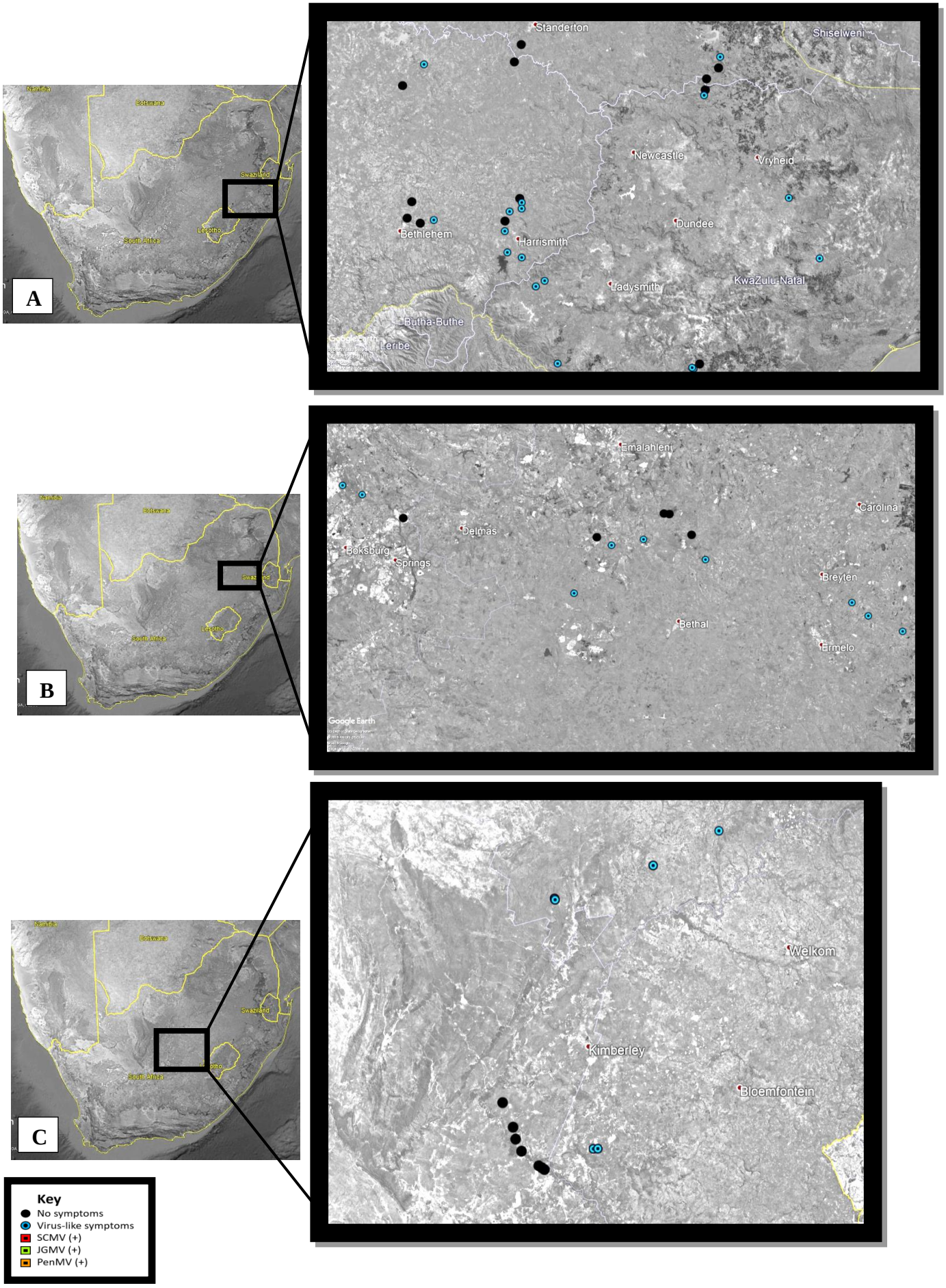

Figure 2.6 Regions of South Africa where potyviruses were not detected. A. northern and western Kwa-Zulu Natal, northern Free State and southern Mpumalanga area. B. central and western Mpumalanga. C. eastern Northern Cape.

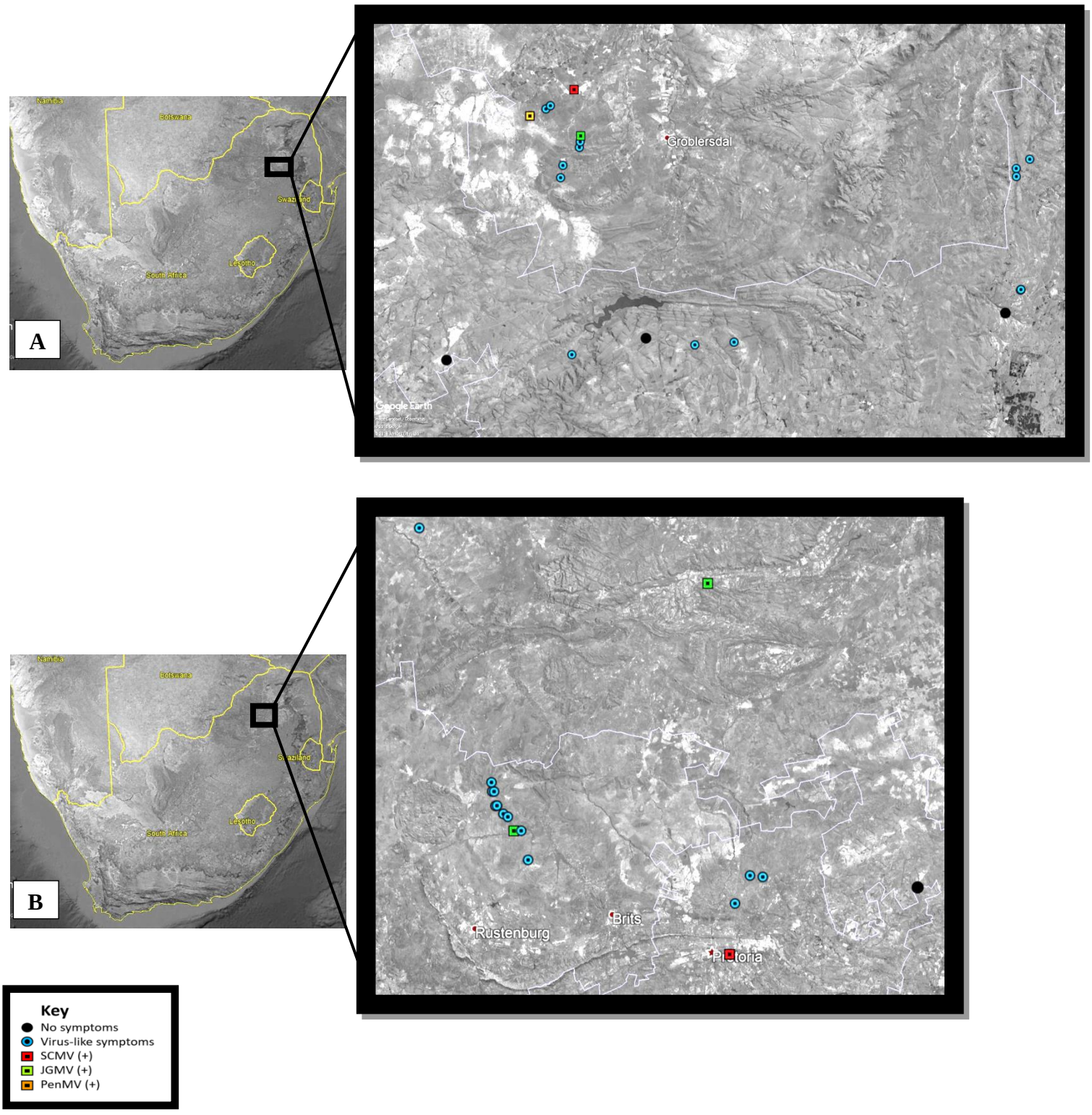

Figure 2.7 North-western regions of South Africa where potyviruses were detected. A. Groblersdal and Loskop area. B. Gauteng and southern North-West Province.

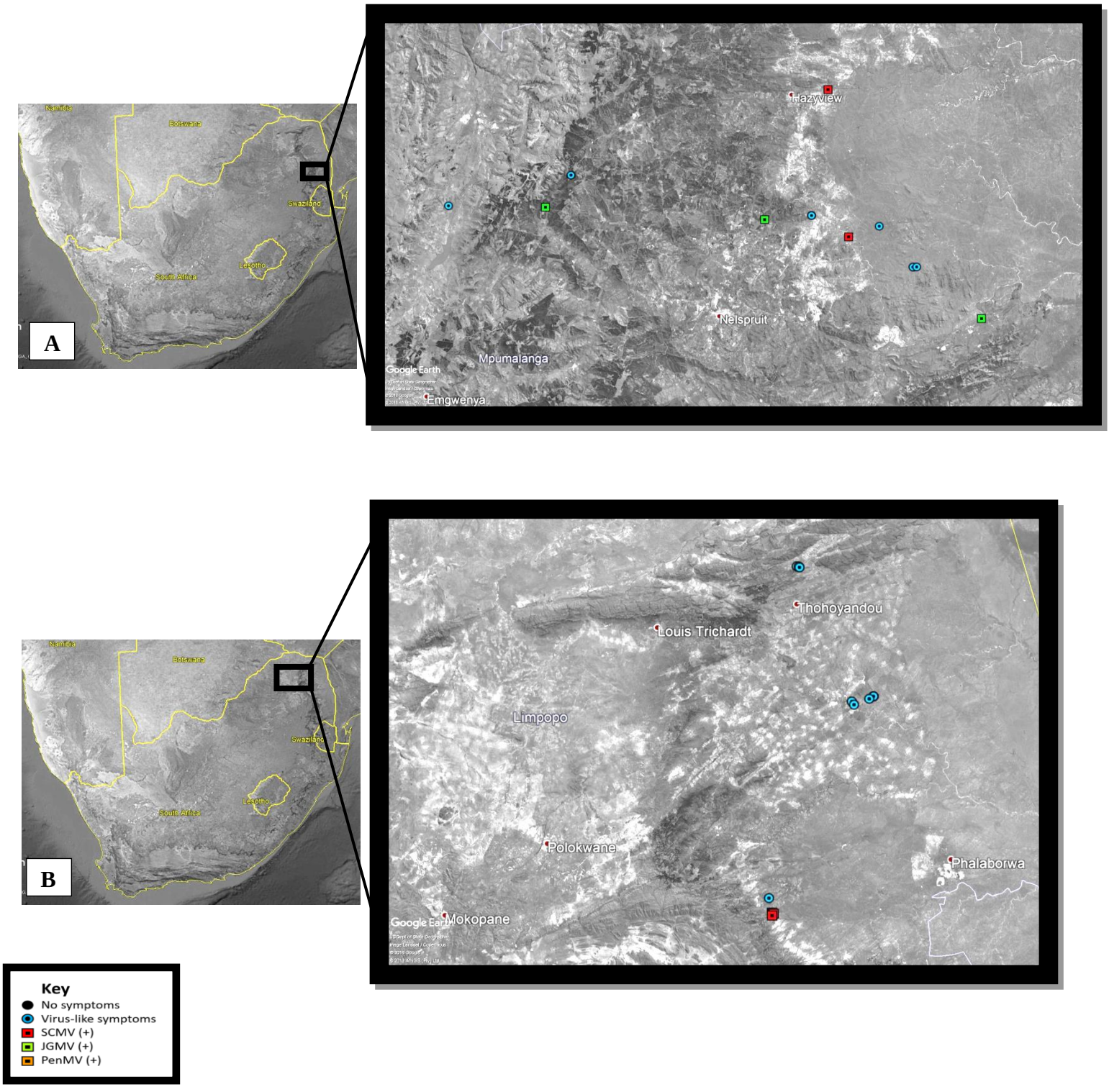

Figure 2.8 North-eastern regions of South Africa where potyviruses were detected. A. Lowveld area: northern and eastern Mpumalanga. B. Limpopo Province.

Appendix B

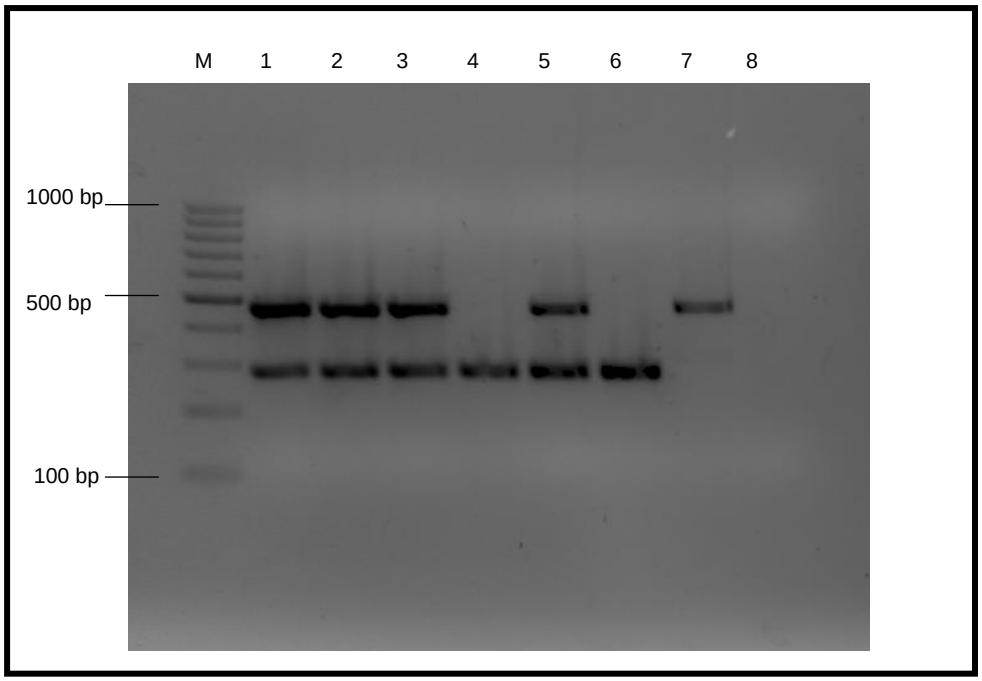

Figure 4.1 Example of how the optimal primer concentration for each pair was established. Optimal MCMV primer concentration establishment to 10µM SCMV primer. Lane M: 100 bp DNA ladder; Lane 1-5: 10µM SCMV-specific primers with 10, 8, 6, 2 and 4µM MCMV-specific primers, respectively; Lane 6: SCMV positive control; Lane 7: MCMV positive control; Lane 8: negative PCR control.

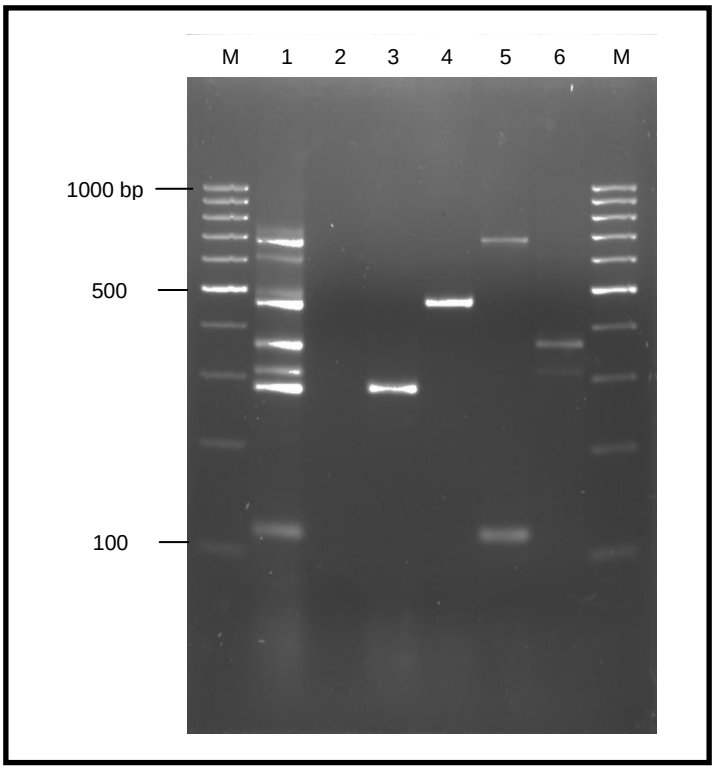

Figure 4.2 Electrophoresis of DNA amplified products from total RNA obtained from 4 symptomatic maize samples by uniplex and multiplex Polymerase Chain Reaction. Lane M: 100 bp DNA size marker; Lane 1: multiplex PCR with mixed viral cDNAs using four primer pairs specific to MSV, SCMV, MCMV and JGMV; Lane 2: no template control; Lanes 3-6: uniplex PCR using primers specific to SCMV, MCMV, JGMV and MSV, respectively.

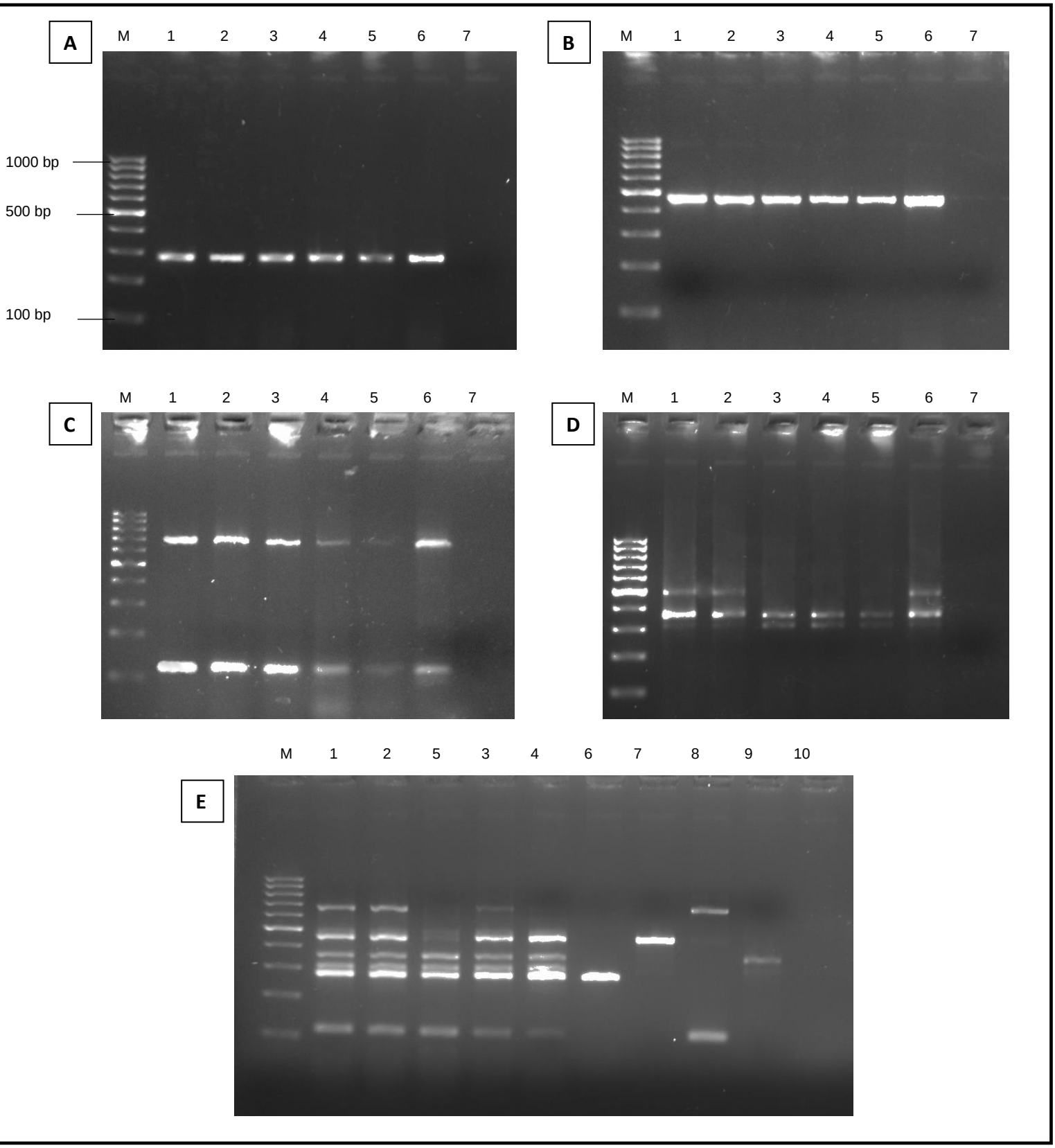

Figure 4.3 Gel images showing establishment of detection limits of primers used in uniplex and multiplex Polymerase Chain Reaction. **A.** SCMV detection limit. **B.** MCMV detection limit. **C**. JGMV detection limit. **D**. MSV detection limit. Images A-D: Lane M, 100 bp DNA size marker; Lane 1- 5: amplicon concentrations of 15.811fg/µl, 5fg/µl, 1.581fg/µl, 0.5fg/µl and 0.158fg/µl, respectively; Lane 6, 50ng/µl amplicon as positive control; Lane 7, no template control. **E.** Detection limit of all 4 primers in multiplex. Lane M, 100 bp DNA size marker; Lanes 1- 5: amplicon concentrations of 15.811fg/µl, 5fg/µl, 1.581fg/µl, 0.5fg/µl and 0.158fg/µl, respectively; Lanes 6-9:

SCMV, MCMV, KGMV and MSV positive controls; Lane 10, 50ng/µl amplicon as positive control; Lane 7, no template control.

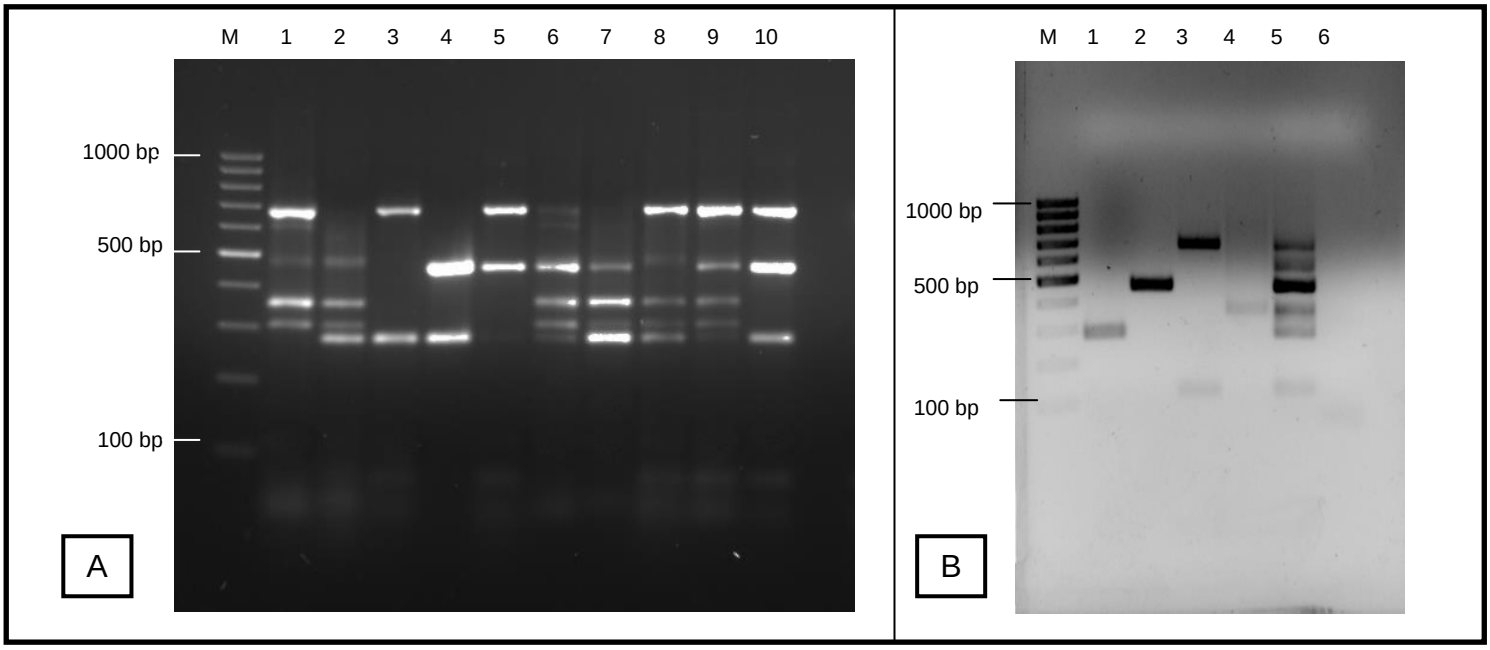

Figure 4.4 Gel electrophoresis of PCR products obtained from mixed cDNA and DNA samples (A) with the controls used, electrophoresed on a separate gel (B). A: Lane M, 100 bp marker. Lane 1, Product of RT-PCR reaction containing: JGMV RNA & MSV DNA; Lane 2, SCMV RNA with MSV DNA; Lane 3, SCMV and JGMV RNAs; Lane 4, MCMV and SCMV RNAs; Lane 5, JGMV and MCMV RNAs; Lane 6, MCMV and SCMV RNAs, MSV DNA; Lane 7, MCMV and SCMV RNA & MSV DNA; Lane 8, JGMV and SCMV RNA, MSV DNA; Lane 9, JGMV, SCMV and MCMV RNAs and MSV DNA; Lane 10, JGMV, MCMV and SCMV RNAs. B: Lane M, 100 bp marker. Lane 1, SCMV positive control; Lane 2, MCMV positive control; Lane 3, JGMV positive control; Lane 4, MSV positive control; Lane 5, amplicon control for all 4 viruses in one reaction (multiplex PCR positive control); Lane 6, no template control.

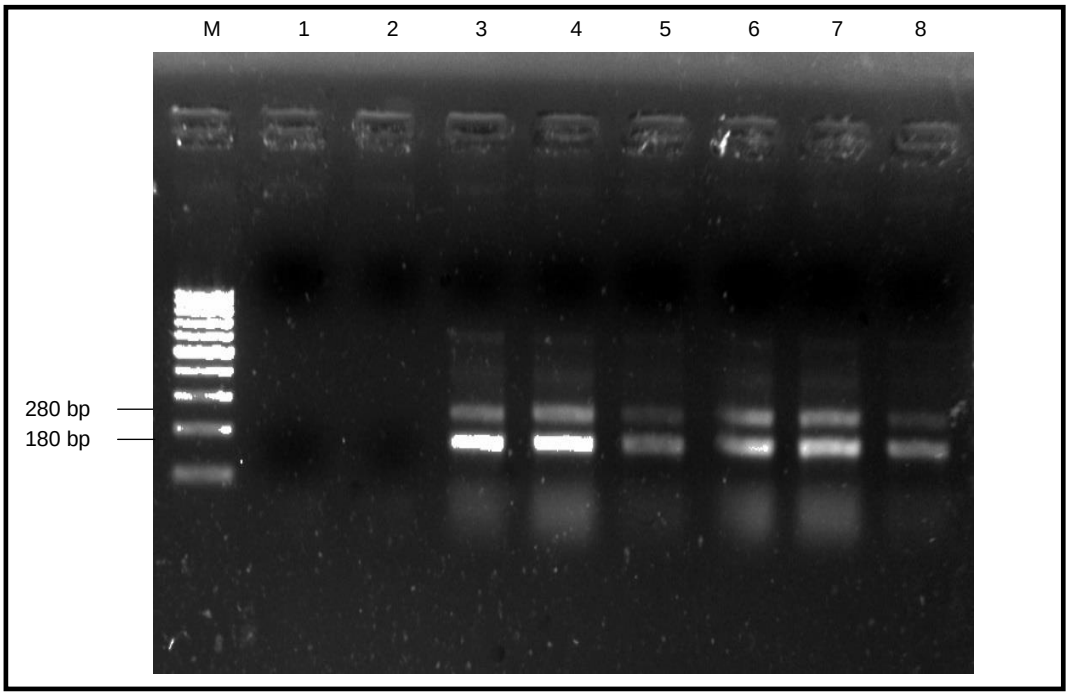

Figure 4.5 Representative gel image of confirmation of RNA integrity. Lane M, 100 bp size marker; Lane 1, RT no template control; Lane 2, PCR no template control; Lane 3, maize healthy control/RNA positive control; Lanes 4-8, samples that tested negative for four viruses tested for in the multiplex RT-PCR system.