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EPIDEMIOLOGY OF TOMATO CURLY STUNT VIRUS AND IT VECTOR IN MOZAMBIQUE

By:

VALTER NUNO ANTÓNIO NUAILA

Dissertation submitted in partial fulfillment for the degree of Master of Science in

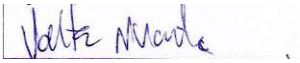
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DECLARATION

I hereby declare that this dissertation is my work and has not been presented for a degree in any other University.

Signature:  Date: 26/09/2013

Valter Nuaila

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LIST OF ABREVIATIONS

ºC – degree Celsius

ACMV -*African cassava mozaic virus*

Ann. - Annals

ANOVA – analysis of variance

Appl. - Applied

Arch. - Archive

Biochem. - Biochemistry

Chem. - Chemistry

CMD - Cassava mosaic disease

CP – coat protein

DAE – Day after emergence

DI – disease incidence

dsDNA – double strand DNA

EACMV – *East African cassava mosaic virus*

EACMV-UG - *East African cassava mosaic virus* - Uganda

ELISA – enzyme linked imunosorbent assay

FAO - Food and Agriculture Organization

FAOSTAT - Food and Agriculture Organization Statistics

FL – Full length

G - group

Gen. – General

ICTV – international community for taxonomy of viruses

IR – intergenic region

min - minute

mtCO1 – Mitochondrial cytochrome oxidase I

nt – nucleotide

NW – New World

ORF – open reading frame

OW – Old World

PCR – Polymerase chain reaction

PpYVV – *Pepper yellow vein virus*

PYMV – *Potato Y mosaic virus*

RAPD – random amplified polymorphism DNA

RCA – Rolling Circle Amplification

RDR – Recombination dependent replication

RCR – Rolling Circle Replication

RFLP – Restriction fragment length polymorphism

SA – South Africa

SCR – satellite conserved region

SI – Severity indices

ssDNA – single strand DNA

TbLCZV – *Tobacco leaf curl Zimbabwe virus*

TNA – Total Nucleic Acids

ToCSD - Tomato Curly Stunt Disease

ToCSV – *Tomato curly stunt virus*

ToLCV – *Tomato leaf curl virus*

TYLCV – *Tomato yellow leaf curl virus*

USA – United States of America

USDA – United States Department of Agriculture

UV – ultra violet

WF – Whitefly

YL – Yield losses

ABSTRACT

Tomato (*Solanum lycopersicum* (L)) is one of the most important horticultural crop grown in Mozambique. The emergence of Tomato Curly Stunt Diseases (ToCSD) is limiting the production of this crop. ToCSD, caused by monopartite begomoviruses *Geminiviridae* family) are worldwide known as important constrain in the tropic and subtropics regions and a broad understanding on the features related with virus, such as the interaction between the virus-vector and host, are key to develop a reliable techniques for diagnostic and management of the disease.

In 2005, a ToCSD was reported for the first time in Moamba district, in Maputo, a southern province of Mozambique, a region bordering the province of Mpumalanga in South Africa, where a ToCSD was previously detected in 1997. In 2006, the disease spread to the district of Chókwè, in Gaza province, one of the major tomato growing areas in the southern region of Mozambique, where it reached epidemic proportions. In both years (2005-2006), surveys conducted in those areas revealed 80 to 100% of incidence.

Thus, aiming to contribute to understand the epidemiology and possible diversity of ToCSD and it vector, *Bemisia tabaci*, in Mozambique, this study was conducted. To fulfill the scope of the study, surveys were conducted in 2 seasons (hot and cold), 6 provinces and 8 districts in which data of incidence and severity of ToCSD as well as whiteflies number and sample collection were performed. The surveys and PCR based screening revealed that in 6 out of 8 districts ToCSD was present, with high intensity (incidence and severity) in the south region of Mozambique.

Suspected samples that tested negative to PCR either using specific ToCSV primers or universal begomovirus primers were used in Rolling Circle Amplification (RCA) followed by restriction digest and the banding pattern suggested the possibility of ToCSD diversity. To confirm the suspicions, core coat protein amplicons and full length amplicons were sequenced and analyzed and at least three new viruses species were detected.

B. tabaci is currently considered a cryptic species complex due to the large number of biotypes worldwide that are found associated with begomoviruses. Thus amplicons of the *mtCO I* gene were digested to discriminate diversity and representative samples were sequenced and the results from this study showed that Q type and other types (non B and non Q) were present in the surveyed areas. This genetic variability of whitefly in Mozambique reinforces the complexity of this cryptic whitefly species collection.

Several management strategies have to be tested to find the better combination and avoid excessive usage of pesticides, and consequently, a trial was performedin order to find the critical period for infection with ToCSD under field conditions. The results showed that the period from 30 – 60 Days After Emergence (DAE) was the critical for the infection with ToCSV, because high disease incidence, severity and Yield losses were observed in this period.

This research has shed some light on ToCSD, but several questions derived from this study need to be pursued in order to improve tomato yields in Mozambique.

RATIONALE FOR STUDY

Background

Tomato (*Solanum lycopersicum* (L). belongs to the family *Solanaceae*, and originates from South America (Peru, Bolivia and Equator), and then was domesticated in Mexico. In the middle of the 16th Century, it was introduced into Europe, from which the plant was disseminated to southern and Oriental Asia, Africa and Middle East (Ribeiro and Rulkens, 1999; AVRDC, 2010).

Tomato is one of the most popular vegetables grown in the home garden, as well as commercially in tunnels and field. It is available in a variety of sizes, shapes, and colors—including red, yellow, orange, and pink. Sizes vary from the bite-sized cherry tomatoes to the giant beefsteak varieties. Tomato fruits may be round, oblate (flattened at the top and bottom), or pear-shaped, and constitute an important source of nutrients, with appreciable quantities of β – carotene, which is converted into vitamin A and C in the human body, and also contains potassium, magnesium, phosphorus and calcium (Varela *et al.,* 2003) .

Tomato plants grow better at temperatures of 20–27°C. Fruit setting is poor when average temperatures exceed 30°C or fall below 10°C, and the plant prefers a well-drained soil because it is sensitive to water logging. The optimal soil pH for tomato is 6.0 to 7.0; disorders such as blossom end rot are common if soil pH is lower than 5.5 (Naika *et al.,* 2006).

Tomato has become one of the most important horticultural crops widely grown in the world. In 2005, the world production was nearly 137 million of tons of fresh fruits, in an estimated area of

about 4.84 millions of hectares, with China being the major producer, with 25.28% of the total production. Egypt was the major African country producing tomato and the 7th in the world with nearly 3 % of total production (FAO 2008). Actually the world production is decreasing nevertheless, Asia and Africa account for about 79% of the global tomato area with about 69% of world output (AVRDC, 2010).

In Mozambique, tomato is grown by small-scale farmers, mainly for household consumption and as income source for commercial farmers. It is grown throughout the country, but mostly in the provinces of Maputo and Gaza (in the southern part of Mozambique), Manica, Tete and Zambezia (in the center) and Niassa (in the north) (Varela *et. al.*, 2003; Dobson et al., 2002).

In 2003, the cultivated area of tomato in Mozambique, was approximately 2000 ha, with an average yield of 9 ton/ha. This value was lower if compared with the tomato production of the neighboring countries, namely South Africa (SA), Swaziland, Zambia and Zimbabwe, that achieve yields around 100 ton/ha.. In 2008, the situation became worse in Mozambique, with the area of tomato production reduced by half (1000 ha), and the yields also reduced to 8.5 ton/ha (FAOSTAT, 2008).

Besides the abiotic and growing practices constrains, in the tropics, tomato production is severally reduced by devastating pests and diseases. The major pests are fruit borer, common armyworm, beet armyworm, whiteflies, leaf miner and spider mites. The major pathogen diseases are whitefly-transmitted geminiviruses, bacterial spot, bacterial wilt, fungal damping

off, early and late bright, fusarium wilt, southern bright and black leaf mold. Pests and diseases contribute to 30-50% of the tomato production losses (Ribeiro *et al.*, 1996; AVRDC, 2010).

In the last two decades, the *Geminiviridae* virus family, especially the begomovirus genus, has become one of the main groups of viruses infecting tomato worldwide (Mubin *et al*, 2010).

Rationale for study

Tomato is one of the most highly produced crops in Mozambique, grown by commercial, small-scale and subsistence farmers. In 1998, a new emerging virus devastated extensive areas of tomato production, first in (SA) , and later in the southern regions of Mozambique, initially in the district of Moamba bordering with SA, where afterwards it spread quickly to Chókwé, one of the most important tomato growing area in Mozambique (unpublished data). Further characterization of this virus revealed that it was a new monopartite begomovirus species, transmitted by *Bemisia tabaci* (Gennadius), and was named as *Tomato curly stunt virus* (ToCSV) (Pietersen and Smith 2002). Later in an updated taxonomy (Fauquet *et al*., 2008) the nomenclature changed to *Tomato curly stunt virus*- [South Africa: Onderberg:1998] (ToCSV-[ZA:Ond:98]). For the purpose of this dissertation, the name ToCSV will be used.

The occurrence of Tomato leaf curl disease (ToCSD) in tomato-growing areas reduces the yield and nutritional advantages provided by this crop, and has a negative impact on the survival of the farmers, grocers and their dependents (families). Since 2005, the first time ToCSD was reported by the farmers to the agricultural officers in Mozambique (Moamba district), the virus, suspected to be ToCSV, has become a serious constraint to the production of tomato.

Symptoms of the disease were similar to those reported in South Africa (SA), and the identity of the virus in Mozambique was confirmed by PCR to be ToCSV (unpublished). However, since the first detection of ToCSV in SA and Mozambique, it is now known that there is more than one begomovirus species, and several variants of ToCSV, are associated with curly stunt disease in SA (Esterhuizen, pers. comm.). Therefore, it is important to provide a reliable method to both identify and detect, as early as possible, the presence of ToCSV or other viruses, in order to design an efficient plan of control and mitigate the negative social impact that are caused by this devastating disease.

The diagnosis of ToCSV in Mozambique has been based on the symptoms observed in the fields. Studies conducted have shown that this method is not accurate and sometimes it is possible to confuse the symptoms, because other non-related viruses can induce similar symptoms. So in order to improve this situation, symptom analysis must be complemented by molecular detection to confirm the presence of ToCSV in these areas. Furthermore, it can no longer be presumed that the symptom-inducing virus is ToCSV based on the recent tomato begomovirus diversity findings in SA, and consequently a more comprehensive study on these viruses is required. Should more than one virus be detected, a simple and rapid diagnostic method to distinguish between these viruses needs to be developed and implemented.

The information reported in Mozambique to date, based on personal observation, is that the vector, *B. tabaci*, that transmits ToCSV, is only restricted to the southern region (Maputo and Gaza provinces). However, tomato is not only grown in this region and it is known that *B. tabaci* is one of the most widely spread and adaptive vector of begomoviruses. It is therefore

important to understand the epidemiology (incidence and severity) of ToCSV and *B. tabaci* in other regions growing tomato in Mozambique. While it is known that type B was the main genetic whitefly type transmitting tomato begomoviruses in SA (Pietersen *et al.,* 2002, 2008), it has been known for decades as being an invasive type that spread globally on many diverse crops (Dinsdale *et al.*, 2010). In addition to the B type, another invasive type (Q), part of the *B. tabaci* cryptic species complex, has spread into many crops, including tomato, and was first reported in the Mediterranean region and was restricted to this region (Ghanim *et al.,* 2007*).* Recent findings have revealed that the Q type is spreading in regions already invaded by the B-type, in some locations, resulted in the rapid displacement of B by Q (Chu *et al.,* 2010a, b; Luo *et al.*, 2010). In many regions of the world, epidemics of plant diseases caused by begomoviruses transmitted by *B. tabaci* occurred soon after the invasion of the B and Q types (Hogenhout *et al.,* 2008). Therefore one cannot assume that it is only the B type spreading the disease in Mozambique, thus, molecular identification of the whitefly vector (s) transmitting tomato-infecting begomoviruses in Mozambique needed to be performed.

Based on the background and rationale described above, the general objective of this study was to learn more about Tomato curly stunt disease and it vector, in Mozambique, using molecular biology tools, so that the information can be disseminated to agricultural officers, farmers and other stakeholders, and that certain strategies can be developed in order to reduce the impact of this disease in the agricultural sector.

General objective

- ✓ Study the epidemiology and genetic diversity of ToCSV, and it whitefly vector, in Mozambique.

Specifics aims

- ✓ Determine the distribution, incidence and severity of ToCSD in selected provinces in Mozambique;
- ✓ Establish a reliable molecular procedure for the diagnosis of ToCVs in Mozambique;
- ✓ Identify possible ToCSV and *Bemisia tabaci* genetic diversity in Mozambique;
- ✓ Determine the critical time of infection with ToCSV under field conditions.

CHAPTER 1

LITERATURE REVIEW

1.1. The Family *Geminiviridae*

The *Geminiviridae* family is one of the largest families of plant viruses, with 209 definite and tentative members. All members of the family have circular single-stranded DNA genome that are approximately 2.7 kb in length and encapsulated within twined (geminate) icosahedral particles (figure 1). Geminiviruses can either be monopartite or bipartite if their genomes contains one or two DNA molecules respectively known as DNA A and/or DNA-B (Stanley *et al*., 2005*;* Fauquet *et al.*, 2008).

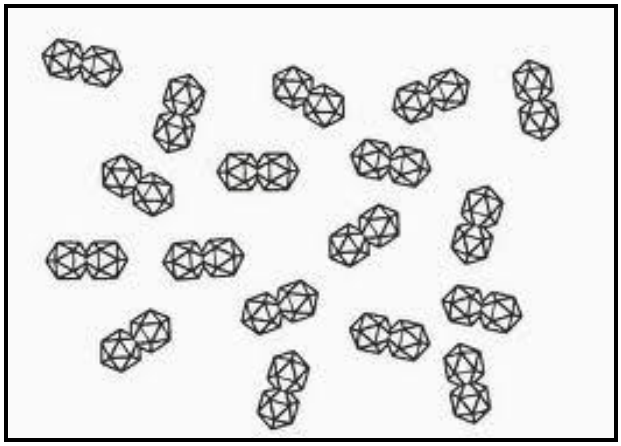


Figure 1. Geminiviruses particles

Based on the genome arrangement, biological properties, insect vector, and host range, geminiviruses are divided into four genera *Mastrevirus, Curtovirus, Topocuvirus* and *Begomovirus* (figure 2). The genus *Begomovirus* is increasingly important with about 185 species, including tentative and definitive members (Stanley *et al.,* 2005; Pandey *et al., 2010*; Mubin *et al., 2010*). Their genetic diversity and the geographical distribution, were used to

classify the geminiviruses into two groups, the Old World (OW- Europe, Africa, Asia and Australia) and the New World (NW – America) (Shah *et al.,* 2009).

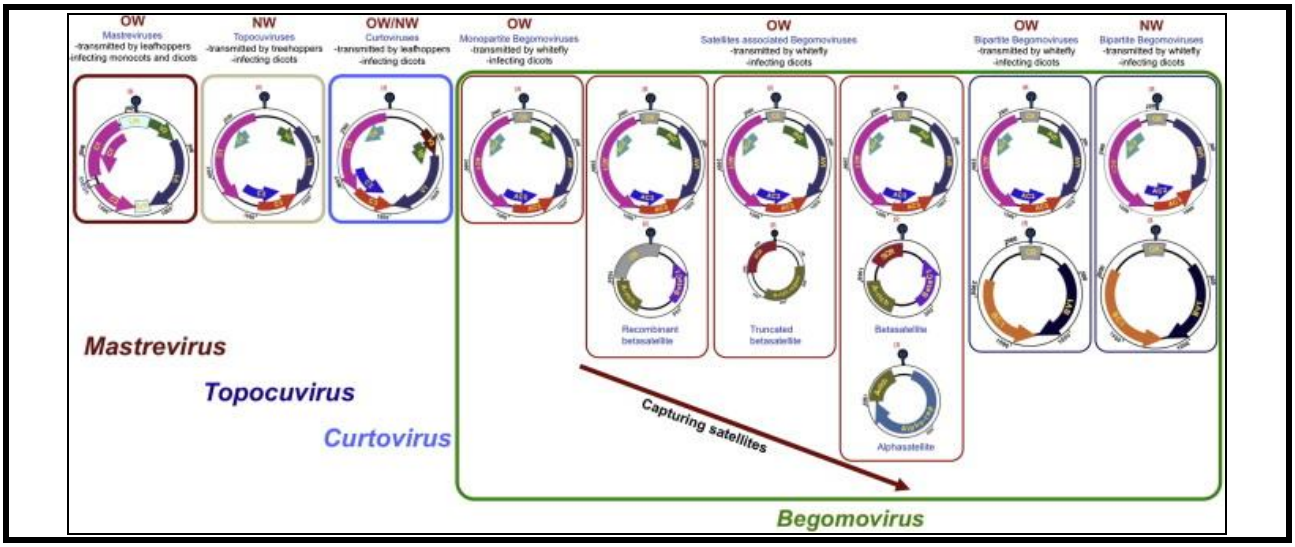


Figure 2. Genomic organization of geminiviruses and their satellites (Shah *et al*., 2009)

1.2 Genus *Begomovirus*

1.2.1 Genomic organization and viral encoded proteins

Begomoviruses are mostly bipartite, but some Old World begomoviruses are monopartite. Bipartite begomoviruses have two ssDNA components, designated A and B. Each component has ~2,600 nucleotides (nt). The genes on the DNA-A component are involved in encapsidation and replication, whereas the genes on the DNA-B component are involved in the movement of virus through the plant, host range, and symptom expression (figure 2) (Gafni and Epel, 2002; Prasama and Rai, 2007; Pandey *et al., 2010*).

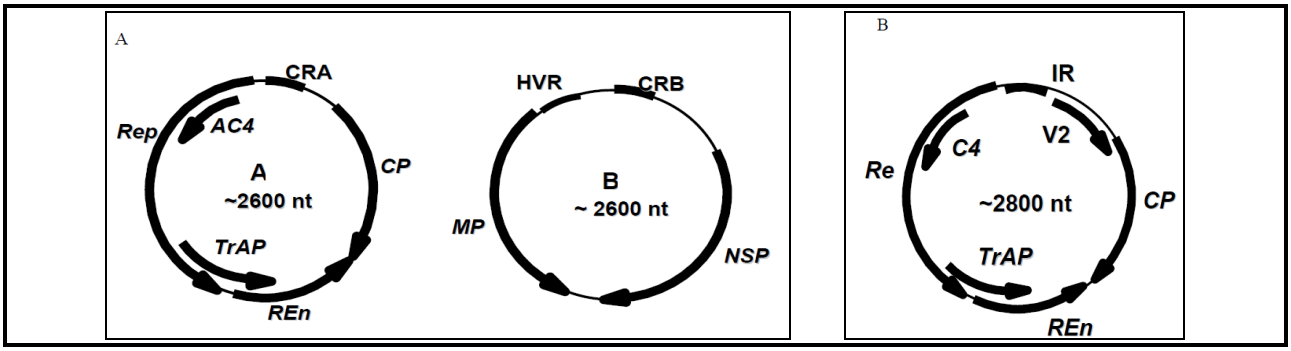


Figure 3. Genome structure of begomoviruses. A) Bipartite begomovirus. B) Monopartite begomoviruses, IR = Intergenic region, CRA = Common region A, CRB = Common region B, CP = Coat protein, TrAP = Transcription activator protein, REn = Replication-enhancer protein, AC4 = AC4 protein, Rep = Replication- associated protein, C4 = Symptoms expression (SE), V2 = Movement protein MP = Movement protein, NSP = Nuclear- shuttle protein (Nava 2003).

One of the five genes on the DNA-A component, the coat protein (*cp*) gene, is transcribed in the viral sense or clockwise direction. The other four genes (*ac1-4*), encoding replication-associated protein (Rep), transcriptional activator protein (TrAP), replication enhancer (REn) and RNA silencing suppressor, respectively, are transcribed in complementary sense or counterclockwise direction (Lazarowitz, 1992; Stanley, 2005).

The two sets of genes overlap and are separated by an intergenic region (IR), which begins with the start codon of the Rep and ends with the start codon of the CP. This region does not encode any protein and its sequence varies widely among begomoviruses, except that there is a conserved GC-rich inverted repeat sequence, which has the potential to form a stem-loop structure (~30 nt) with the invariant nanomeric TAATATTAC sequence or loop of the stem-loop structure. The nanomeric sequence contains the initiation site of rolling circle DNA replication (Gutierrez, 2000; Laufs *et al.*, 1995a), while the IR region also contains the TATA box, and the forward and inverted repeats which is are binding motifs of the rep associated protein. In

bipartite begomoviruses, the IR also contains an identical sequence of ~200 nt in the A and the B components called the common region (CR). The CR sequence is different among different begomoviruses and is used to identify the A and B components of the same virus (Lazarowitz, 1992, Castillo *et al.*, 2004).

The CP is required for encapsidation of progeny virions, vector transmission, virion structure, and host specificity. For bipartite begomoviruses, the CP is not required for either local or systemic viral spread. In contrast, in all monopartite begomoviruses, the CP is essential for viral spread (Gafni and Epel, 2002). The Rep is the only gene essential for replication, being required for transcription of both A and B components (Argüello-Astorga *et al*. 1994). Begomoviruses replicate in the nucleus of infected cells through a double-stranded DNA intermediate via a rolling circle mechanism. This motif functions as a major recognition element of the replication origin in begomoviruses. These cis-acting elements belong to a series of iterate DNA motifs called iterons (Argüello-Astorga and Ruiz-Medrano, 2001).

A functional organization of the replication origin in begomoviruses has been hypothesized. The Rep protein binds to the iterons associated with the TATA box, where a TATA-binding protein was previously bound. A transcription factor binds to a cis-regulatory element, associated with the 5′ border of the stem-loop sequence, and creates a nucleosome-free region in its neighborhood. The transcriptional factor interacts with the TATA binding protein, by means of its activation domain, looping the intervening DNA. This event would place the stem-loop structure in an accessible position so that the Rep complex can nick the viral (+) strand in the loop of the hairpin structure. The stem-loop structure may acquire a cruciform structure as a

consequence of the interactions with the transcription factor or/and Rep (Argüello-Astorga *et al.*, 1994).

TrAP is a transactivator of the expression of the CP and the nuclear shuttle protein (NSP) genes (Sunter *et al*. 1990; Sunter and Bisaro, 1991; Gutierrez, 2000). TrAP along with the two proteins encoded by the B component are indirectly involved in the systemic movement of the virus through the plant (Gafni and Epel, 2002). The REn protein is not essential for viral replication. However, viral DNA replicates at higher levels when REn is present (Sunter *et al*., 1990). The AC4 gene is involved in symptom expression of monopartite begomoviruses and is a strong suppressor of host RNA silencing, an innate immunity mechanism (Gutierrez *et al.*, 2004; Shah *et al.*, 2009).

The DNA-B component has two genes: the *nsp* gene, which is transcribed from the viral-sense strand, and a movement protein (MP), which is transcribed from the complementary-sense strand. The NSP is implicated in nuclear shuttling of the viral genome, and MP is involved in cell-to-cell movement of the virus via plasmodesmata (Gafni and Epel, 2002). The MP appears to be a symptom-inducing element or a determinant of pathogenicity of bipartite begomoviruses. Mutation studies suggest that the 3' region of the MP gene is associated with symptom development (Gafni and Epel, 2002).

Bipartite begomoviruses often spontaneously produce approximately half-sized defective DNA B components that function as defective interfering (DI) DNA. The DI DNA may have a

biological role during infection to reduce the severity of the disease by competing with the genomic components for cellular resources (Stanley *et al*., 1990, 2005).

1.2.2 Replication

Genomic DNA replication of begomoviruses follows a rolling circle mechanism, known as rolling circle replication (RCR), which can be divided into two phases (figures 3 top panel):

- Conversion of ssDNA into dsDNA forms on entering the nucleus of the infected cells.
- Rolling circle phase to replicate viral ssDNA on dsDNA templates. This step requires the participation of Rep, which is the only viral protein absolutely required for RCR, as it is responsible for initiating the DNA replication. Once Rep binds and nicks the circle, host DNA polymerase carries out RCR (Lanfs *et al.*, 1995a).

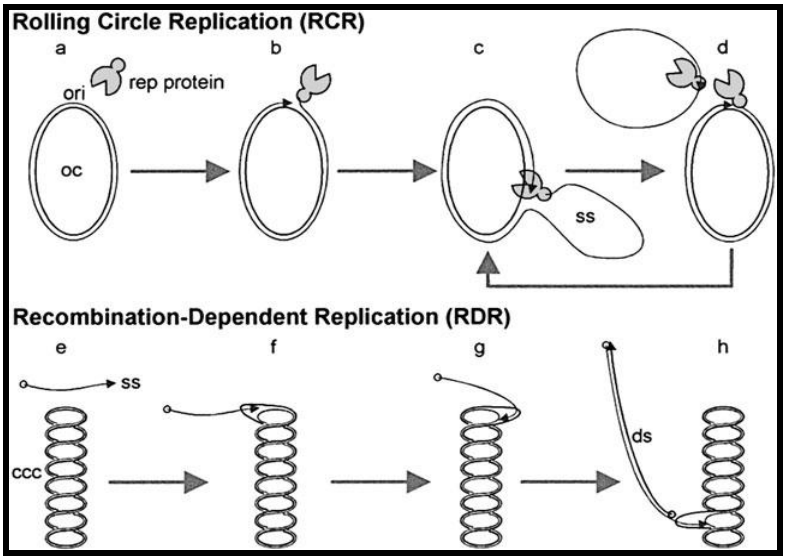


Figure 4. Models of RCR and RDR. Step a: binding of a replication associated protein (Rep; corresponding to ORF AC1, C1 or C1-2 for geminiviruses), respectively, to the origin of replication (ori). S*tep b: n*icking of DNA and covalently binding of Rep to the 5¢-end of DNA. Step c: ssDNA displacement and replication. Step d: new nicking,ssDNA closing and Rep release. Step e: incomplete ssDNA interacts with cccDNA at homologous sites. Step f: homologous

recombination. Step g: loop migration and ssDNA elongation. Step h: ssDNA elongation and complementary strand synthesis resulting in dsDNA (Jeske *et al.*, 2001).

Recently, an additional model of replication of begomoviruses (geminiviruses in general) and their satellites has been proposed Jeske *et al.,* 2001. This model, known as recombination-dependent replication (RDR), and was based on analysis of replication intermediates of reference geminiviruses *Tomato yellow leaf curl virus* (TYLCV), *Beet curly top virus* (BCTV), *African cassava mosaic virus (*ACMV), *Tomato leaf curly virus* (ToLCV), *Abutilon mosaic virus* (AbMV) and one satellite molecule (DNA β), using two dimensional gel electrophoresis and electron microscope. Apart of the previously identified RCR, a range of intermediates were suggested on RDR pathway. The RDR (figure 3 (bottom panel)) model has three steps (Kreuzer, 2000; Mosig *et al.,* 2001):

- Processing of the broken dsDNA to produce the 3´end ssDNA required for DNA strand invasion;
- Invasion of a homologous duplex 3´end ssDNA to form a structure know as the `` displacement loop´´ (D-loop or bubble loop). DNA strand invasion by the 3´end of the ssDNA allows it to serve as a potential primer for DNA replication;
- DNA heteroduplex extension (branch migration). At this step, the protein-directed branch migration occurs at the rear of the loop as DNA polymerase extends the leading-strand products at the front of the loop. Because both reactions occur at a similar rate, the size of the loop is roughly unchanged.

This type of RDR does not need a topoisomerase, even when the circular DNAs are supercoiled, and the two parents strands do not need to separate from each other (Kreuzer, 2000, Jeske *et al.*, 2001). RDR apparently does not require the participation of Rep in terms of its cognate virus recognition and nicking of ssDNA at the nanonucleotide sequence for mutation of replication. A recent study conducted by Lin *et al.* (2003), also supported this possibility, where mutants of ToLCV and its satellite were inquired in their ability to bind *in vitro* and were still infectious.

1.2.3 Recombination

One of the earliest piece of evidence for recombination amongst geminiviruses was obtained from studies of a severe mosaic disease of cassava in Uganda (Zhou, *et al.*, 1997) and the sequence analysis revealed that a virus responsible for the disease, *East african cassava mosaic virus-Uganda* (EACMV-UG) had arisen by interspecific recombination between EACMV and ACMV in the coat protein.

Recombination is now known to be a common event, and in fact the main driver of genetic diversification and evolution of geminiviruses (Nawaz-ul-Rehman and Fauquet, 2009). In a study by Padidam et al., 1999, representative sequences of 64 distinct species of geminivírus were searched for recombination events among geminiviruses, using software that can detect gene conversion (GENE CONV). In total, the analysis identified 420 statistically different recombinants fragments distributed across the viral genome. More than 90% of the detected fragments either from viruses between different continents or between begomoviruses and curtoviruses, were located in the N-terminal region of the Rep, suggesting that they are old events occurring presumably before the geographical isolation (Padidam *et al 1999)*. This

analysis also suggested that interspecific nucleic acid exchange has resulted in remarkable diversity among geminiviruses and could be the cause of the emergence and increase of new geminiviruses diseases (Fauquet *et al.,* 2005; Idris and Brown 2005).

1.4 Satellites

Recently, monopartite begomoviruses have been associated with small circular single-stranded DNA satellites (ssDNA) molecules, earlier known as DNA α and β (alpha and betasatellites, respectively). The alphasatellites are self-replicating molecules, dependent on the helper virus for movement encapsidation and vector transmission, but no specific function of these molecules is known (Shah *et al.,* 2009). The betasatellites are half the size of their helper begomovirus (~1.4 knp) and are required to induce symptom in their host (Mansoor *et al*., 2003; Mubin *et al.,* 2010; Sivalingam *et al.,* 2010). These molecules were found in many monopartite begomoviruses infecting a diverse range of plant families, such as *Malvaceae, Coprifololiaceae, Solannaceae, Curbitaceae* and *Asteraceae* (Briddon *et al.*, 2003). The DNA satellites attracted the attention of virologists since it has been showed that typical symptoms of Ageratus yellow vein and Cotton leaf curl diseases occurred only when their respective virus were co-inoculated with their DNA β components (Saunders *et al.* 2000; Briddon *et al*,. 2001; Sivalingam *et al.,* 2010).

The genome of DNA β molecules have approximately 1400 nt in the full-length form or ~700 nt for the deleted form and contains three characteristic regions (Briddon *et al.* 2003):

- **Satellite conserved region (SCR)**. This is a region of 200 nt containing a putative stem-loop that contains the nanonucleotide (TAG/ATATTAC) sequence typical of

geminiviruses, and a very highly conserved region of over 100 nt located on the 5´side of the stem-loop, with high content of GC (~70%) (Briddon *et al.* 2003; Zhon *et al.* 2003).

- **Adenine rich region (A rich region)**. This region has typically 160-180 nt and 60% of A located between nucleotide ±700 and ±1000. It was suggested that this region may be present to increase the size of these molecules to become a fraction (either half or quarter) of the typical size of geminiviruses. Thus, the nucleotide can be tolerated during systemic movement which operates through a stringent size-selective mechanism (Etessami *et al.* 1989; Rojas *et al.*, 1998).

- **Potential coding region (PCR).** The DNA β contains an Opening Reading Frame (ORF, βC1) on the complementary strand on 3´side of the stem-loop. This ORF encodes a protein of ~118 amino acids. Through mutation analysis Zhon *et al.* (2003) demonstrated that the βC1 gene product is associated with symptoms induction. The βC1 protein of DNA β satellite (Yβ10) associated with TYLCV-China Y10 isolate (TYLCVNV-Y10), is nucleophilic and is able to suppress RNA silencing activity (Cui *et al.* 2005).

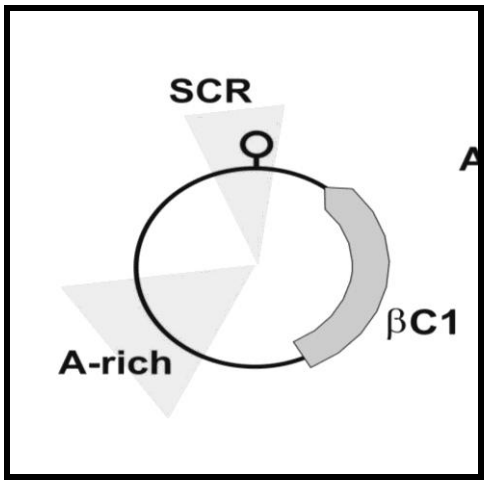


Figure 5. DNA β structure. SCR (satellite conserved region) A-rich (Adenine rich region) and βBC1 Open reading frame (Cui *et al.* 2005).

1.5. Criteria for Species Demarcation

Several taxonomic criteria for demarcating species of begomoviruses have been proposed by the International Committee on Taxonomy of Viruses (ICTV), based on the reliability and applicability of these criteria to the large number of characterized begomoviruses (Fauquet *et al*. 2003, 2008). Nucleotide sequence comparison plays a much greater role in determining taxonomic status. Thus, for comparative analyses, only full-length DNA-A sequences were considered, based on recombination events that readily occur among begomoviruses (*Fauquet et al.* 2003; Pita *et al.*, 2001). A cut-off value of 89% of nucleotide sequence identity (NSI) of the A component was established to distinguish different species from strains (Fauquet *et al*., 2008).

1.6 . Variability of begomovirus species

High diversity among begomovirus species is associated with mixed infections, in which recombination and pseudorecombination events may explain the frequent emergence of new

begomoviruses. Recombination is the exchange of DNA between similar DNA components, and pseudorecombination is the exchange of DNA-A or B components between viruses (Polston and Anderson, 1997; Shah *et al.*, 2009). Both events have been demonstrated in the laboratory (Hill et al. 1998; Garrido-Ramirez et al. 2000) and under natural conditions (Pita et al. 2001). Some isolates of cassava mosaic virus (ACMV), TYLCV and Potato yellow mosaic virus (PYMV) are good examples of recombination and pseudorecombination (Monci *et al.* 2002; Pita et al. 2001; Umaharan et al. 1998).

1.7 Detection and Discrimination of Begomoviruses

Begomoviruses have been detected in plants or insects by different techniques, such as visualization of nuclear inclusion bodies by light microscopy, ultrastructural localization of virions in plant cells by transmission electron microscopy, serological assays using polyclonal or monoclonal antibodies (Hunter et al. 1998; Konate et al. 1995; Pico et al. 1999; Polston et al. 1989), DNA hybridization assays (Lotrakul et al. 1998), Polymerase chain reaction (PCR) (Deng et al. 1994; Ghanim et al. 1998; Lotrakul et al. 1998; Mehta-Prem *et al*. 1994; Pico *et al*. 1999; Rosell *et al*. 1999), immunocapture PCR (Rampersad and Umaharan, 2003), and print-PCR (Navas-Castillo *et al*. 1998), among others. Molecular cloning and DNA sequencing of viral genomes have become the tools of choice, allowing virus identification and evaluation of relationships with other virus isolates (Brown *et al*. 2001; Padidam *et al*., 1995; Paximadis *et al*. 1999; Rybicki, 1994).

The circular replication intermediates and products, prone the geminiviral DNA to be amplified by the bacteriophage φ 29 polymerase, an enzyme that combines polymerase and strand-

displacement activities (Blanco *et al.*, 1989, referred by Jeske, 2007) in a technique known as rolling circle amplification (RCA). RCA (figure 6) is being widely used for detection and diversity studies of geminiviruses and is proving to be helpful to circumvent many bacterial cloning steps, since their products can be sequenced, modified and inoculated directly in a cell-free system (Jeske, 2007).

RCA has a high potential to replace most of the PCR and ELISA techniques used to diagnose geminiviruses, due to many reasons, such as: easiness to handle (no thermal cycler, specific primers are needed), amplification of all DNA components of a virus without the knowledge of their sequences including defective DNAs and satellites, no contamination, no false positives are revealed (virtually), working on died samples and multi-infected plants (Jeske, 2007; Schubert *et al.*, 2007).

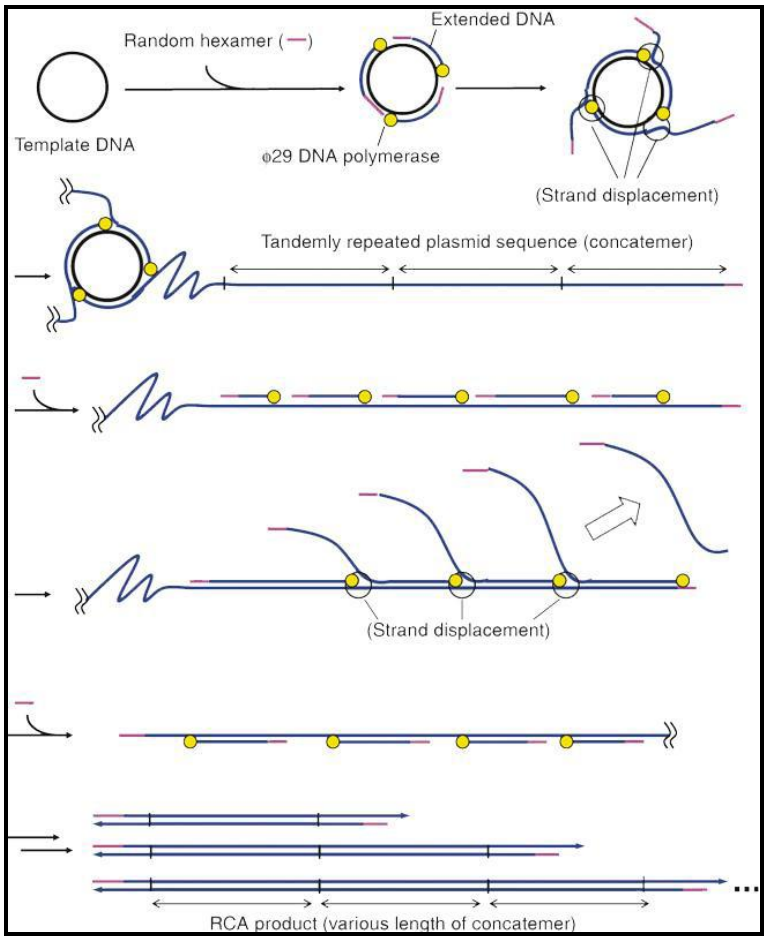


Figure 6. Schematic representation of RCA (extracted from Wyant, 2011).

More recently, high-throughput deep sequencing using new non-Sanger technologies has revolutionized the number of sequences that can be obtained in a short space of time (Kreuze *et al*., 2009).

1.8. Impact of Begomoviruses and their whitefly vectors on Agriculture

The global emergence of begomovirus diseases in most commercially-grown crops over the past three decades has had an enormous impact in agriculture. Proliferation and rapid dissemination of begomoviruses that infect important food and industrial crops in Latin America have been the

consequence of drastic changes in traditional cropping systems (Morales and Anderson, 2001), along with the introduction of the B type of *Bemisia tabaci* beginning in the mid-1980s (Polston and Anderson, 1997). *B. tabaci* type B has displaced many indigenous biotypes, because of its broader host range, higher fecundity, dispersal capacity, virus-transmission efficiency, and resistance to insecticides traditionally used against whiteflies (Brown *et al*. 1995; Luo *et al.*, 2009). Begomoviruses have been reported as limiting factors in the production of several crops in the Americas such as cotton, common bean, tomato, pepper, and cucurbits, among others.(Morales and Anderson, 2001; Polston and Anderson, 1997).

In the 1990s, Cassava mosaic begomoviruses (CMBs) caused a major regional pandemic of Cassava mosaic disease (CMD) (affecting parts of at least five countries in Africa) that led to massive economic losses and destabilization of food security (Legg and Thresh, 2000). A key factor in the genesis and spread of the pandemic was the recombination of two distinct cassava mosaic begomoviruses (ACMV and EACMV) to produce a novel and more virulent hybrid (Pita et al. 2001).

Tomato yellow leaf curl virus (TYLCV) is another example of an emerging virus that has caused epidemics worldwide with frequent losses of up to 100% (Moriones and Navas-Castillo, 2000). In tomato, the virus causes prominent upward curling of leaflet margins, reduction of leaflet area, yellowing of young leaves, and stunting of plants. These symptoms were first reported in tomato crops from Israel in the late 1930's, then in Middle Eastern countries from the 1960s to the present. Damage to tomato crops attributed to TYLCV has been reported in the Middle East and

Far East, Africa, Europe, Caribbean Islands, Central America, Mexico, and the United States of America (Moriones and Navas-Castillo, 2000).

1.9 Management of Begomoviruses

Begomovirus management strategies have been implemented in several locations in Central America and the Caribbean after the occurrence of serious agricultural and economic crises caused by begomovirus infection of many crops in the mid 1980s (Hilje, 2002). In the first decade of implementation, area-wide plant-protection campaigns were initiated. These involved quarantine regulations and host-free periods in the Dominican Republic, Mexico, and Cuba. Cultural practices (such as production of seedlings under netting and the use of living ground covers in production fields) are the most novel contributions of this action plan (Hilje, 2002).

Several practices have been implemented to control begomoviruses in tomato crops, such as the destruction of infected crops at the end of the production cycle using herbicide combined with oil to kill plants and whiteflies, and then burning the plants; use of virus-free transplants; and removal of infected plants at the first sign of begomovirus symptoms (Schuster and Polston, 1999). In greenhouses, the use of ultraviolet (UV)-absorbing plastic sheets or (UV)-absorbing screens of 50-mesh density (Antignus, 2000) (which interferes with the visual behavior of *B. tabaci*) has been shown to reduce begomovirus transmission and crop losses. The use of a 50-mesh screen prevents vector entry, but it produces poor ventilation and overheating of the closed structures (Morriones and Navas- Castillo, 2000). A good practice is to eradicate plants that can be sources of inoculum, making it possible to reduce the primary spread of the virus (Kashina *et al*., 2002; Schuster and Polston, 1999). One practice more accepted by growers in many locations

is chemical control of the insect vector, but it has a detrimental environmental impact; and whitefly vector populations rapidly develop resistance to insecticides. Insecticides that are not toxic to non-target species reduce the impact on important natural enemies such as *Eretmocerus sp.* and *Diglyphus sp*. parasitoids compared to conventional insecticides (*Hanafi et al*., 2002).

Host resistance to begomoviruses is another means of management. Development of resistant cultivars by classical plant breeding or genetic engineering requires time, a good scale for evaluating symptom severity, inoculation protocols, and constant adjustment of the resistance due to changes in begomovirus populations (Lapidot and Friedmann, 2002). Some genes for resistance to certain begomoviruses that infect tomato have been identified in wild species of *Lycopersicon,* and have been transferred to cultivated tomato. Thus, resistant cultivars and breeding lines have been generated. Recently, immunity to infection to TYLCV was obtained from *L. hirsutum*. This immunity was shown to be controlled by three additive recessive genes (Vidavsky and Czosnek, 1998).

Another option is to obtain resistance is using genetic engineering via pathogen-derived resistance approaches, such as CP-mediated resistance (Kunik *et al*. 1994; Sinisterra *et al*. 1999); MP-mediated resistance (Duan *et al*. 1997a; Hou *et al*. 2000), defective interfering viral DNA (Stanley *et al*. 1990); *Rep* gene in antisense orientation (Bendahmane and Gronenborn, 1997); and expression of truncated viral Rep protein (Brunetti *et al*. 2001; Polston *et al*. 2001). The two first approaches involve expression of the CP and MP to inhibit viral proliferation. The last three approaches inhibit viral replication by disrupting the activity of the *Rep* gene. Induction of RNA

silencing to suppress viruses using transgene-expressed hairpin or inverted RNA repeats designed from virus gene targets is currently the favored method (Lapidot *et al* 2006).

Currently, for optimal control of begomoviruses in tomato, it is necessary to use several practices simultaneously. Development of new and improved methods of control for begomoviruses depends on our understanding of the mechanisms involved in virus-vector and virus-host plant recognition, and knowledge of the variant forms of the virus in natural populations.

1.10. *Bemisia tabaci* (Gennadius)

1.10.1. Taxonomy and morphological characterization of *B. tabaci*

Bemisia tabaci (Gennadius) (Hemiptera: *Aleyrodidae*) (whiteflies) is a plant sap-sucking insect. the puparium appears translucent, cream to distinctly yellow, without evident adorning wax secretion. The dorsum has a thin, transparent wax layer, which is 0.55–0.87 mm long and 0.35–0.64 mm wide in size. The shape is sub oval, often strongly tapered to posterior. When slide-mounted, the cuticle is evenly pale. The margin is finely crenulated. (EPPO, 2004). *B. tabaci* is an oviparous insect and from the deposition of the eggs, there are two more stages, the nymphal and puparial (figure. 7).

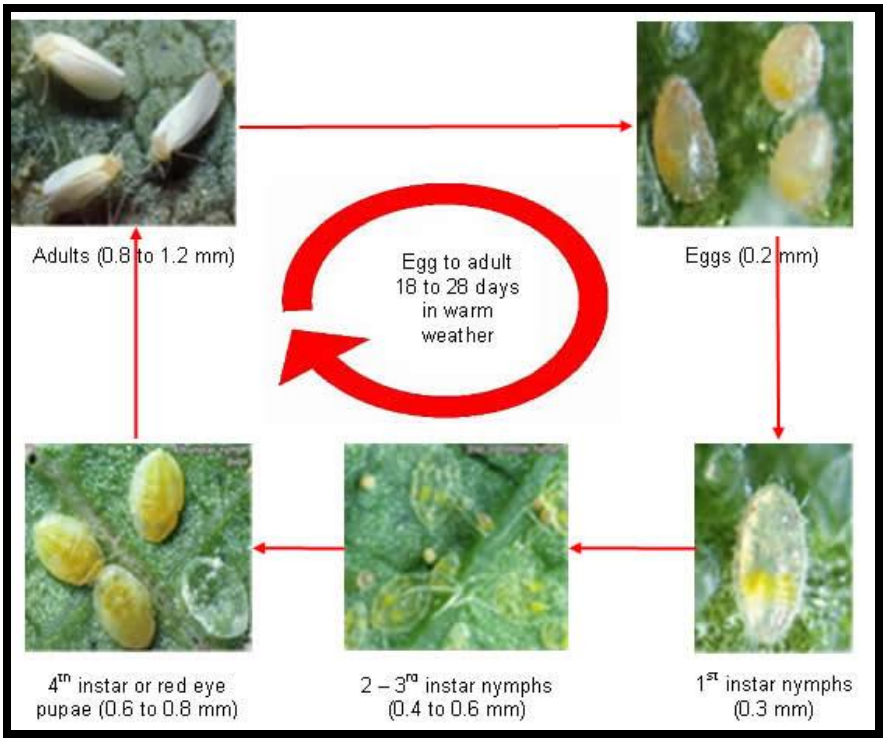


Figure 7. *B. tabaci* life cycle from egg to adult (Subramaniam *et al*., 2008)

1.10.2 Genetic diversity of *B. tabaci*

B. tabaci has been known for a several years to exhibit host- plant related races or biotypes (De Barro, 2005). During the late 1908s, a particular fecund race was identified from the southern US, which demonstrated high level of insecticide resistance, transmitted previously unknown begomoviruses, and induced a physiological change in squash known as "squash silver leafing". Later it was found to exhibit a unique electrophorm pattern for general esterases and became designated B-type. The indigenous American *B.tabaci*, exhibited a different esterase pattern and was named the A-Biotype (Costa and Brown, 1991; Burban *et al.*, 1992; Perring *et al.*, 1992; Costa *et al*,, 1993). Since then, 20 different types have been identified, the most widespread being the B-type which has spread around the world owing to its association with ornamentals and the world trade in this commodity (De Barro, 2005).

Genetic differentiation between the various genotypes has been further supported by numerous molecular methods involving Random Amplified Polymorphic DNA – polymerase Chain Reaction (RAPD-PCR) (De Barro, 2005), and later a PCR based method targeting the mitochondrial Cytochrome Oxidase 1 (*mtCO1*) gene yielding a product of approximately 870 bp was developed (Frohlich, *et al.*, 1999; Berry *et al*, 2004) that is widely used for *B. tabaci* identification.

More recently, the *B. tabaci* species complex has been divided phylogenetically into 12 clades or groups based on statistical parsimony networking analysis (figure 8) of a portion of the *mtCOI* gene (De Barro and Almed, 2011; De Barro *et al,*. 2011).

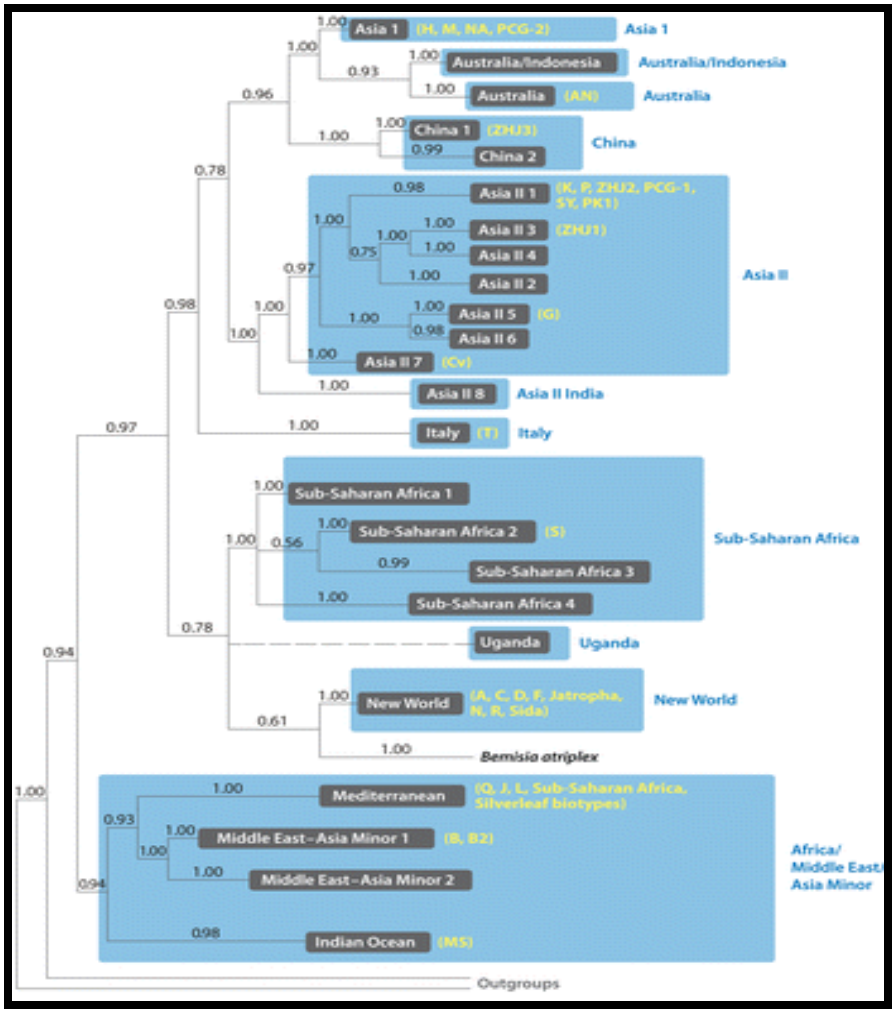


Figure 8. Evolutionary relationship of the cryptic species complex of *B. tabaci* (De Barro *et al.*, 2011)

1.10.3 Detection of whitefly on plants

Whiteflies are usually detected by close examination of the undersides of leaves (figure 9) to search for the tiny yellow/cream scale-like larval instars. They also occasionally occur on the upper surfaces of the leaves and vary from being widely scattered to dense clusters. Shaking the plant may disturb the small white adults, which flutter out and quickly resettle. Adults may also be found on sticky traps placed above infested plants. Samples of larvae should be collected

while still attached to the leaves and stored dry or in phials of 70% ethanol for examination in the laboratory (EPPO, 2004).



Figure 9. Adult whiteflies feeding on tomato leaf.

1.10.3. Damage to plants

B. tabaci is broadly polyphagus, feeding on an estimated 600 plant species. Since the early 1980s, it has caused escalating problems to both field and protected agricultural crops and ornamental plants. High infestations of *B. tabaci* may reduce host vigor and growth, cause chlorosis and uneven ripening, and induce physiological disorders. The larvae produce honeydew on which sooty moulds grow, reducing the photosynthetic capabilities of the plant, resulting in defoliation and stunting. *B. tabaci* is a vector of over 100 plant viruses in the genera *Begomovirus* (*Geminiviridae*), *Crinivirus* (*Closteroviridae*) and *Carlavirus* or *Ipomovirus* (*Potyviridae*) (Jones, 2003). Begomoviruses are the most numerous of the *B. tabaci* transmitted viruses and can cause crop yield losses of between 20% and 100% (Brown & Bird, 1992).

1.11 Tomato-infecting begomovirus diseases

1.11.1. Origin and distribution

Begomovirus - induced diseases of tomato have been known for more than 40 years, but only since the end of the eighties has it become widespread in all important tomato growing areas around the Mediterranean basin (Moriones and Navas-Castillo, 2000). In this part of the world, which includes the Near-East, Northern Africa and Southern Europe, tomato is an important protected crop, particularly in the winter season (Davino, *et al* , 2007). Tomatoes suffer from several distinct begomovirus diseases worldwide, but the two most widely spread are tomato leaf curl and tomato yellow leaf curl diseases, both of which are caused by more than 200 distinct geminivirus, within species, strains and variants (Fauquet *et al*., 2008).

In 1991, the causal agents of yellow leaf curl disease at that time were described and their sequences determined: one from Israel, *Tomato yellow leaf curl virus* (TYLCV-IL; Navot *et al*., 1991; Fauquet *et al*., 2008), and the other from Italy, *Tomato yellow leaf curl Sardinia virus* (TYLCSV-Sar (Fauquet *et al*., 2005, 2008), and are both monopartite belonging to the genus *Begomovirus*, family *Geminiviridae* and were transmitted by *B. tabaci*, B type (Fauquet *et al*., 2005). Several other isolates were later reported, and their DNA sequences indicated that they could easily be classified as strains or variants of either TYLCV or TYLCSV. In the past two decades, many new distinct begomoviruses have been identified, that cause disease in tomatoes (Czonesk *et al.,* 1998; Davino *et al.,* 2000; Fauquet *et al.,* 2003, 2005, 2008).

1.11.2 Identification

Early diagnosis of TYLCV and TYLCSV was essentially based on symptom observation, although symptoms vary greatly as function of soil, growth conditions and climate (Czosnek and Laterrot, 1997). Several methods have been described to identify these two viruses, which are found on the same host plant (tomato) and produce very similar symptoms. A simple and robust one was described in 2000 (Accotto *et al*., 2000) and later tested and suggested in an EPPO standard (EPPO, 2005): it is a Polymerase Chain Reaction/restriction fragment length polymorphism (PCR/RFLP) based assay, which results in the amplification of a portion of the coat protein gene with a nonspecific primer pair, followed by digestion with *Ava*II restriction enzyme. This yields two different patterns for isolates of TYLCV and TYLCSV, with the two patterns superimposed in the case of mixed infections. Similarly, for other begomoviruses species and their variants, PCR-specific primers have been developed, or the virsuses are sequenced (Polson and McGovern, 1999; Massumi *et al.*, 2007; Fernandez *et al.*, 2010; Pandey *et al.*, 2010).

Although this method is useful, new problems are posed by frequent recombination events where the presence in the same area of more than one begomovirus species exists. For example, both TYLCV and TYLCSV occur in Spain, and a recombinant virus was found (*Tomato yellow leaf curl Malaga virus* (TYLCMalV) (Monci *et al.*, 2002), whose genome is composed of portions from each parent species. Another recombinant from Spain has been described recently (Garcia-A. *et al.*, 2006) and new ones are being detected in Italy (Accotto and Davino, unpublished), where TYLCV has colonized areas endemically infected by TYLCSV (Davino *et al*., 2006). TYLCV and TYLCSV can colonize the same plant and even the same cell (Morilla *et al.*, 2004),

creating the conditions for appearance of new viral types through recombination. Thus it has now become almost standard practice to sequence the entire DNA-A of unknown tomato-infecting begomoviruses in order to detect new or recombinant geminiviruses.

1.11.3 Tomato Curly Stunt Virus –[South Africa:Onderberg: 1998]

1.11.3.1. Characterization

During 1997 a new disease of tomato plants emerged in tomato production areas in the Onderberg region, South Africa. The affected tomato plants showed a foliar symptom similar to those induced by TYLCV, including foliar chlorosis, leaf curling and reduced fruit set. Since the first report in 1997 (Pietersen and Smith 2002), the disease has spread to additional tomato growing locales throughout South Africa and also has been identified in the southern region of Mozambique Maputo and Gaza Provinces) (unpublished).

The Tomato Curly Stunt Disease (ToCSD) name was based on observed field symptoms on tomato plants. A preliminary investigation was undertaken to detect a suspected begomovirus in affected tomato plants, and this revealed the association of a provisional new monopartite begomovirus species, referred to as *Tomato Curly Stunt Virus* (ToCSV) (Pietersen *et al.,* 2000). This conclusion was based on analysis of nucleotide sequence of the core coat protein sequence (GenBank AF261885) (figure 8), which shared less than 80% nt sequence similarity with the most closely related begomoviruses. ToCSV has been shown to be experimentally transmissible by the B-type whitefly *B. tabaci*, a recent introduction to the tomato-producing region of South Africa (Brown, 2000). The virus has recently renamed *Tomato Curly Stunt Virus* –[South

Africa:Onderberg: 1998] (ToCSV- [ZA:Ond:98]) in the new taxonomy (Fauquet *et al., 2005*), but for the puposes of this dissertation will be referred to as ToCSV.

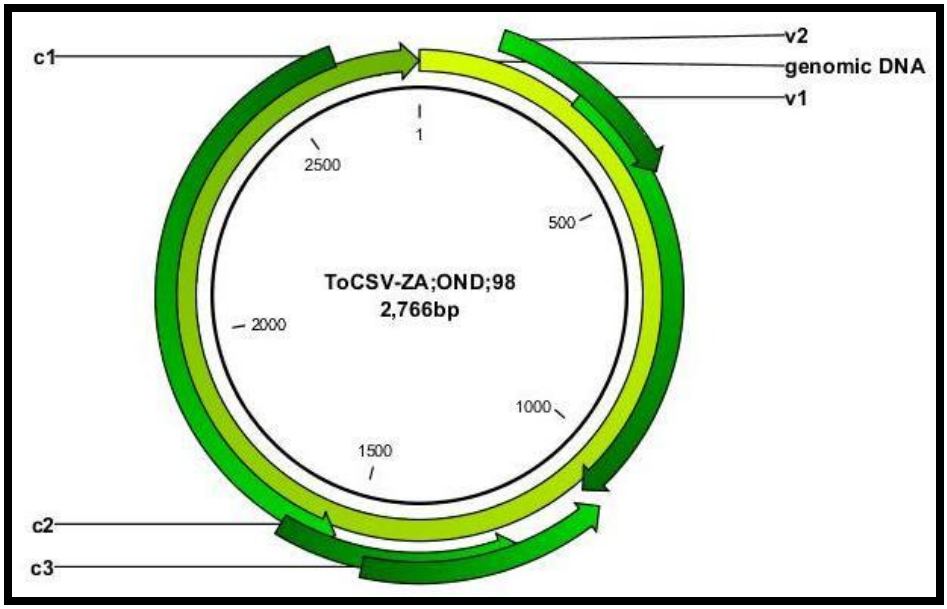


Figure 10. Genomic organization of ToCSV ORFs: V1 (*Cp*), V2 (*Mp*), C1 (*Rep*), C2 (*Trap*), C3 (*Ren*).

Sequence comparisons of the full length genome showed that ToCSV shared the highest nucleotides identity with *Tobacco Leaf Curl Zimbabwe virus* (TbCZV) (AF350330), *Pepper Yellow vein virus* (PpYVV), from Mali (AY502935) and *Tomato Leaf curl Mali Virus* (ToLCMLV) (AY502936) at 84%, 82% and 78%, respectively. Phylogenetic analysis (figure 10), suggest that ToCS] (AF261885) is probably indigenous to south western Africa and is not an introduced virus. Recombination with begomovirus-infected indigenous wild species or weeds is speculated (Esterhuizen and Rey, pers.com.).

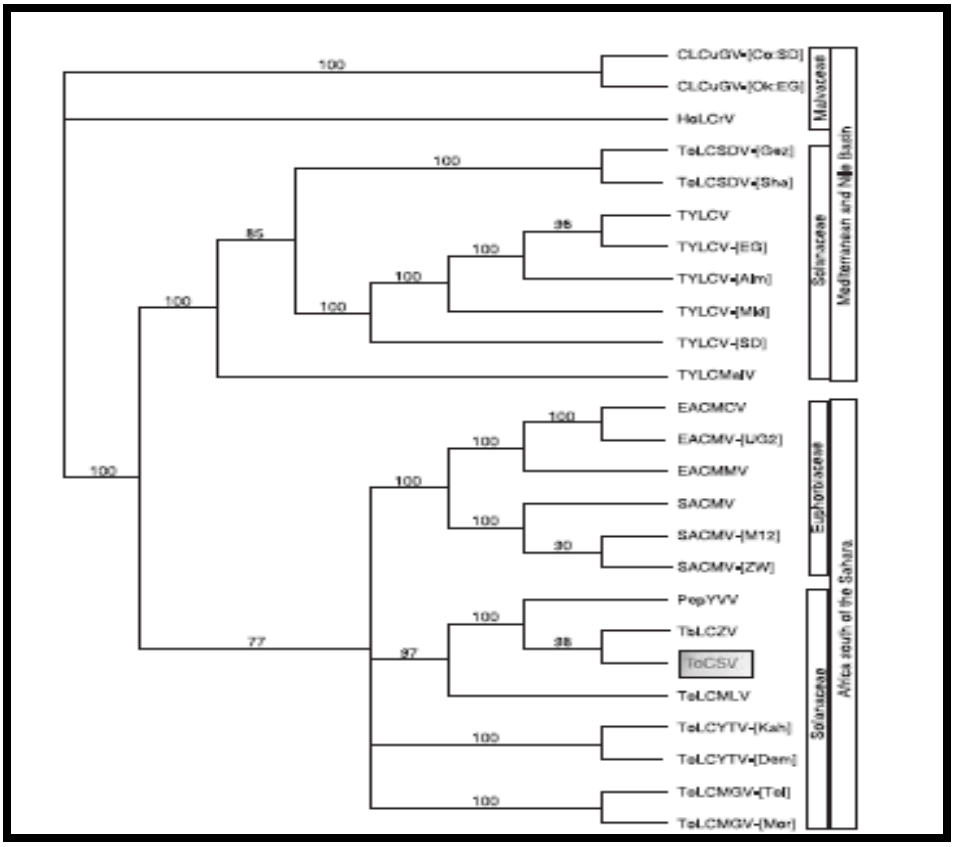


Figure 11. Phylogenetic tree indicating the relationships for Tomato curly stunt virus (ToCSV) and selected begomoviruses using maximum parsimony, available in PAUP 4·0bv10. The vertical and horizontal branch lengths are arbitrary. Bootstrap (1000 replicates) values are placed at the major nodes on the tree (Pietersen *et al* 2008).

1.11.3.2. Disease control

Control measures in the affected regions are traditionally based on limiting vector population. Chemical control has been only partially effective, especially under high disease pressure, and in addition to its deleterious effects on the environment, the vector has been shown to develop pesticide resistance (Hilje *et al,* 2001; Palumbo *et al* 2000; Lapidot *et al.,* 2002). Cultivation of tomatoes with resistance to ToCSV can reduce the insecticide required and increase the likelihood of sustained control (Vidasky and Czonesk, 1998; Lapidot *et al.,* 2001;). The assessment of cultivars with resistance to ToCSV must be supported by other studies because the

response of the pathogen may be affected by test conditions such as temperature, light, growth conditions, inoculation pressure and plant age (or development stage) at the time of inoculation (Loebenstein, 1972). In some instances, it has been shown that mature plants resist or tolerate virus infection much better than plants infected at an early stage of development, leading to what appears to be increased viral resistance (Garcia and Murphy, 2001; Moriones *et al* 1998; Smit and Parevliet, 1990; Soler *et al.*, 1998; Lapidot & Levy, 2007).

1.11.3.3. Host range

According to Pietersen *et al*, 2008, ToCSV infect a suite of species (table 1), similar for those reported for TYLCV-IL (Ying & Davis 2000), including the ability to infect bean. TYLCV has been reported to cause economic loss in commercial bean (*Phaseolus vulgaris)* in Spain (Navas-Castillio *et al*, 1999), and disease symptoms in bean were reported to range from severe to symptomless, depending on the cultivar (Lapidot, *et al.*, 2002).

In the host range screening described by Pietersen *et al.,* 2008, three of the tested beans developed obvious symptoms of ToCSV. Interestingly, symptoms of ToCSV were not observed in commercial bean fields in close proximity to ToCSV-ZA:Ond:98- infected tomato fields (Pietersen, *unpublished observations)* suggesting a barrier for spread and parallels a report from Israel (Lapidot, 2002), where 42 commercial varieties of common bean were screened for TYLCV-IL resistance and more than 50% (24 varieties) showed severe symptoms and accumulated high levels of virus. Interestingly, pepper (*Capsicum sp.*) was not a host for ToCSV in the experimental host range study, and indeed pepper crops planted in the proximity of tomato fields with high incidence of ToCSV disease did not show ToCSV or TYLCV-IL like symptom,

even although certain pepper cultivars are known to be susceptible to TYLCV-IL (Reina *et al*, 1999).

Table 1. Experimental host range study for ToCSV

Test plant[a]	**No of symptomatic plants/No of inoculated plants**	**PCR virus detection in test plants**
Brassica pelonensis	0/10	0/10
Capsicum annum Anaheim	0/10	0/10
C. annum Capistrano	0/10	0/10
C. annum Golden Wonder	0/10	0/10
Cucurbita maxima Big Man	0/10	0/10
Datura stramonium	2/10	10/10
Glycine max Rampage	0/10	0/10
***G. max* Buffalo**	2/10	2/10
Gossypium hirusuntu Delta Pine 90	0/10	0/10
Lycopersicon esculentum Star 9006	10/10	10/10
Malva parviflora	0/10	0/10
Manihot esculentum	0/10	0/10
Nicotiana benthamiana	0/10	5/10
N. clevalandii	0/10	4/10
N. glutinosa	2/10	1/10[b]
N. tabacum	0/10	4/10
Phaseolus vulgares Black turtle soup	0/10	0/10
***P. vulgaris* Bonus**	2/10	2/10
***P. vulgaris* Bountiful**	5/10	7/10
Pisum sativa Green feast	0/10	0/10
Solannum melongena	0/10	0/10
Vicia faba	0/10	0/10
Vigna unguiculata California blackeye	0/10	0/10
V. unguiculata tvu 1582	0/10	0/10

[a]test species indicated in bold are susceptible to ToCSV-ZA:Ond:98 [b]Only one of the two symptomatic *N glutinosa* plants was PCR positive. Source: Pietersen *et al.,* 2008.

1.11.3.4. ToCSV in Mozambique

In 2005, a new disease was reported for the first time by the farmers to the agricultural officers, in Moamba district, in Maputo, a southern province of Mozambique, a region bordering the province of Mpumalanga in South Africa, where ToCSV was previously detected in 1997 by Pietersen.

In 2006 the disease spread quickly to the district of Chókwè, in the province of Gaza, one of the main tomato growing areas in the southern region of Mozambique. ToCSD reached epidemic proportions in both years (2005-2006) and high yield losses, ranging from 50 – 80& were observed. Leaf samples were sent for PCR and the results revealed that ToCSV-[ZA:Ond:98] was the causal agent (unpublished). Since this time, and supported by the study of Pietersen and Smith (2002), they concluded that varieties showing tolerance to TYLCV can also be tolerant to ToCSV. Field trials aiming to select resistant\tolerant varieties to include in a management strategy, were conducted in Moamba, and Chókwè, and promising lines such as LLANERO from Guatemala were identified (*unpublished results*). The lines/varieties used in the trials, were those that showed good performance against TYLCV in country such as Guatemala, India, USA, etc. and were provided by Favi Vidasky (Israel) and Douglas Maxwell (USA).

CHAPTER 2

DISTRIBUTION, INCIDENCE AND SEVERITY OF ToCSD IN MOZAMBIQUE

2.1. Abstract

In 2005, a ToCSD was reported for the first time in Moamba district, in Maputo, a southern province of Mozambique, a region bordering the province of Mpumalanga in South Africa, where a ToCSD was previously detected. In 2006, the disease spread quickly to the district of Chókwè, in Gaza province, one of the major tomato growing areas in the southern region of Mozambique, where it reached epidemic proportions. In both years (2005-2006), surveys conducted in those areas revealed 80 to 100% of incidence due to the quick spread of ToCSD and because tomato is grown through Mozambique, two surveys were conducted to determine the distribution and disease index (DI) (incidence and severity) of ToCSD in the major tomato growing provinces, namely, Maputo, Gaza, Manica, Tete and Zambezia. During the surveys, tomato leaf samples and whiteflies were collected. The leaf samples were used to screen for the presence of ToCSD by PCR using the partial coat protein primers designed by Pietersen and Smith (2002), which only picks up ToCSV. The surveys results revealed that ToCSV was occurring with high incidence in Maputo and Gaza provinces, Moamba and Namaacha and Chokwe districts, ranging from 62.5 – 74.5% in the dry season and 66-78% in the rain season. The severity indices was also high in the same locals and ranged 2-2.5 in both seasons. Some of the symptomatic tomato samples were negative in the PCR suggesting that mixed infections of different virus species or ToCSV genetic variants virus could be causing ToCSD.

Keywords: Epidemiology, Tomato, Begomovirus, ToCSD, and Mozambique

2.2. Introduction

The outbreak and spread of certain aggressive *B. tabaci* types, such as B and Q, which are capable of transmitting viruses more efficiently than the local populations, over the past decade, combined with the high recombination of geminiviruses, have been correlated with the quick spread, increase and emergence of whitefly-transmitted geminiviruses (including new species) in several crop species worldwide (Polston and Anderson, 1997; Rybicki and Pietersen, 1999).

Tomato curly stunt disease (ToCSD) is caused by a whitefly-transmitted monopartite begomovirus infecting tomato plants in tropical and subtropical countries. The name was based on observed field symptoms which appears several weeks after infection and vary according to the region, but generally include severe stunting, marked reduction of leaf size, upward cupping and the consequent yield reduction (Pietersen *et al.*, 1998; 2008).

The surveys conducted in SA from 1997-2003 revealed that ToCSD were present in all tomato growing regions (Pietersen *et al* 2008). The screening for the presence of ToCSD was done using a PCR based assay using the core CP primers ((Wyatt and Brown, 1996). The results showed that ToCSD quickly spread throughout the country reaching epidemics proportions with yield losses near to 100 % (Pietersen and Smith 2002).

In 2005, ToCSD-like symptoms were reported for the first time to the agricultural officers, in Moamba district, in Maputo, a southern province of Mozambique, a region bordering the province of Mpumalanga in SA In 2006 the disease spread to the district of Chókwè, in Gaza province, one of the major tomato growing areas in the southern region of Mozambique, and

further research confirmed that was the same virus found in Onderberg, the south African region where the ToCSV was observed for the first time (Pietersen and Smith 2002). Based on the results from SA, surveys and molecular based approaches were conducted in this study to understand the epidemiology and diversity of ToCSD and it vector (*B. tabaci*) in the major tomato growing provinces in Mozambique.

2.3. Materials and methods

2.3.1. Surveys and sample collection

To determine the distribution, incidence and severity of ToCSD in the major tomato growing provinces in Mozambique, two surveys were carried on in two seasons, Survey 1 cold/dry season 2009 and Survey 2 hot/rain season 2009 in the following provinces (district in the brackets):

- Maputo (Moamba and Namaacha);
- Gaza (Chokwe and Xai-Xai);
- Manica (Manica and Sussundenga);
- Tete (Angonia);
- Zambezia (Nicuadala).

In each district, 10 fields were randomly selected, 20 plants/field, were observed and scored using the severity scale (table 2) adapted from Pietersen and Smith (2002). Leaf tomato samples from the observed plants, either symptomatic or not, were collected for ToCSV screening by PCR. All data were registered in the sheet prepared for this propose (**appendix 1**). The selected fields were not the same from one survey to the other. Whiteflies were counted and collected.

Table 2. ToCSD severity score scale.

Severity score	Symptoms description
1	Healthy plant
2	Initial symptoms slightly appearing
3	Curl well developed and slight capping
4	Curl and capping well developed and slight stunt
5	High capping, curling and stunt

Adapted from Pietersen & Smith, 2002

2.3.2. Data analysis

2.3.2.1. Severity and incidence

Data of severity and incidence were arranged per district. Disease incidence (DI(and severity indices (SI) (formula 1 and 2) were calculated as below:

$$DI(\%) = \frac{NSP}{TNOP} * 100$$

(1) Where,

DI – Disease incidence in percentage
NSP –Number of symptomatic plants
T N O P – Total number of observed plants
M S – Maximum score observed
100 – Conversion factor

$$S.I = \frac{\sum SS * Fr}{T.N.O.P * M.S.}$$

(**2**) Where,

SI – Severity indices
SS – Severity score
Fr – Frequency of observation of each severity score
T N O P – Total number of observed plants
M S – Maximum score observed

3.2.2. Confirmation of infection by Polymerase chain reaction (PCR)

The collected leaf samples were submitted to a Total Nucleic Acid (TNA) extraction using the protocol described by Accoto *et al.,* (2000), where 0.15gr of leaf material were ground liquid nitrogen, added 500ul of extraction buffer (Tris-HCl p^H 8, 50mM EDTA, 500mM, NaCl, 1% SDS and 10mM citric acid). Steps with potassium acetate, ispropanol and ethanol followed and the elution with ddH_2O, ended the TNA extraction procedure (appendix 2).

A PCR was set to screen for the presence of ToCSV in all 3200 samples, using the partial coat protein (PCP) primers designed by Pietersen and Smith (2002), yielding a fragment of approximately 305 bp. The reaction conditions were 1x PCR buffer, 0.5mM of $MgCl_2$, 0.2mM of each dNTP, 0.1U of DreamTaq DNA polymerase (all reagents from Fermentas), and 0.4µM of each primer (ToCSV sense 5´TCTGACCCATCGCACGGGT 3´ and ToCSV anti sense 5´CGCTTCACAAGAGCCTGCTCC 3´). The cycling conditions were the following: initial denaturation at 94ºC for 1 min, 35 cycles of 92ºC for 30 sec., 62ºC for 20 sec. and 72ºC for 30 sec ending with a final extension at 72ºC for 5 min. The PCR products were then ran in 1% agarose gel in 1x TAE buffer, stained with Ethidium bromide (10mg/ml), visualized under UV light (Tran illuminator), photographed and images saved on the Gel Doc system (Bio-Rad).

The DI, SI, whitefly number and PCR results were statistically analyzed using one way ANOVA, at 5% of probability (p value) and significant means were tested using Tukey test, using the statistical package STATISTICA 10 (Statsoft).

2.4. Results and Discussion

2.4.1. Disease incidence and Severity indices of ToCSV in Mozambique

The surveys revealed that ToCSV-like symptoms were found in 6 of the 8 districts surveyed. The ToCSD incidence (DI) and severity indices (SI) were, in general, higher in the second survey (hot/rainy season), compared to the first cooler/drier season in 2009 (table 3), and the highest values of DI, and SI respectively, were from Moamba (**78 %, 2.38**), followed by Chókwè (**74.5%, 2.015**) and Namaacha (**66%, 2.055)**) all districts from the south of Mozambique (table 3 and appendix 3).

Table 3. Means comparison of DI, SI, PCR positives of ToCSD and Whiteflies

Province	District	Survey 1 cold/dry season 2009				Survey 2 hot/rain season 2009			
		DI (%)	SI	% of pos PCR	Nr of WF	DI (%)	SI	% of pos PCR	Nr of WF
MAPUTO	Moamba	**74.5 a**	2.200a	31.0 b	2.360 a	78.00 a	2.380 a	47.0 a	2.930 a
	Namaacha	**62.5 b**	1.905 b	25.0 c	1.835 b	66.00 a	2.035 b	39.5 a	2.330 b
GAZA	Xai-Xai	**0.0 d**	1.000 d	0.0 d	0.000 d	0.92 c	1.020 d	0.0 b	0.290 d
	Chokwe	**74 .0 a**	2.125 a	41.5 a	1.885 b	74.50 a	2.015 b	43.5 a	2.420 b
MANICA	Manica	**0.0 d**	1.000 d	0.0 d	0.000 d	40.00 b	1.445 c	2.5 b	0.850 c
	Sussundenga	**0.0 d**	1.000 d	0.0 d	0.000 d	0.00 c	1.000 d	0.0 b	0.000 d
ZAMBEZIA	Nicuadala	**29.0 c**	1.370 c	0.0 d	0.565 c	33.00 b	1.380 c	5.5 b	0.765 c
TETE	Angonia	**0 .0 d**	1.000 d	0.0 d	0.075 d	5.50 c	1.055 d	0.0 b	0.68 c

Means followed by the same letter in the column are not statistically different

The district of Manica, which had no symptomatic plants in the survey 1, had higher DI and SI in survey 2 (**40%** and **1.445**) than Nicoadala, which had a DI increase of 6 % from the first to the second survey. The ANOVA and means comparisons of DI and SI of Manica and Nicoadala revealed no significant differences in survey 2, while in survey 1 the difference was significant.

Xai-Xai, Angonia and Sussundenga showed no significant differences on DI and SI in both surveys (table 3).

Moamba and Namaacha had an increase of 3.5% of DI, from survey 1 to 2, compared to Chokwe, which increased 0.5% of DI. The ANOVA and means comparison, indicated no significant differences between Moamba, Namaacha and Chokwe in survey 2, while in survey 1 Moamba and Chokwe differed significantly from Namaacha (table 3).

The observed results suggests that hot/rainy season is critical for ToCSD transmission and spreading due to many reasons such as movement of infected seedlings, high population of whiteflies, neighboring between fields growing tomato, poor cropping management, such as no crop rotation, what maintain the inoculums source on alternative hosts and, even when the vector population reduce, the disease still transmissible, because a single whitefly can transmit the virus as found by De Barro, 2005; Ghanim, *et al.*, 2007; Czonesk *et al.*, 2000. Subramaniam *et al*., 2008 demosntrated that the temperature has been shown to be a key factor on whiteflies population dynamics and temperatures around 29 ºC (hot season in Mozambique) a boom of whiteflies population can be observed as demonstrated.

Czosnek and Laterrot (1997) conducted a survey on TYLCV, another monopartite begomovirus infecting tomato worldwide and found that in 6 years the incidence of TYLCV increased significantly. Pietersen and Smith, (2002), reported that TYLCV and ToCSV, besides sharing about 60-85% of nucleotide similarity they also share most of the epidemiological characteristics, what also helps to understand the results found in this study.

The results from Xai-Xai, Angonia and Sussundenga shows that areas with low pressure, in terms of tomato growing and very disperse fields, as was the case, may need more time to have significant DI and SI because Angonia and Sussundenga are very cold areas, what helps to reduce whitefly population, and the tomato was introduced recently (around 2006), Xai-Xai has the same situation in terms of time of tomato growing. A new survey is needed to update and follow up.

Looking at the percentage of positive samples screened by PCR and the number of whiteflies found per district, were not statistically different between districts with high values of DI and SI, namely, Moamba, Namaacha and Chokwe as well as among those with lower values of DI and SI, Manica and Nicoadala (table 3). Besides being expected and concordant with other reported studies (Ghanim, *et al.*, 2007; Czonesk *et al.*, 2000) on other whiteflies-transmitted begomoviruses, the results obtained in this study showed that one whitefly/plant and a source of virus inoculums can be enough to have infective begomoviruses.

2.4.2. PCR screening

In positive samples to PCR, using the PCP primers, the expected fragment of about 305 bp was obtained (figure 12). In survey 1, only 12.18% were positive (195 out of 1600 samples) and in survey 2 the number of positives samples increased only about 5%, 276 out of 1600 samples. Chokwe had more positive samples in survey 1 (83 samples = 41.5%) than the other districts and in survey 2, Moamba had more (94 samples = 47%) (table 3). These results contrasted with the DI values which were 25-40% higher, than the number of positives samples in both surveys (figures 13 and 14). Showing that not in all symptomatic samples was detected ToCSV.

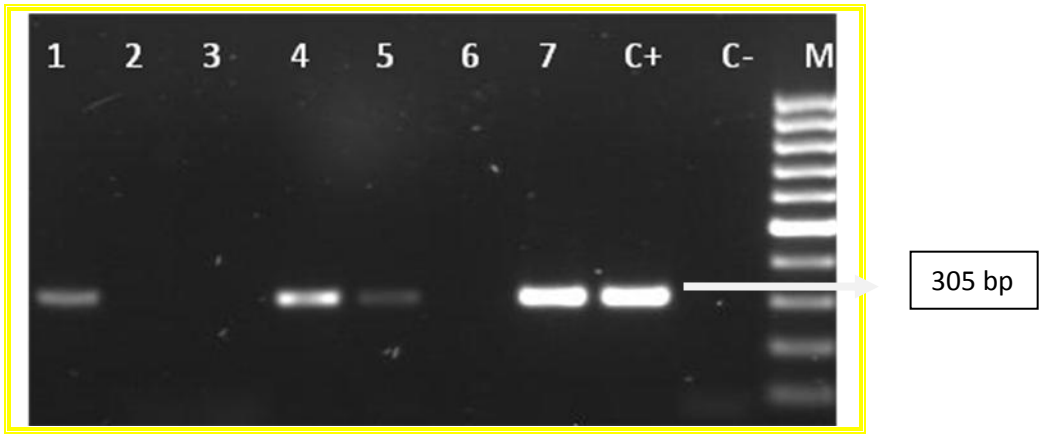


Figure 12. 1% agarose gel depicting amplified PCR products from some representative leaf samples Lane 1-7: experimental samples, lanes 8 and 9, controls (positive and negative respectively), lane 10 is a 100 bp DNA ladder (Fermentas).

These results could have been observed due to many factors, such as inhibitors in the PCR mix, or failure of the primers to amplify due to the presence of different binding sites, suggesting the presence of new species, strains or variants (Brown, *et al.*, 2000*;* Fauquet, *et al*, 2005, 2008; Czosnek and Laterrot, 1997). The observed symptoms could also have been caused by other biotic or abiotic agents, such as bacterial, fungal or soil nutritional deficiency (Collarico, 2003).

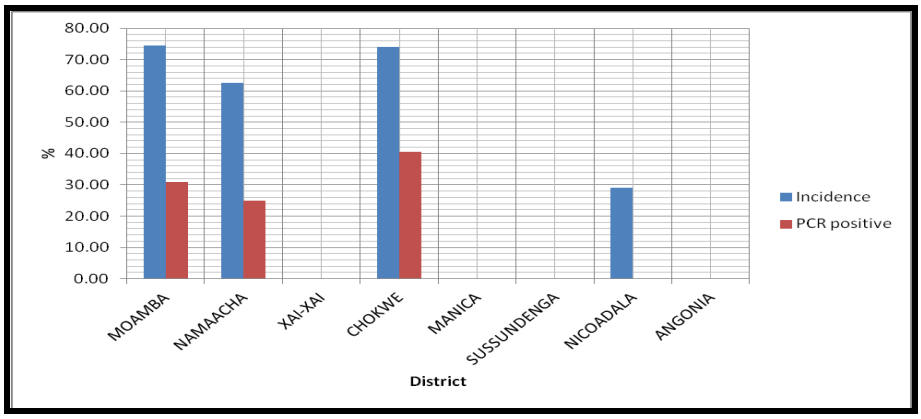


Figure 13. ToCSV DI and PCR positive in survey 21

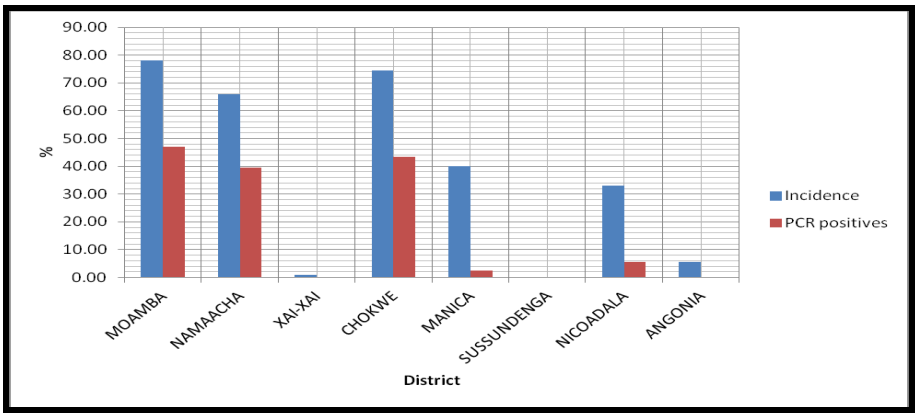


Figure 14. ToCSV DI and PCR positive in survey 2

Failure to amplify ToCSV in many symptomatic plants suggested that further molecular screening tests were needed, and a further study using tools as RCA-RFLP to detect possible diversity of ToCSD in Mozambique was performed (chapter 3) This was followed by cloning and sequencing of selected virus samples showing different RCA-RFLP patterns in order to determine if variability in ToCSD-associated geminivirsues occurring in Mozambique.

In conclusion, the districts in the south of Mozambique (Moamba, Namaacha and Chokwe) had the highest values, in both seasons, for the four evaluated parameters, namely, ToCSV incidence and severity, number of whiteflies (*B. tabaci*) and PCR positive samples, than the other districts surveyed, being the hot/rainy season the most critical for ToCSV transmission, spreading and increasing of whiteflies numbers.

The survey and the PCR based screening tool also brought the overall situation in terms of incidence, severity of ToCSV, whiteflies presence and the reliability of PCR as a screening tool for ToCSV and led to other studies to understand ToCSD and *B. tabaci* diversity in Mozambique.

CHAPTER 3

DIVERSITY OF ToCSD-ASSOCIATED BEGOMOVIRUSES AND *BEMISIA TABACI* IN MOZAMBIQUE

3.1. Abstract

The outbreak of different *B. tabaci* biotypes/types worldwide is associated with the emergence and spreading of monopartite begomoviruses, which are currently believed to be more widely spread geographically than the bipartite viruses, and are associated with significant yields losses in tomato. The attributes responsible for establishment and emergence of begomoviral diseases in tomato, and the large numbers of begomovirus species and strains associated with tomato, can be due to several factors such as multiple infections, mutations and recombination within and between tomato and wild plants, resulting in new species/strains which overcome resistance barriers and adapt to changing environmental conditions. Symptoms, ELISA, DNA hybridization and PCR based methods have been shown to be less efficient to track the changes that occur in begomoviruses. A RCA-RFLP based method has been used for detection and identification of begomoviruses and their satellites. Since ToCSV was detected in South Africa, new virus species and variants, and whitefly types, were recently identified. In order to understand the epidemiology of ToCSD and its whitefly vector, a diversity study was conducted in Mozambique which aimed to establish new strains or species of ToCSV and *B. tabaci* occur in Mozambique. Leaf and whitefly samples collected in the surveys were used for begomovirus and the whitefly type identification using RCA-RFLP and PCR–RFLP, respectively. CP primers were used to amplify the core coat protein (CCP) gene, and full-length genomes were obtained by using the overlapping primers (prAV1134, prAC344: and prV2644, prAC1154). A partial fragment (~810 bp) of the *(mtCOI)* gene (marker), followed by restriction digestion with *Bfa I*, to discriminate

whitefly haplotypes.. Eighteen positives were obtained using the CP primers, 5 different patterns representing variants of ToCSV or new species were found based on RCA-RFLP. Three full length genomes were assembled and shared 70- 82% of nucleotide similarity with ToCSV. The sequence analyses of the CCP revealed three groups (G1, G2 and Manica 71), G1 and G2 were sharing 94-99% of nucleotide similarity within them and Manica 71 was completely isolated with 25-36% of similarity with the other two groups. G1 was closely related to ToCSV from SA in about 74%, G2 and Manica 71 in ~28%. The mtCOI sequence analysis showed that some samples were related to B type, with 86% of similarity, others were related also in 86% with the Q type and others only shared 28-40% of similarity, either with B or Q type, suggesting to be other types.

Keywords: Diversity recombination, sequencing, RCA and RFLP

3.2. Introduction

Bemisia tabaci (Gennadius) (Insecta: Hemiptera:Homoptera: Sternorrhyncha: Aleyrodoidea: Aleyrodidae: Aleyrodinae) is a phloem-feeding, largely polyphagous insect that lives predominantly on herbaceous species. It is a considerable pest of ornamental, vegetable, grain legume, and cotton production, causing damage directly through feeding and indirectly through the transmission of plant pathogenic viruses, primarily begomoviruses. *B. tabaci* is known as a cryptic species complex due its genetic diversity and ability to transmit different viruses. The characterization of new species either by PCR, targeting the mtCO I gene, followed by RFLP or by microsatellite based approaches, have been shown to be strong tools to study *B. tabaci* diversity worldwide (Dinsdale, *et al.,* 2010; De Barro and Ahmed, 2011).

Begomoviruses are an important group of whitefly (*Bemisia tabaci*)-transmitted viruses in the family *Geminiviridae*. They inflict significant economic losses in many dicotyledonous crops including beans, cassava, cotton, melon, pepper, potato and tomato. *Tomato yellow leaf curl virus* (TYLCV), *Tomato leaf curl virus* (ToLCV) and *Tomato curl stunt virus* (ToCSV) are the begomoviruses severely constraining tomato production in many tropical and sub-tropical regions of the world (Czonesk *et al.,* 1997; Pandey *et al.,* 2010; Pietersen *et al.,* 2000) Begomoviruses exhibits great geographic-dependent but host-independent genomic variation in which recombination, especially interspecific homologous recombination, occurs. The devastating cassava mosaic disease (CMD) epidemic caused by recombinant East African cassava mosaic viruses in Uganda and neighboring countries (Polson *et al.,* 1994; Hammed and Robinson, 2004), and the pathogenic recombinant, *Tomato yellow leaf curl Malaga virus*, in Spain (Padidam *et al.,* 1998),Cotton leaf curl disease epidemic in Pakistan (caused by a species complex including a variety of mostly recombinant begomovirus species (Delatte *et a.*, 2005)), are good examples among the others that have been reported worldwide.

Begomovirus genomes are composed of either one (monopartite) or two (bipartite) single stranded circular DNA molecules ranging in size between 2500 and 2800 nucleotides. Most TYLCVs of the Old World, and almost all known New World begomoviruses are bipartite with genomes comprising DNA-A and DNA-B molecules. Monopartite Old World begomoviruses, which are now believed to be the predominant begomovirus form, have only a DNA-A like genome component.

Although many natural begomoviruses recombinants have been reported so far, the biological significance of begomovirus recombination is not clearly understood. Besides the apparent importance of recombination in begomovirus evolution, this can have major implications when we attempt to use these sequences to infer the evolutionary histories of begomoviruses (Garcia-Andre, *et al.*, 2006; 2007). Consequently, the detailed characterization of recombination amongst tomato-infecting begomoviruses is a prerequisite for understanding how these important pathogens are evolving.

PCR, ELISA and hybridization based assays have been shown to be less effective to detect recombinants due the limitation of these methods, such as the previous knowledge of the sequence, need of machines, and high possibility of false positives To overcome these inconveniences and keep tracking and understanding the dynamic of begomoviruses, a technique known as rolling circle amplification (RCA) was developed to amplify circular DNA. RCA is an isothermal method which uses the DNA polymerase of the *Bacillus subtilis* bacteriophage phi29 (Φ29), a monomeric enzyme that possesses a polymerase activity located on the C-terminal domain and a 3'–5'-exonuclease activity within its N terminal domain involved in proofreading function, resulting in an error rate of only 1 in 106–107, approximately 100 times more accurate than *Taq* DNA polymerase, (Jeske 2007, Schubert *et al.*, 2007).

During begomovirus rolling circle replication and recombinant-dependent replication, circular intermediates are produced, that makes RCA ideal for begomovirus detection. RCA products can be used for RFLP to discriminate the species, strains or variants, or can be sequenced directly for whole genome sequencing.

Following the initial identification of ToCSV in SA and Mozambique, further molecular and field work was carried out and a new begomoviral species and several ToCSV strains/variants were found in SA (Esterhuizen *et al., unpublished*). Due to this finding, and the fact that several symptomatic tomato plants did not amplify ToCSD-associated viruses, using the specific primers for the ToSCV-type virus, a diversity study of ToCSV and it vector was conducted in Mozambique.

3.3. Materials and methods

3.3.1. ToCSV diversity

3.3.1.1. Screening for other tomato begomoviruses

TNA of 60 symptomatic samples that were negative in the screening for ToCSV (Chapter 2) were used to screen for the presence of potentially other begomoviruses, using the degenerated universal primers (Wyatt and Brown, 1996), which yield a product of ~570 bp, corresponding the core of the coat protein (CP) gene not overlapping with other ORFs.

The PCR reaction was as follow: 1x PCR buffer, 0.5mM of $MgCl_2$, 0.2mM of each dNTP, .1U of DreamTaq DNA polymerase (Fermentas, Thermo Scientific, USA) and 0.4µM of each primer (CPR 1 5´- GCC CAT GTA (T/C) CG(A/G) AAG CC-3´ and CPF 2 5´-GG(A/G) TTA GA(A/G) GCA TG(A/C) GTA C-3´). The cycling conditions were: initial denaturation at 94ºC for 1 min, 35 cycles of 94ºC for 30 sec, 57ºC for 20 sec and 72ºC for 30 sec ending with a final extension at 72ºC for 5 min. The PCR products were then ran on a 1% agarose gel in 1x TAE buffer, stained with Ethidium Bromide (10mg/ml), visualized in an UV light, and photographed on a Gel Doc system.

3.3.1.2. Rolling Circle Amplification (RCA) and RFLP

Positive samples, and TNA from 12 symptomatic samples that were still negative in the screening using the CCP universal primers, were used to perform RCA, using the TempliPhi™ Kit (GE Healthcare, Munich, Germany) following the manufacturer's protocol. Briefly, 2µl (10ng to 20ng) of total nucleic acids were dissolved in 5µl of sample buffer, denatured for 3 min at 95 °C and cooled down for 1 min on ice followed by addition of 5µl reaction buffer and 0.2µl enzyme mix, in a final volume of 10µl. The amplification was for 16-20 h at 30°C and stopped for 10min at 65°C. Aliquots of the RCA products corresponding to 300ng were digested with *Hpa*II restriction enzyme (Fermentas, Thermo Scientific, USA) and ran in 2% agarose gel in 1x TAE buffer, stained with Ethidium Bromide (10mg/ml), visualized in an UV light, photographed and managed in a Gel Doc system.

3.3.1.3. Full length amplifications

TNA of 3 representative samples showing different patterns on RCA-RFLP, were used to amplify the full length genome, using overlapping primers designed by Idris and Brown (1998) which cover the entire genome of most of the monopartite begomoviruses (~2.8 kbp). Two PCR reactions were set as follow: 1x PCR buffer, 0.5mM of $MgCl_2$, 0.2mM of each dNTP, 0.1U of DreamTaq DNA polymerase (Fermentas, Thermo Scientific, USA) and 0.4µM of each primer (**pr AV 2644**: 5´- ATT ACC GGA TGG CCG C -3´, and **pr AC 1154**: 5´ CT(G/C) AA(C/T) TTC (A/C)AA GT(C/T) TGG ACG -3´, for one PCR and **pr AC 344**: 5´ - CT(G/T) GGC TT(C/T) CT(A/G) TAC AT(A/G) GGC - 3´ and **pr AV 1134**: 5´- CGT CCA (A/G)AC TT(G/T) GAA (A/G)TT (G/C)AG - 3´ for the other PCR). The cycling conditions were: initial denaturation at 94ºC for 1 min, 35 cycles of 94ºC for 30 sec, 57ºC for 20 sec and 72ºC for 30 sec

ending with a final extension at 72ºC for 5min. The PCR products were then ran on a 1% agarose gel in 1x TAE buffer, stained with Ethidium Bromide (10mg/ml), visualized in an UV light, photographed and managed in a Gel Doc system.

3.3.2. *Bemisia tabaci* diversity

3.3.2.1. DNA extraction

DNA was extracted from each single whitefly collected during the surveys by grinding in 40µl of lyses buffer (10mM Tris, 1mM EDTA, pH 8.0, Tween-20 and ddH_2O) followed by two incubation, first at 65ºC and the second at 95ºC for 20 and 5 minutes, respectively, then placed on ice for 5 minutes and stored at -20ºC until needed.

3.3.2.2. PCR and RFLP

Four (4)µl of the DNA extract were used in a 20µl PCR reaction, using the mtCOI marker primers, with an expected size of ~810 bp. The conditions were the following: 1x PCR buffer, 0.2mM of each dNTP, 0.4µM of each primer (C1-2195 5´ TTGATTTTTTGGTCATCCAGAAGT 3´ and TL2-N-3014 5´ TCCAATGCACTAATCTGCCATATTA 3´) and 0.1U of DreamTaq DNA polymerase (Fermentas, Thermo Scientific, USA). The cycling conditions were the following: initial denaturation at 94ºC for 2 min, 35 cycles of 92ºC for 90 sec, 60ºC for 45 sec and 72ºC for 60 sec ending with a final extension at 72ºC for 5min. Five (5)µl of the PCR product were ran on 1% agarose gel in 1x TAE buffer, stained with Ethidium bromide (10 mg/ml), visualized in an UV light, and photographed and saved on the Gel Doc system. The remaining PCR product was digested using *Bfa I* restriction enzyme (Fermentas, Thermo Scientific, USA), by incubation for

16h at 37ºC. The digestion products were run on 2.5 % agarose gel and treated as described before.

3.3.3. Cloning and sequencing

3.3.3.1. Virus samples

PCR products with a single band of ~570 bp, were cleaned up using a kit (Roche), following the manufacturer instruction and cloned into pTZ57 R/T cloning vector (Fermentas, Thermo Scientific, USA) using the protocol of the manufacturer. Inserts were checked by PCR using CP primers and sent for sequencing.

PCR amplicons derived from the primer pairs **pr AV 2644, pr AC 1154** and **pr AC 344, pr AV 1134** with an expected sizes of 1156 bp and 1747 bp, respectively, were cloned into pTZ57 R/T cloning vector (Fermentas, Thermo Scientific, USA) using the protocol of the manufacturer. Inserts were checked by PCR using the vector primers and were sent off for sequencing in triplicates.

3.3.3.2. Whiteflies samples

Samples representing the different RFLP patterns following digestion with *Bfa I*, were selected. PCR products of these samples, with a single band of ~810 bp, were cleaned up using a commercial kit (Roche), following the manufacturer instruction, and cloned into pTZ57 R/T cloning vector (Fermentas, Thermo Scientific, USA) using the protocol of the manufacturer. Inserts were checked by PCR using mtCO I primers and sent off for sequencing in triplicates.

3.3.4. Sequence analysis

The sequences obtained were trimmed and edited, blasted, using NCBI BLAST query and aligned. From the alignment, multiple comparisons and phylogenetic trees with 1000 bootstrap were created using the neighbor joining and maximum likelihood methods. All the analyses, excluding the BLAST, were performed using CLC Bio workbench and Geneious softwares. Relevant reference sequences (appendix 4) either for the virus as well as for the whiteflies studies were accessed and obtained in the Genebank and used to do the alignment and phylogenetic analysis.

3.4. Results and Discussion

3.4.1. Screening for ToCSD diversity

From the 60 samples tested using the CCP primers, 18 tested positive in PCR (figure 15). From this number, 17 were from the south of Mozambique and one from Manica district (centre of Mozambique), Moamba, the district bordering with Mpumalanga, SA, where ToCSD was observed for the first time, had the highest number of CCP positives (7), followed by Chokwe and Namaacha, with 5 each.

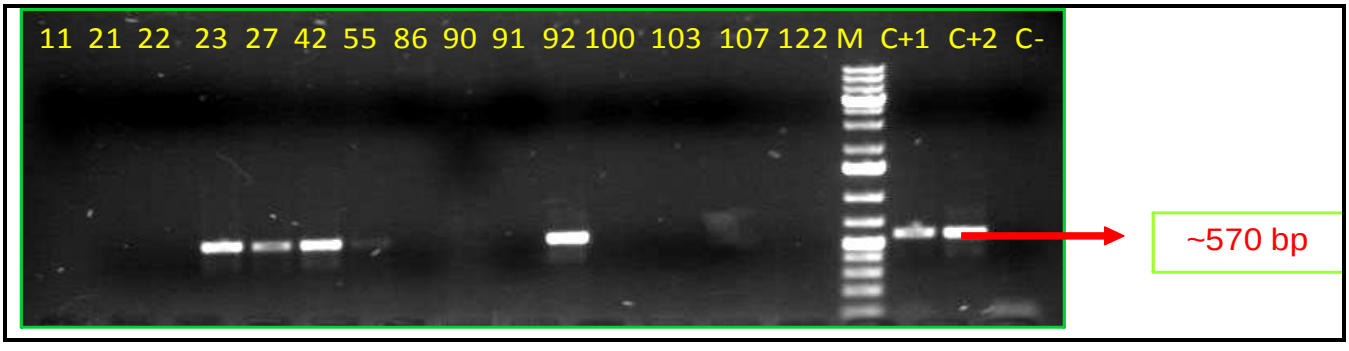


Figure 15. 1% agarose gel showing the 570 bp PCR virus amplicons from representative samples using CCP primers. All lanes before the ladder (M) are the selected symptomatic samples. C+1 and C+2 – clones of positive samples given by Dr Esterhuizen, Johannesburg University, SA), C- - negative control.

The PCR results suggest that the CCP primers. are useful to pick more diversity, although they can miss or fail to amplify if the binding site of the primers changes. Besides the change on the primer binding region, as result of a recombination or pseudo recombination or other genetic variation, PCR inhibitors and DNA quality can also affect the detection, mainly in degenerated primers, as are the CCP. So other tools such as RCA followed by RFLP, and sequencing can resolve some of the PCR limitation and be helpful to detect and identify begomoviruses diversity (Jeske 2007).

3.4.2. RCA and RFLP to detect ToCSD diversity

The RCA-RFLP analysis revealed 5 different DNA patterns (figure 16 and 17). Three patterns with 4 to 5 bands, were from Moamba, Chokwe and Namaacha (south of Mozambique) (figure 16) and 10 samples from Manica and Zambezia (centre of Mozambique) that were negative in all PCR´s ran, showed 2 patterns (figure 20) completely different (with 7-10 bands), indicating that other circular genomes were present and amplified using RCA and have a *Hpa II* restrictions sites.

In all analyzed samples it was possible to observe that despite some differences in terms of RFLP patterns, in the south of Mozambique, there was a dominant pattern (figure 16, lanes 1-4,6,8 and 12), which probably represents strains closely related to the ToCSV – type virus. The other RFLP patterns, with 3 - 5 bands (figure 16, lanes 5, 9, 10 and 11), might be new species deriving from ToCSV, event that is common on begomoviruses, being this results concordant to Zhou, *et al.,* (1997); Padidam *et al.* (1999); Nawaz-ul-Rehman and Fauquet, (2009), which found novel species originated from recombination.

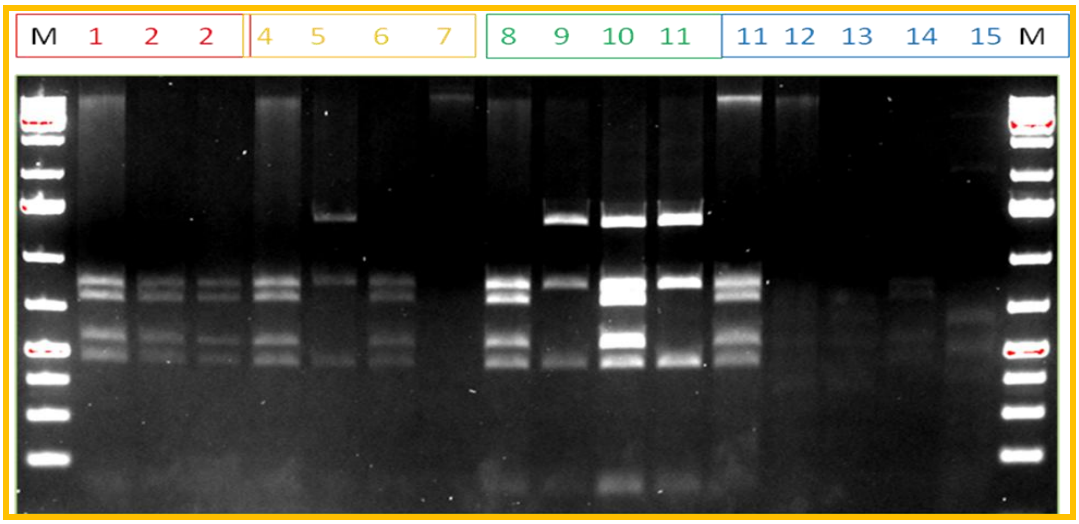


Figure 16. 2% agarose gel showing 3 different representative patterns of representative samples, after digestion of RCA products with *Hpa II*. Lanes 1-3, samples from Boane district, lanes 4-7, samples from Namaacha district, lanes 8-11, samples from Chokwe, lanes 12-15, samples from Moamba, M- 1 kb DNA ladder.

Interestingly, is samples from the same districts, Chokwe, Namaacha and Moamba (figure 16), showed different patterns, which can be an indication that more than one strain, variant or specie can occur in the same location. Suggesting that the capacity to adapt and variation can originate multi infections detectable by RCA-RFLP, what is in concordance with Wyatt *et al*, (2011), which reported new viruses associated to tomato based on RCA/RFLP.

The banding pattern observed in the figure 17 (lanes 1, 3, 4, 6-9) suggest that a different virus isolate is causing a ToCSD-like in the centre of Mozambique, because the pattern is completely different from the one found on samples from the south of Mozambique (figure 16). A comparison with samples from South Africa and Zimbabwe that are the neighboring countries with most of the areas where the samples were collected could be helpful to understand the differences and clarify other issues related with ToCSD diversity and virus spreading.

The detection of genetic diversity, illustrated by different or polymorphic RFLP banding patterns following RCA-RFLPs, is not surprising if one considers that begomovirus evolution and spreading is driven by recombination and mutational events, which is also helped by the different haplotypes of *B. tabaci* biotypes, which are associated with begomoviruses, throughout the world, as found in several studies related to begomovirus variability (Brown, *et al.*, 1996, 2000, 2005, Paddidam, *et al.*, 2010; Davino *et al.*, 2008; De Barro, *et al.*, 2011; Wyatt *et al.*, 2011; Rey *et al.*, 2012;).

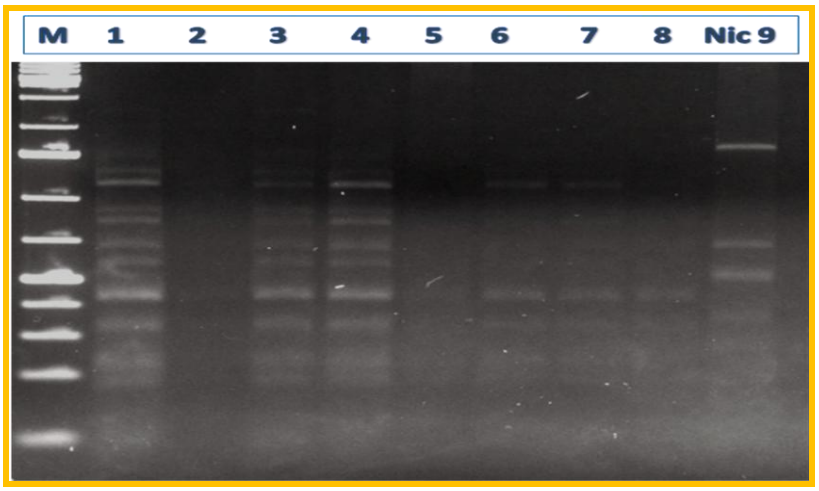


Figure 17. 2% agarose gel showing 2 different representative patterns after digestion of RCA amplified virus products with *Hpa II*. Lanes 1-8, samples from Manica, lane 9, sample from Nicoadala, M- 1 kb DNA ladder.

The first approach to sequence the full length of those different patterns, was the digestion of the RCA products using either *BamH I or Hind III*, expecting a single band of ~ 2.8 kbp, according to the restriction maps analysis performed on related begomoviruses. The samples from the south Boane, Namaacha and Moamba (south of Mozambique) behaved, in general, as expected for *BamH I*, yielding a single band of ~2.8 kbp (figure 18).

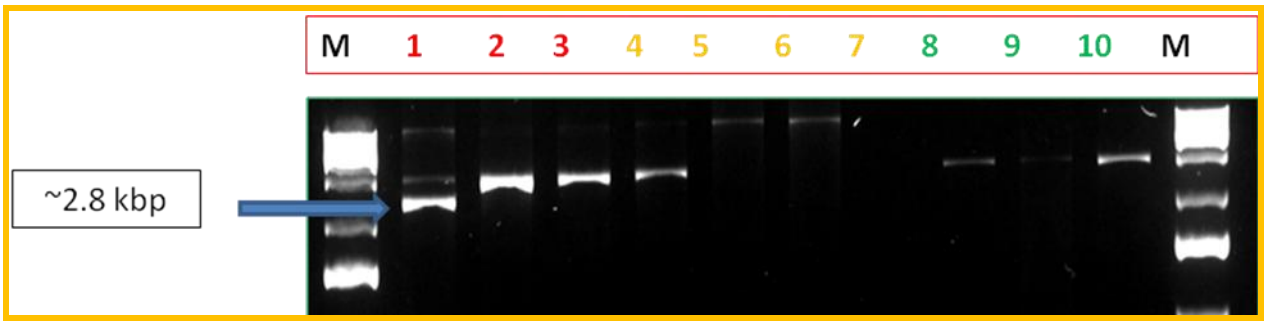


Figure 18. 1% agarose gel showing RCA products digested with BamH I. lanes 1-3, samples from Boane, lanes 4-7 samples from Namaacha, lanes 8-10, samples from Moamba, M- 1 kb DNA ladder.

The samples from Manica and Nicuadala, failed to be cut with *BamH I*, but with *Hind III*, were possible to obtain the ~2.8 kbp fragment (figure 19). This suggests that their genome lack a *BamH I* restriction site, indicating that may be coming from different begomovirus source inducing ToCSD-like symptoms (Fauquet *et al.*, 2009) or is a different pathogen.

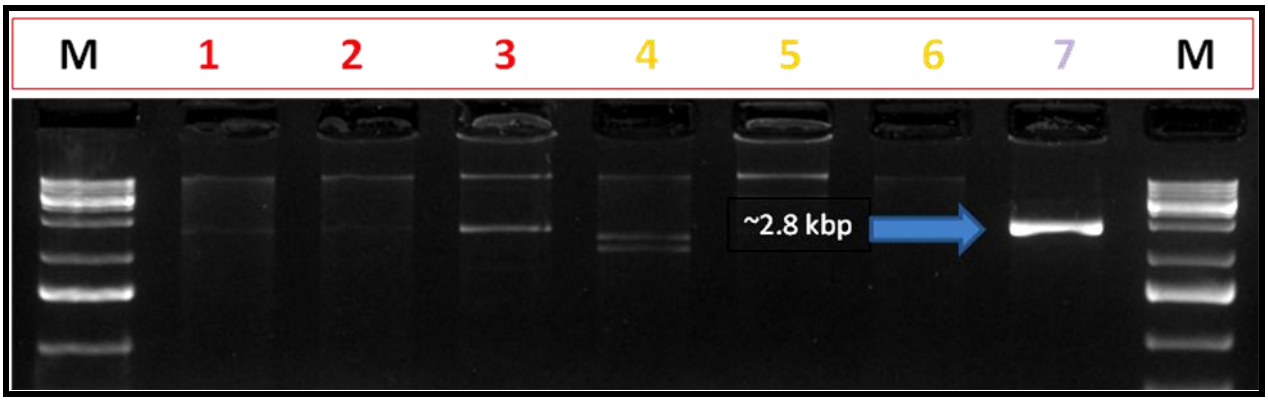


Figure 19. 1% agarose gel showing RCA amplified virus products digested with Hind III lanes 1-3, samples from Manica, lanes 4-7 samples from Nicuadala, lane 7, M- 1 kb DNA ladder

The sequencing of these ~2.8 kbp fragments obtained either using *BamH I* or *Hind III*, failed to be done. No transformed colonies were obtained, thus the approach had to be changed, and the full length sequences for some of the samples were generated using the overlapping primers. In

some samples it was possible to amplify with only one of the primer pair and those were discarded for the purpose of this dissertation.

3.4.3. B. tabaci identification by PCR and RFLP

A total of 80 samples from the surveyed districts were analyzed by PCR and a fragment of ~810 bp was obtained in 57 samples (figure 20). From the 57 samples, 30 were digested with *Bfa I*, and showed a complex of DNA banding patterns (figure 21), and the most predominant was the *B. tabaci* Q type and related (figure 21, lanes 1,2, 4), which was for a long time known to be endemic in the Mediterranean basin (Ghanim *et al*, 2001a), but recent studies revealed that this type is spreading and displacing the B type in tropical countries including South Africa (De Barro *et al.*, 2011; Esterhuizen *et al*, 2012), and is now reported for the first time in Mozambique.

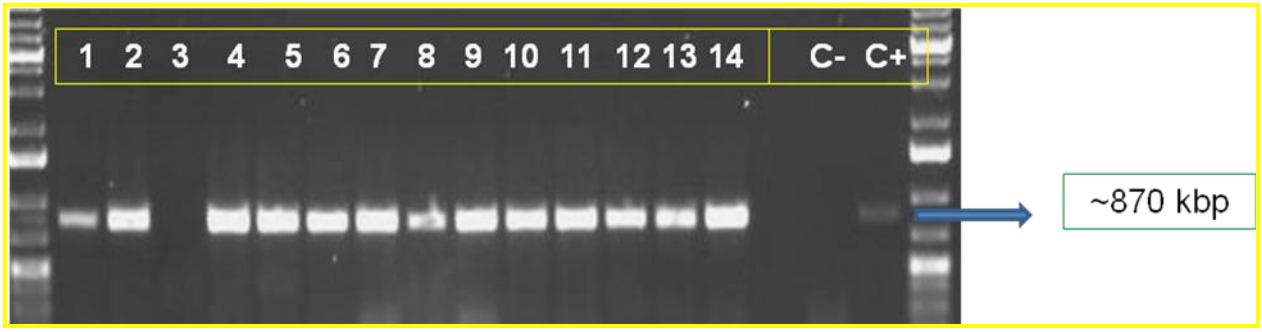


Figure 20. 1% agarose gel, showing *mtCO I* amplicons from whitefly samples. Lanes 1-14, test samples, C-, negative control. C+, positive control, 1kb DNA ladder.

So far no B type was found in the analyzed samples (figure 21), suggesting that this type may be being replaced by Q and other haplotypes. The B, Q types, and a new subclade, SSAF 5 (Esterhuizen *et al*.,2012), has been found in South Africa, and whitefly types are able to transmit

ToCSD It also speculated that the B biotype, so prevalent in the 1980's in South Africa, may have been displaced in some regions in the country (Esterhuizen *et al*.,2012).

Since the Mozambique and South Africa "corridor" border on each other, and both are toamt-growing areas, it is not unreasonable to speculate a similar scenario in the two countries.

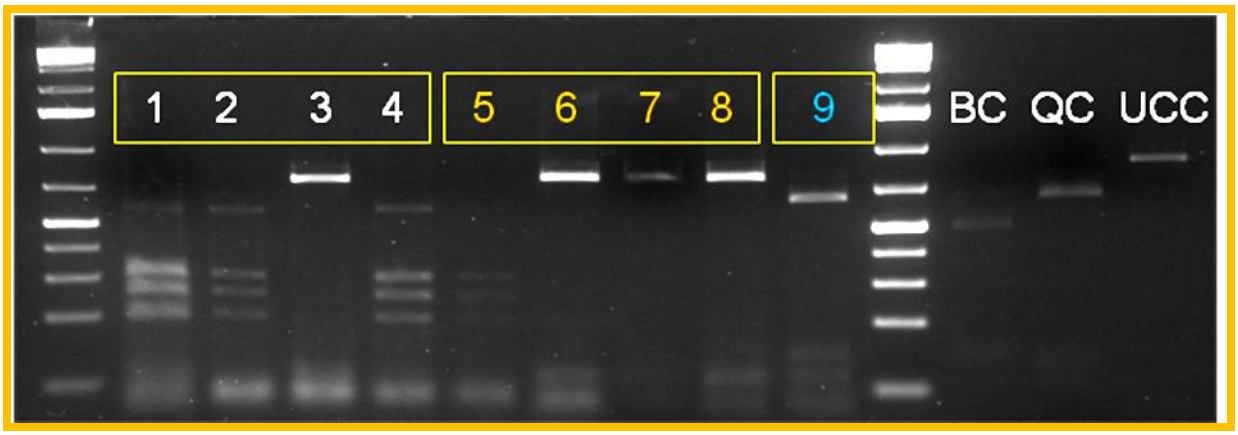


Figure 21. 1% agarose gel showing the banding pattern, from representative whitefly samples, of *mtCOI* amplicons digested with *Bfa I.* Lanes 1-9, test samples. BC, B type control. QC, Q type control, UCC, uncut control, 1kb DNA ladder.

Pesticide resistance is one of the events that drive the emergence of new genetic types with the ability to displace, colonize and transmit viruses more efficiently than native or local populations (De Barro *et al*., 2011; Deying, *et al*, 2008; Luo, *et al.*, 2009), could be one of the reasons for the increase in ToCSD distribution and spread in SA and Mozambique over the past decade. Inappropriate crop management, including excessive pesticide application, is observable in both SA and Mozambique (personal observations). The exotic Q type was most certainly introduced through contaminated ornamental crops as this is not found in southern Africa. In SA, it is thought the Q type entered via contaminated plant material in Port Elizabeth (Esterhuizen *et al*., 2012). However larger sampling in more districts and provinces in Mozambique is required to

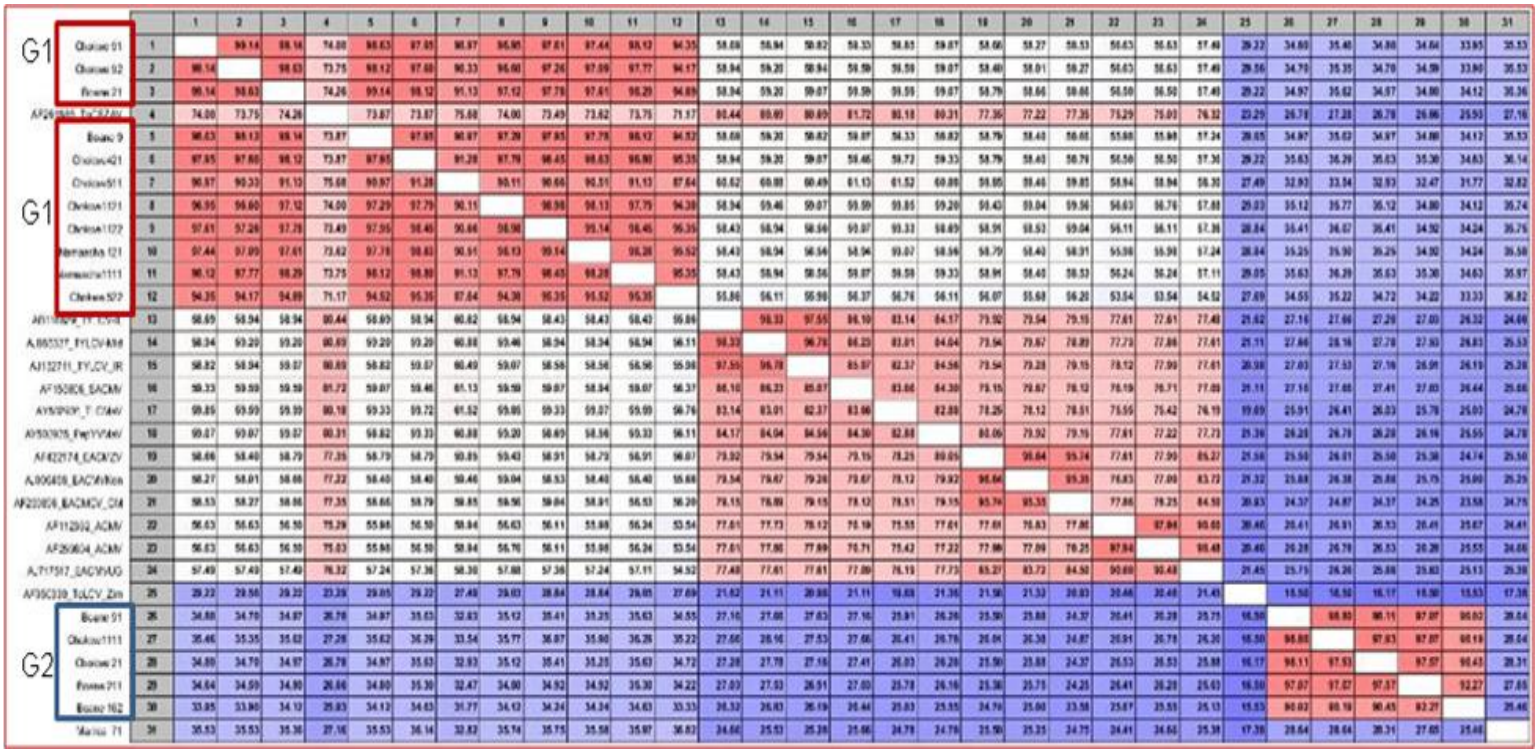
conclude that the B type has been replaced and find out if other types are transmitting ToCSD in Mozambique.

3.4.4. Sequence analysis

3.4.4.1. Virus sequences

The pairwise comparisons of the core coat protein (CCP) sequence, revealed three groups named as G1, G2 and Manica 71 (last sequence in the table 4) which are genetically divergent in nucleotide sequence with about 27-35% of similaritybetween G1 with G2 and Manica 71 and about 25-29% between G2 and Manica 71 (table 4). Within G1 and G2, the nucleotide similarity was about 92-99%, indicating that whatever nucleotide changes occurred, and the divergence between the groups, the primers binding sites were still conserved. G1 was closely related to ToCSV (AF261885, Pietersen *et al.,* 2008) sharing 71-76% and this similarity could indicate the presence of new species and strains, according to the threshold of 85% - 90%, established to demarcate begomovirus strain and species (Fauquet *et al,* 2009), but the CCP region is not long enough to conclude if the divergence is due to speciation or strain variation, although Wyatt and Brown, 1996, indicates otherwise. G2 and Manica 71 were only sharing 23-28% of similarity with ToCSV (table 4) and similar assumption can be done and looking the nucleotide similarity is likely more probable to be a new species.

Table 4. Pairwise nucleotide comparison between reference coat protein virus sequences and Mozambican isolates



G1, G2 and Manica 71 shared about 60% and 21-28% nucleotide sequence similarity with other begomoviruses, such as *Tomato yellow leaf curl virus* - Israel (TYLCV-IL), TYLCV-mld, *East African cassava mosaic virus* (EACMV) (and related strains), and *Pepper Yellow mosaic virus* (PepYMV). Sequence analysis based on the CCP is limiting, and full-length sequencing is key to determine the overall similarity between putative virus species/strains, and classify the suspected new types according to the ICTV criteria (ICTV, 2009).

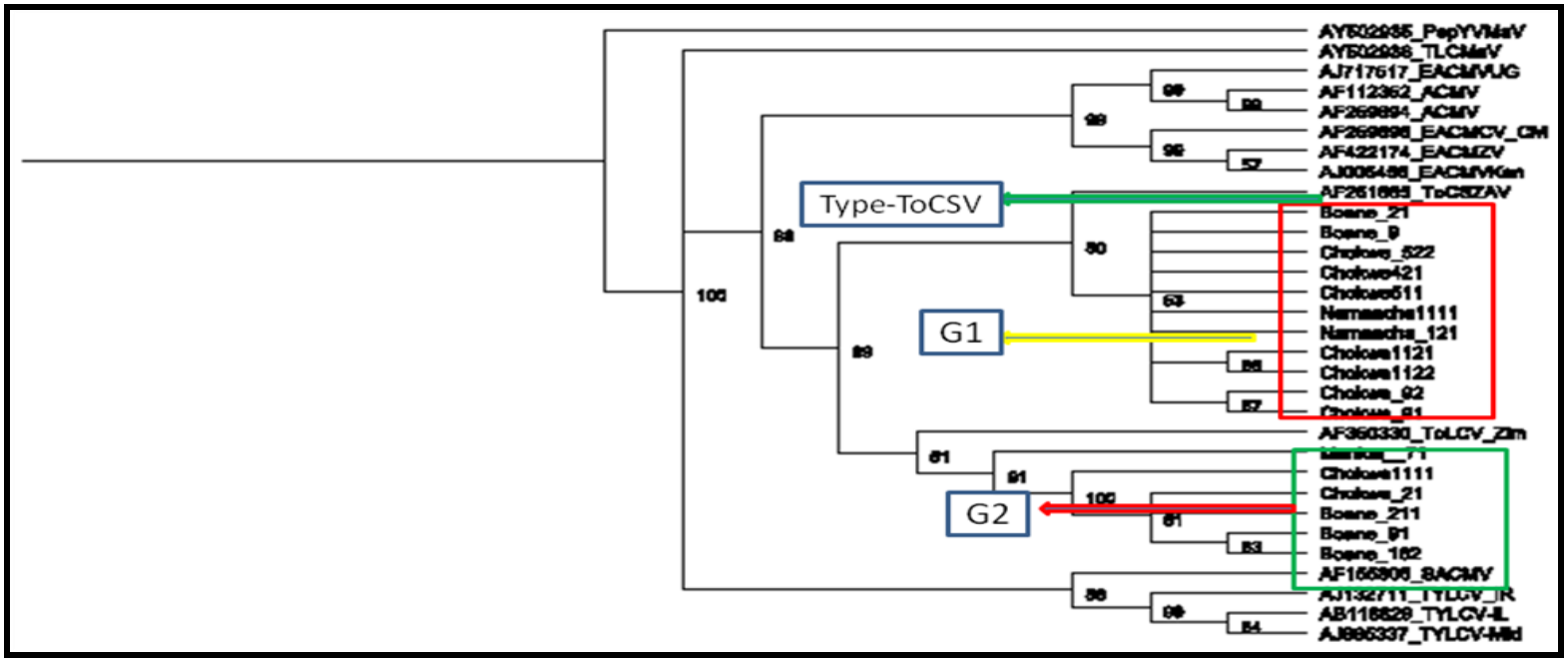


Figure 22. Phylogenetic tree showing the relationship between Mozambique virus isolates (G1 and G2) and reference virus sequences using neighbor joining method

The phylogenetic tree (figure 22), based on neighbor joining method, illustrate better the previous analysis based on the pairwise comparison (table 4), showing that G1 was closely related to ToCSV and the oldest begomoviruses, G2 and Manica 71 are more related to begomoviruses in the southern African region, such as *Tobacco Leaf curl Virus-Zimbabwe* (ToLCZbV) and *East African Cassava Mosaic Virus-Uganda* (EACMV-UG), which are considered new viruses when compared to others. The node support of 61% at the branch between ToLCZbV and the G2 group suggests that some event, possibly recombination, led to the emergence of these new viruses (Rey *et*

al., 2012). The tree also shows that the TYLCV, ACMV/EACMV and other tomato viruses have a common ancestor. However full-length sequences will shed more light on the evolution of these groups of southern African begomovirus clades.

Table 5. Nucleotide sequence similarity between reference full length virus sequences and Mozambican isolates

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
ToCSV Moz 1	1		79.70	81.28	69.83	67.47	68.40	65.06	66.77	64.92	61.71	61.40	59.02	64.33	65.09	61.01	61.23	65.18
ToCSV MOZ 3	2	79.70		82.83	77.05	69.57	70.14	60.62	68.33	66.45	61.82	61.66	59.19	64.73	65.03	61.75	61.90	66.82
AF261885_ToCSZAV	3	81.28	82.83		74.66	82.81	80.03	68.30	76.66	74.66	70.55	70.27	67.49	73.43	74.19	70.36	70.50	75.86
ToCSV Moz 2	4	69.83	77.05	74.66		65.06	65.10	56.09	63.42	61.51	56.92	57.49	54.65	59.74	60.27	57.09	57.30	62.20
AF350330_ToLCV_Zim	5	67.47	69.57	82.81	65.06		81.08	67.11	75.53	73.73	68.92	68.22	66.62	70.93	72.35	68.87	69.01	74.23
AY502936_TLCMaV	6	68.40	70.14	80.03	65.10	81.08		69.50	77.88	77.01	71.45	70.86	67.89	74.50	76.07	71.37	71.44	77.02
AB116629_TYLCV-IL	7	65.06	60.62	68.30	56.09	67.11	69.50		82.92	82.52	65.84	65.64	63.46	67.94	70.66	65.47	65.47	69.42
AJ865337_TYLCV-Mld	8	66.77	68.33	76.66	63.42	75.53	77.88	82.92		89.32	71.16	70.77	67.78	73.38	77.10	71.94	72.09	76.47
AJ132711_TYLCV_IR	9	64.92	66.45	74.66	61.51	73.73	77.01	82.52	89.32		70.92	70.71	67.71	72.76	75.19	70.24	70.49	75.29
AJ006458_EACMVKen	10	61.71	61.82	70.55	56.92	68.92	71.45	65.84	71.16	70.92		92.43	87.15	86.21	79.52	67.69	68.08	70.40
AJ717517_EACMVUG	11	61.40	61.66	70.27	57.49	68.22	70.86	65.64	70.77	70.71	92.43		84.63	82.97	78.63	71.32	71.74	70.37
AF259896_EACMCV_CM	12	59.02	59.19	67.49	54.65	66.62	67.89	63.46	67.78	67.71	87.15	84.63		77.87	71.17	65.43	65.67	69.16
AF422174_EACMZV	13	64.33	64.73	73.43	59.74	70.93	74.50	67.94	73.38	72.76	86.21	82.97	77.87		80.96	69.66	70.12	71.90
AF155806_SACMV	14	65.09	65.03	74.19	60.27	72.35	76.07	70.66	77.10	75.19	79.52	78.63	71.17	80.96		71.88	72.34	74.51
AF112352_ACMV	15	61.01	61.75	70.36	57.09	68.87	71.37	65.47	71.94	70.24	67.69	71.32	65.43	69.66	71.88		96.76	69.55
AF259894_ACMV	16	61.23	61.90	70.50	57.30	69.01	71.44	65.47	72.09	70.49	68.08	71.74	65.67	70.12	72.34	96.76		70.05
AY502935_PepYVMaV	17	65.18	66.82	75.86	62.20	74.23	77.02	69.42	76.47	75.29	70.40	70.37	69.16	71.90	74.51	69.55	70.05	

The overlapping primers failed to amplify the manica 71 sample, reinforcing it difference in term of virus source that caused the ToCSD-like symptoms. Analyzing the nucleotide similarity of the three full length genomes (from the South of Mozambique), generated by assembling the overlapping fragments, named as ToCSV Moz 1 (Moz 1), ToCSV Moz 2 (Moz 2), both from Chokwe and ToCSV Moz 3 (Moz 3), from Moamba, was possible to notice that they were sharing 79.70% between Moz 1 and Moz 3, 69.83% between Moz 1 and Moz

2 and 77.02% between Moz 2 and Moz 3 (table 5). Comparing Moz 1, Moz 2 and Moz 3 with the type ToCSV (AF261885, Pietersen *et al.*, 2008) the nucleotide similarity was 81.28%, 74.66% and 82.83%, respectively indicating that there are at least three new and distinct viruses inducing ToCSD-like symptoms in Mozambique, according to the threshold of 88% of nucleotide similarity, defined by ICTV for species demarcation (ICTV, 2009; Fauquet *et al.,* 2009). The type ToCSV, was much closer to Moz 1 and Moz 3 (81.28%, and 82.83%, respectively) than Moz 2 (74.66%).

The phylogenetic tree suggests that the three viruses evolved from the type-ToCSV (figure 23) and the alignment showed that the nucleotide variations were random and occurred in all genes. The full length results indicated that changes had occurred through the time via one of the event that drives Begomovirus evolution, as well as the adaptability of *B. tabaci* to new niches, especially those derived from excessive use of pesticides and poor crop management (Collarico 2003; De Barro, *et al.,* 20011; Esterhuizen, *et al.*, 2012; Rey *et al.,* 2012). Though, more full lengths sequences are needed to have a broad picture and have a strong conclusion. Interestingly, in Chokwe two different species were found and this led to other study to know if they can mix infect the same host.

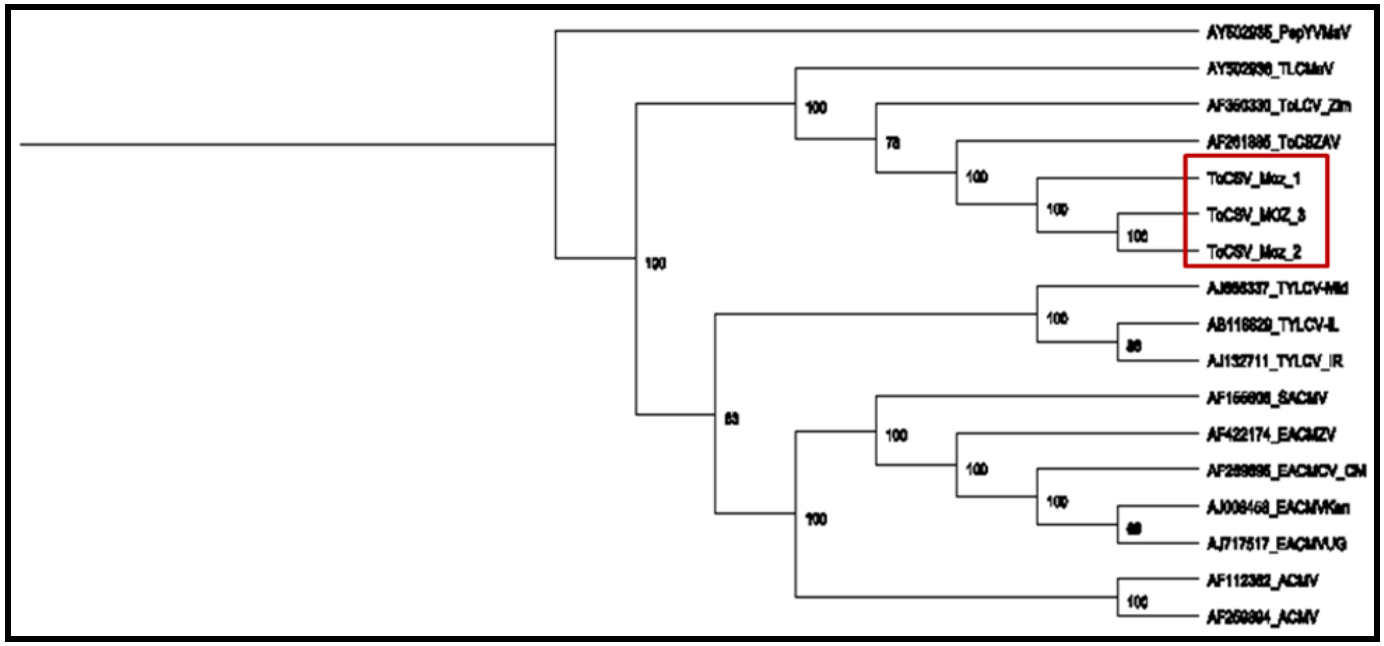


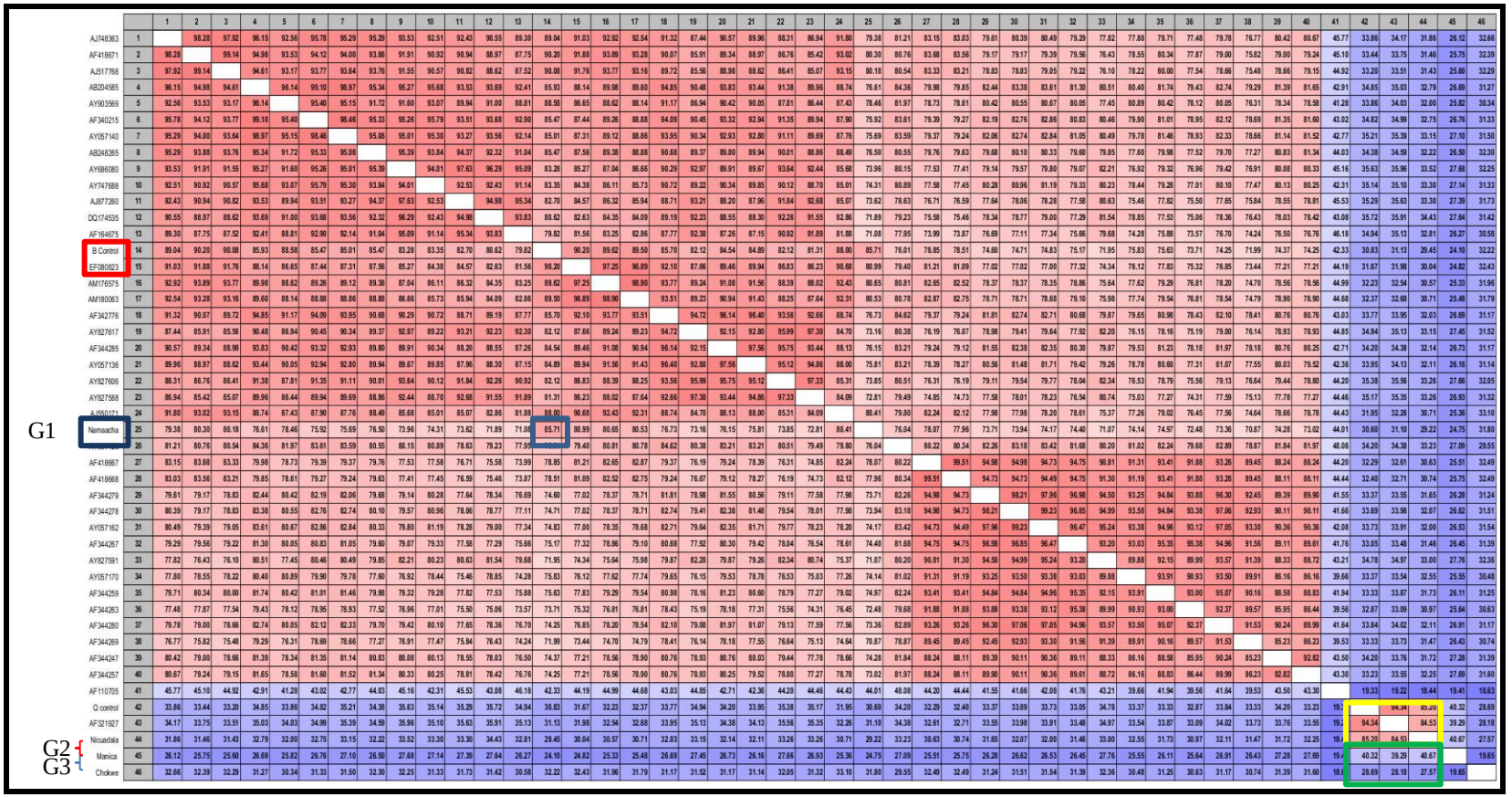
Figure 23. Phylogenetic tree showing the relation between Mozambique virus isolates (G1 and G2) and reference sequences neighbor joining method

3.4.4.2. Whiteflies sequences

The nucleotide comparison of the *mtCOI* sequences (table 6) revealed that the tested whiteflies belong to three different groups, G1, G2 and G3. The first group (G1) shared a nucleotide similarity of about 85% with the B type (B control), 34.20% with the Q type (Q control), what are percentages bellow the accepted (95%) to consider either B or Q types. G1 also share ~34% with G2 and 27-30% with G3. G2 shares 84-95% with the Q type, what is consistent to the patterns obtained on the RFLP analysis. G3 is an isolate group that did not share

significant similarity within the group as well as with the others two groups. The results analysis of the whiteflies sequences from this study reinforce the reports by De Barro *et al*, 2011 and Esterhuizen *et al.*, 2012, where they showed that there are at least four distinct sub-Saharan clades of whiteflies implicated in begomoviruses transmission, and that the two whitefly isolates on G3 could be one of these. However, infectivity tests and wider sample analyses are needed to make any further conclusions. Nonetheless, the results obtained in this survey are good indication and confirmation that of genetic complexity of the genus *Bemisia*, and in particular the species complex *Bemisia tabaci*. There is currently no consensus amongst the whitefly taxonomy groups (DE Barro *et al., 2011*; Dinsdale *et al.*, 2010; BROWN *et al.*, 2009) as to what nucleotide sequence divergence cutoff constitutes a new species, and while 3% has been suggested by De Barro *et al.*, 2011. More molecular marker and biological data will be required to resolve this problem.

Table 6. Nucleotide sequence similarity between reference *B. tabaci* sequences and isolates from Mozambique



The phylogenetic tree (figure 24) grouped the tested whiteflies samples in two groups, G1 and G2, which are closely related to the B type and Q type, although the branch nodes is not supportive for the B type related group.

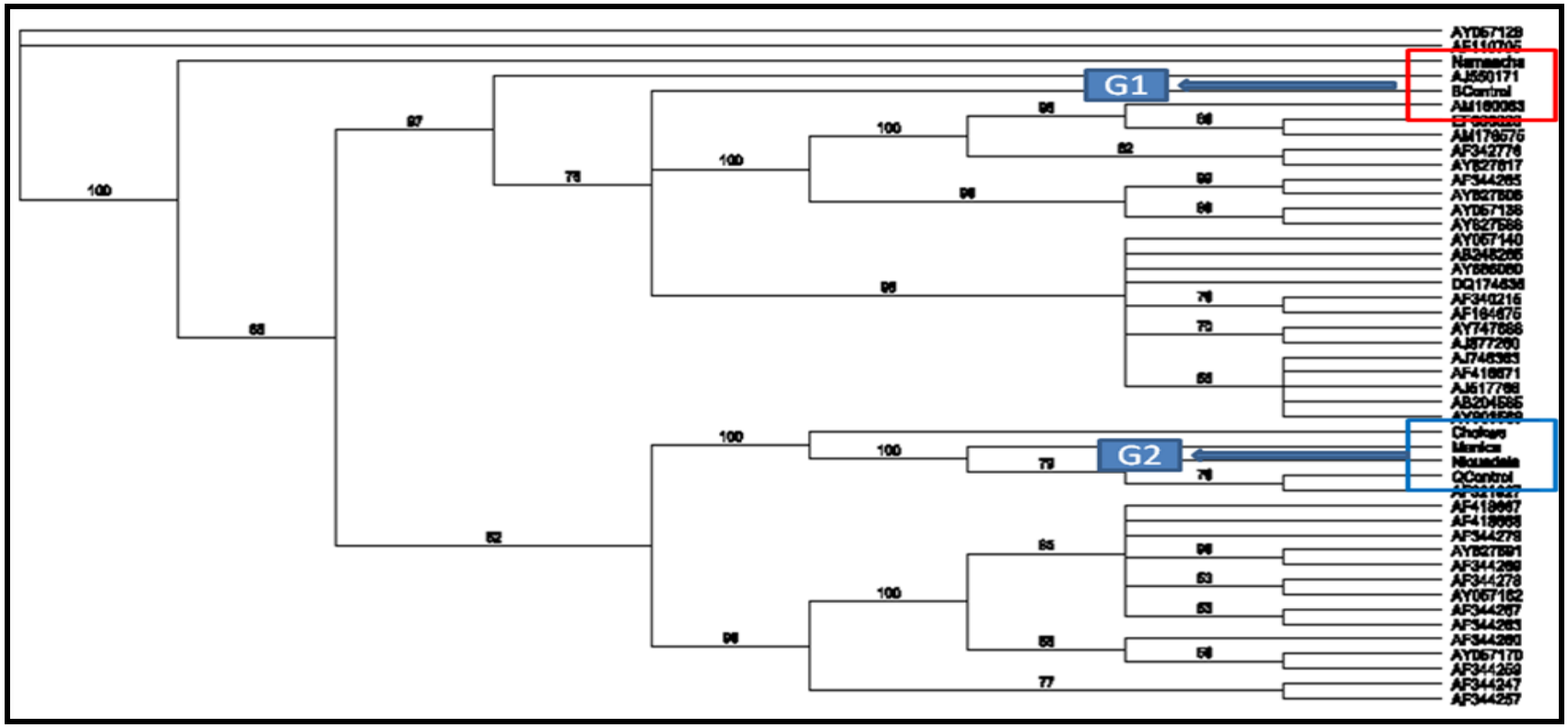


Figure 24. Phylogenetic tree showing the relation between Mozambique whiteflies isolates (G1 and G2) and reference sequences neighbor joining method

Interesting is that the samples labeled as Namaacha and Chokwe from the south of Mozambique, were genetically related to two different types of whiteflies, B (less related) and Q, respectively, and two of the new viruses found (previous section), were from this region, suggesting a possibility of Q, B and related types may be involved in ToCSD transmission in Mozambique, and studies in SA done by Esterhuizen *et al*., (2012) support this hypothesis.

In conclusion, the use of CCP universal primers and RCA/RFLP followed by sequencing brought more light to look for virus diversity related to ToCSD. The CCP sequence analysis gave an indication of possible virus diversity and the full length analysis revealed that three new viruses (Moz1,2 and 3) were present in Mozambique.

Using the PCR/RFLP on *mtCO I* amplicons, was found that Q type and related of *B. tabaci* were more predominant than the B type and related, suggesting a possible replacement of the B type in Mozambique as was found in SA.

Wider sampling, transmission tests and sequence analysis is required, either for the virus, as well as for the whiteflies to make more robust the findings of this study and contribute on the understanding of the tomato infecting begomoviruses and it vector diversity.

CHAPTER 4

EFFECT OF TIMING OF VIRUS INOCULATION ON YIELD AND ToCSD PROGRESSION IN FIELD TRIALS

4.1. Abstract

Integrated disease management is a worldwide known and applied strategy to control the significant yield losses caused by the pathogens. This approach has been for decades used on tomato infecting begomoviruses. ToCSD, an associated begomovirus disease, has been considered one of the major challenges for tomato production in SA and Mozambique. Means such as the use of tolerant/resistant varieties, chemical control of the whitefly vector (*B. tabaci*) are applied to reduce the devastating effect of ToCSD. Although promising results were found with those methods, they still need to be improved and complemented with others. Thus, a trial was set, aiming to understand how the timing of inoculation of ToCSV can affect the yields and the progression of ToCSD in field conditions. The trial was conducted using the completely randomized experimental design with 8 treatments, namely: non-infected, 5, 10, 15, 25, 35, 50 and 65 days after emergence (DAE), with 3 repetitions of 10 plants each per treatment. In each time of infection, seedlings of the test plants were taken for infection to a pre selected field, (known as infected by symptoms and PCR ckeck) and the trays were left surrounded by infected plants. Seven (7) days of after exposure to the virus and about 10 whiteflies per plant, the plants were sprayed to kill the whiteflies and then inspected for whiteflies presence before being transferred to a greenhouse (insect free and with an insect proof net). The virus source and the test plants were checked by PCR, using Pietersen and Smith (2002) primers. ToCSV incidence,

severity and yields losses were the parameters measured. The results revealed that the plants infected from 35-65 DAE had high values of DI, SI and yield losses.

Keywords: **Begomovirus, control, Mozambique, ToCSD**

4.2. Introduction

Tomato infecting begomoviruses are one of the most devastating viruses in tropical and subtropical regions, reducing the yields in about 20-80% (Naika *et al.*, 2006; polston *et al.*, 1997). ToCSD symptoms vary from area to area in terms but typically are characterized by leaf curling upward with or not some yellowing and in severe infection, stunting can be observed (Varela *et al*., 2003).

In 1997 and 1998, a typical ToCSD emerged in SA with incidence varying from 0-50% and in 2000 the disease spread to new areas reaching 100% of incidence (Pietersen and Smith, 2002). Similar scenery was observed in Mozambique from 2005-2007 (*unpublished observations*)

To control ToCSD, several strategies such as chemical control of the whitefly vector, UV absorbing plastics, use of tolerant/resistant cultivars, etc, either combined or not have been applied. But those methods somehow failed to be effective, due to many reasons like, environmental deleterious effect, whitefly resistance, soil overheating, cultivars susceptibility, time consuming and costs)Antignus *et al.*, 2001; Palumbo *et al.*, 2001; levy and Lapidot, 2007).

An interesting approach to complement those that are applied is to monitor symptoms and the effect on yields components comparing non-infected and infected test plants, using promising resistant cultivar or a known susceptible cultivar (Levy and Lapidot, 2007).

Thus, this study was performed to determine the possible effect of plant age of a known susceptible cultivar, when inoculated with ToCSV, under field conditions, would have on DI, SI and yield loss.

4.2. Materials and methods

4.2.1. General description

A field from Boane district, in Maputo province, one of the affected area with ToCSV in Mozambique was selected. In this field, 15 plants showing symptoms ranging from mild to severe (2- 5), according to the scale adapted from Pietersen and Smith (2002) (Chapter 3) were used as virus source material and were confirmed as infected by PCR using the Pietersen and Smith (2002) primers: (ToCSV sense 5´TCTGACCCATCGCACGGGT 3´ and ToCSV anti sense 5´CGCTTCACAAGAGCCTGCTCC 3´). Samples from 5 plants non-symptomatic were also collected for PCR check. Soil and other crop management practices and conditions were carefully considered

4.2.2. Treatments and inoculation procedures

A susceptible cultivar named HTX 14 was used. Eight (8) inoculation times were chosen as treatments (non-infected (control), 5, 10, 15, 25, 50 and 65 DAE). Each treatment was replicated 3 times and with 10 plants (seedlings). The plants were exposed 7 days for virus inoculation in

the field, with an average of 10'15 whiteflies per plant, then, were treated with an insecticide to remove all whiteflies and transferred to an insect proof greenhouse. Samples were collected from the test plants for PCR using Pietersen and Smith primers (2002).

4.2.3. Mmeasured parameters and data analysis

After 7 days of virus exposition, the test plants were inspected and based on the severity scale, adapted from Pietersen and Smith (2002), were scored, and each 15 days were monitored and the data was registered in a sheet to determine the severity indices, the incidence was also registered. Yield and yield losses were also calculated. Disease Incidence, severity indices (formula 1 and 2) and yield losses (formula 3) were submitted to one way ANOVA using the SAS package, and means with significant differences were compared, using Tukey test. Correlation tests using Pearson model were also ran.

$$YL)\%) = \frac{TWfC - TWfTP}{TWfC} * 100$$ **(3)** Where,

YL – Yield losses in percentage
TWfC –Total weight of fruit from the control
TWfTP – Total weight of fruits from the test plants

4.3. Results and Discussion

4.3.1. Confirmation of ToCSD presence in the virus source plants by PCR

From the 20 samples collected to confirm the presence of ToCSV in the plants chosen as virus source plants and to check the specificity of the primers and the sensibility of the PCR, resulted a 100% of match, i.e. the suspected as infected were positive and the suspected as negative were negative (table 7 and figure 25)**,** showing that either the plants chosen as virus source as well as the PCR were appropriate for the study.

Table 7. Severity score and PCR results of infected plants

Sample ID	Severity	PCR	Sample ID	Severity	PCR
1	3	+	11	3	+
2	3	+	12	4	+
3	2	+	13	3	+
4	4	+	14	4	+
5	3	+	15	5	+
6	4	+	16	1	-
7	4	+	17	1	-
8	4	+	18	1	-
9	5	+	19	1	-
10	4	+	20	1	-

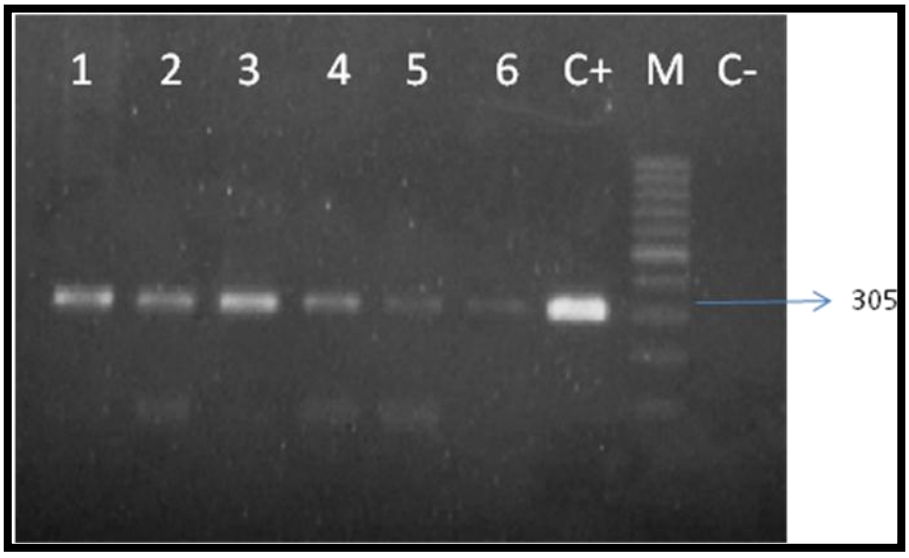


Figure 25. 1% Agarose gel showing the screening of field samples from Boane by PCR. Lane 1-6, test samples, C+, positive control, C-, negative control, M, 100 bp DNA ladder.

4.3.2. Disease incidence, severity indices and yield losses

The ANOVA, using 5% of significance (p = 0.05), revealed that the timing of inoculation had significant differences in the means of disease incidence (DI) and severity indices (SI). However, plants inoculated from 5-15 DAE did not show any symptoms, while the other treatments had the DI and SI ranging from 91 -100 % and 1.87-3.97, respectively. Plants inoculated on 25, 35 and 65 DAE had the highest average DI (100%) and the highest average of SI was from plants inoculated 65 DAE (3.97(**(**Table **8).**

Table 8. Average comparison of DI and SI per treatment

Treatment	Mean of DI (%)	Mean of SI	Mean of YL (%)
Control	0.00 c*	0.00 c	0.00 b
5 DAE	0.00 c	0.00 c	8.99 b
10 DAE	0.00 c	0.00 c	42.24 a
15 DAE	0.00 c	0.00 c	16.19 b
25 DAE	100 a	1.90 b	29.96 a
35 DAE	100 a	1.87 b	16.23 b
50 DAE	91.66 b	2.50 b	29.44 a
65 DAE	100 a	3.97 a	67.21 a

*- means followed by the same letter are statistically equal

The results in the table **8** contrast with those found by Levy and Lapidot (2007), which found a SI of 4 in all inoculation days (14, 28 and 45 days after sowing (DAS)) in a susceptible cultivar, meaning that the time of inoculation had no effect in the SI. However, the results found in this study are consistent to the scenery that happen in the field because the 30 – 60 DAE correspond to the transplantation and flowering, stage that are considered critical for tomato growing and is pointed by Hanifi *et al., (*2022a) and Jones, (2003), as the stage of high preference of the whiteflies and that explain why the stages before 30 DAE were less affected.

The means of yield loss (YL) also show the tendency observed with DI and SI, although, plants inoculated at 10 DAE and 35 DAE, unexpectedly, had a significant and non significant YL, respectively. Two factors can explain this scenery, one is that parts of the plants inoculated 10 DAE were infected by a bacterium (personal observation), which reduced the number and fruit size. Secondly, the SI of the plants inoculated 35 DAE was the lowest (1.87) and this represent the stage of infection in which the plant can produce close to it potential. This has been pointed out in other studies. For example in Vidasky and Czonesk 1998 was found that plants showing initial symptoms can still produce as those symptomless even in susceptible cultivars.

Pearson correlation between DI and SI showed a strong and positive correlation (table 9), while, DI and SI when correlated with the YL, showed a positive but weak relation, revealing that the YL observed were not only due to ToCSV infection, what suggests a repetition of the trial to evaluate the consistency of the results.

Table 9. Pearson correlation between DI, SI and YL

Variable 1	Variable 2	Correlation factor
DI	SI	.8948
DI	YL	.1342
SI	YL	.2113

In summary, this study showed that managing the time of plantation of tomato seedlings can help on the control of ToCSV. 35 – 60 DAE in this study was the critical for ToCSV infection and this period corresponded to the transplantation and flowering times. However, was not possible to correlate with precision the DI, SI and YL, due to unexpected external factors that affected the trial, mainly the fruits.

CHAPTER 5

CONCLUDING SUMMARY

Tomato curly stunt disease (ToCSD) is challenging tomato production in Mozambique mainly in Maputo and Gaza provinces)Moamba, Namaacha and Chokwe districts), where the disease incidence ranged from 62.5 - 74.5% in the Cold/Dry season and from 66 – 78% in the Hot/Rain season and the severity indices was mild (around 2) in those areas and seasons. During the Hot season symptoms of ToCSD were observed in Manica, Zambezia and Tete provinces (Manica, Nicuadala and Angonia districts, respectively). The *B. tabaci* abundance was directly related to the disease incidence.

The PCR based test as a screening tool for ToCSD either using specific or universal primers showed to be a good approach and helped to respond symptoms doubts . The use of RCA-RFLP, as a complementary tool to the PCR, besides helping to resolve DNA quality issues and PCR sensibility, brought much more information in term of possible diversity of ToCSV. 5 patterns were found, indicating that recombination or other evolutionary events may be occurring and variants or species can be found co-existing in the same area in Mozambique.

The *mtCOI* gene of *B. tabaci*, confirmed to be a great marker for characterization of this insect and showed that other biotypes can be implicated on ToCSD transmission in Mozambique, although none study of infectivity was conducted so far.

The sequence analysis of partial coat protein (PCP), full length (FL) and mtCOI, was key to understand the phylogenitical position of the isolates from Mozambique to other reference

sequences. Most of the PCP and FL of the virus sequence analysis showed that the Mozambican isolates are strongly related with the ToCSV (AF281885). Interesting is that the CP sequences were more related, genetically, to the virus considered new, such as, TbMV –Zimbabwe and EACMV –Uganda, which are result of recombination events, confirming this event as a source of new variants or species. And three new species, causing ToCSD-like symptoms are pointed to be occurring in Mozambique.

Many strategies to control ToCSD can be applied, either by controlling the vector, tomato or spontaneous plants, that act as reservoir of virus and vectors. With this study was possible to contribute with information to improve the control strategies, because was observed that manipulating the time of transplantation and control virus sources, the inoculums can be reduced significantly and a special care as to be addressed within the transplantation and flowering periods.

This study provided indication and material for more studies to complement and clarify unclear issues. The number of samples of infected plants and whiteflies need to be increased to have a broader picture of the real scenery. More and representative sequences of FL are needed to understand the differences and to perform recombination studies.

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APPENDIXES

Appendix 1. Field data collection sheet

Country		Date	
Province		Field Nr	
District		Variety	
Latitude		Farming porpose	
Longitude			

Plant nr	INC	SEV	WF nr	Observation
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

INC – Incidence, SEV – Severity, WF-Whitefly

Appendices 2 DNA Extraction method based on the method of Accotto *et. al.* (2000)

The method can be used for TYLCV and ToMoV DNA extraction. Leaf material can be easily collected using an eppendorf tube without touching the leaf with fingers, thus avoiding cross-contamination. The method can be carried out using either 10 mM *B*-mercaptoethanol in the extraction buffer or 10 mM citric acid.

1. Grind 0.15 grams of leaf material to a fine powder in liquid nitrogen using either a pestle and mortar, or plastic grinding bag and wallpaper roller, (ensure the tissue does not thaw once frozen). Add 500 µl of extraction buffer (100 mM Tris-HCl pH= 8, 50 mM EDTA, 500mM NaCl, 1% SDS and 10 mM ***B***-mercaptoethanol or citric acid 10 mM).

2. Mix tubes by vigorous shaking and then incubated at 65^{o}C for 5 minutes. Add 150 µl of 5 M K acetate and shake tubes vigorously to mix then incubated at 0^{o} C for 10 minutes. (This step removes most proteins and polysaccharides as a complex with the insoluble potassium dodecyl sulphate precipitate).

3. Tubes are then spun at 13000 rpm for 10 minutes and then the supernatant (500 µl) withdrawn to a tube containing 350 µl of cold isopropanol, then mixed well and centrifuged at 13000 rpm for 10 minutes.

4. Wash the precipitate by addition of 500 µl of 70% ethanol and centrifuge for 5 minutes at 13000 rpm. Carefully discard ethanol and desiccate for 15 minutes in a vacuum chamber or thoroughly air dry. Resuspend pellets in 100 µl of 10 mM Tris, 1 mM EDTA pH = 8

Appendix 3. Data base summary of the 3200 analyzed samples

			Survey 1				Survey 2			
Province	District	Field Nr	Incidence (%)	Severity	% of pos PCR	Nr of WF	Incidence	Severity	% of pos PCR	Nr of WF
MAPUTO	MOAMBA	1	95	2.5	45	2.6	95	2.5	55	2.85
		2	65	1.9	30	2.3	65	2	35	3.05
		3	85	2.3	30	1.6	95	2.5	50	1.95
		4	85	2.3	35	1.95	90	2.35	55	2.8
		5	55	1.7	25	2	65	2	50	2.8
		6	45	1.65	15	2.3	55	1.8	40	3.3
		7	70	2.25	30	2.8	70	2.4	45	3.45
		8	75	2.4	25	2.1	75	2.4	45	2.05
		9	90	2.7	35	3.8	90	3.1	60	4.3
		10	80	2.3	40	2.15	80	2.75	35	2.75
		Mean	**74.5**	**2.2**	**31**	**2.36**	**78**	**2.38**	**47**	**2.93**
	District	Field Nr	Incidence (%)	Severity	% of pos PCR	Nr of WF	Incidence	Severity	% of pos PCR	Nr of WF
	NAMMACHA	1	55	1.65	20	1.35	55	1.9	90	2.2
		2	65	2.25	35	2	70	2.35	40	3.2
		3	40	1.4	15	1.75	60	1.75	25	2.95
		4	60	1.7	45	3	60	1.85	35	2.7
		5	60	1.6	25	1.65	60	1.6	40	1.8
		6	50	1.55	5	1.45	55	1.7	30	1.95
		7	75	2.15	0	1.9	75	2.2	45	2.45
		8	90	2.65	25	2.05	90	2.65	25	2.15
		9	60	1.95	40	1.55	65	2.2	20	1.75
		10	70	2.15	40	1.65	70	2.15	45	2.15
		Mean	**62.5**	**1.905**	**25**	**1.835**	**66**	**2.035**	**39.5**	**2.33**

	District	Field Nr	Incidence (%)	Severity	% of pos PCR	Nr of WF	Incidence	Severity	% of pos PCR	Nr of WF
GAZA	XAI-XAI	1	0	1	0	0	1	1	0	1.6
		2	0	1	0	0	1	1	0	0
		3	0	1	0	0	1	1	0	0
		4	0	1	0	0	0.2	1.2	0	1.3
		5	0	1	0	0	1	1	0	0
		6	0	1	0	0	1	1	0	0
		7	0	1	0	0	1	1	0	0
		8	0	1	0	0	1	1	0	0
		9	0	1	0	0	1	1	0	0
		10	0	1	0	0	1	1	0	0
		Mean	**0**	**1**	**0**	**0**	**0.92**	**1.02**	**0**	**0.29**
	District	Field Nr	Incidence (%)	Severity	% of pos PCR	Nr of WF	Incidence	Severity	% of pos PCR	Nr of WF
	CHÓKWE	1	95	2.4	55	2.45	90	2	55	3.25
		2	80	2.65	60	1.5	55	1.55	40	1.75
		3	65	1.95	35	1.95	85	1.9	35	1.15
		4	45	1.65	20	0.95	75	2.15	50	1.15
		5	65	1.95	35	1.95	85	1.9	35	1.15
		6	45	1.65	20	0.95	75	2.15	50	1.15
		7	95	2.15	40	2.55	45	1.65	30	2.2
		8	65	1.8	30	1.45	90	2.2	50	3.65
		9	90	2.05	35	2.6	45	2.1	30	2.35
		10	95	3	75	2.5	100	2.55	60	3.2
		Mean	**74**	**2.125**	**40.5**	**1.885**	**74.5**	**2.015**	**43.5**	**2.1**

	District	Field Nr	Incidence (%)	Severity	% of pos PCR	Nr of WF	Incidence	Severity	% of pos PCR	Nr of WF
MANICA	MANICA	1	0	1	0	0	65	1.75	0	1.45
		2	0	1	0	0	55	1.6	0	1.4
		3	0	1	0	0	40	1.45	0	1.15
		4	0	1	0	0	20	1.2	0	0
		5	0	1	0	0	25	1.3	0	0
		6	0	1	0	0	75	1.95	25	1.9
		7	0	1	0	0	35	1.35	0	0.65
		8	0	1	0	0	20	1.2	0	0
		9	0	1	0	0	30	1.3	0	0.7
		10	0	1	0	0	35	1.35	0	1.25
		Mean	**0**	**1**	**0**	**0**	**40**	**1.445**	**2.5**	**0.85**
	District	Field Nr	Incidence (%)	Severity	% of pos PCR	Nr of WF	Incidence	Severity	% of pos PCR	Nr of WF
	SUSSUNDENGA	1	0	1	0	0	0	1	0	0
		2	0	1	0	0	0	1	0	0
		3	0	1	0	0	0	1	0	0
		4	0	1	0	0	0	1	0	0
		5	0	1	0	0	0	1	0	0
		6	0	1	0	0	0	1	0	0
		7	0	1	0	0	0	1	0	0
		8	0	1	0	0	0	1	0	0
		9	0	1	0	0	0	1	0	0
		10	0	1	0	0	0	1	0	0
		Mean	**0**	**1**	**0**	**0**	**0**	**1**	**0**	**0**

	District	Field Nr	Incidence (%)	Severity	% of pos PCR	Nr of WF	Incidence	Severity	% of pos PCR	Nr of WF
ZAMBÉZIA	NICUADALA	1	5	1.05	0	0.75	35	1.5	0	1.1
		2	5	1.05	0	0.25	25	1.25	10	1.1
		3	0	1	0	0	0	1	0	0
		4	50	1.6	0	1.2	0	1	0	0
		5	45	2	0	1.3	50	1.45	0	0.6
		6	70	1.8	0	0	45	1.6	15	1.5
		7	40	1.4	0	0.65	70	1.9	10	1.05
		8	15	1.15	0	0	55	1.6	10	1.2
		9	35	1.4	0	0.75	50	1.5	10	1.1
		10	25	1.25	0	0.75	0	1	0	0
		Mean	**29**	**1.37**	**0**	**0.565**	**33**	**1.38**	**5.5**	**0.765**
	District	Field Nr	Incidence (%)	Severity	% of pos PCR	Nr of WF	Incidence	Severity	% of pos PCR	Nr of WF
TETE	ANGÓNIA	1	0	1	0	0.3	0	1	0	1.3
		2	0	1	0	0	0	1	0	0.7
		3	0	1	0	0	0	1	0	0.9
		4	0	1	0	0.1	0	1	0	0.6
		5	0	1	0	0	15	1.15	0	0.5
		6	0	1	0	0	10	1.1	0	0.25
		7	0	1	0	0	15	1.15	0	0.95
		8	0	1	0	0.35	15	1.15	0	0.4
		9	0	1	0	0	0	1	0	0.85
		10	0	1	0	0	0	1	0	0.35
		Mean	**0**	**1**	**0**	**0.075**	**5.5**	**1.055**	**0**	**0.68**

Appendix 4 Accession numbers of reference virus and whiteflies sequences used in this study.

Virus sequences

>AF261885 ToCSV-ZA V2 AM701768 ToLCToV >AJ865340 ToLCMaV >AM701759 ToLCCo >AJ865341 ToLCCoV2 >AY036009 HoLCrVca >AF014881 HoLCrVGz >AM701763 ToLCMohV >FN600540 ToLCNamakely >FR751146 CoLFGezira >FR751145 CoLCGezira2 >AF155064 CoLCGezira2 REPFORM >AY036010 CLCGzrVEgpt >FR751143 CoLCGzR2 >GU945265 CoLCJor2 >AJ620916 ToMaybegovirus pV1 >DQ127170 ToLCUgaV >JN604484 TYLCV isoKW1 >AF329235 Casgemvirus >FJ956702 TYLCV iso Alb13 >FJ956701 TYLCV_isoAlb12> DQ644565 TYLCV isoAl-Batinah >JF451352 TYLCV KISR >FJ956705 TYLCV Alb34 >FM210276 CoLCGzr Camron >AM701766 ToLCV Antsir >FN555173 PepVMaV >FM876849 PepVMaV_iso brkna >JN604488 TYLCV ISODT2 >JN604486 TYLCV ISO KW3

***Bemisia tabaci* sequences**

FJ710456 FJ710471 FJ710469 AF321927 HM802268 FJ710455 HM802266 EU192071 EU255281 EU000312 AJ867555 HM802267 JN855568 FJ710457 EU760719 JN855575 EU255278 JN855571 EU255284 EU255283 AB204585 AB248265 AF164675 AF340215 AF418671 AJ517768 AJ748363 AJ877260 AY057140 AY686080 AY747688 DQ174535 AF344247 AF344257 AY057170 AF344285 AY827588 AY057136 AY827606 AF110705 AY057128 AF321927 AY903569 EF080823 AF342776 AY827617 AM180063 AM176575 AF344259 AF344263 AF344267 AF344269 AJ550171 AF344279 AF344278 AF344280 AY057162 AF418667 AF418668 AY827591