Determining the distribution and genetic diversity of *Coguvirus eburi* in South African citrus and development of a citrus-infecting coguvirus detection assay

by

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Declaration

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ABSTRACT

Citrus virus A (CiVA) is a novel negative-sense single-stranded RNA virus discovered with high-throughput sequencing (HTS). CiVA is classified as a member of the species *Coguvirus eburi* and is closely related to a member of the species *Citrus coguvirus*, named citrus concave gum-associated virus (CCGaV). CCGaV and CiVA are members of the genus *Coguvirus* in the family *Phenuiviridae* in the order *Bunyavirales*. CiVA has a bipartite genome encoding an RNA-dependant RNA polymerase (RdRp) on RNA 1 and a nucleocapsid protein (NP) and a putative movement protein (MP) on the ambisense RNA 2. The confirmed presence of CiVA in cultivars of grapefruit (*Citrus paradisi* Macf.), sweet orange (*C. sinensis* (L.) Osb.) and clementine (*C. reticulata* Blanco) in South Africa initiated a study to determine the distribution, genetic diversity and symptom association of CiVA in three provinces and seven citrus production regions. CiVA was detected in six citrus production regions in symptomatic and asymptomatic sweet orange trees. In three citrus production regions, CiVA was detected in sweet orange trees displaying a fruit rind symptom similar to citrus impietratura. CiVA was also detected in grapefruit trees with typical citrus impietratura symptoms and in symptomless clementine trees. The three encoded gene regions of CiVA were Sanger sequenced to investigate the genetic diversity between isolates from the six citrus production regions and three citrus species. Phylogenetic analysis of the nucleotide sequences (nt) of each encoded gene region was performed through the construction of Maximum-likelihood (ML) phylogenetic trees and nucleotide identity matrices. Phylogenetic analysis and nt identity matrices indicated a higher genetic diversity within the NP than the MP and RdRp. More genetic diversity was observed between isolates from the three citrus species than between isolates from the different citrus production regions. A real-time RT-PCR detection assay targeting the RdRp was also developed to simultaneously detect CiVA and CCGaV. Two cDNA synthesis methods for reverse transcription (RT) were compared and a degenerate, dual priming oligo (DPO) reverse primer was designed to improve the specificity of the detection assay. Two PCR assays that utilised the DPO reverse primer with two different forward primers were compared. The cDNA synthesis method and choice of primer pair had an influence on the amplification efficiency, specificity and sensitivity of the real-time detection assay. A tissue specificity assay was also performed and CiVA was detected throughout the plant in leaf midribs, leaf lamina, green bark and roots. Lower Ct values were consistently associated with the green bark and leaf midribs. The detection assay was implemented for pathogen screening within the Citrus Improvement Scheme (CIS) of South Africa, ensuring the release of CiVA free budwood to the citrus industry.

OPSOMMING

Sitrus virus A (CiVA) is ontdek met die gebruik van hoë-deurset volgordebepaling gebaseerde opsporing (HTS). CiVA is ‘n lid van die spesie *Coguvirus eburi* en is nouverwant aan sitrus-konkaaf gom-geassosieerde virus (CCGaV), ‘n lid van die spesie *Citrus coguvirus*. Beide virusse is lede van die genus *Coguvirus* wat deel is van die familie *Phenuiviridae* in die orde *Bunyavirales*. CiVA is ‘n negatiewe-string RNA virus en besit twee genome. Die RNA 1 genoom kodeer vir die RNA-afhanklike RNA polimerase (RdRp) en die RNA 2 genoom kodeer vir ‘n nukleokapsiedproteïen (NP) en ‘n vermeende bewegingsproteïen (MP). Die bevestiging van die teenwoordigheid van CiVA in variëteite van pomelo (*Citrus paradisi* Macf.), soetlemoene (*C. sinensis* (L.) Osb.) asook clementine (*C. reticulata* Blanco) in Suid-Afrika, het gelei na ‘n opname in drie provinsies en sewe sitrus produksie gebiede. In drie sitrus produksie gebiede is CiVA in soetlemoen bome met vrugskil simptome soortgelyk aan sitrus-impietraturasiekte geïdentifiseer. CiVA is ook in soetlemoen bome met geen vrugsimptome geïdentifiseer. Pomelo bome met tipiese sitrus-impietraturasiekte simptome was geïnfekteer met CiVA, maar pomelo bome met geen simptome in dieselfde sitrus boord was vry van CiVA. Clementine bome met geen vrugskil simptoom was ook geïnfekteer met CiVA. Die nukleotied (nt) volgorde van die drie proteïne van CiVA was bepaal met behulp van Sanger-volgorde bepaling om die genetiese diversiteit tussen die isolate van die ses produksie gebiede en drie sitrus tipes te ondersoek. Filogenetiese analises van die nt volgorde van die drie proteïne was uitgevoer deur die konstruksie van ‘n maksimum waarskynlikheid (ML) filogenetiese boom. Additionele nukleotied identiteit matrikse was ook uitgevoer. Filogenetiese analise en nt identiteit matrikse het 'n hoër genetiese diversiteit binne die NP aangedui as in die MP en RdRp. Meer genetiese diversiteit is waargeneem tussen isolate afkomstig van die drie sitrus spesies as tussen isolate afkomstig van die verskillende sitrus produksie gebiede. ‘n Tru-transkripsie polimerase-kettingreaksie (RT-PCR) opsporings toets, wat in werklike tyd waargeneem kan word, is ook ontwikkel vir die gelyktydige identifikasie van CCGaV en CiVA. Twee cDNA sintese metodes vir tru-transkripsie was gevergelyk en ‘n gedegenereerde omgekeerde inleier met dubbele inleier aktiwitiet (DPO) is ontwerp om die spesifisiteit van die opsporingstoets te verbeter. Twee PCR toetse wat gebruik maak van die DPO omgekeerde inleier en twee verskillende vorentoe inleiers was gevergelyk tydens amplifikasie. Die cDNA sintese metode en keuse van inleier paar het 'n invloed gehad op die amplifikasie doeltreffendheid, spesifisiteit en sensitiwiteit. 'n Weefselspesifisiteitstoets is uitgevoer en CiVA is regdeur die plant opgespoor in die hoofaar van die blaar, blaarskyf, groen bas en wortels. Laer Ct-waardes was geassosieer met die groen bas en hoofaar van die blaar. Die opsporingstoets is geïmplementeer in die sitrusverbeteringskema (CIS) van Suid-Afrika om die vrystelling van CiVA vrye plant materiaal aan die sitrusbedryf te verseker.

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1. INTRODUCTION

1.1 GENERAL INTRODUCTION

The production of citrus occurs throughout more than 100 tropical and sub-tropical countries where the export of citrus contributes financially to the economy (Vapnek, 2009). South Africa has been the second largest fresh citrus exporter in the world since 2006 and exports to more than 40 countries (Edmonds, 2021). Over the past two years, South African citrus exports have grown by 22%. In 2019, 130 million cartons were exported, followed by 146 million cartons in 2020. The estimated export for 2021 is a record breaking 161.6 million cartons (Chadwick, 2021). It is evident that the production of citrus plays is a significant role in the agricultural sector of South Africa.

Citrus production in South Africa occurs throughout all provinces except Gauteng and only 0.012% in the Free State province. The largest production regions are situated in the Limpopo province (39%) and the Eastern Cape province (27%). In 2020, 65% of the South African citrus production was exported, 29% was processed and 6% was consumed by the local market. South Africa was ranked as the 12th largest producer of fresh citrus and remained the second largest fresh citrus exporter worldwide in 2020. The majority of South African citrus is exported to European markets (38%) followed by the Middle East (17%) (Edmonds, 2021).

The production of citrus can be negatively impacted by the range of citrus pathogens causing disease including viruses, bacteria and fungi. Most often, tree vigour and fruit yield are affected when infected with a pathogen, resulting in poor tree growth and fruit production. Some pathogens induce fruit rind symptoms, resulting in a reduction of fruit quality that is not suitable for export, while other pathogens can cause premature fruit drop, reducing the amount of citrus that can be exported. It is therefore essential to prevent pathogen spread or the establishment of these diseases to maintain the health of the industry.

Citrus trees are vegetatively propagated through the budding of a specific citrus cultivar onto a rootstock (Albrecht et al., 2017). Occasionally, pathogen-infected budwood material is used during the propagation process. Subsequently, the infected trees are distributed, potentially spreading disease. It is therefore imperative that pathogen-free budwood material is used during the propagation process as a citrus tree has the potential to be productive for decades (Lee et al., 1999). The implementation of budwood certification programmes, that provide pathogen-free plant propagation material to nurseries and growers, has limited the spread of infected budwood material (Childs, 1968).

Despite the recent advances in molecular detection and the discovery of novel plant viruses with the use of high-throughput sequencing (HTS) (review by Roossinck et al., 2015), biological indexing is still required for the detection of some systemic pathogens with an unknown aetiology. PCR-based detection assays are specific and sensitive, but require prior knowledge of pathogens and can therefore not detect uncharacterised pathogens. Biological indexing is, however a lengthy process that can take up to a year to complete. HTS has been compared with biological indexing and was demonstrated to be equal to or more reliable in detecting viruses than biological indexing (Rott et al., 2018). The use of HTS can improve certification programmes by reducing the time and costs needed to release budwood to the industry.

1.2 PROBLEM STATEMENT

South African citrus is susceptible to a variety of citrus viruses, viroids and bacteria that negatively impact production and exportation. However, the implementation of the Citrus Improvement Scheme (CIS) of South Africa contributed towards the current health status of the industry. Viruses cause a variety of citrus diseases affecting various production factors and in some instances, a disease has the ability to destroy an entire industry. Around the world, budwood certification programmes were introduced to limit the spread of graft transmissible diseases and to protect citrus industries.

HTS has the potential to detect novel viruses that can be used to resolve the aetiology of diseases. The identification of novel viruses allows for the development of molecular detection assays, reducing the reliance on biological indexing. Recently, citrus concave gum-associated virus (CCGaV) and citrus virus A (CiVA) was discovered with the use of HTS and these viruses were associated with citrus concave gum disease and citrus impietratura, respectively. Investigating the possible association of CiVA with citrus impietratura in South Africa and determining its genetic diversity can generate valuable information that can be applied to manage the virus.

Currently, the only molecular detection assay available for the detection of CiVA within the CIS is an end-point RT-PCR. It would therefore be of value to the CIS to have a single real-time RT-PCR detection assay for citrus-infecting coguviruses that can simultaneously detect CCGaV and CiVA. This will reduce the need for biological indexing and the time required to perform multiple tests. It is the purpose of this study to determine the incidence and genetic diversity of CiVA in South Africa and to develop a real-time RT-PCR detection assay for two citrus coguviruses.

1.3 PROJECT AIM AND OBJECTIVES

The aim of this study was to investigate the association of CiVA with citrus impietratura on grapefruit and fruit rind symptoms similar to citrus impietratura on sweet oranges, determining the genetic diversity of CiVA in various citrus production regions and citrus species and to develop a detection assay for the simultaneous detection of citrus infecting coguviruses.

The following objectives were set out achieve this aim:

- Conduct a survey in commercial citrus orchards in different production regions to determine the incidence of CiVA in South Africa and to determine the association of CiVA with citrus impietratura and similar fruit rind symptoms.
- Evaluate the nucleotide sequence diversity between CiVA isolates from different citrus production regions and citrus species through the analysis of sequences from the three CiVA encoded proteins using identity matrices and phylogenetic analysis.
- Develop a real-time RT-PCR detection assay for the simultaneous detection of CiVA and CCGaV.
- Validate the developed detection assay by evaluating sensitivity and specificity.

1.4 CHAPTER LAYOUT

This thesis contains five chapters. The general introduction is followed by a literature review, two research chapters and a general conclusion.

Chapter 1: Introduction

General introduction, problem statement, aims and objectives and chapter layouts of the thesis are provided. The scientific outputs generated during this study and the contributions of the authors are stated.

Chapter 2: Literature review

An overview of literature pertaining to the graft transmissible pathogens of South Africa, their management and detection is provided with a focus on diseases of unknown aetiology. The discovery of novel citrus viruses, possibly associated with diseases of unknown aetiologies are discussed.

Chapter 3: Determining the distribution and genetic diversity of Coguvirus eburi in South Africa and the likely association with citrus impietratura

In this chapter, a survey was conducted in the citrus production regions of South Africa to determine the distribution of CiVA and the association with citrus impietratura and similar fruit rind symptoms. Genetic variability was determined through phylogenetic analysis and nucleotide identity matrixes.

Chapter 4: Development of a real-time RT-PCR for the simultaneous detection of two citrus-infecting coguviruses

In this chapter, a real-time RT-PCR detection assay was developed for the simultaneous detection of CiVA and CCGaV. Different primer combinations were compared in terms of PCR efficiency, specificity and limit of detection. A dual-priming reverse primer was designed for increased assay specificity.

Chapter 5: Conclusion

General concluding remarks are provided and includes future prospectives.

1.5 RESEARCH OUTPUTS

1.5.1 Manuscript from thesis

Manuscript in :

De Bruyn, R., Bester, R., Cook, G., Steyn, C., Breytenbach, J.H.J., and Maree, H.J. 2022. Distribution and genetic diversity of *Coguvirus eburi* in South African citrus and the development of a real-time RT-PCR assay for citrus-infecting coguviruses. *Plant Disease*. DOI: 10.1094/PDIS-11-21-2409-RE

This paper combines the results of Chapter 3 and Chapter 4 and is the work of the first author who was responsible for the processing of all survey samples, primer design and sequencing of the three gene regions of CiVA, analysis of the sequence diversity between CiVA isolates and the development and optimisation of a real-time RT-PCR detection assay. *Writing of the manuscript and data analysis was conducted by RDB and GC. HJM and RB participated in a large-scale survey and assisted with manuscript writing and phylogenetic analysis. JHJB participated in surveys and performed biological indexing. CS participated in a large-scale survey and assisted with the processing of samples collected in the mentioned survey.*

1.5.2 Manuscripts co-authored during duration of study

- Bester, R., Cook, G., Breytenbach, J. H., Steyn, C., De Bruyn, R., & Maree, H. J. (2021). Towards the validation of high-throughput sequencing (HTS) for routine plant virus diagnostics: measurement of variation linked to HTS detection of citrus viruses and viroids. *Virology Journal, 18*(1), 1-19.

This paper highlights the possibility of implementing HTS for routine pathogen screening, using validated standard operating procedures. RDB contributed towards the RNA extraction of inoculated plant material and confirmation of the infection status using RT-PCR.

- Cook, G., Coetzee, B., Bester, R., Breytenbach, J. H., Steyn, C., **de Bruyn, R**., & Maree, H. J. (2020). Citrus tristeza virus isolates of the same genotype differ in stem pitting severity in grapefruit. *Plant Disease, 104*(9), 2362-2368.

This manuscript reports the biological and genetic characterization of the mild and severe stem pitting isolates of the T68 genotype from citrus tristeza virus (CTV) derived from the same infection source. RDB assisted with RNA extraction of plant material, transmission confirmation using RT-PCR, stem pitting evaluation and assisted with full-genome Sanger sequencing.

- Cook, G., Steyn, C., Breytenbach, J. H., **de Bruyn, R**., & Fourie, P. H. (2020). No detection of seed transmission of citrus tatter leaf virus in 'Meyer' lemon. *Journal of Plant Diseases and Protection, 127*(6), 895-898.

This paper investigated the transmission of citrus tatter leaf virus (CTLV) through seed. RDB contributed towards the plant RNA extraction and assisted with the molecular testing of the samples using RT-PCR.

- Fourie, P.H., Kirkman, W., Cook, G., Steyn, C., **de Bruyn, R**., Bester, R., Roberts, R., Bassimba, D.D., José, C.M. and Maree, H.J., 2021. First report of 'Candidatus Liberibacter africanus' associated with African Greening of Citrus in Angola. *Plant Disease, 105*(2), pp.486-486.

This disease note reports the first detection of Candidatus Liberibacter africanus (Laf) in Angola. RDB assisted with the DNA extraction of plant material as well the detection of Laf using real-time PCR.

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2. LITERATURE REVIEW

2.1 GRAFT TRANSMISSIBLE DISEASES OF SOUTH AFRICAN CITRUS

Citrus trees are woody perennial crops in the genus *Citrus* and members of the family *Rutaceae* (Talón et al., 2020) that can be productive for decades. Commercial citrus cultivars usually consist of a cultivar specific scion grafted onto a rootstock to prevent root diseases (Albrecht et al., 2017). Most often, commercial citrus is propagated through vegetative propagation using budding or grafting techniques (Singh, 2018).

Citrus crops are susceptible to a range of pathogens that can be transmitted through grafting and budding of infected plant propagation material, exchanged between countries and production regions (Conejero et al., 2013; Vapnek, 2009). Some of these pathogens are also spread through mechanical transmission (Alas et al., 2019; Belabess et al., 2020; Barbosa et al., 2005; Dauthy & Bovè, 1965; Garnsey & Muller, 1988; Liu et al., 2020) and arthropod vectors (Liu et al., 2020; Roistacher & Bar-Joseph, 1987). These pathogens are referred to as graft transmissible pathogens (GTPs). GTPs of citrus include viruses, viroids and some bacteria. Graft transmissible diseases (GTD) can either be destructive, causing decline and death of the infected tree or less severe, causing a reduction in fruit quality and yield, stunting and a reduction in growth, resulting in long term losses (Roistacher, 1991). Most often, budwood is infected with more than one GTP (Vidalakis et al., 2004), potentially resulting in the spread of multiple pathogens. Most GTDs induce characteristic symptoms in the field that can be used to identify the disease; however, some field trees are symptomless and can only be detected through biological indexing (Roistacher, 1991). Management of these diseases are difficult (Roistacher, 2004) as there is no treatment available for the infected trees (Gergerich & Dolja, 2006). GTPs can however, be eliminated from infected budwood (discussed in 2.2). The GTPs that have been detected in South Africa are discussed in Table 2.1 (Adapted from Annexure 9 of the Citrus Improvement Scheme (CIS) procedural guide).

Table 2.1: Graft transmissible pathogens present in South African citrus and their effect on citrus production.

Pathogen	Distribution in SA	Symptoms	Consequences
Citrus tatter leaf virus (CTLV)	Occurs at very low incidence (Da Graça 1977; Marais and Lee 1986). Not recently detected in commercial orchards	○ Severe stunting; bud-union incompatibility on trifoliate types ○ Pitting and fluting of rootstock (Calavan et al. 1963).	Current main rootstocks in South Africa are affected (Calavan et al. 1963; Marais and Lee 1986).

Citrus Psorosis virus (CPsV)	Under official eradication until 1967. Only few instances are known in older plantings (Da Graça 1978). Not recently detected in commercial orchards	○ Bark scaling. ○ Wood staining (Da Graça 1978).	Reduced production (up to 75%) (Wallace 1953).
Citrus tristeza virus (CTV)	Endemic (McClean 1963)	○ Quick decline ○ Stem pitting (McClean 1960)	Decline, small fruit and reduced productive lifespan (McClean 1963)
Citrus vein enation virus (CVEV)	Endemic (McClean 1954)	○ Galls on stems ○ Enations on leaves (Only rough lemon and lime) (McClean 1954)	None
Citrus exocortis viroid (CEVd)	More common in older orchards (Basson and Schwartz 1964).	○ Bark scaling ○ Stunting	Unproductive trees, early decline of trees and reduced crop (Vernière et al. 2006)
Citrus bent leaf viroid (CBLVd)	Occasionally detected in older orchards	○ Pits in trunk with corresponding pegs in bark	Reduction in yield (Hashemian et al. 2009) and poor tree performance (Hashemian et al. 2010).
Hop stunt viroid (HSVd)	Occurs commonly in older orchards (Basson & Schwartz 1964)	○ Tree stunting ○ Phloem discoloration (gumming) ○ Cachexia disease (Serra et al., 2008; Vidalakis et al. 2010)	Stunting resulting in yield reduction. Poor tree performance (Hashemian et al. 2010).
Citrus dwarfing viroid (CDVd)	Occurs commonly in older plantings.	○ Tree stunting ○ Gum pocket with certain rootstocks (Van Vuuren et al. 2005)	Reduced crop due to smaller tree size (Vidalakis et al. 2010).
Citrus bark cracking viroid (CBCVd)	Reported present (Cook et al. 2012).	○ Bark cracking of trifoliate orange rootstocks.	Unknown
Liberibacter africanus (Laf)	Present throughout SA, except Northern Cape, Free State and regions of Western Cape and Eastern Cape.	○ Mottling leaves ○ Greened fruit ○ Decline (McClean and Schwartz 1970)	Reduced production and unmarketable fruit (McClean and Schwartz 1970).

Some graft transmissible diseases within the South African citrus industry are not associated with a causal agent. These citrus diseases of unknown aetiology include citrus impietratura, citrus concave gum disease and cristacortis. It has been suspected that these diseases are caused by viruses due their graft transmissible nature. Since there is no treatment for plant virus infections, a tree will remain

infected for the entirety of its life span (Villamor et al., 2019). Detection of these viruses is therefore an essential part in the management of GTDs.

2.2 CITRUS IMPROVEMENT SCHEME OF SOUTH AFRICA

The first citrus eradication and certification programmes were implemented due to the proven graft transmissibility of CPsV through infected propagation material as reviewed by Belabess et al. (2020). Various graft transmissible pathogens of citrus have been reported since the initial report of the graft transmissibility of CPsV. Subsequently, various citrus-producing countries implemented budwood certification programmes to limit the spread of infected propagation material, effectively controlling these diseases (Ferguson, 1993; Juárez et al., 2015).

The citrus improvement scheme (CIS) of South Africa was established in 1973 to provide pathogen-free propagation material to the South African citrus industry and the first certified tree of the scheme was produced in 1976 (Von Broemsen & Lee, 1988). The purpose of the scheme is to provide the South African citrus industry with high-quality nursery trees that are made from the best genetic citrus material and are free from any harmful pathogens (https://www.citrusres.com/divisions/cis).

The elimination of GTPs from infected budwood occurs through a process called shoot tip grafting (STG). STG has been used successfully for many years to produce pathogen-free propagation material from mature infected trees (Juárez et al., 2015; Roistacher et al., 1976). Prior to STG, pathogen-free budwood was generated through nucellar embryony and nucellar tissue culture; however, this process resulted in plants that were thorny and late bearing with fruit that varied in quality. The application of STG can, however produce pathogen-free budwood within one year after grafting and is therefore a more time-efficient method to use in certification programmes (Roistacher et al., 1976). STG was introduced into South Africa in 1977 (von Broemsen & Lee, 1988) and is the aseptic grafting of the apical meristem tip, often pathogen-free, onto a virus-free seedling rootstock that was grown *in vitro*. The implementation of the STG process in South Africa resulted in the successful production of a wide variety of pathogen-free citrus cultivars.

The CIS consists of four phases. Phase 1 includes cultivar introduction, pathogen elimination through STG, biological indexing and pre-immunization with a mild CTV source. Phase 2 is the supply of the pathogen-free cultivar to the Citrus Foundation Block (CFB) followed by cultivar evaluations. Phase 3 is the establishment of mother trees at the CFB mother block, followed by the production of multiplication trees. Phase 4 is the supply of certified budwood to nurseries for the production of trees supplied to the commercial growers. A certificate is issued by the CIS for each delivery of certified nursery trees to a commercial grower that confirms that the supplied trees meet the requirements of

the CIS. The reference number contained on the certificate can allow budwood source trees to be traced. The ability to trace certified trees within the scheme is one of the major strengths of the South African CIS and has helped trace genetic and disease problems back to the source plant (Von Broemsen & Lee, 1988).

2.3 CITRUS DISEASES WITH UNKNOWN AETIOLOGY

2.3.1 Citrus impietratura

Citrus impietratura was first described in Israel on the fruit of lemon (*Citrus limon* (L.) Osb.), grapefruit (*C. paradisi* Macf.) and occasionally sweet orange (*C. sinensis* (L.) Osbeck) citrus types (Reichert & Hellinger, 1930). Initial fruit rind symptoms reported on lemon include discoloured yellow lesions on unripe, green fruit and orange lesions on yellow, ripe fruit. Symptoms observed on grapefruit and occasionally oranges included green (Fig. 2.1 A) or reddish lesions on the fruit rind and the presence of gum in the albedo (Fig. 2.1 B) of symptomatic grapefruit and lemon fruit (Reichert & Hellinger, 1930).

Subsequently, citrus impietratura was reported by a number of countries including Sicily, Cyprus, Morocco, Lebanon, Turkey and Greece (Chapot, 1961; Ruggieri, 1955; Papasolomontos, 1965). Additional symptoms reported for citrus impietratura included abnormal fruit drop, small fruit with skin hardening and fruit rind lesions that were either sunken or protruding (Chapot, 1961; Papasolomontos, 1965; Terranova & Scuderi, 1968). The severe fruit drop associated with citrus impietratura (Fig. 2.1 C) can most likely be attributed to the presence of gum in and around the stem of the fruit (Wallace, 1978). In most instances, the only indication of the presence of citrus impietratura is the severe fruit drop and the presence of symptomatic fruit in an otherwise healthy tree (Papasolomontos, 1965; Pappo & Oren, 1974).

Although citrus impietratura was studied extensively, the causal agent remained elusive. Graft transmission experiments reproduced disease symptoms, demonstrating the systemic nature of citrus impietratura and suggesting a virus as the causal agent. Young grapefruit trees were inoculated with citrus impietratura infected bark and the resulting fruit in the following season displayed symptoms of citrus impietratura (Papasolomontos, 1965). Similarly, graft transmission tests were conducted by using sweet orange trees that displayed symptoms of citrus impietratura, topworked with healthy grapefruit cv. ‘Marsh’ buds and the subsequent fruit produced from these grapefruit shoots showed symptoms of citrus impietratura (Ruggieri, 1965). The probable viral nature of citrus impietratura was confirmed through transmission experiments in 1968, but it was noted that fruit symptoms and transmission of the disease was highly variable (Scaramuzzi et al., 1968). Symptom expression was not

observed every year and the severity of symptoms varied between different citrus types (Cartia & Catara, 1972; Cartia & Catara, 1974; Scaramuzzi et al., 1968). These observations complicated research to identify the aetiology of the disease.

Indexing for the presence of citrus impietratura was reliant on fruit symptom production; however, many fruits needed to be observed for at least one year after graft inoculation (Bar-Joseph & Loebenstein, 1970). The presence of leaf-flecking symptoms (Fig. 2.1 D) was noted on young leaves of sweet orange cv. 'Madam vinous' as well as sour orange (*C. aurantium*) indicator seedlings three weeks after graft inoculation with citrus impietratura infected material. No leaf-flecking symptoms were observed on indicator seedlings inoculated with budwood from symptomless trees (Bar-Joseph & Loebenstein, 1970). Due to the consistent production of leaf-flecking symptoms on indicator seedlings grafted with citrus impietratura infected budwood, a new indexing method for the detection of citrus impietratura was considered (Bar-Joseph & Loebenstein, 1970).

Symptoms of citrus impietratura were first reported in South Africa in 1976 and were observed on grapefruit in the Kwazulu-Natal province; however, confirmation of the disease was unsuccessful (Moll et al., 1976). Shortly after, Marsh grapefruit and navel sweet orange cv. 'Washington' in the Cape Province were reported with similar symptoms. Graft inoculations from these trees induced oak-leaf patterns in sour orange and sweet orange seedlings in the glasshouse (Da Graça, 1978). The presence of citrus impietratura in South Africa was confirmed in 1984 through biological indexing and transmission studies. Budwood from symptomatic citrus impietratura 'Marsh' grapefruit trees, as well as symptomless 'Marsh' grapefruit trees from Nkwaleni, were used. Buds of infected trees were grafted to rough lemon (*C. jambhiri* Lush) seedlings for biological indexing. In the transmission study buds of symptomatic trees were grafted to Marsh grapefruit trees which were planted in the field. In both the transmission and indexing experiments, the subsequent fruit produced displayed citrus impietratura symptoms (van Vuuren et al., 1984).

The natural spread of citrus impietratura was suggested when the percentage of trees displaying citrus impietratura symptoms increased over a three-year period (Scaramuzzi et al., 1968). However, several other studies concluded that citrus impietratura was not naturally spread (Cartia & Catara, 1974; Pappo & Oren, 1974; van Vuuren et al., 1984). To date, citrus impietratura has no known insect vector and the only means of transmission is through grafting. The management of this disease can therefore be achieved through the supply of disease-free budwood through the CIS where the viral agent is removed through STG. Confirmation of elimination of the causal agent is done by biological indexing on an indicator host and the absence of flecking symptoms indicate the absence of the pathogen (Roistacher, 2004).

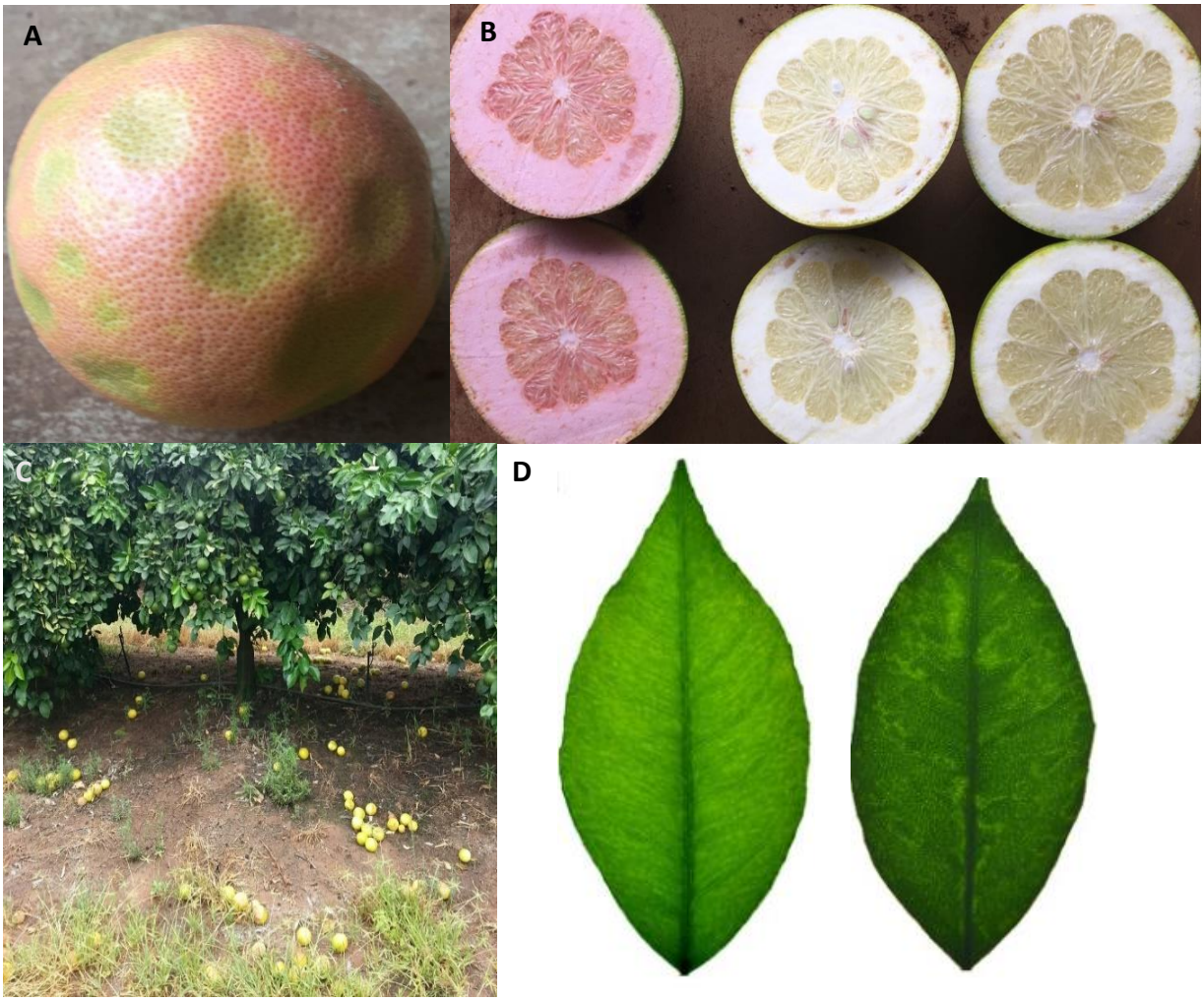

Figure 2.1: Green sunken spots present on a Star Ruby grapefruit from a citrus impietratura infected tree (A). Gumming present in the albedo of Star Ruby (left) and Marsh (right) grapefruits of a citrus impietratura infected tree (B). Characteristic fruit drop of a Marsh grapefruit tree infected with citrus impietratura (C) (A-C: Brian Chedzey - Farmers Agri-Care). Leaf flecking (left) and oak leaf pattern (right) present on Madam vinous biological indicator plants (D) (Navarro et al., 2018a).

2.3.2 Citrus concave gum disease

Citrus concave gum disease was first reported in 1926 (Fawcett, 1926). Most often the tree trunk and branches are affected (Vogel, 1961). Susceptible hosts include sweet orange, mandarins (*C. reticulata*), tangelos (*C. reticulata x C. paradisi* Macf.) and tangors (*C. reticulata x C. sinensis* (L.) Osb), while other citrus types remain symptomless. In the field, concave gum can be identified through three symptoms, including the presence of concavities in the trunk (Fig. 2.2 A and B), concentric gum rings in the cross-sections of large branches and twigs (Fig. 2.2 C) and symptoms of leaf flecking or oak leaf patterns on young flush (Roistacher, 1991). Prior to the presence of concavities in the trunk and branches, gum layers develop after two to three years and can be observed in shoots with a one-centimetre diameter

(Vogel & Bové, 1974). During the formation of the concavities, the bark often cracks and gum exudes from these cracks.

The presence of leaf flecking or oak leaf patterns (Fig. 2.1 D) in young flush of susceptible cultivars such as grapefruit and sweet orange can be used for diagnosis in the field and may appear on each side of the midvein as thin and translucent lines or as a full translucent oak leaf imprint (Roistacher, 1991). Initial symptom expression of an infected tree is mild leaf flecking in young flush followed by the development of an oak leaf pattern on the larger leaves. Foliar symptom expression can be observed on the spring flush (Vogel, 1961). These symptoms can also be used as a positive indication when performing biologically indexing on Dweet tangor seedlings which have been found to be the most sensitive (Roistacher, 1991).

Transmission tests were conducted through graft-inoculation of material from trees displaying oak leaf patterns. After two months in the glasshouse a gum exude was observed at the inoculation point (Ieki & Ito, 1996). In another experiment, infected material from a tree showing severe symptoms of citrus concave gum disease was used to inoculate healthy trees. After two years, bark cracking and gum exudation was observed. Cross-sections of branches that were located far from the inoculation point also displayed gum exudation. These results confirmed that the pathogen causing citrus concave gum disease is graft-transmissible (Ieki & Ito, 1996). To this day, the spread of citrus concave gum disease has not been associated with an insect vector.

The severity of the disease increases with the age of the infected trees (Vogel, 1961) and causes a reduction in fruit quality and production yield (Wallace, 1978). Concave gum induces alternate fruit-bearing and a reduction in tree vigour, followed by tree death (Ieki & Ito, 1996; Vogel & Bové, 1974).

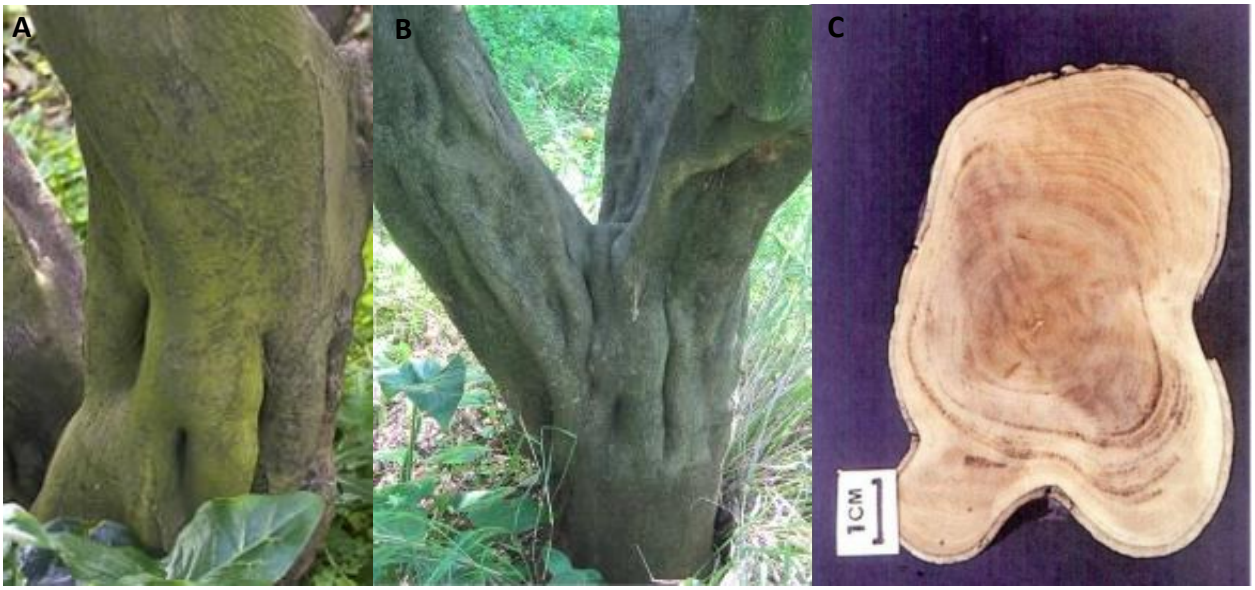

Figure 2.2: Deep concavities present on the trunks of sweet orange trees caused by citrus concave gum disease (A and B) (Navarro et al., 2018a). Concentric gum rings in a cross section of a branch from a tree displaying citrus concave gum disease symptoms (C) (Roistacher, 1991).

2.3.3 Cristacortis

Cristacortis was first reported in 1964 on sour orange and sweet orange cv. 'Tarocco' as a severe stem-pitting disorder and subsequently observed in various citrus-producing countries (Vogel & Bové, 1968). Initially, small depressions on the trunk of a three-year-old 'Tarocco' sweet orange tree was noted (Fig. 2.3 A), resembling symptoms of concave gum; however, depressions were observed on both scion and rootstock (Vogel & Bové, 1964). Removal of the bark from the concavity revealed deep and vertical pits that were most often accompanied with gum present at the base of the pits (Roistacher, 1991; Vogel & Bové, 1968).

Initial symptom development appeared on trunks and was later found on the main limbs and secondary branches. Symptoms observed on the tree trunk was not limited to certain areas, occurring anywhere on the trunk from the ground up (Vogel & Bové, 1968). Depressions in the trunk formed as a result of sharp protuberances from the cambial side of the bark, penetrating the wood and forming pits which often presented as multiple peaks (Fig. 2.3 B and C). Discoloured lines were observed in the cross-sections of trunks/branches, through a pit, which extended from the bottom of the pit towards the centre of the trunks/branches. Branches that were severely pitted appeared weak and bent towards the ground (Vogel & Bovè, 1964). Similar to citrus impietratura and citrus concave gum, leaf flecking or oak leaf patterns are induced on the young flush of infected trees (Roistacher, 1991).

The agent causing cristacortis was shown to be easily transmitted through grafting (Vogel & Bovè, 1968). The successful transmission of cristacortis to sour orange indicator seedlings suggests that the disease is likely caused by a virus (Servazzi et al., 1968). Although the individual pits resembled those of concave gum, it appeared that the symptoms were not caused by the same pathogen (Vogel & Bovè, 1968). It was shown later that the pathogen was unrelated and different from other known diseases that affect citrus (Vogel & Bovè, 1974).

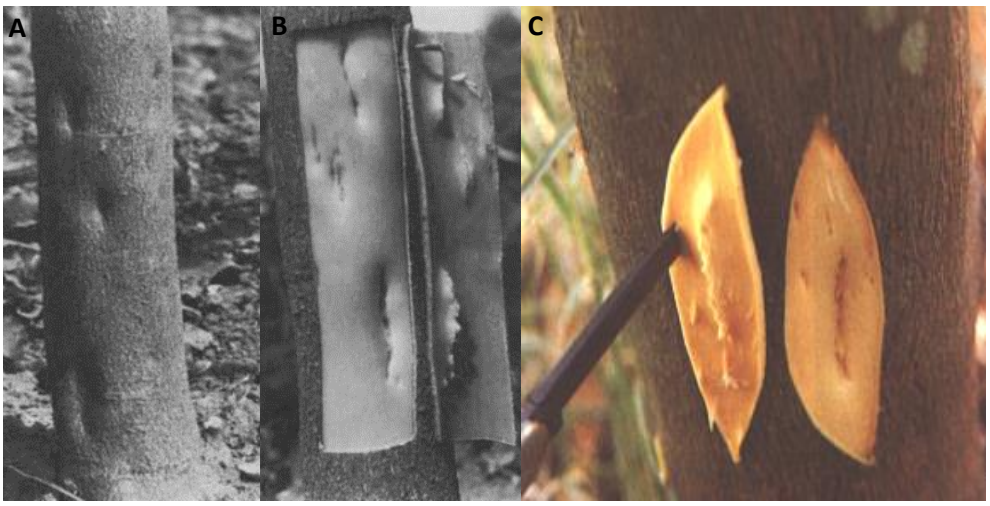

Figure 2.3: Cristacortis pit formation on a sweet orange tree (A) (Vogel & Bovè, 1968). Sharp peaks are present on the inside of the bark on a Cristacortis infected tree (B) (Vogel & Bovè, 1968). Formation of pegs on the cambial side of the bark on a cristacortis infected tree (C) (Roistacher, 1991).

2.4 DIAGNOSING A CITRUS DISEASE

The basis for a successful budwood certification programme is the reliable and accurate diagnosis of citrus diseases. For known viruses, molecular detection assays are available (Vidalakis et al., 2004); however, for some uncharacterized pathogens, such as those inducing citrus impietratura, cristacortis and citrus concave gum disease, diagnosis is solely based on biological indexing.

2.4.1 Biological indexing

Biological indexing has been used for decades as the basis for pathogen detection in citrus budwood certification programmes and relies on the expression of symptoms when certain citrus types that are sensitive to a specific virus, referred to as an indicator host, is inoculated with diseased plant material and monitored for symptom expression (Legrand, 2015; Skaria, 2000). The type of symptom and time till symptom expression varies depending on the disease, chosen indicator and growth conditions (Roistacher, 1991; Skaria, 2000). It is essential to determine the sensitivity of an indicator host to a specific disease for optimal symptom expression.

Biological indexing is sensitive and has the ability to detect pathogens that went undetected with more specific tests (Legrand, 2015). However, biological indexing is time-consuming as the indicators need to be inspected weekly over a period of one to eight months due to the transient nature of symptom expression associated with some citrus diseases (Legrand, 2015; Vidalakis et al., 2004). Careful planning is also required to establish a supply of well maintained, high-quality indicator plants (Martín et al., 2002; Skaria, 2000). A temperature-controlled insect-proof glasshouse and skilled personnel are also required for the successful identification of diseases present (Iftikhar et al., 2021; Martín et al., 2002).

The established protocols for the biological indexing of graft transmissible pathogens of citrus were put in place without experimentally evaluating the potential effects of a mixed infection (Vidalakis et al., 2004). Symptom expression can be affected by different pathogens interacting with each other and can result in false negatives due to symptom expression of other viruses being suppressed or reduced (Legrand, 2015; Singh & Ready, 2003). Biological indexing tests can also be negatively affected by the spatial distribution of the pathogen in the plant, leading to potential false-negative results.

Citrus impietratura, cristacortis and citrus concave gum disease all produce similar leaf flecking or oak leaf patterns when biologically indexed therefore, these leaf symptoms cannot be used to diagnose the specific disease (Vogel & Bovè, 1972); however, it does indicate the presence of a disease (Roistacher, 1991). The oak leaf pattern or leaf flecking symptoms induced by these viruses can fade from young leaves as the leaves mature and may not reappear in later flushes, it is therefore essential

to monitor the first three flushes after inoculation (Roistacher, 1991). It should be noted that the absence of symptoms does not indicate the absence of a virus (Legrand, 2015).

Biological indexing of field samples can be difficult to implement due to the requirement for available indicator plants, glasshouse space and the need for long-term plant maintenance and monitoring (Legrand, 2015). Although diagnostics techniques that are more specific and faster are required for specific detection of graft transmissible diseases (Martín et al., 2002), it is still recommended that biological indexing be applied within budwood certification programmes to enable detection of uncharacterised pathogens (Legrand, 2015).

2.4.2 Serological and Molecular detection

Over the last few decades, advances in molecular biology have led to the development of fast, accurate and sensitive techniques for the detection of plant viruses, including immunological and nucleic acid-based methods (Makkouk & Kumari, 2006). Serological virus detection is a method of choice in many certification schemes; however, nucleic acid-based detection of plant viruses offers increased sensitivity and specificity (Pallás et al., 2018). These detection methods have successfully been established in various laboratories globally for the detection of plant viruses (Boonham et al., 2014; López et al., 2003; Pallás et al., 2018).

2.4.2.1 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a simple and fast detection method that has been used for the past 30 years (Makkouk & Kumari, 2006). ELISA was first introduced in the 1970s (Engvall & Perlmann, 1971; Engvall & Perlmann, 1972) and was applied for the detection of plant viruses in 1976 (Clark et al., 1976). ELISA is based on the binding of antibodies, specific to an antigen. Detection is visualized by the modification of a substrate that induces a colour change in the presence of an antigen-antibody-enzyme complex (Gan & Patel, 2013).

The most common type of ELISAs are sandwich ELISAs, consisting of a primary unlabelled-antibody immobilized to the microtitre plate, allowing the binding of the antigen. A secondary enzyme linked-antibody is added which also binds to the antigen, sandwiching the antigen between the two antibodies (Catherine, 2021; Gan & Patel, 2013; Garnsey & Cambra, 1993). Indirect ELISAs consist of the binding of a primary unlabelled-antibody to an immobilized antigen, followed by the binding of a secondary labelled-antibody which detects the primary antibody (Catherine, 2021; Gan & Patel, 2013). Commercially, universal secondary labelled-antibodies are available (Naidu & Hughes, 2003). Indirect ELISAs are therefore more appropriate for plant virus detection in disease management programmes due to increased sensitivity and reduced costs of using a single universal secondary labelled-antibody

for various virus detection assays (Catherine, 2021; Garnsey & Cambra, 1993; Nascimento et al., 2017; Serçe & Ayyaz, 2020).

The introduction of ELISA transformed virus detection through simplifying detection and shortening the time needed to obtain results (Torrance & Jones, 1981). The sensitivity and specificity of ELISAs were improved with the use of monoclonal antibodies (Boonham et al., 2014). Although ELISA has been widely used for the routine testing of viruses, there are some disadvantages. Compared to molecular detection assays, the development of ELISAs are expensive as high-quality antisera, allowing sensitive and specific binding to viral antigens, increases costs due to the requirement of specialised laboratories and virologist expertise to produce these antisera (Boonham et al., 2014). Closely related viruses can be difficult to differentiate since the virus coat protein can be conserved within a genus and the species determining factor is not always reflected in the coat protein (Boonham et al., 2014). Although ELISA is suitable and still widely used as a diagnostic tool, the method is no longer used in the South African CIS and was replaced by nucleic acid-based methods due to increased sensitivity and ease of assay development.

2.4.2.2 Polymerase chain reaction

Nucleic acid-based detection methods, targeting the viral nucleic acids present in a sample, are more commonly used for virus detection largely due to increased sensitivity (Mehetre et al., 2021). The polymerase chain reaction (PCR) was first reported in 1986 (Mullis et al., 1986) and has since been implemented as the new standard for virus detection (Mehetre et al., 2021; Makkouk & Kumari, 2006). During PCR, the target virus is amplified through the binding of primers that are complementary to the target sequence and the extension of the primed single-stranded DNA by a DNA polymerase (Makkouk & Kumari, 2006). The incorporation of a reverse transcription (RT) step allows the molecular detection of RNA viruses (Makkouk & Kumari, 2006; Mehetre et al., 2021; Rubio et al., 2020).

Comparatively, RT-PCR is more sensitive, specific and less expensive to develop than ELISA (López et al., 2009) and have therefore been developed for the detection of many plant viruses (Serçe and Ayyaz 2020). Generally, two types of RT-PCRs are used for the detection of plant viruses and include end-point RT-PCR and real-time RT-PCR. End-point PCR requires a post RT-PCR step where the amplified products are visualized on an agarose gel (Rubio et al., 2020). The post RT-PCR step introduces a contamination risk due to the opening of tubes after amplification (Boonham et al., 2014).

Real-time RT-PCR assays detect the accumulation of amplicons in real-time through the use of a fluorescent reporter (Smith & Osborn, 2009). The fluorescent signal produced by the reporter increases as double-stranded DNA products are formed and therefore can reflect the starting amount

of target product present in the sample (Kubista et al., 2006; Makkouk & Kumari, 2006). The elimination of the post RT-PCR step and the reduction in cycle times, due to the amplification of smaller DNA fragments, enables a real-time RT-PCR assay to be more time and cost-effective and reduces the risk of contamination (Makkouk & Kumari, 2006). The two types of fluorescent chemistries used in a real-time RT-PCR assays include DNA intercalating dyes and hydrolysis probes (Smith & Osborn, 2009).

SYBR green is a DNA binding dye that intercalates between adjacent base pairs and fluoresces as it binds to any double-stranded DNA. Primers highly specific to their target region are required as SYBR Green binds non-specifically to double-stranded DNA (Fittipaldi et al., 2010). SYBR Green assays can differentiate between target and non-specific amplicons with the application of melt curve analysis as each double stranded amplicon will have a melting temperature determined by their DNA sequence (Smith & Osborn, 2009). A hydrolysis probe real-time RT-PCR assay includes a probe labelled with a fluorophore at the 5'-end and a quencher at the 3'-end (Livak et al., 1995). The presence of the quencher inhibits the fluorescence of the fluorophore; however, extension of the primer by the DNA polymerase cleaves the probe, releasing the fluorophore and resulting in fluorescence (Makkouk & Kumari, 2006). The presence of the probe provides greater specificity as it ensures that the fluorescence detected is only from the amplification of the target sequence (Smith & Osborn, 2009).

The detection of multiple viral species simultaneously in a single reaction is more time and cost-effective, reduces the need for lengthy biological indexing and can be useful in the selection and propagation of pathogen-free material (Pallás et al., 2018; Serçe & Ayyaz, 2020; Varveri et al., 2015). Simultaneous detection can either occur with the use of degenerate primers or a multiplex PCR (Pallás et al., 2018). A probe-based multiplex real-time RT-PCR can be developed by labelling probes with different fluorophores which enables the detection of different targets in one reaction (Baldwin et al., 2008). These are valuable benefits for certification schemes in order to decrease the time taken to release cultivar budwood to the industry (Varveri et al., 2015).

Although end-point and real-time PCR methods are highly sensitive, they are generally either species- or genus-specific and can fail to detect divergent variants of a virus, a poorly characterized virus or an unknown virus (Candresse et al., 2014).

2.4.2.3 High-throughput sequencing

High-throughput Sequencing (HTS) is an alternative approach for the detection of known and unknown viruses. HTS can reveal the complete phytosanitary status of a plant (Maree et al., 2018) through the sequencing of large amounts of nucleic acids (Adams et al., 2018), enabling the detection

of a broad range of viruses simultaneously (Al Rwahnih et al., 2015; Kreuze et al., 2009; Maree et al., 2018; Villamor et al., 2019). HTS is one of the most significant advancements in molecular biology since the introduction of PCR and requires no prior knowledge of a virus for detection (Adams et al., 2018).

Since the initial report of using HTS in plant virology (Wren et al., 2006), numerous novel plant viruses have been discovered (Adams et al., 2009; Al Rwahnih et al., 2009; Barba et al., 2014; Bejerman et al., 2020; Elbeaino et al., 2014; Espach et al., 2012; Kreuze et al., 2009; Navarro et al., 2018a; Navarro et al., 2018b; Marais et al., 2015; Rott et al., 2018; Villamor et al., 2016; Villamor et al., 2017). The discovery of novel viruses has increased the understanding of the role that viruses play in disease aetiology, plant-virus interactions as well as the evolution of these viruses (Maree et al., 2018; Villamor et al., 2019).

Most often in citrus, rapid identification of viruses associated with damaging diseases are required for effective control (Bester et al., 2021). Traditional virus identification methods, such as biological indexing and electron microscopy is time-consuming and has limitations (Kreuze et al., 2009). Multiple assays are required to screen for pathogens when performing ELISAs, end-point and real-time RT-PCRs. In contrast, HTS can detect all viruses present in a sample with a single test (Maree et al., 2018). The sequence data generated through HTS can also be used for the development of new detection assays (Bester et al., 2021) and improving current detection assays to detect variants of a virus (Marais et al., 2015).

HTS has transformed plant virus research and can potentially be implemented as a reliable standalone virus detection assay if optimised and validated properly (Bester et al., 2021). HTS for pathogen screening within the CIS can be implemented to reduce the need for biological indexing as well as reduce the number of tests that need to be performed.

2.5 TWO NOVEL CITRUS-INFECTING COGUVIRUSES

The discovery of new viruses is often led by an investigation into virus and virus-like diseases of agricultural crops. The application of HTS in citrus led to the discovery of novel viruses that resolved diseases with unknown aetiologies (Maliogka et al., 2018; Vives et al., 2013). These include the discovery of citrus vein enation virus (CVEV) (Vives et al., 2013), citrus yellow vein clearing virus (CYVCV) (Loconsole et al., 2012a), citrus chlorotic dwarf-associated virus (CCDaV) (Loconsole et al., 2012b). More recently, an investigation into citrus concave gum disease has led to the discovery of a novel virus associated with the disease, called citrus concave gum-associated virus (CCGaV) (Navarro et al., 2018a). CCGaV was identified in trees from Italy displaying typical citrus concave gum symptoms.

CCGaV has subsequently been reported in apples in the United States of America (Wright et al., 2018) and Brazil (Nickel et al., 2020).

CCGaV is a negative-sense single-stranded RNA virus and was classified as a member in the order *Bunyavirales* due to the following features: (i) a bipartite genome, (ii) identical stretch of 26 nucleotides at the 5' and 3' ends of both genomic RNA's, allowing for the formation of a panhandle structure, (iii) an ambisense RNA containing an AU rich intergenic region (IR) and (iv) an IR sequence stretch resembling a terminal transcription signal (TTS) previously reported for members of this order (Abudurexiti et al., 2019). Within the order, CCGaV is more closely related to members of the genus *Tenuivirus* due to the presence of non-enveloped virions; however, members of the genus *Tenuivirus* have four to six genomic RNAs. Although the presence of the IR resembling the TTS suggests a closer relationship to members of the genus *Phlebovirus*, members within this genus have tripartite genomes, differing from the bipartite genome reported for CCGaV. Due to the mentioned differences, CCGaV could not be classified within these genera and a new genus was proposed to include CCGaV within the order *Bunyavirales*. The new genus *Coguvirus* was created to accommodate CCGaV, a member of the species *Citrus coguvirus*.

A second novel virus closely related to CCGaV was discovered with HTS and was named citrus virus A (CiVA) (Navarro et al., 2018b). CiVA, classified as a member of the species *Coguvirus eburi*, is the second member of the genus *Coguvirus* and was discovered in a symptomless tree in Italy. Subsequently, CiVA has been detected in citrus and pears in various countries (Bester et al., 2021; Beris et al., 2021; Bougard et al., 2021; Minutolo et al., 2020; Svanella-Dumas et al., 2019). There has been a report associating CiVA with citrus impietratura on grapefruit in Greece (Beris et al., 2021). Typical citrus impietratura symptoms have been reported on grapefruit trees in the Nkwaleni region in the Kwazulu-Natal province of South Africa and fruit rind symptoms on 'Delta' Valencia sweet orange trees have also been reported and is possibly linked to the presence of CiVA in South Africa (Chapter 3).

The two members of the new genus *Coguvirus* has a bipartite genome consisting of a negative sense RNA1 and an ambisense RNA2. (Navarro et al., 2018a; Navarro et al., 2018b). The negative-sense RNA 1 contains a single open reading frame (ORF) encoding the RNA-dependant RNA polymerase (RdRp) and needs to be replicated into a positive sense viral complementary (vc) RNA before transcription into mRNA (Fig. 2.4). The ambisense RNA 2 contains two ORF's, one on each polarity strand. ORF 1 encoding the movement protein is located on the viral negative-sense RNA and is directly transcribed into mRNA. ORF 2 encoding the nucleocapsid protein is located on the positive sense vcRNA which is transcribed into mRNA (Fig. 2.5) (Navarro et al., 2018a).

Both CCGaV and CiVA do not encode for a glycoprotein (Navarro et al., 2018a; Navarro et al., 2018b). The presence of a glycoprotein is most often associated with negative-sense RNA viruses transmitted by insects (Navarro et al., 2018a). The absence of an encoded glycoprotein likely indicates that CCGaV and CiVA are not transmitted by an insect vector, corresponding with the literature regarding the absence of natural spread of CCGaV and CiVA.

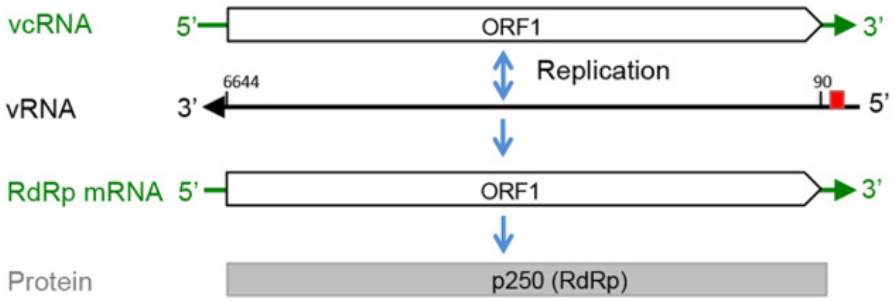

Figure 2.4: Graphic illustration of the negative-sense single-stranded RNA 1 of CCGaV (Navarro et al., 2018a). The negative sense RNA is replicated into a positive-sense viral complementary RNA for translation.

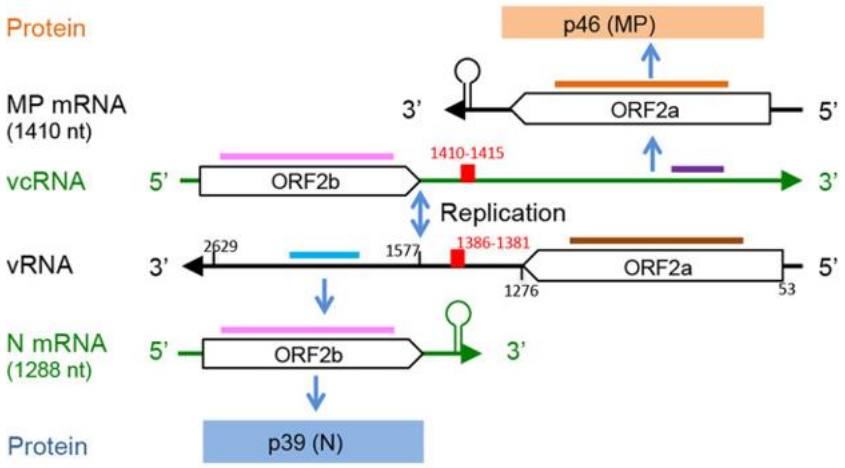

Figure 2.5: The ambisense RNA 2 of CCGaV, encoding a putative movement protein on the negative-sense viral RNA and a nucleocapsid protein on the positive-sense viral complementary RNA (Navarro et al., 2018a).

2.6 CONCLUSION

Graft transmissible diseases of citrus have been studied extensively; however, the aetiology of some diseases remain unclear. High-throughput sequencing allows for the detection of novel viruses associated with diseases of unknown aetiology and the generated sequence data can be used for the swift development of detection assays for these viruses. In this study, the association of CiVA with citrus impietratura on grapefruit and similar fruit rind symptoms on sweet orange was investigated. The genetic diversity of CiVA was evaluated and a real-time RT-PCR detection assay, targeting the RdRp was developed for the simultaneous detection of CiVA and CCGaV. This assay was implemented in the South African CIS for pathogen screening to reduce the number of tests that need to be performed on suspected infected plant material and contribute to the release of pathogen-free plant material to the CIS.

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3. DETERMINING THE DISTRIBUTION AND GENETIC DIVERSITY OF *COGUVIRUS EBURI* IN SOUTH AFRICA AND ASSOCIATION WITH CITRUS IMPIETRATURA

3.1 INTRODUCTION

Some citrus diseases, including citrus impietratura and citrus concave gum disease have unknown aetiologies dating back to the 1930s (Fawcett, 1926; Reichert & Hellinger, 1930). Symptoms of citrus impietratura include abnormal fruit drop, gumming in the albedo, fruit hardening and sunken or raised lesions on the fruit rind (Moreno et al., 2000). Fruit rind symptoms of citrus impietratura have been reported on grapefruit (*Citrus paradisi* Macf.), sweet oranges (*C. sinensis* (L.) Osb.), clementine (*C. reticulata* Blanco) and lemon (*C. limon* (L.) Osb.) (Bar-Joseph & Loebenstein, 1970; Cartia & Catara, 1974; Ruggieri, 1965; Scaramuzzi et al., 1968; Terranova & Scuderi, 1968). Symptoms similar to citrus impietratura was first reported in South Africa in 1976 on grapefruit cv. 'Marsh' and navel sweet orange cv. 'Washington' (Moll et al., 1976; da Graça, 1978) and it was confirmed that a graft transmissible agent was associated with the observed citrus impietratura symptoms (da Graça, 1978).

Detection of citrus impietratura within the citrus improvement scheme (CIS) of South Africa is reliant on biological indexing on a sensitive indicator host. However, the transient nature and sometimes poor or no expression of symptoms could easily be missed. Biological indexing is also a lengthy process, delaying the release of propagation material to the citrus industry (Vidalakis et al., 2010). The spread of citrus impietratura infected budwood was limited through shoot tip grafting (STG), where graft transmissible pathogens are eliminated from infected budwood. Although the spread was limited, the aetiology of the disease needed to be resolved. This proved difficult due to the inability to fulfil Koch's postulates and identify a pathogen. Citrus impietratura symptoms are not observed every year and symptom expression can be irregular (Papasolomontos, 1965; Scaramuzzi et al., 1968; Terranova & Scuderi, 1968), increasing the difficulty of studying the disease. Resolving the aetiology of citrus impietratura will allow improved disease management through the implementation of molecular detection assays that could replace long-term biological indexing.

The use of high throughput sequencing (HTS) has been instrumental in resolving the aetiology of some citrus diseases. Recently, citrus concave gum-associated virus (CCGaV) was discovered with HTS and reported to be associated with citrus concave gum disease (Navarro et al., 2018a). Citrus virus A (CiVA), closely related to CCGaV, was discovered in a symptomless sweet orange tree in Italy with the use of HTS and was subsequently detected in symptomless mandarin, clementine and lemon citrus types (Navarro et al., 2018b) and also in pear (Bougard et al., 2021; Svanella-Dumas et al., 2019). Recently, CiVA was reported to be associated with citrus impietratura (Beris et al., 2021).

CiVA and CCGaV are negative-sense single-stranded RNA viruses with a bipartite genome. An RNA-dependent RNA polymerase (RdRp) is encoded on the negative-sense RNA 1 and a nucleocapsid protein (NP) and putative movement protein (MP) on the ambisense RNA 2 (Navarro et al., 2018a; Navarro et al., 2018b). CCGaV was classified as a member of the species *Citrus coguvirus* and CiVA as a member of the species *Coguvirus eburi*. Both virus species belong to the genus *Coguvirus* in the family *Phenuiviridae* (Kuhn et al., 2020). CiVA and CCGaV are phylogenetically related to other members in the order *Bunyavirales*; however, their unique molecular characteristics, including the absence of an external envelope and lack of encoded glycoproteins, initiated the establishment of the genus Coguvirus (Navarro et al., 2018).

Determining the distribution and genetic diversity of CiVA in South African can lead to improved disease control through the early detection of these viruses. Phylogenetic analysis has become a significant tool in comparing molecular data and studying the evolutionary relationships among organisms (Hillis, 1997; Pavlopoulos et al., 2010). A phylogenetic tree is regarded as the most informative method to graphically illustrate relationships and similarities between organisms (Pavlopoulos et al., 2010). Nucleotide identity matrices are informative pairwise comparisons between the nucleotide bases of two or more sequences and indicates the percentage similarity between these sequences (Campanella et al., 2003).

The presence of CiVA in South Africa was confirmed in 2018 with the use of HTS (Bester et al., 2021). Subsequently, a field survey was conducted to establish the distribution of CiVA in the various citrus production regions of South Africa and to determine the association of CiVA with citrus impietratura. The genetic diversity of CiVA in the various citrus production regions was established through phylogenetic analysis.

3.2 MATERIALS AND METHODS

3.2.1 Detection of CiVA in field samples

3.2.1.1 'Delta' Valencia sweet orange

Field surveys were conducted in sweet orange cv. 'Delta' Valencia orchards in three provinces and seven citrus production regions (Limpopo province: Hoedspruit, Letsitele, Tshipise; Mpumalanga province: Komatipoort, Hectorspruit, Malelane; Eastern Cape province: Kirkwood) during 2019 in South Africa. Orchard planting dates in these regions ranged from 1984 to 2011. The 155 trees sampled were categorized as symptomatic or asymptomatic based on the presence of a fruit rind symptom, but for some trees the fruit symptoms were not definitive and symptom expression was classed as uncertain (Table 3.1). Current CIS budwood source trees for 'Delta' Valencia were also sampled for testing.

Table 3.1: 'Delta' Valencia samples collected during a survey in seven citrus productions regions of South Africa and classified according to the rind symptom observed.

Province	Production region	Symptomatic	Uncertain	Asymptomatic	Total	CiVA positive
Limpopo						
	Hoedspruit	63	20	30	113	113
	Tshipise	5	9	1	15	15
	Letsitele	4	0	4	8	4
Eastern Cape						
	Kirkwood	0	0	6	6	4
Mpumalanga						
	Malelane	0	3	2	5	0
	Hectorspruit	0	1	1	2	2
	Komatipoort	0	0	6	6	1

3.2.1.2 'Marsh' grapefruit

Budwood sampled from ten citrus impietratura symptomatic and eight asymptomatic 'Marsh' grapefruit trees from three orchards in Nkwaleni, KwaZulu-Natal province were received in 2018. RNA was extracted, stored and subsequently used in this study.

3.2.1.3 Clementine

A CiVA positive clementine cultivar was identified during routine screening of gene source accessions with primer pair CiVA_5148F (GAGAATGACAAGTATGACCA)/ CiVA_6023R (ACAGAAGAATGACAATAGCA). Field samples of this cultivar from the Hoedspruit production region were also tested for the presence of CiVA.

3.2.1.4 Total RNA extraction

Total RNA was extracted from the collected survey samples using an acid-phenol extraction (Cook et al., 2016). Macerated leaf midribs were incubated on ice for 10 min, followed by centrifugation for 5 min at 13400 rcf and 4°C. Two phase separations were performed by transferring 1600 µl and 1000 µl supernatant to 350 µl and 500 µl chloroform, respectively, followed by centrifugation at 4°C for 10 min at 13400 rcf. Precipitation of 800 µl supernatant with 200 µl 4 M LiCl and 200 µl isopropanol took place at room temperature for 30 min, followed by a 20 min centrifugation (13400 rcf) at 15°C. The supernatant was discarded and the pellet was washed with 500 µl 75% (v/v) EtOH, followed by centrifugation at 13400 rcf for 15 min at 4°C. The pellets were air dried for 15 min and resuspended in 100 µl nuclease-free water.

3.2.1.5 Molecular detection

A two-step end-point RT-PCR was performed using primer pair CiVA_5148/CiVA_6023R, designed from available CiVA genomes of South African isolates (Bester et al., 2021) to confirm the presence of CiVA.

Complementary DNA (cDNA) was synthesized using random hexamers and RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA). Random hexamers (ThermoFisher Scientific) (0.2 µg/µl) and total RNA (1 µg) was incubated for 3 min at 65°C followed by 1 min on ice. Additional components added include 5x Reaction Buffer (ThermoFisher Scientific), 1 mM dNTP Mix (ThermoFisher Scientific), 20 units RT enzyme and 5 units RiboLock RNase Inhibitor (ThermoFisher Scientific) with a total reaction volume of 10 µl. Reverse Transcription was performed at 25°C for 5 min, 42°C for 60 min and reaction termination at 70°C for 10 min.

End-point PCR was performed using GoTaq G2 Hot Start Green Master Mix (Promega Corp., Madison, WI, USA) with 0.2 µM forward primer and 0.2 µM reverse primer and 1 µl cDNA with a total reaction volume of 10 µl. Cycling parameters were 94°C for 2 min followed by 40 cycles of 94°C for 20 s, annealing for 30 s at 55°C, extension at 72°C for 20 s and final extension of 72°C for 10 min. PCR products were visualized via gel electrophoresis on a 1.5% (w/v) agarose gel and the presence of a 876 bp product indicated CiVA detection. CiVA positive samples were further tested for the presence of citrus tristeza virus (CTV), citrus bent leaf viroid (CBLVd), hop stunt viroid (HSVd), citrus dwarfing viroid (CDVd), citrus bark cracking viroid (CBCVd), citrus viroid V (CVd-V), citrus exocortis viroid (CEVd), citrus tatter leaf virus (CTLV) and citrus psorosis virus (CPsV) as described previously (Cook et al., 2016).

3.2.2. Biological indexing

Fifteen 'Madam vinous' sweet orange seedlings were graft inoculated with bark patches from symptomatic 'Delta' Valencia trees from the Hoedspruit region that contained CiVA and CTV. The seedlings were maintained in an insect-free polycarbonate tunnel with wet-wall cooling with average day temperatures ranging from 24°C - 28°C. Plants were tested after four months as above to confirm virus transmission. Seedlings were monitored for oak leaf and leaf flecking symptoms as these symptoms were associated with the first CiVA isolate found in citrus in South Africa and also reported for other coguviruses (Beris et al., 2021; Navarro et al., 2018a).

3.2.3 Sequence diversity of three encoded CiVA proteins

A sequence diversity study was performed by Sanger sequencing a partial region of the RdRp of forty-two samples from six production regions and three citrus species, using the same primer pair (CiVA_5148F/CiVA_6023R) used for the detection of CiVA in field samples. The amplified PCR product spans over a 515 bp region of the conserved RdRp domain found in members of the order *Bunyavirales* (Navarro et al. 2018a) and 229 bp over a non-conserved region. The complete MP and NP of eight samples, one from each production region and citrus species, were Sanger sequenced with primers designed on the RNA 2 of available South African isolates MT720884 and MT720886 (Table 3.2).

Table 3.2: Primer pairs used for the Sanger sequencing of the RNA 2 proteins of CiVA.

Primer pair	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)	Region on RNA 2
CiVA2 1F CiVA2 571R	ACACAAAGATCCCATAACTTT TCGTGTAGAGACATTCCTG	570	48	Movement protein
CiVA2 1F CiVA2 1383R	ACACAAAGATCCCATAACTTT GTTCAGCAGAGCRCAC	1382	48	Movement protein
CiVA2 389F CiVA2 912R	ACTCTGCCTTTGACCAAGATTCC CTCYCTYACCTTTCTCCTTTG	523	54	Movement protein
CiVA2 389F CiVA2 1383R	ACTCTGCCTTTGACCAAGATTCC GTTCAGCAGAGCRCAC	994	49	Movement protein
CiVA2 899F CiVA2 2132R	AGGAGAAAGGTGAGGGAGATGTATGAG CTCTCGACTGAGGGGCAGTCAACAG	1285	60	Movement + nucleocapsid protein
CiVA2 1214F CiVA2 2132R	GGTGTGAAGTTTAATGAGTTTGGAG CTCTCGACTGAGGGGCAGTCAACAG	918	54	Movement + nucleocapsid protein
CiVA2 1214F CiVA2 2694R	GGTGTGAAGTTTAATGAGTTTGGAG ACACATAGAACCCATAACTTTTG	1480	52	Movement + nucleocapsid protein
CiVA2 2044F CiVA2 2694R	GCAGGTTCACTCCTACC ACACATAGAACCCATAACTTTTG	650	48	Nucleocapsid protein

3.2.3.1 Sanger sequencing

Complimentary DNA was synthesized using reverse primer CiVA_6023R for the partial RdRp region and the respective reverse primers for the MP and NP regions (Table 3.2). RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific) and of total RNA (1 µg) was incubated with 10 µM reverse primer for 3 min at 65°C followed by 1 min on ice. Additional components added include 5x Reaction Buffer (ThermoFisher Scientific), 1mM dNTP Mix (ThermoFisher Scientific), 20 units RT enzyme and 5 units RiboLock RNase Inhibitor (ThermoFisher Scientific) with a total reaction volume of 10 µl Reverse Transcription was performed at 50°C for 60 min and reaction termination at 85°C for 10 min.

End-point RT-PCR was performed as above with the respective primer pairs (CiVA_5148F/CiVA_6023R and Table 3.2). PCR products were gel-purified using the Zymoclean Gel DNA recovery kit (Zymo Research, Irvine, CA, USA). Direct sequencing was performed in both directions by Inqaba Biotechnical Industries (Pty) Ltd. Low quality bases were removed and ambiguities were manually edited. Overlapping sequences of the forward and reverse sequences were used to create a consensus sequence for each sample and primer pair (Table 3.2) using BioEdit (Hall, 1999). The overlapping consensus sequences of each primer pair was aligned in CLC Sequence Viewer 8 (QIAGEN Aarhus., Prismet, Denmark) in order to assemble the complete MP and NP gene sequences.

3.2.3.2 Phylogenetic analysis

Phylogenetic analysis was performed on the nucleotide (nt) sequences, obtained with Sanger sequencing of the partial RdRp and the complete MP and NP sequences of the selected South African CiVA isolates. A CCGaV isolate was included in the phylogenetic analysis as the outgroup. MAFFT online version 7, set with default parameters (Katoh & Standley, 2013), was used to generate sequence alignments of the partial RdRp and complete MP and NP nt sequences. The ends of the sequences were trimmed to obtain sequences of equal length. Maximum-likelihood (ML) phylogenetic trees were created in MEGA 7 (Kumar et al., 2016) using the respective alignments. Bootstrap support of 1000 replications were applied, followed by the removal of bootstrap values below 50%. The Jukes-Cantor substitution model was used during the phylogenetic analysis (Jukes & Cantor, 1969). Additionally, nt similarity matrices for each gene region was constructed in BioEdit with BLOSUM 62 (Hall, 1999).

3.3 RESULTS

3.3.1 Detection of CiVA in field samples

3.*3.1.1 'Delta' Valencia sweet orange*

Symptoms on 'Delta' Valencia fruit were observed as different sized green blotches that were either depressed lesions on the rind (Fig. 3.1 B, C and E) or flat discoloured areas (Fig. 3.1 A and D). These symptoms were observed in the early fruit ripening stage. The green blotches were no longer visible after fruit ripening unless depressions were present in the fruit rind (Fig. 3.1 F). Typically, fruit symptoms were observed on a single branch of an infected tree; however, severe symptom expression was also observed where the entire tree displayed fruit rind symptoms. Gumming in the albedo was not observed in fruits displaying rind symptoms. No fruit drop was observed for the trees where the rind symptom was expressed.

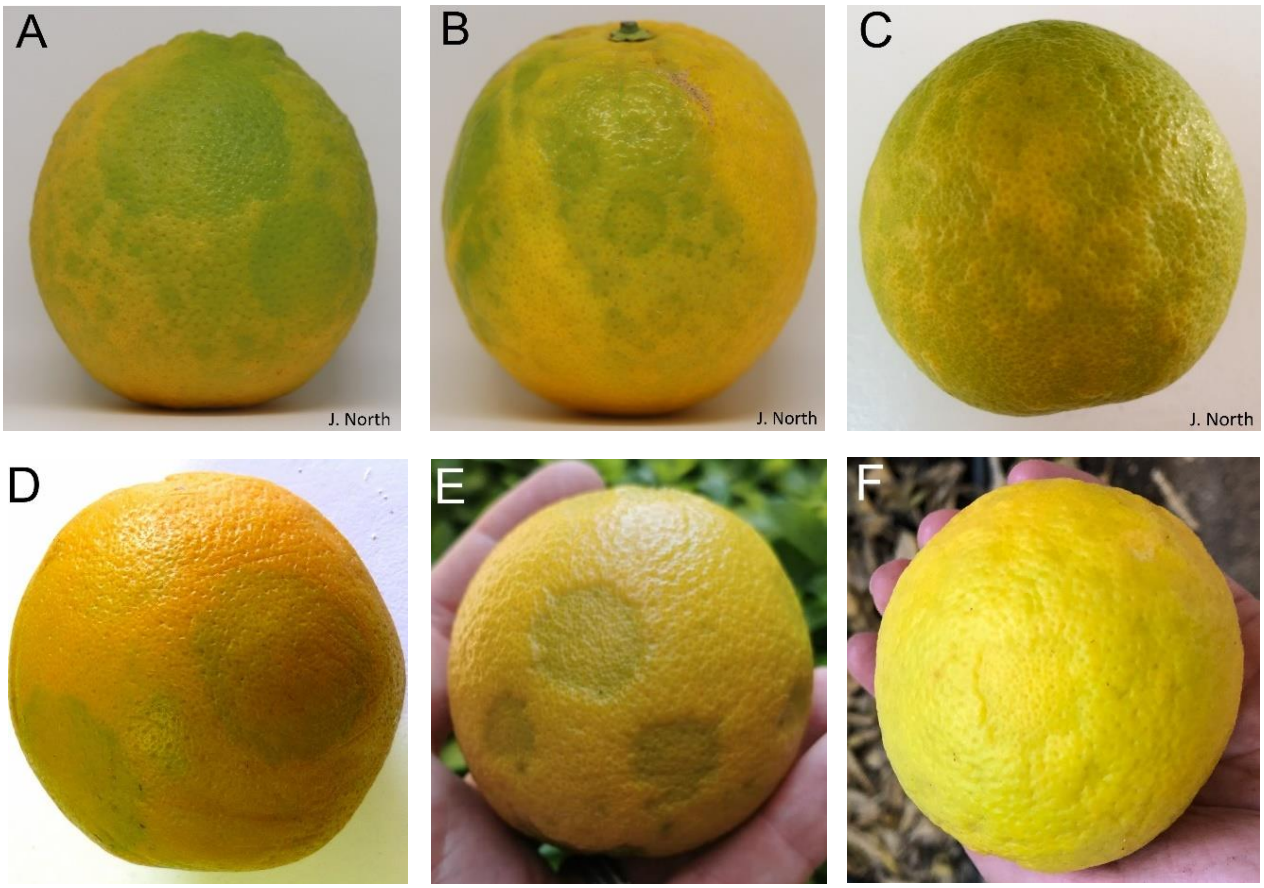

Figure 3.1: Symptoms on 'Delta' Valencia fruit in the early fruit ripening stage is seen as green blotches of various sizes (A - E), that are either depressed lesions in the rind (B, C and E) or occur as discoloured areas without depressions (A and D). As the fruit ripens, the green blotching diminishes (B and D) until lesions are no longer visible unless the lesions were expressed as depressions in the rind which remain visible (F).

All symptomatic 'Delta' Valencia trees were observed in the three production regions of the Limpopo province and the most (113) samples were collected in the Hoedspruit production region. The initial report of citrus impietratura-like symptoms on the rind of 'Delta' Valencia fruit originated within the Hoedspruit region in 2011. The grower associated symptom expression with periods of cooler night temperatures during the fruit ripening stage and symptom expression was not observed every year. All symptomatic trees from the Letsitele and Tshipise citrus production regions, within the Limpopo province, were CiVA positive; however, samples classified as asymptomatic and uncertain from the Tshipise region were also CiVA positive. No correlation between symptom expression and a specific rootstock were observed as symptoms were observed on trees grafted on Rough lemon (*C. jambhiri* Lush), Carrizo citrange (*C. sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.) and Swingle citrumelo (*C. paradisi* Macf. × *P. trifoliata* L. Raf.) rootstocks.

Samples collected from the three citrus production regions in the Mpumalanga province were classified as uncertain or asymptomatic. CiVA was not detected in the Malelane citrus production region. CiVA was detected in the two samples, one uncertain and one asymptomatic, from the Hectorspruit region and only one asymptomatic sample from the Komatipoort region. Six asymptomatic trees were sampled in the Kirkwood citrus production region of the Eastern Cape province and four were confirmed CiVA positive.

Fifty-two percent of the CiVA positive trees were symptomatic while the remaining 48% were classified as uncertain or asymptomatic. 139 samples (90%) collected from orchards planted between 1990 and 2004 tested positive for CiVA. CiVA was not detected in 16 samples collected from orchards planted in 1984, 1990, 1992 and 2011 and no fruit rind symptoms were observed on the sampled trees. The only other citrus pathogen detected in CiVA positive and CiVA negative 'Delta' Valencia samples were CTV.

3.3.1.2 'Marsh' grapefruit

Typical citrus impietratura symptoms were observed on the 'Marsh' grapefruit samples received in 2018 including severe fruit drop, gumming in the albedo and sunken green areas on the fruit rind. Samples were collected from three different orchards in Nkwaleni, Kwazulu-Natal and included ten symptomatic and eight asymptomatic samples. The ten symptomatic samples tested positive for CiVA while the eight asymptomatic trees were CiVA negative. CTV, HSVd and CDVd was detected in all symptomatic samples while eight samples additionally contained CEVd. No symptoms were observed in the same orchard in 2019.

3.3.1.3 Clementine

CiVA was detected in the three clementine citrus species from the Hoedspruit production region; however, no fruit rind or leaf symptoms were observed. Only one field site, planted in 2011, was visited.

3.3.2 Biological indexing

Eight of the 15 inoculated 'Madam vinous' sweet orange indicator seedlings tested positive for CiVA and CTV four months after inoculation. One seedling tested positive for only CTV and the graft inoculations to six plants were unsuccessful. Leaf flecking symptoms were observed on the CiVA positive seedlings (Fig. 3.2). The presence of fruit rind symptoms could not be monitored due to the absence of fruit.

Figure 3.2: Leaf flecking (right) on a 'Madam vinous' sweet orange seedling inoculated with bark from a 'Delta' Valencia CiVA positive tree from the Hoedspruit region in South Africa. Symptomless leaf (left) from an un-inoculated seedling.

3.3.3 Sequence diversity of three encoded CiVA proteins

3.3.3.1 Sanger sequencing

A sequence of 744 bp, representing a partial RdRp sequence at nucleotide positions 726 to 1468, was generated for forty-two CiVA positive samples from six citrus production regions and three citrus species. The complete MP (1188 bp) and NP (1113 bp) sequences were assembled for eight CiVA positive samples from each production region and citrus species. The sequences produced during the Sanger sequencing of each gene region was submitted to GenBank and GenBank accession numbers are indicated in Table 3.3.

Table 3.3: GenBank accession numbers and sample information of South African citrus virus A (CiVA) isolates used in the nucleotide analysis of the RNA-dependant RNA polymerase (RdRp), the movement protein (MP) and the nucleocapsid protein (NP).

Genbank Accession	Sample	Encoded protein	Citrus Type	PROVINCE Region	Rootstock	Year planted	Rind Symptoms
				MPUMALANGA			
MZ055887	Kmp	Partial RdRp	Valencia	Komatipoort	Swingle	1990	Asymptomatic
MZ055888	Hcs1	Partial RdRp	Valencia	Hectorspruit	Swingle	1997	Uncertain
MZ055889	Hcs2	Partial RdRp	Valencia	Hectorspruit	Swingle	1997	Asymptomatic
MZ055882	Cmp	Partial RdRp	Clementine		Carizzo	Gene source	Asymptomatic
MZ020555	Kmp	Complete MP	Valencia	Komatipoort	Swingle	1990	Asymptomatic
MZ020551	Hcs	Complete MP	Valencia	Hectorspruit	Swingle	1997	Uncertain
MZ020552	Cmp	Complete MP	Clementine		Carizzo	Gene source	Asymptomatic
MZ020564	Kmp	Complete NP	Valencia	Komatipoort	Swingle	1990	Asymptomatic
MZ020560	Hcs	Complete NP	Valencia	Hectorspruit	Swingle	1997	Uncertain
MZ020561	Cmp	Complete NP	Clementine		Carizzo	Gene source	Asymptomatic
				EASTERN CAPE			
MZ055883	Kwd1	Partial RdRp	Valencia	Kirkwood	Swingle	1990	Asymptomatic
MZ055884	Kwd2	Partial RdRp	Valencia	Kirkwood	Troyer	1990	Asymptomatic
MZ055885	Kwd3	Partial RdRp	Valencia	Kirkwood	Troyer	1990	Asymptomatic
MZ055886	Kwd4	Partial RdRp	Valencia	Kirkwood	Troyer	1990	Asymptomatic
MZ020554	Kwd	Complete MP	Valencia	Kirkwood	Troyer	1990	Asymptomatic
MZ020563	Kwd	Complete NP	Valencia	Kirkwood	Troyer	1990	Asymptomatic
				LIMPOPO			
MZ055890	Lts1	Partial RdRp	Valencia	Letsitele	Carizzo	1996	Symptomatic
MZ055891	Lts2	Partial RdRp	Valencia	Letsitele	Carizzo	1996	Symptomatic
MZ055892	Lts3	Partial RdRp	Valencia	Letsitele	Carizzo	1996	Symptomatic
MZ055893	Lts4	Partial RdRp	Valencia	Letsitele	Carizzo	1996	Symptomatic
MZ055894	Tsp1	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Uncertain
MZ055895	Tsp2	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Uncertain
MZ055896	Tsp3	Partial RdRp	Valencia	Tshipise	Rough Lemon	2004	Uncertain
MZ055897	Tsp4	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Symptomatic
MZ055898	Tsp5	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Symptomatic
MZ055899	Tsp6	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Uncertain
MZ055900	Tsp7	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Uncertain
MZ055901	Tsp8	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Symptomatic
MZ055902	Tsp9	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Symptomatic
MZ055903	Tsp10	Partial RdRp	Valencia	Tshipise	Rough Lemon	1999	Asymptomatic
MZ055904	Hds1	Partial RdRp	Valencia	Hoedspruit	Carizzo	1995	Symptomatic
MZ055905	Hds2	Partial RdRp	Valencia	Hoedspruit	Carizzo	1995	Symptomatic
MZ055906	Hds3	Partial RdRp	Valencia	Hoedspruit	Carizzo	1995	Symptomatic
MZ055907	Hds4	Partial RdRp	Valencia	Hoedspruit	Carizzo	1995	Symptomatic
MZ055908	Hds5	Partial RdRp	Valencia	Hoedspruit	Carizzo	1995	Symptomatic
MZ055909	Hds6	Partial RdRp	Valencia	Hoedspruit	Swingle	1995	Symptomatic
MZ055910	Hds7	Partial RdRp	Valencia	Hoedspruit	Swingle	1995	Symptomatic
MZ055911	Hds8	Partial RdRp	Valencia	Hoedspruit	Swingle	1995	Symptomatic
MZ055912	Hds9	Partial RdRp	Valencia	Hoedspruit	Swingle	1995	Symptomatic
MZ055913	Hds10	Partial RdRp	Valencia	Hoedspruit	Swingle	1995	Asymptomatic
MZ020556	Lts	Complete MP	Valencia	Letsitele	Carizzo	1996	Symptomatic
MZ020557	Tsp	Complete MP	Valencia	Tshipise	Rough Lemon	1999	Asymptomatic
MZ020565	Lts	Complete NP	Valencia	Letsitele	Carizzo	1996	Symptomatic
MZ020566	Tsp	Complete NP	Valencia	Tshipise	Rough Lemon	1999	Asymptomatic
MZ020562	Hds	Complete NP	Valencia	Hoedspruit	Swingle	1995	Asymptomatic
				KWAZULU-NATAL			
MZ055914	Nkw2C.1	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055915	Nkw2C.2	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055916	Nkw2C.3	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055917	Nkw2C.4	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055918	Nkw2C.5	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055919	Nkw2C.6	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055920	Nkw3A	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055921	Nkw3B	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055922	Nkw10A	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ055923	Nkw10B	Partial RdRp	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ020559	Nkw	Complete MP	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura
MZ020568	Nkw	Complete NP	Grapefruit	Nkwaleni	Not recorded	2003	Impietratura

3.3.3.2 Phylogenetic analysis

Phylogenetic analysis of the partial RdRp is illustrated in Figure 3.3. The 'Delta' Valencia and grapefruit isolates including South African isolate 1.5 (MT720883) and 1.8 (MT720885) formed a clade with 85% bootstrap support. Within the clade, isolate 1.5 and 'Delta' Valencia isolates (MZ055883 to MZ055912) grouped together with bootstrap support of 89%. A single 'Delta' Valencia isolate (MZ055910) separated from isolate 1.5 and the other 'Delta' Valencia isolates with 60% bootstrap support. The grapefruit isolates also grouped together within the clade, apart from isolate Nkw10B (MZ055923) with 94% bootstrap support. Although the clementine isolate (MZ055882) appeared to be separated from the clade, it was without significant bootstrap support. Nucleotide identities of the partial RdRp (Table 3.4) between 'Delta' Valencia isolates (MZ055885 to MZ055913) and isolate 1.5 (MT720883) ranged from 99.5% to 100%, supporting the grouping of these isolates. The grapefruit isolate (MZ055916) shared 98.5% to 98.6% identity to the 'Delta' Valencia isolates and 96.3% to the clementine isolate. The clementine isolate shared the lowest nt identity to other South African citrus isolates at 95.8%.

Figure 3.4 illustrates the phylogenetic analysis of the complete MP nt sequences. The formation of two subclades was supported by a bootstrap value of 62%. Isolates from 'Delta' Valencia (MZ020551 and MZ020553 to MZ020557) and South African isolate 1.5 (MT720884) grouped together in the first subclade with strong bootstrap support of 98%. The second subclade was formed with 79% bootstrap support, containing the clementine isolate (MZ020552) and South African isolate 1.8 (MT720886). An isolate from the USA (MT922053) and the South African CiVA isolate from pear (MZ463040) also formed part of the second subgroup. The isolate from grapefruit (MZ020559) separated from the two subclades, however without significant bootstrap support. The formation of the first subclade is supported by the results of the nt identity matrix (Table 3.5). 'Delta' Valencia isolates and South African isolate 1.5 shared high nt identities between each other at 99.6% to 99.9%. Isolates from the second subclade shared ranging between 96% to 97.2%. The grapefruit isolate (MZ020559) shared 98.4% to 98.6% nt identity to the 'Delta' Valencia isolates and 96.1% nt identity to the clementine isolate (MZ020552). Similar to the partial RdRp identity matrix, the clementine isolate shared the lowest nt identity to other South African citrus isolates at 96%.

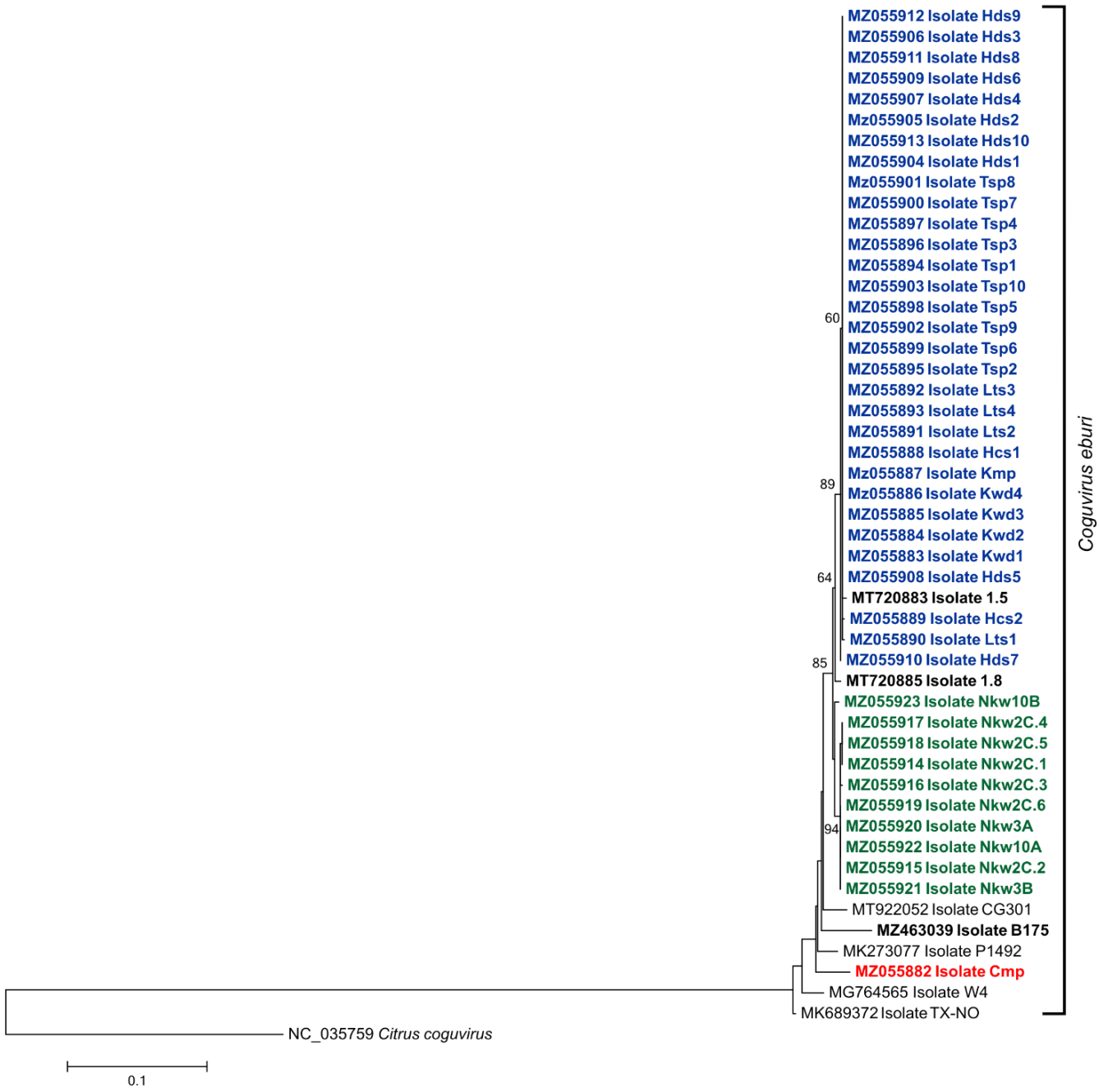

Figure 3.3: Maximum-likelihood phylogenetic tree illustrating the evolutionary relationship between citrus virus A (CiVA) isolates based on a partial RNA-dependent RNA-polymerase nucleotide sequence (744bp). South African CiVA isolates from citrus and pear (MZ463039) are indicated in bold. Selected international CiVA isolates are included and a CCGaV isolate (NC_035759) was used as the outgroup. The tree was drawn to scale and bootstrap value below 50% were removed. 'Delta' Valencia isolates are indicated in blue, 'Marsh' grapefruit isolates are indicated in green and the clementine isolate is indicated in red.

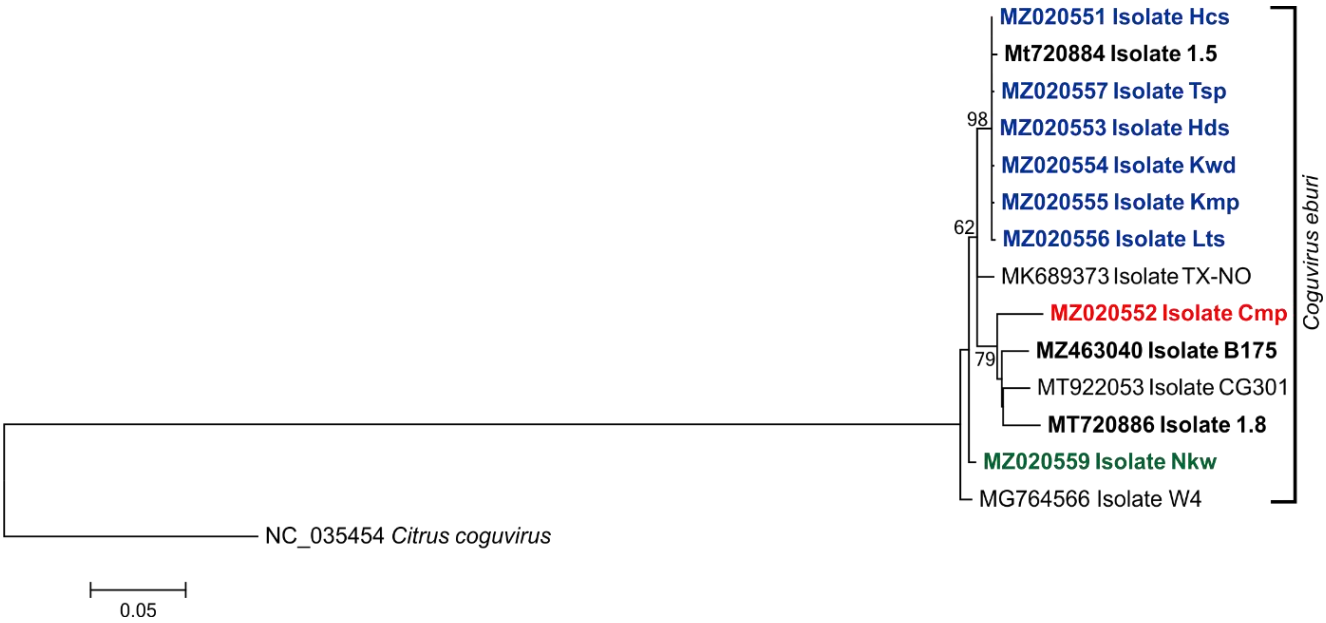

Figure 3.4: Maximum-likelihood phylogenetic tree illustrating the evolutionary relationship between citrus virus A (CiVA) isolates based on the complete movement protein nucleotide sequence. South African CiVA isolates from citrus and pear (MZ463040) are indicated in bold. Selected international CiVA isolates are included and a CCGaV isolate (NC_035454) was used as the outgroup. The tree was drawn to scale and bootstrap value below 50% were removed. 'Delta' Valencia isolates are indicated in blue, the 'Marsh' grapefruit isolate is indicated in green and the clementine isolate is indicated in red.

Two subclades formed in the NP phylogenetic tree (Fig. 3.5) with high bootstrap support of 97%. Similar to the MP phylogenetic tree, the first subclade contained 'Delta' Valencia isolates (MZ020560 and MZ020562 to MZ020566) and the South African isolate 1.5 (MT720884) with bootstrap support of 81%. Within the first subclade, isolate Lts (MZ020565) and isolate Tsp (MZ020566) grouped together with 60% bootstrap support. The second subclade contained an isolate from the USA (MK689373), the grapefruit isolate (MZ020568) and the isolate from Italy (MG764566). The grapefruit and Italy isolates grouped together in the subclade with strong bootstrap support of 91%. The clementine isolate (MZ020561) separated from the two subclades with South African isolate 1.8 (MT720886) without significant bootstrap support. The nt identity matrix of the complete NP sequences (Table 3.6) supported the formation of the first subclade. 'Delta' Valencia isolates and isolate 1.5 shared nt identities ranging from 98.3% to 99.9%. Isolate Lts and Tsp shared high nt identities between each other at 99.8%, supporting their grouping within the subclade. The grapefruit isolate shared nt identities ranging from 97.5% to 98.8% to the 'Delta' Valencia isolates and 94.8% to the clementine isolate. The clementine isolate shared the lowest nt identities of 94.1% to 95.5% with the 'Delta' Valencia isolates, similar to the partial RdRp and complete MP identity matrices.

The consistent grouping of 'Delta' Valencia isolates in the phylogenetic trees and the high nt similarities between these isolates based on the partial RdRp (99.5% to 100%) and the complete MP (99.6% to 99.9%) indicate low sequence diversity within this group. The NP was the least conserved with nt identities between these isolates ranging between 98.3% to 99.9%. The clementine isolate consistently had the lowest nt identity to the 'Delta' Valencia and grapefruit isolates and separated from these isolates and was more closely related to the South African CiVA isolate from pear (isolate B175) in the three phylogenetic trees.

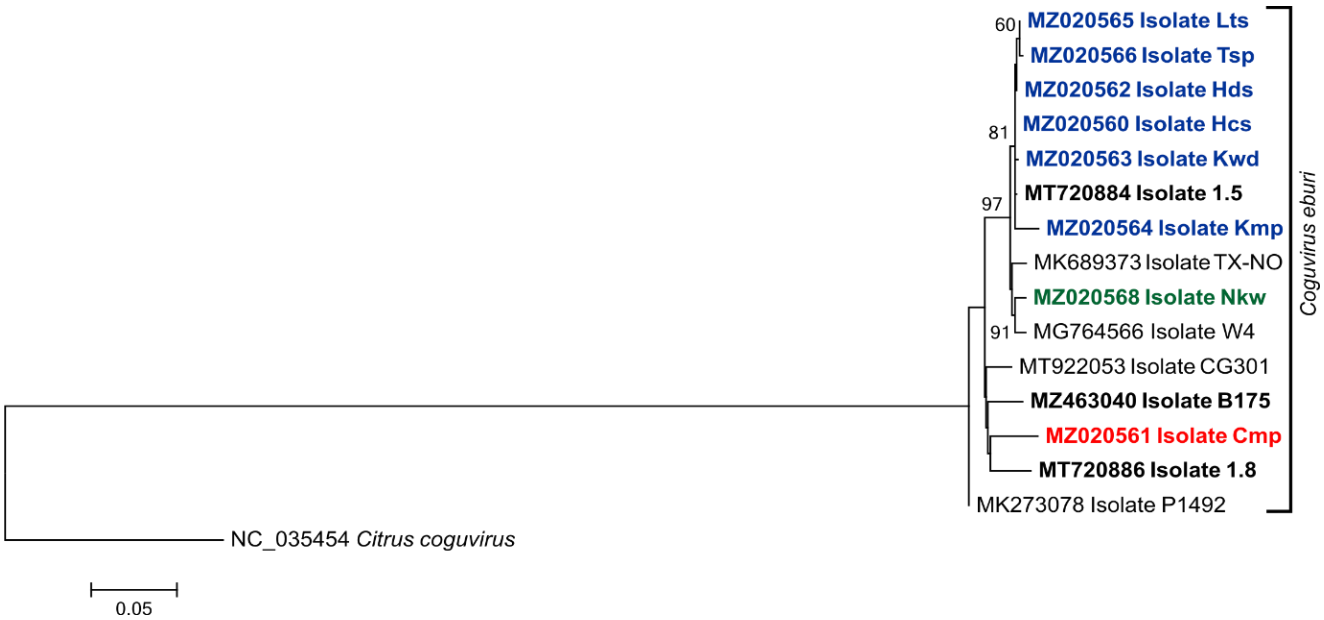

Figure 3.5: Maximum-likelihood phylogenetic tree illustrating the evolutionary relationship between citrus virus A (CiVA) isolates based on complete nucleocapsid protein nucleotide sequences. South African CiVA isolates from citrus and pear (MZ463040) are indicated in bold. Selected international CiVA isolates are also included and a CCGaV isolate (NC_035454) was used as the outgroup. The tree was drawn to scale and bootstrap value below 50% were removed. Delta' Valencia isolates are indicated in blue, the 'Marsh' grapefruit isolate is indicated in green and the clementine isolate is indicated in red.

Table 3.4: Nucleotide sequence identity matrix of the partial RNA-dependant RNA polymerase of South African citrus virus A (CiVA) isolates and selected isolates from GenBank**.**

	MZ055882 CMP	MZ055885 Kwd	MZ055887 Kmp	MZ055888 Hcs	MZ055890 Lts	MZ055903 Tsp	MZ055913 Hds	MZ055916 Nkw	MT922052 CG301	MK273077 P1492	MK689372 TX-NO	MG764565 W4	MT720883 1.5	MT720885 1.8	MZ463039 B175
MZ055882 CMP	ID	0,959	0,959	0,959	0,958	0,959	0,959	0,963	0,954	0,961	0,958	0,959	0,959	0,961	0,939
MZ055885 Kwd	0,959	ID	1	1	0,998	1	1	0,986	0,97	0,971	0,969	0,956	0,997	0,99	0,951
MZ055887 Kmp	0,959	1	ID	1	0,998	1	1	0,986	0,97	0,971	0,969	0,956	0,997	0,99	0,951
MZ055888 Hcs	0,959	1	1	ID	0,998	1	1	0,986	0,97	0,971	0,969	0,956	0,997	0,99	0,951
MZ055890 Lts	0,958	0,998	0,998	0,998	ID	0,998	0,998	0,985	0,969	0,97	0,967	0,955	0,995	0,989	0,95
MZ055903 Tsp	0,959	1	1	1	0,998	ID	1	0,986	0,97	0,971	0,969	0,956	0,997	0,99	0,951
MZ055913 Hds	0,959	1	1	1	0,998	1	ID	0,986	0,97	0,971	0,969	0,956	0,997	0,99	0,951
MZ055916 Nkw	0,963	0,986	0,986	0,986	0,985	0,986	0,986	ID	0,973	0,973	0,973	0,96	0,983	0,987	0,956
MT922052 CG301	0,954	0,97	0,97	0,97	0,969	0,97	0,97	0,973	ID	0,969	0,969	0,954	0,967	0,971	0,951
MK273077 P1492	0,961	0,971	0,971	0,971	0,97	0,971	0,971	0,973	0,969	ID	0,967	0,96	0,969	0,97	0,951
MK689372 TX-NO	0,958	0,969	0,969	0,969	0,967	0,969	0,969	0,973	0,969	0,967	ID	0,977	0,969	0,97	0,947
MG764565 W4	0,959	0,956	0,956	0,956	0,955	0,956	0,956	0,96	0,954	0,96	0,977	ID	0,956	0,958	0,938
MT720883 1.5	0,959	0,997	0,997	0,997	0,995	0,997	0,997	0,983	0,967	0,969	0,969	0,956	ID	0,987	0,948
MT720885 1.8	0,961	0,99	0,99	0,99	0,989	0,99	0,99	0,987	0,971	0,97	0,97	0,958	0,987	ID	0,955
MZ463039 B175	0,939	0,951	0,951	0,951	0,95	0,951	0,951	0,956	0,951	0,951	0,947	0,938	0,948	0,955	ID

Table 3.5: Nucleotide sequence identity matrix of the complete movement protein of South African citrus virus A (CiVA) isolates and selected isolates from GenBank.

	MZ020551 Hcs	MZ020552 Cmp	MZ020553 Hds	MZ020554 Kwd	MZ020555 Kmp	MZ020556 Lts	MZ020557 Tsp	MZ020559 Nkw	MT922053 CG301	MK689373 TX-NO	MG764566 W4	MT720884 1.5	MT720886 1.8	MZ463040 B175
MZ020551 Hcs	ID	0,961	1	0,999	0,999	0,997	0,999	0,986	0,968	0,984	0,978	0,997	0,961	0,968
MZ020552 Cmp	0,961	ID	0,961	0,96	0,96	0,96	0,96	0,961	0,965	0,96	0,957	0,958	0,96	0,962
MZ020553 Hds	1	0,961	ID	0,999	0,999	0,997	0,999	0,986	0,968	0,984	0,978	0,997	0,961	0,968
MZ020554 Kwd	0,999	0,96	0,999	ID	0,998	0,996	0,998	0,985	0,968	0,983	0,977	0,996	0,96	0,967
MZ020555 Kmp	0,999	0,96	0,999	0,998	ID	0,996	0,998	0,985	0,968	0,983	0,977	0,996	0,96	0,967
MZ020556 Lts	0,997	0,96	0,997	0,996	0,996	ID	0,996	0,984	0,966	0,981	0,975	0,994	0,96	0,965
MZ020557 Tsp	0,999	0,96	0,999	0,998	0,998	0,996	ID	0,985	0,968	0,983	0,977	0,996	0,962	0,967
MZ020559 Nkw	0,986	0,961	0,986	0,985	0,985	0,984	0,985	ID	0,97	0,984	0,986	0,984	0,962	0,968
MT922053 CG301	0,968	0,965	0,968	0,968	0,968	0,966	0,968	0,97	ID	0,968	0,963	0,966	0,968	0,972
MK689373 TX-NO	0,984	0,96	0,984	0,983	0,983	0,981	0,983	0,984	0,968	ID	0,977	0,981	0,963	0,965
MG764566 W4	0,978	0,957	0,978	0,977	0,977	0,975	0,977	0,986	0,963	0,977	ID	0,975	0,957	0,961
MT720884 1.5	0,997	0,958	0,997	0,996	0,996	0,994	0,996	0,984	0,966	0,981	0,975	ID	0,958	0,967
MT720886 1.8	0,961	0,96	0,961	0,96	0,96	0,96	0,962	0,962	0,968	0,963	0,957	0,958	ID	0,967
MZ463040 B175	0,968	0,962	0,968	0,967	0,967	0,965	0,967	0,968	0,972	0,965	0,961	0,967	0,967	ID

Table 3.6: Nucleotide sequence identity matrix of the complete nucleocapsid protein of South African citrus virus A (CiVA) isolates and selected isolates from GenBank.

	MZ020560 Hcs	MZ020561 Cmp	MZ020562 Hds	MZ020563 Kwd	MZ020564 Kmp	MZ020565 Lts	MZ020566 Tsp	MZ020568 Nkw	MZ463040 B175	MT720884 1.5	MT720885 1.8	MK689373 TX-NO	MT922053 CG301	MG764566 W4	MK273078 P1492
MZ020560 Hcs	ID	0,955	0,999	0,998	0,986	0,997	0,997	0,988	0,963	0,999	0,959	0,988	0,967	0,988	0,973
MZ020561 Cmp	0,955	ID	0,954	0,953	0,941	0,954	0,954	0,948	0,954	0,954	0,952	0,951	0,956	0,948	0,964
MZ020562 Hds	0,999	0,954	ID	0,997	0,985	0,998	0,996	0,987	0,962	0,998	0,958	0,987	0,966	0,987	0,973
MZ020563 Kwd	0,998	0,953	0,997	ID	0,984	0,995	0,995	0,986	0,961	0,997	0,957	0,986	0,965	0,986	0,973
MZ020564 Kmp	0,986	0,941	0,985	0,984	ID	0,983	0,983	0,975	0,949	0,985	0,946	0,976	0,956	0,974	0,96
MZ020565 Lts	0,997	0,954	0,998	0,995	0,983	ID	0,998	0,985	0,962	0,996	0,96	0,985	0,966	0,987	0,971
MZ020566 Tsp	0,997	0,954	0,996	0,995	0,983	0,998	ID	0,985	0,962	0,996	0,96	0,985	0,966	0,987	0,971
MZ020568 Nkw	0,988	0,948	0,987	0,986	0,975	0,985	0,985	ID	0,957	0,987	0,952	0,983	0,961	0,987	0,967
MZ463040 B175	0,963	0,954	0,962	0,961	0,949	0,962	0,962	0,957	ID	0,962	0,957	0,959	0,965	0,956	0,97
MT720884 1.5	0,999	0,954	0,998	0,997	0,985	0,996	0,996	0,987	0,962	ID	0,958	0,989	0,966	0,987	0,973
MT720885 1.8	0,959	0,952	0,958	0,957	0,946	0,96	0,96	0,952	0,957	0,958	ID	0,954	0,961	0,954	0,967
MK689373 TX-NO	0,988	0,951	0,987	0,986	0,976	0,985	0,985	0,983	0,959	0,989	0,954	ID	0,967	0,983	0,97
MT922053 CG301	0,967	0,956	0,966	0,965	0,956	0,966	0,966	0,961	0,965	0,966	0,961	0,967	ID	0,961	0,975
MG764566 W4	0,988	0,948	0,987	0,986	0,974	0,987	0,987	0,987	0,956	0,987	0,954	0,983	0,961	ID	0,967
MK273078 P1492	0,973	0,964	0,973	0,973	0,96	0,971	0,971	0,967	0,97	0,973	0,967	0,97	0,975	0,967	ID

3.4 DISCUSSION

Initially, no pathogen could be associated with citrus impietratura; however, the disease was suspected to be caused by a virus due to the graft transmissibility of the disease (Caruso et al., 1993; Ruggieri, 1965). Resolving the aetiology of citrus impietratura has recently advanced with the discovery of CiVA (Navarro et al., 2018b) as one report suggests an association of CiVA with citrus impietratura (Beris et al., 2021). In this study, a field survey was conducted to establish the distribution of CiVA, determine the association of CiVA with citrus impietratura and evaluate the genetic diversity of CiVA.

The 'Marsh' grapefruit samples, displaying typical citrus impietratura symptoms, were received from Nkwaleni in the Kwazulu-Natal province where citrus impietratura was first reported in South Africa (Moll et al., 1976). CiVA detection correlated with symptomatic trees and asymptomatic trees were CiVA negative. Citrus viroid's and CTV were also detected in the symptomatic trees along with CiVA; however, these other pathogens have not been associated with citrus impietratura symptoms. It is possible that the stress induced by multiple pathogens intensified the severity of symptom expression as previous reports indicate that co-infection can alter disease severity (Abdullah et al., 2017; Rubio et al., 2013). These results suggest the association of CiVA with citrus impietratura in grapefruit and support the previous report and association of CiVA with citrus impietratura (Beris et al., 2021). The 'Marsh' grapefruit orchards in Nkwaleni are 17 years old and was most likely propagated from infected source trees, which has been phased out with the implementation of the CIS. Citrus impietratura on grapefruit in South Africa is therefore currently a rare occurrence.

CiVA was detected in six of the seven citrus production regions, mostly in 'Delta' Valencia orchards planted between 1990 and 2004. Subsequent to the implementation of the CIS, cultivars went through the STG process. CiVA was therefore not detected in the current 'Delta' Valencia budwood source trees or newer orchard plantings. Supply of CiVA free 'Delta' Valencia budwood was initially supplemented with budwood from the open-ground source trees as sufficient supply of the new budwood source was not available, explaining the detection of CiVA in orchards planted between 1990 and 2004.

CiVA was detected in the 'Delta' Valencia trees displaying impietratura-like symptoms on the fruit rind, without gumming in the albedo. CiVA was also detected in 'Delta' Valencia trees with no fruit rind symptom; however, all CiVA negative trees were asymptomatic. These results would suggest that CiVA is likely associated with the citrus impietratura-like symptoms observed on the fruit rind; however, symptom expression is erratic. Historic reports indicate that citrus impietratura symptoms were not

observed every year; however, when observed, symptom expression was inconsistent (Papasolomontos, 1965; Scaramuzzi et al., 1968; Terranova & Scuderi 1968). Similarly, in the 'Marsh' grapefruit orchards monitored in this study, erratic symptom expression was observed. Symptom expression is most likely influenced by environmental conditions (Scaramuzzi et al., 1968; Van Vuuren et al., 1984). The development of citrus impietratura symptoms on the fruit rind was experimentally associated with cooler temperatures (20°C - 24°C) during fruit development and when temperatures were increased to 27°C - 33°C, gumming was observed in the albedo (Bar-Joseph, 1976). Symptom expression on 'Delta' Valencia fruit was most likely also associated with cooler night temperatures during fruit development, as noted by the grower. Previous reports of sweet oranges with dark green areas and rind depressions, without gumming in the albedo, were referred to as citrus impietratura (Cartia and Catara 1974). Therefore, the disease observed on the rind of 'Delta' Valencia fruit is most likely the same disease, based on the symptom description.

The severity of symptom expression was reported to vary between citrus types due to their susceptibility to citrus impietratura (Cartia & Catara, 1972; Cartia & Catara, 1974; Scaramuzzi et al., 1968). Sweet oranges and grapefruit were reported to be highly susceptible to citrus impietratura (Cartia & Catara, 1972); however, symptoms observed on 'Delta' Valencia sweet oranges were less severe than symptoms observed on 'Marsh' grapefruit. It is possible that a combination of environmental conditions and a higher tolerance of 'Delta' Valencia trees resulted in less severe symptom expression. The absence of fruit drop in infected 'Delta' Valencia trees could be due to the absence of gum production in the affected fruit. The presence of gum in the peduncle of fruit from a citrus impietratura infected tree was associated with fruit drop as it was suspected that the localized gumming occluded the vessels, resulting in the drop of fruit (Caruso et al., 1993). No symptoms were observed on the clementine field samples and CIS budwood sources that tested positive for CiVA. Previous reports indicate the failure of symptom expression in clementine trees inoculated with infected material, suggesting that clementine has a higher tolerance to citrus impietratura (Cartia & Catara, 1972; Scaramuzzi et al., 1968).

The 'Delta' Valencia samples were from trees that were grafted on Rough lemon, Carrizo citrange and Swingle citrumelo rootstocks while the clementine samples were from trees that were grafted on Carizzo citrange. The rootstocks of the 'Marsh' grapefruit samples are unknown. No association between symptom expression and a specific rootstock was made in this study although historical reports indicated that Rough Lemon was initially thought to be more tolerant to citrus impietratura as no symptoms or only mild symptoms developed when inoculated with infected material (Ruggieri, 1965; Terranova & Scuderi, 1968). However, further investigations indicated that Rough lemon rootstocks were in fact susceptible to citrus impietratura (Caruso et al., 1993).

The natural spread of citrus impietratura was initially suspected (Scaramuzzi et al., 1968) but further investigations concluded that natural spread did not occur (Cartia & Catara, 1974; Pappo & Oren, 1974; van Vuuren et al., 1984). The natural spread of CiVA has not been confirmed as no insect vector has been identified. This is possibly due to the absence of an encoded glycoprotein (Diaz-Lara et al., 2019; Navarro et al., 2018a). Glycoproteins interact with the arthropod host receptors, allowing invasion of the host cell (Hulswit et al., 2021; Kormelink et al., 2021). There were no reports of CiVA spreading to adjacent orchards in the surveyed production regions, suggesting that CiVA is not transmitted by a natural vector or that no vector was present in the surveyed regions. Currently, the only known means of transmitting CiVA is through grafting and control of the virus can be achieved through the supply of virus-free budwood.

Biological symptom expression is a vital part of describing a virus and is supportive to any genetic data. Prior to any associated viruses, leaf flecking and/or oak leaf pattern on young leaves of sweet orange indicator seedlings were used to indicate the presence of graft-transmissible pathogens of citrus impietratura, concave gum and cristacortis infections (Bar-Joseph & Loebenstein, 1970; Roistacher, 1991; Vogel & Bové, 1974). Recently, two citrus-infecting coguviruses, CCGaV and CiVA, were associated with citrus concave gum disease (Navarro et al., 2018a) and citrus impietratura (Beris et al., 2021), respectively. Leaf flecking was observed during the biological indexing of CiVA positive material from sweet orange and grapefruit trees with citrus impietratura disease (Beris et al., 2021) and from CCGaV positive trees displaying concave gum disease (Navarro et al., 2018a). Similarly, in this study, leaf flecking was observed on sweet orange seedlings inoculated with CiVA positive material from the 'Delta' Valencia sources (Fig. 3.2) and although CTV was also detected in the CiVA positive trees, leaf flecking is not a symptom associated with CTV infection. However, no leaf flecking or oak leaf symptoms were observed during the biological indexing of CiVA positive samples by Navarro et al., (2018b), but expression of these symptoms are influenced by environmental conditions and poor symptom expression can occur under unfavourable conditions. (Roistacher, 1991). These symptoms are also transient and can be missed (Navarro et al., 2018a; Roistacher, 1991). The expression of leaf flecking or oak leaf symptoms can therefore be indicative of the presence of citrus infecting coguviruses and biological indexing is an important verification of disease presence.

The genetic diversification of species enables viruses to adapt to changing environments and hosts. This evolving diversification is an important consideration in the development of molecular detection assays (Rubio et al., 2020). Since CiVA was detected in six different geographical regions, three different citrus species as well as in pears in South Africa, the genetic diversity of CiVA was investigated to understand the origin of these infections as well as the possible influence of hosts and geographical distribution. Phylogenetic analysis is used to indicate relatedness between organisms and provides

information regarding the evolution of species (Soltis & Soltis, 2003). Nucleic acid sequences are used for the phylogenetic analysis of closely related viral species and variants (Michu, 2007), whereas analysis of amino acid sequences is used to analyse distantly related viruses (Simmons & Freudenstein, 2002). Therefore, in this study, the nucleotide sequences of three CiVA encoded proteins obtained for various CiVA isolates in South Africa were analysed as CCGaV has not been detected in South Africa.

The 'Delta' Valencia isolates grouped as clades in the three phylogenetic trees of the CiVA genes analysed and were supported by bootstrap values above 70%, indicating reliable groupings (Michu, 2007). The short branch lengths observed between the isolates from the different citrus production regions indicated high sequence similarity between these isolates. Nucleotide identity matrices supported the phylogenetic analyses and showed high percentage sequence similarities between the isolates from the different citrus production regions, suggesting that the geographical distribution of CiVA was not associated with genetic diversity. Results therefore indicate that these isolates were most likely derived from a common source. Before the implementation of the CIS, 'Delta' Valencia budwood material was supplied from open ground source trees, which would have been the most probable source of the detected field infections.

The 'Delta' Valencia isolates were more closely related to the 'Marsh' isolates as these isolates grouped together in a clade in both the RdRp and NP phylogenetic trees with bootstrap support values above 70%. However, within each clade these isolates separated from each other into subclades, indicating a more distant common ancestor. The separation of the 'Marsh' grapefruit and 'Delta' Valencia isolates for the MP were not significant. The percentage nucleotide similarities also indicated that the 'Delta' Valencia and 'Marsh' grapefruit isolates are more closely related to each other and that the clementine isolate is more distantly related to both of the above mentioned. Even though higher sequence diversity was observed between the clementine isolate and the 'Delta' Valencia and 'Marsh' grapefruit isolates in the nucleotide similarity matrices, these differences were not supported with reliable bootstrap values using the phylogenetic analyses. Lower bootstrap values may be due to the presence of uninformative sites in the alignment (Soltis & Soltis, 2003) and the diversity indicated in the nucleotide matrices is therefore not clearly observed in the phylogenetic trees. It is also of interest to note that the nucleotide sequences of the isolate described in pears in South Africa does not differ phylogenetically from those described from citrus and that the host species does not seem to have a significant effect on the genetic diversification of CiVA. In summary, the sequence diversity detected is most likely due to the origin of the various infected budwood sources. Historically, 'Marsh' grapefruit was imported in 1938 from Florida in the USA and again in 1952 from California while the clementine isolate was imported from Spain in 1995. 'Delta' Valencia was selected from a branch mutation of an old-clone Valencia tree in 1952 in South Africa.

Identification of conserved gene regions are important for the development of reliable diagnostic assays. Nucleotide identity percentages indicated that the MP was the most conserved gene followed by the RdRp with the NP being the least conserved, based on the similarity percentages between the isolates. Similarly, the RdRp of other members of the order *Bunyavirales* was shown to be more conserved than the NP (Rott et al., 2018). False negatives were associated with the NP during the development of a CiVA detection assay, which was attributed to highly variable NP sequences (Beris et al., 2021). Most often, the NP of plant viruses are multifunctional and plays a role in viral replication, movement between cells and expansion of the host range, therefore NP diversity would be present between members of the same order, family and genus (Callaway et al., 2001). Results of this study indicate the preferred use of the RdRp or the MP as target regions for diagnostic assays.

The information provided by this study provides a better understanding of the symptom expression observed on the fruit of 'Delta' Valencia and 'Marsh' grapefruit and the possible association with CiVA. It also allows the implementation of effective control strategies. The distribution of CiVA in South Africa was not due to natural spread, but rather the use of infected budwood material. CiVA-free budwood source material is now available within the CIS. Genetic variation does not seem to be linked to the geographical distribution of the virus, but rather the source of infection. The MP and RdRp gene regions of CiVA is the most conserved and suitable targets for detection assay development. The spread of CiVA in South Africa can effectively be controlled through the release of CiVA free material, confirmed CiVA negative with biological indexing and molecular detection.

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4. DEVELOPMENT OF A REAL-TIME RT-PCR FOR THE SIMULTANEOUS DETECTION OF TWO CITRUS-INFECTING COGUVIRUSES

4.1 INTRODUCTION

Plant virus detection is an essential part of plant disease management. Once an infection has been established, the plant remains infected for the entirety of its lifespan. Various detection methods are available; however, molecular detection methods such as reverse transcription (RT) followed by the polymerase chain reaction (PCR) is used most often for RNA viruses (Alemu, 2015; Rubio et al., 2020). RT-PCRs can either be end-point, which requires the visualization of the PCR products on an agarose gel or real-time where the accumulation of PCR products is monitored in real-time. A real-time PCR is less time consuming as the need for post PCR visualizations are not required. Real-time PCRs are also one of the most widely used techniques for the detection of plant viruses and are accurate and reliable with increased sensitivity (Bester et al., 2012; James et al., 2006; Khurana & Marwal, 2016; Rubio et al., 2020).

Simultaneous detection of viruses in a single real-time RT-PCR reaction is more beneficial as it is more cost-effective and time-efficient (Pallás et al., 2009). Multiple PCR-based strategies for simultaneous detection exist, including the use of degenerate primers (Rubio et al., 2020). Degenerate primers allow for the detection of multiple viruses within a genus using a single primer pair. These primers can be used with either a fluorescently-labelled probe or a DNA binding dye for detection (Bubner and Baldwin 2004). These assays can potentially also differentiate between different species of a virus with probes labelled with differently coloured fluorophores or when using a DNA binding dye through melt curve analysis (Bester et al., 2012; Pallás et al., 2018; Vaerman et al., 2004). Melt curve analysis can also indicate the specificity of a detection assay by distinguishing between specific and non-specific amplicons.

Primer design is essential as primers influence the efficiency and specificity of the reverse transcription process, subsequently influencing the accuracy, stability, and sensitivity of the detection assay (Feng et al., 2012; Stahlberg et al., 2004; Zhang & Byrne, 1999). Detection assays should therefore be optimised for amplification efficiency, limit of detection and specificity (Olmos et al., 2007; Svec et al., 2015). The use of a dual priming oligo (DPO) system was reported previously to increase amplification efficiency, specificity, and sensitivity (Chun et al., 2007; Su & Li, 2019; Xu et al., 2015; Xu et al., 2017) and can be used for the simultaneous detection of multiple viruses in a single PCR assay (Ma et al., 2015; Xu et al., 2017). The DPO contains two priming regions separated by a polydeoxyinosine linker. The longer 5'-segment (18-25bp) of the primer provides stable annealing, allowing the shorter 3'-

segment (6-12bp) to bind and provide target-specific extension, subsequently reducing non-specific extension. The separation of the two priming segments by the polydeoxyinosine linker prevents the formation of hairpin structures within the primer itself. The shorter 3'-segment is unable to form stable secondary structures due to its length therefore effectively blocking the formation of these secondary structures (Chun et al., 2007).

Plant virus detection assays are most often developed subsequent to the discovery of novel viruses (Villamor et al., 2019). Citrus concave gum-associated virus (CCGaV) and citrus virus A (CiVA) are two novel viruses discovered in citrus with the use of high-throughput sequencing (HTS) (Navarro et al., 2018a; Navarro et al., 2018b). CCGaV is a member of the species *Citrus coguvirus* and CiVA a member of the species *Coguvirus eburi.* Both virus species are classified within the genus *Coguvirus* in the family *Phenuiviridae* in the order *Bunyavirales* (Kuhn et al., 2020). CiVA was subsequently detected in several countries, including South Africa, in citrus and pears (Bester et al., 2021; Beris et al., 2021; Bougard et al., 2021; Minutolo et al., 2020; Svanella-Dumas et al., 2019).

In South Africa, citrus diseases are managed through the propagation of pathogen-free budwood within the Citrus Improvement Scheme (CIS). Detection of citrus viruses is therefore essential in the management of these diseases. Due to the confirmed presence of CiVA in South Africa (Bester et.al 2021) and historical reports of concave gum disease (Schwarz & McClean, 1969), a reliable detection assay for both CiVA and CCGaV was required for pathogen screening within the CIS. Detection assays for CiVA and CCGaV have been reported and include an end-point multiplex RT-PCR targeting the nucleocapsid protein (NP) of both viruses (Minutolo et al., 2020), a real-time SYBR-green RT-PCR targeting the movement protein (MP) of CiVA (Beris et al., 2021) and a probe-based real-time RT-PCR targeting the RNA-dependant RNA polymerase (RdRp) of CiVA and CCGaV for the individual detection of each virus (Diaz-Lara et al., 2021). A serological detection assay targeting the NP of CCGaV has also been developed (Minutolo et al. 2021). The development of a real-time RT-PCR for the simultaneous detection of CCGaV and CiVA would be more beneficial as it would limit the number of tests needed to confirm the presence of these viruses.

In this study, a real-time RT-PCR detection assay was developed for the simultaneous detection of CCGaV and CiVA. The RdRp, situated on the negative-sense single-stranded RNA 1 was targeted and a DPO was designed to increase the specificity of the assay. The influence of primers during cDNA synthesis and the subsequent amplification efficiency, limit of detection and specificity of the detection assay was investigated using a SYBR Green DNA binding dye and melt curve analysis. The most efficient and specific assay with the lowest limit of detection was implemented within the CIS for the simultaneous detection of CiVA and CCGaV.

4.2 MATERIALS AND METHODS

4.2.1 Primers and controls

4.2.1.1 Primer design

Degenerate primer pairs were designed based on a nucleotide alignment containing RNA 1 sequences for CCGaV (MH038042, MK940540, NC_035759 and MH137740) and CiVA (MG764565, MT720883 and MT720885) that was available at the time of primer design (Table 4.1). Additionally, a DPO reverse primer was designed to increase specificity (Fig. 4.1). Two forward primers were designed for comparison using the online Oligo Analyzer Tool from Integrated DNA Technologies (IDT).

Table 4.1: Real-time RT-PCR degenerate primer pairs designed to amplify a region of the RNA dependant RNA-polymerase situated of CCGaV and CiVA.

Primer pair	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
Cogu F2 Cogu R2	GCTTCATCWGCAGTKWGATC TGATWGTGTCYCCATCYAG	145	52
Cogu F2 Cogu DPR	GCTTCATCWGCAGTKWGATC TGATWGTGTCYCCATCYAGIIIIIIITGCAATTTTG	159	52
Cogu F_GC3 Cogu DPR	ACATCTTTDACAATYGCTTCATCWGCAGT TGATWGTGTCYCCATCYAGIIIIIIITGCAATTTTG	174	57

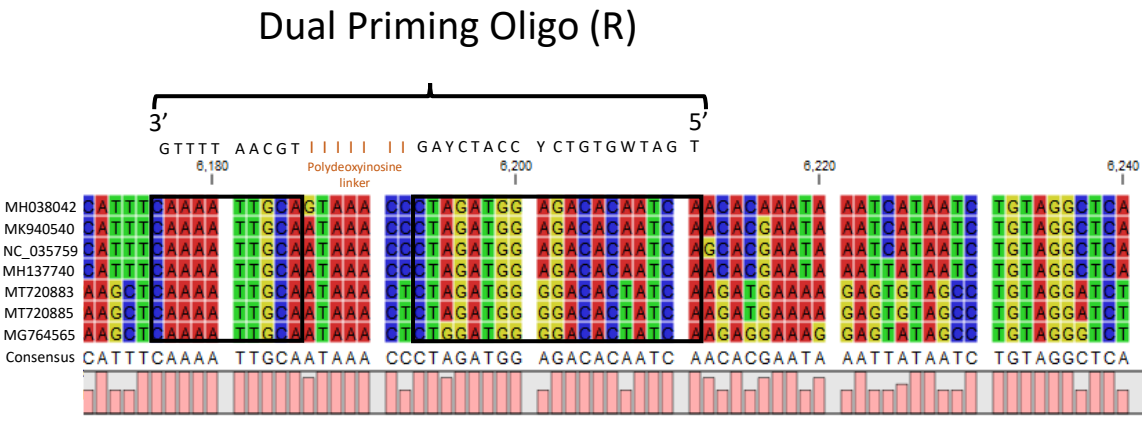

Figure 4.1: The design of the dual priming oligo reverse primer, Cogu DPR illustrated on the RNA 1 alignment used for primer design. The longer 19 bp 5'-region is separated from the shorter 10 bp 3'-region by seven inosine bases.

4.2.1.2 Control samples

Survey samples obtained from Chapter 3, confirmed CiVA positive with primer pair CiVA_5148F (GAGAATGACAAGTATGACCA)/CiVA_5023R (ACAGAAGAATGACAATAGCA), were used to validate the designed primer pairs for the real-time RT-PCR detection assay (Table 4.1). A total nucleic acid (TNA) CCGaV positive control was obtained from Dr Navarro, National Research Council of Italy, to confirm

CCGaV detection. The CCGaV positive control was excluded from the real-time RT-PCR validation experiments due to the limited concentration available. The detection of CCGaV was confirmed with the final real-time assay.

4.2.1.3 Complimentary DNA synthesis

Complimentary DNA (cDNA) was synthesized with the virus specific Cogu DPR reverse primer (VS-cDNA) and random hexamers (RH-cDNA). These two cDNA synthesis methods were applied throughout the study.

Synthesis of VS-cDNA was performed using RevertAid H Minus Reverse Transcriptase (ThermoFisher Scientific Inc., Waltham, MA, USA). One microgram of total RNA was incubated with 10 µM reverse primer for 3 min at 65°C followed by 1 min on ice. Additional components added include 5x Reaction Buffer (ThermoFisher Scientific), 1mM dNTP Mix (ThermoFisher Scientific), 20 units RT enzyme and 5 units RiboLock RNase Inhibitor (ThermoFisher Scientific) with a total reaction volume of 10 µl. Reverse Transcription was performed at 50°C for 60 min and reaction termination at 85°C for 10 min.

Synthesis of RH-cDNA was performed using RevertAid H Minus Reverse Transcriptase (ThermoFisher Scientific). Random Hexamers (ThermoFisher Scientific) (0.2 µg/µl) and total RNA (1 µg) was incubated for 3 min at 65°C followed by 1 min on ice. Additional components added include 5x Reaction Buffer (ThermoFisher Scientific), 1 mM dNTP Mix (ThermoFisher Scientific), 20 units RT enzyme and 5 units RiboLock RNase Inhibitor (ThermoFisher Scientific) with a total reaction volume of 10 µl. Reverse Transcription was performed at 25°C for 5 min, 42°C for 60 min and reaction termination at 70°C for 10 min.

4.2.1.4 Primer validation

RNA from CiVA positive samples and the CCGaV positive control was used for cDNA synthesis using the respective virus specific reverse primers (Table 4.1) and RevertAid H Minus Reverse Transcriptase (ThermoFisher Scientific) as above for VS-cDNA.

End-point PCR was performed using GoTaq G2 Hot Start Green Master Mix (Promega Corp., Madison, WI, USA) with 0.2 µM forward and 0.2 µM reverse primer and 1 µl cDNA with a total reaction volume of 10 µl. Cycling parameters were 94°C for 2 min followed by 40 cycles of 94°C for 20 s, annealing for 30 s (Table 4.1), extension for 20 s at 72°C and final extension of 72°C for 10 min. PCR products were viewed on a 2% (w/v) agarose gel to confirm detection of CiVA and CCGaV.

4.2.2 Real-time protocol

Real-time PCR was performed with both VS-cDNA and RH-cDNA. Complimentary DNA synthesized by both methods were amplified by forward primer Cogu F2 or Cogu F_GC3 in combination with reverse primer Cogu DPR and referred to as:

VS_F2: VS-cDNA synthesis and amplification with primer pair Cogu F2/Cogu DPR.

VS_GC3: VS-cDNA synthesis and amplification with primer pair Cogu F_GC3/Cogu DPR.

RH_F2: RH-cDNA synthesis and amplification with primer pair Cogu F2/Cogu DPR.

RH_GC3: RH-cDNA synthesis and amplification with primer pair Cogu F_GC3/Cogu DPR.

Real-time PCR was performed in a Rotor-gene Q thermocycler (Qiagen) using 1X SensiFAST SYBR No-ROX Mix (Bioline Reagents Ltd, United Kingdom) with 0.4 µM of forward and reverse primer and 1 µl cDNA in a total reaction volume of 15 µl. Cycling parameters were 95°C for 3 min followed by 40 cycles of 95°C for 15 s, annealing for 20 s (Table 4.1) and extension at 72°C for 15 s followed by a melt curve analysis with temperatures ranging from 56°C to 95°C with a 1°C increase in temperature every 5 s. Each sample was tested in triplicate.

This real-time RT-PCR protocol was applied to all optimisation experiments of the four assays that follow.

4.2.3 Validation experiments

4.2.3.1 Amplification efficiency

Amplification efficiency of the four assays was determined through standard curve construction. Complimentary DNA of a CiVA positive control was used to create a 5-times dilution series with six dilution points. Each cDNA dilution point was amplified in triplicate. The cDNA concentrations ranged from 20 ng/µl to 0.0064 ng/µl and was calculated from the initial RNA input concentration of 1 µg, assuming a one-to-one conversion. The Rotor-gene Q software version 2.2.3 (Qiagen) was used to calculate amplification efficiency and the linearity correlation coefficient (R^2).

4.2.3.2 Limit of detection

The limit of detection for the four assays was determined using four RNA samples, previously confirmed CiVA positive, from different citrus species. These included a sweet orange (*Citrus sinensis* (L.) Osbeck.) cv. 'Delta Valencia', a clementine sample (*C. reticulata* Blanco), a grapefruit (*C. paradisi* Macf.) cv. 'Marsh' and a sweet orange (*C. sinensis* (L.) Osbeck.) cv. 'Madam vinous'.

The RNA of each of the four citrus samples was used to create a 10-times dilution series with six dilution points. Aliquots (1 µg) of total RNA were used as the first point of the 10-times RNA dilution series. RNA input concentrations for cDNA synthesis ranged from 1000 ng to 0.01 ng. For each cDNA reaction 1 µl RNA of each RNA dilution point was used. The second cDNA dilution point of the standard curve from each assay was included in all runs to adjust for inter-assay variation. The detection limit was determined by the lowest total RNA input for cDNA synthesis at which all three technical replicates produced a Ct-value below 35.

4.2.3.3 Specificity

The reaction specificity of the four assays were tested using RNA obtained from eleven citrus species (pummelo, lime, limequat, sweet orange, lemon, mandarin and grapefruit) free from CiVA, citrus tristeza virus (CTV), citrus bent leaf viroid (CBLVd), hop stunt viroid (HSVd), citrus dwarfing viroid (CDVd), citrus bark cracking viroid (CBCVd), citrus viroid V (CVd-V), citrus exocortis viroid (CEVd), citrus tatter leaf virus (CTLV) and citrus psorosis virus (CPsV). The real-time reaction was visualized on a 2% (w/v) agarose gel for confirmation of the absence of non-specific amplicons.

4.3.4 Melt curve analysis

Melt curve analysis was performed as part of the real-time optimisation experiment for each of the four assays to determine the melting temperature of the CiVA amplicons produced by each primer pair. Differences in melting temperatures of CiVA amplicons from different citrus species were also investigated. Melt curve analysis was used to distinguish between non-specific amplicons and CiVA positive samples. The Rotor-gene Q software version 2.2.3 (Qiagen) was used to perform the melt curve analysis.

4.3.5 Tissue specificity

Tissue specificity was investigated using the PCR assay with highest efficiency, lowest limit of detection and highest specificity. Four ‘Madam vinous’ sweet orange seedlings, confirmed CiVA positive four months after patch graft-inoculation with a CiVA field source was used. One un-inoculated ‘Madam vinous’ sweet orange seedling was used as a negative control. Different tissue types were sampled including leaf midrib, leaf lamina, green bark and roots. Total RNA was extracted using an acid-phenol method as described previously by Cook et al., (2016) and shortly explained in Chapter 3. Melt curve analysis was performed to confirm the absence of non-specific amplification in the negative control.

4.3 RESULTS

4.3.1 Samples and controls

4.3.1.1 Primer validation

The three primer pairs designed for the real-time RT-PCR detection assay (Table 4.1) detected CiVA and CCGaV. However, non-specific amplicons were detected in CiVA positive samples when using primer pair Cogu F2/Cogu R2, which was subsequently excluded from further optimisation assays. The use of the Cogu DPR reverse primer effectively eliminated the non-specific amplicons observed when using reverse primer Cogu R2.

4.3.2 Validation experiments

4.3.2.1 Amplification efficiency

The results of the standard curve construction for the four assays are indicated in Table 4.2. Both cDNA synthesis strategies produced stable and reliable assays as indicated by the R^2 and standard deviation values of each assay. Detection of CiVA remained reliable at the 0.32 ng/µl cDNA dilution for all four assays; however, detection became unreliable for assays RH_F2 and RH_GC3 at the 0.0064 ng/µl cDNA dilution point. An increase in standard deviation values was observed as the concentration of the cDNA decreased from 0.16 ng/µl to 0.0064 ng/µl.

Amplification efficiencies ranged between 83% to 94%. An 11% difference in amplification efficiency was observed between assays VS_F2 and RH_F2, both amplified by forward primer pair Cogu F2/Cogu DPR. Similar amplification efficiencies were observed for assays VS_GC3 and RH_GC3 with a 2% difference between the two cDNA synthesis methods. Differences in amplification efficiencies were observed between the two forward primers used with a 10% difference between assays VS_F2 and VS_GC3 and a 3% difference between assays RH_F2 and RH_GC3. Data points for assays VS_F2 and VS_GC3 remained linear at all cDNA concentrations (Fig. 4.2 A and B). However, data points for assays RH_F2 and RH_GC3 deviated from the standard curve at the lowest cDNA concentration (Fig. 4.2 C and D).

Cycle threshold (Ct) value differences were observed between the two cDNA synthesis methods. Lower Ct values were observed for assays VS_F2 and VS_GC3 compared to the higher Ct values of assays RH_F2 and RH_GC3. Ct value differences were also observed between the two primer pairs used. Lower Ct values were observed for cDNA amplified with primer pair Cogu F_GC3/Cogu DPR compared to primer pair Cogu F2/Cogu DPR, irrespective of the cDNA synthesis method. A consistent increase in Ct values, at an average of every 2.58 cycles, were observed for assays VS_F2 and RH_F2

between the cDNA dilution points. Ct value increases between the cDNA dilution points of assays VS_GC3 and RH_GC3 were less consistent, increasing every 2.27 to 3.18 cycles.

Table 4.2: Standard curve results for the four real-time RT-PCR assays to determine the efficiency percentage and linearity correlation coefficient values for each assay.

			cDNA 5x dilution series					
	Efficiency (%)	**R^2**	**20ng/µl**	**4ng/µl**	**0.8ng/µl**	**0.16ng/µl**	**0.32ng/µl**	**0.0064ng/µl**
			Average Ct (standard deviation)					
VS_F2	83%	0.99	17.61	20.20	22.78	25.41	28.24	31.28
			(0.05)	(0.06)	(0.06)	(0.09)	(0.23)	(0.25)
VS_GC3	93%	0.99	16.12	18.39	20.93	23.39	25.94	28.28
			(0.10)	(0.05)	(0.15)	(0.13)	(0.17)	(0.20)
RH_F2	94%	0.99	21.82	24.38	26.58	29.38	31.35	33.98
			(0.06)	(0.06)	(0.11)	(0.05)	(0.12)	(0.79)
RH_GC3	91%	0.98	18.13	20.21	22.81	25.11	27.52	30.70
			(0.05)	(0.02)	(0.04)	(0.05)	(0.33)	(1.00)

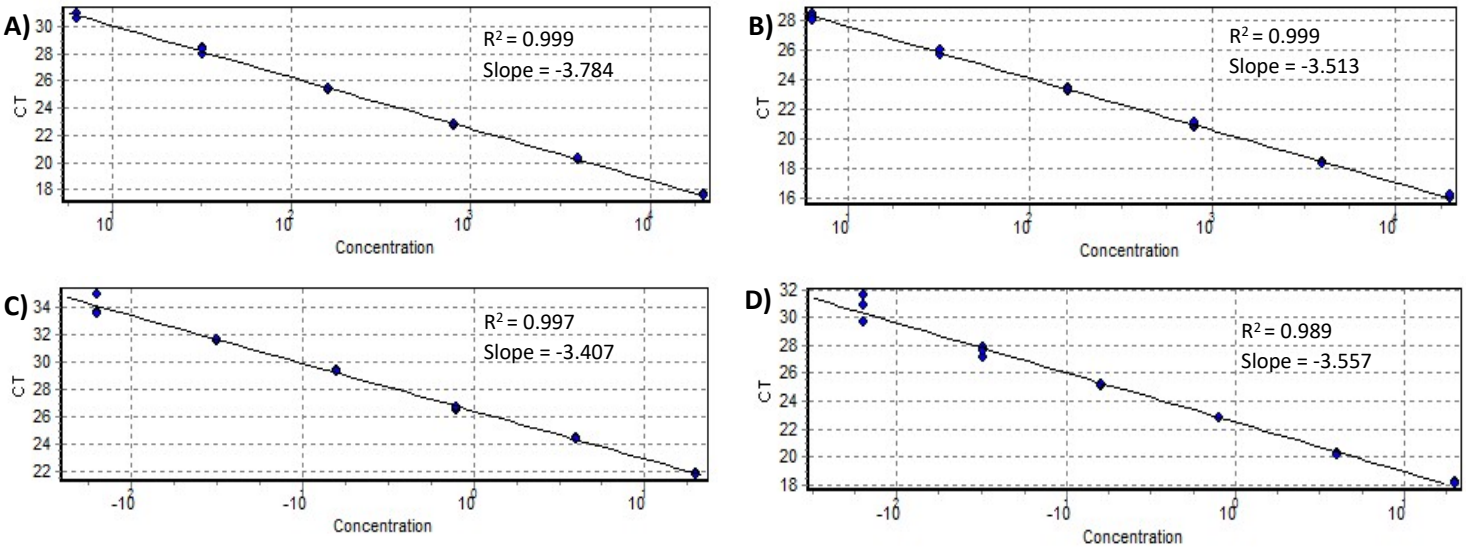

Figure 4.2: Standard curves showing the linearity of each real-time RT-PCR assay over a dynamic range of 20 ng/µl to 0.0064 ng/µl. Synthesis of cDNA with the virus specific Cogu DPR primer is illustrated for Assay 1 (A) and Assay 2 (B). Synthesis of cDNA for Assay 3 (C) and Assay 4 (D) was performed using random hexamers. The efficiency of each assay corresponds to the slope of the standard curve and should be between -3.6 and -3.1. The standard curve slope of Assay 1 does not fall within the recommended range.

4.3.2.2 Limit of detection

Detection was observed in all three technical replicates of the four citrus samples when using 1µg to 1 ng of input RNA for cDNA synthesis (Table 4.3). Detection at 1 ng of total RNA input for cDNA synthesis was reliable for the four RT-PCR assays with standard deviation values between the technical replicates below 0.5, apart from the clementine sample and the 'Marsh' grapefruit sample for assay VS_GC3. These two samples had standard deviation values of 0.73 and 0.58, respectively. Detection at 0.1 ng and 0.01 ng of total RNA input for cDNA synthesis was less sensitive as detection occurred in only one or two of the three technical replicates with standard deviation values above 0.5.

Similar to the amplification efficiency experiment, lower Ct values were observed for assays VS_F2 and VS_GC3. However, these lower Ct values were only observed for the first two dilution points of 1000 ng and 100 ng. The subsequent Ct values at the 10 ng total RNA dilution point was similar for all four assays. The similar Ct values observed at the 10 ng total RNA dilution point was as a result of only a 1 cycle increase, during real-time amplification, between the 1000 ng and 100 ng total RNA dilution points used for RH-cDNA synthesis, followed by an average increase of only 2 cycles between the 100 ng and 10 ng total RNA dilution points. Assays RH_F2 and RH_GC3 produced Ct values lower than assays VS_F2 and VS_GC3 at the 1 ng total RNA input for cDNA synthesis, except for the 'Delta' Valencia and clementine samples of RH_F2. Assay RH_GC3 produced the lowest Ct values observed at the 0.1 ng RNA input for cDNA synthesis. More technical replicates were detected with Ct values below 35 for assay RH_GC3 at the 0.1 ng and 0.01 ng total RNA input for RH-cDNA synthesis.

Lower Ct values were observed in the 'Delta' Valencia sample, followed by the 'Marsh' grapefruit and 'Madam vinous' samples, which had similar Ct values. This was observed in all four assays. Higher Ct values were observed for the clementine sample in all four assays and no detection was observed at a total RNA input for cDNA synthesis of 0.1 ng for assay VS_GC3.

Table 4.3: Limit of detection was determined for four citrus cultivars by serial dilution of the input RNA for cDNA synthesis. Values in bold indicate detection in two technical replicates and values in italics indicate detection in one replicate.

	Total RNA dilution series for cDNA synthesis					
Assay	**1000 ng**	**100 ng**	**10 ng**	**1 ng**	**0.1 ng**	**0.01 ng**
	Average cycle threshold (standard deviation)					
VS_F2						
'Delta' Valencia	18.81 (0.04)	21.51 (0.05)	25.14 (0.03)	29.34 (0.26)	32.34 (0.58)	**34.72 (1.17)**
Clementine	22.53 (0.06)	24.47 (0.05)	28.97 (0.23)	33.99 (0.27)	**34.66 (0.16)**	
'Marsh' grapefruit	19.72 (0.06)	22.57 (0.04)	26.9 (0.04)	29.91 (0.15)	33.8 (0.59)	*33.97*
Madam vinous	19.79 (0.04)	22.63 (0.03)	26.6 (0.07)	30.93 (0.04)	34.33 (1.14)	
VS_GC3						
'Delta' Valencia	17.02 (0.12)	20.03 (0.17)	23.26 (0.02)	27.43 (0.43)	31.15 (0.41)	
Clementine	19.69 (0.03)	22.49 (0.03)	26.47 (0.19)	31.11 (0.73)		
'Marsh' grapefruit	17.78 (0.03)	20.88 (0.02)	24.29 (0.19)	28.17 (0.58)	**32.34 (0.76)**	
Madam vinous	17.83 (0.02)	20.67 (0.17)	24.21 (0.16)	27.73 (0.35)	**32.1 (0.08)**	**32.79 (0.14)**
RH_F2						
'Delta' Valencia	23.35 (0.03)	24.25 (0.08)	26.34 (0.07)	29.69 (0.03)	33.82 (0.64)	*36.28*
Clementine	25.77 (0.04)	26.74 (0.04)	28.77 (0.01)	32.16 (0.41)	**37.41 (1.07)**	
'Marsh' grapefruit	24.71 (0.12)	25.24 (0.12)	27.27 (0.09)	30.61 (0.31)	34.76 (0.27)	**36.99 (0.89)**
Madam vinous	24.26 (0.09)	25.04 (0.04)	27.03 (0.13)	29.86 (0.48)	33.58 (0.91)	*36.65*
RH_GC3						
'Delta' Valencia	19.55 (0.03)	20.27 (0.01)	22.49 (0.12)	25.91 (0.11)	29.64 (0.45)	31.99 (0.22)
Clementine	22.92 (0.06)	23.9 (0.15)	26.16 (0.07)	28.51 (0.20)	**34.12 (0.46)**	
'Marsh' grapefruit	20.79 (0.03)	21.00 (0.02)	23.16 (0.08)	26.11 (0.06)	30.42 (0.98)	*32.00*
Madam vinous	20.56 (0.05)	21.04 (0.02)	22.98 (0.22)	26.21 (0.13)	29.85 (0.96)	*31.55*

4.3.2.3 Specificity

Non-specific amplification was observed in the healthy citrus samples of assays VS_F2 and VS_GC3. Non-specific amplification of VS_F2 produced Ct values ranging between 30 and 34; however, no peaks corresponding to these Ct values were observed on the melt curve. Visualisation of the real-time RT-PCR products on an agarose gel revealed a faint 450 bp amplicon in two of the citrus samples.

Increased non-specific amplification was observed for assay VS_GC3 with Ct values ranging between 27 and 31 cycles. The no-template control produced a Ct value of 33.57. Non-specific amplicons of 350bp were observed in eight of the citrus samples after visualisation on an agarose gel. Non-specific amplicons of 900 bp were also observed in three of the citrus samples.

No non-specific amplicons were observed for assay RH_F2 during the real-time amplification or on the agarose gel. For assay RH_GC3, two of the citrus samples produced Ct values of 32.59 and 34.07; however, no non-specific amplicons were observed on the agarose gel.

Primer dimers were visible on the agarose gels of all four assays.

4.3.3 Melt curve analysis

The melting temperatures produced by the CiVA positive sample used for standard curve construction was similar for assay VS_F2 and RH_F2 and ranged between 80.0°C and 80.2°C. Assay VS_GC3 and RH_GC3 produced peaks on the melt curve at higher temperatures ranging from 80.7°C to 81°C. No differences in melting temperatures of CiVA amplicons were observed between the two cDNA synthesis methods.

The melting temperatures of CiVA amplicons from the four different citrus species were different and were observed in all four assays. Melting temperatures of CiVA amplicons from the 'Delta' Valencia and 'Marsh' grapefruit samples were similar at a temperature of 81.3°C. The clementine sample produced a peak at 80.7°C on the melt curve while the 'Madam vinous' sample had a melting temperature of 80.9°C.

Melt curve analysis was used to distinguish between non-specific amplicons and the CiVA positive control during the specificity experiment. Peaks observed on the melt curve of assay VS_GC3 ranged between 59°C and 66°C. Additionally, peaks on the melt curve ranging between 85.3°C and 86°C were also observed. The melting temperature of the CiVA positive amplicon was similar in all four assays at 81.0°C, apart from assay VS_GC3 where the melting temperature of the CiVA amplicon was 80.7°C.

Based on the melt curve analysis of the validation experiments, CiVA positive samples had a melting temperature range of 80.0°C to 81.3°C when tested with primer pair Cogu F2/Cogu DPR and 80.7°C to 81.3°C for primer pair Cogu F_GC3/Cogu DPR, irrespective of cDNA synthesis method. The melting temperature of the CCGaV positive control amplicon was 79.3°C when tested with assay RH_F2.

4.3.4 Tissue specificity

CiVA was detected reliably in the four tissue types sampled from the four CiVA positive 'Madam vinous' indicator seedlings when tested with assay RH_F2. CiVA was consistently detected with a lower Ct value in bark tissue, followed by leaf midribs. Lower Ct values were observed for trees 3A, 3B and 3C in the bark, leaf midribs and leaf lamina tissue types compared to the higher Ct values observed for the same tissue types of tree 5B (Table 4.4). Ct values above 35 were produced in the leaf lamina, bark and root tissue of the un-inoculated 'Madam vinous' seedling. No amplification was observed in the no template control (NTC).

Similar melting temperatures of the CiVA amplicon was observed between the four tissue types. Peaks on the melt curve were observed at an average temperature of 81.6°C.

Table 4.4: Tissue specificity results presented as cycle threshold (Ct) obtained for four CiVA positive seedlings sampled from bark, leaf midribs, leaf laminar and roots tested with assay RH_F2.

	Cycle threshold value (standard deviations)			
	Midribs	Lamina	Green Bark	Roots
Tree 3A	24.51 (0.05)	25.09 (0.35)	24.33 (0.03)	28.92 (0.22)
Tree 3B	25.38 (0.04)	26.82 (0.03)	24.91 (0.05)	26.53 (0.04)
Tree 3C	24.47 (0.03)	27.19 (0.14)	23.95 (0.04)	25.93 (0.06)
Tree 5B	28.41 (0.08)	29.57 (0.12)	27.14 (0.19)	27.32 (0.26)
Negative		36.76	37.77 (0.20)	36.86

4.4 DISCUSSION

The discovery of CiVA and CCGaV subsequently led to the development of PCR based detection assays for both viruses. An end-point RT-PCR for the simultaneous detection of CiVA and CCGaV, targeting the NP of both viruses have been developed (Minutolo et al., 2020). False negatives were reported when targeting the NP of CiVA; however, targeting the RdRp and MP was more reliable (Beris et al., 2021). The real-time RT-PCR by Beris et al., (2021) was developed for the specific detection of CiVA targeting the MP. It was reported that the RdRp of other members of the order *Bunyavirales* was more conserved compared to the MP and was the most reliable target region for detection (Rott et al., 2018). More recently, probe-based real-time RT-PCR assays for the specific detection of CiVA and CCGaV targeting the RdRp was developed (Diaz-Lara et al. 2021). Similarly, the CiVA diversity results from Chapter 3, based on nucleotide identity percentages and phylogenetic analysis, indicated that the MP and RdRp gene regions were most conserved.

The real-time RT-PCR detection assay developed in this study targets a region of the RdRp gene. The primers designed were degenerate to allow for the detection of both CiVA and CCGaV as nucleotide differences occurred between the CiVA and CCGaV RNA 1 genomes at the primer binding site. The degeneracy of each primer was limited as the sensitivity and specificity can be reduced due to a decrease in binding between the primer and target template or the partial binding of primer to target template (Rubio et al., 2020). The use of modified nucleotides such as inosine is a suggested approach to limit the degeneracy level of a primer (Rubio et al., 2020). Seven inosine bases were incorporated into the dual-priming system of the Cogu DPR reverse primer as a polydeoxyinosine linker, separating the two priming regions and creating a bubble-like structure that prevents the formation of secondary structures (Chun et al., 2007). The unique structure of the Cogu DPR reverse primer increased specificity through the elimination of the non-specific amplicons that were observed in the CiVA positive samples when replacing reverse primer Cogu F2 with Cogu DRP during amplification.

The real-time RT-PCR assay was optimised and validated to ensure the accurate and reliable simultaneous detection of CiVA and CCGaV. Previous reports indicate that the use of gene-specific or random hexamers as reverse primer during cDNA synthesis has an influence on the amplification efficiency, reliability, stability, sensitivity, and specificity of a real-time RT-PCR detection assay (Deprez et al., 2002; Feng et al., 2012; Johnson et al., 2012; Stahlberg et al., 2004; Zhang & Byrne, 1999). The selection of primers used during real-time amplification also has an influence on the parameters stated above (Schaad & Friederick, 2002; Svec et al., 2015). The influence of primers used during cDNA synthesis and real-time amplification were both observed in this study.

Both VS-cDNA and RH-cDNA produced stable assays with R^2 values of 0.98 and above and standard deviation values below the recommended 0.5 (Johnson et al., 2012). The stability of the four real-time RT-PCR assays were not influenced by the cDNA synthesis methods; however, the amplification efficiency and sensitivity of the assays were. Complimentary DNA synthesized with Cogu DPR and random hexamers produced different amplification efficiencies when amplified with the same primer pair. Assays VS_F2 and VS_GC3 remained linear at the six dilution points (Fig. 4.2 A and B) while assays RH_F2 and RH_GC3 were only linear over five dilution points (Fig. 4.2 C and D). It was reported previously that the amplification of cDNA synthesized with random hexamers is linear over a narrower range (Bubner & Baldwin, 2004; Bustin & Nolan, 2004). Assays VS_F2 and VS_GC3 also appeared more sensitive with lower Ct values. Increased sensitivity has been associated with cDNA synthesis using gene-specific primers (Deprez et al., 2002).

Similar to the amplification efficiency experiment, increased sensitivity was observed for assays VS_F2 and VS_GC3 during the limit of detection experiment. The increased sensitivity was, however only observed for the 1000 ng and 100 ng total RNA dilution points, after which similar Ct values were observed for VS-cDNA and RH-cDNA at the 10 ng total RNA input for cDNA synthesis. Most often, when an assay has an efficiency of 100%, a 10-times dilution series is associated with an average Ct value increase of 3.32 between dilution points (Johnson et al., 2012). This was not observed for the 10-times diluted total RNA input amounts of 1000 ng, 100 ng and 10 ng. The increase in Ct values observed between these RNA input amounts is most likely due to the overestimation of copy numbers most often associated with the use of random hexamers (Zhang & Byrne, 1999). Random hexamers bind at multiple points throughout the target template, resulting in the production of more than one cDNA transcript per original target (Bustin & Nolan, 2004; Bustin et al., 2005). The decrease in total RNA input for cDNA synthesis from 10 ng to 1 ng resulted in the expected average Ct value increase of 3.23 for assay RH_F2 (94% efficiency) and 2.99 for assay RH_GC3 (91% efficiency) as less original target transcripts were available for random hexamers to bind to. Although the cDNA priming strategies had an influence on the sensitivity, both cDNA priming strategies reliably detected CiVA down to 1 ng total RNA input for cDNA synthesis. The overestimation of copy numbers most likely occurred during the RH-cDNA synthesis for the amplification efficiency experiment, however; it was not observed due to the creation of a cDNA dilution series for standard curve construction, where cDNA synthesis was performed with 1000 ng total RNA input.

The two primer pairs compared in this study had an influence on the sensitivity of the four real-time RT-PCR assays. Increased sensitivity was observed for primer pair Cogu F_GC3/Cogu DPR. The increased sensitivity can most likely be due to the longer 174 bp PCR product, enabling the intercalation of more SYBR Green molecules compared to the shorter 159 bp PCR product generated

by primer pair Cogu F2/Cogu DPR, resulting in an increased and more intense fluorescence (Mouillesseaux et al., 2003; Thornton & Basu, 2011).

It is essential to determine the specificity of a detection assay as it indicates the accuracy with which the assay detects the targeted virus (Johnson et al., 2012). The specificity of an assay is most often increased with the use of gene-specific primers during reverse transcription (Bustin & Nolan, 2004; ThermoFisher Scientific, 2016) and the higher temperature (50°C) associated with VS-cDNA synthesis can eliminate the formation of non-specific products (Pfaffl, 2004). However, amplification of VS-cDNA with both primer pairs resulted in non-specific amplification. High Ct values were observed for some samples in assays VS_F2 and VS_GC3, although more for assay VS_GC3. The absence of non-specific amplification when amplifying RH-cDNA with both primer pairs suggests that a mispriming event, the annealing of primers to sequences that are only partially complementary (Boyle et al., 2009), most likely occurred during the reverse transcription process of VS-cDNA. Gene-specific and random hexamers as reverse primers for reverse transcription have been compared previously; however, the performance of each strategy was template dependant (Nolan et al., 2006; Stahlberg et al., 2004).

Forward primer Cogu F2 appeared more specific as less non-specific amplicons were observed for assay VS_F2. Two faint non-specific amplicons were observed on the agarose gel of assay VS_F2; however, no peaks on the melt curve, apart from the CiVA positive control was observed. Decreased specificity was observed for forward primer Cogu F_GC3. The larger sized non-specific amplicons observed on the agarose gel of assay VS_GC3 most likely occurred due to a mispriming event and can be associated with the peaks on the melt curve ranging between 85.3°C and 86°C. The presence of primer dimers was confirmed by peaks observed on the melt curve of assay VS_GC3, corresponding to temperatures between 59°C and 66°C as melting temperatures lower than that of the intended target amplicon was observed (Denman & McSweeney, 2005; Pfaffl, 2001; Park et al., 2020). Previous reports indicate that non-specific bands can occur in the absence of a target template (Park et al., 2020; Ruiz-Villalba et al., 2017).

The advantage of melt curve analysis was demonstrated in this study. Non-specific amplification and primer dimers could be distinguished from CiVA amplicons based on the melting temperatures of PCR products. In some instances, high Ct values were produced by samples with no corresponding peak on the melt curve. Subsequently, the samples were regarded negative as non-specific amplification often occurs between the amplification reagents and background material in the sample during the plateau phase of a real-time RT-PCR, resulting in high Ct values (Lacroix et al., 2016). It has been recommended that a real-time reaction should not exceed 30 to 35 cycles to avoid the non-specific amplification associated with the plateau phase (Ruiz-Villalba et al., 2017; Sachse, 2004).

The results of the validation experiments indicated that assay RH_F2 was the most efficient and specific assay that could reliably detect CiVA down to 1ng of total RNA input for cDNA synthesis. Tissue specificity was therefore determined with assay RH_F2. CiVA was detected reliably in all four tissue types. Lower Ct values were consistently associated with bark tissue, suggesting a higher virus titre in this tissue type. The higher Ct values observed for plant 5B suggests a lower virus accumulation within the plant and can be attributed to the source plant material used for inoculation. Plants 3A to 3C were inoculated from the same field source material; however, plant 5B was inoculated from a different field source. The detection of CiVA throughout the plant indicates the long-distance movement of the virus, confirming a systemic infection (Garcia-Ruiz, 2018).

Optimisation of a detection assay is essential to establish a standard operating procedure for consistent and accurate routine virus detection. Assay RH_F2 was selected for application in the CIS for the simultaneous detection of CiVA and CCGaV. The RH-cDNA synthesised for assay RH_F2 would be more ideal for pathogen screening within the CIS as most citrus samples are infected with more than one virus/viroid. Multiple tests can therefore be performed from a single cDNA reaction. Total RNA should be extracted from bark or leaf midribs as these tissue types produced lower Ct values. Complimentary DNA should be synthesized using random hexamers with one microgram of total RNA. Subsequently, the cDNA should be amplified with primer pair Cogu F2/Cogu DPR and the reaction should not exceed 35 cycles. Samples can be confirmed CiVA positive if a melting temperature between 80.0°C to 81.6°C is observed and a CCGaV positive sample will produce a melting temperature of 79.3°C. It is essential to always include a positive and negative control to verify the results obtained.

The development of accurate, fast and reliable detection assays is essential for the management of citrus diseases in order to maintain a healthy industry. In this study, a real-time RT-PCR detection assay was developed for the simultaneous detection of two currently known citrus-infecting coguviruses that was implemented within the CIS for pathogen screening.

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5. CONCLUSION

Citrus is an economically important worldwide crop that is prone to diseases caused by viruses. The causal agents of citrus impietratura, cristacortis and citrus concave gum disease remained unidentified; however, the application of high throughput sequencing (HTS) led to the discovery of novel citrus viruses associated with two of these diseases. Citrus concave gum-associated virus (CCGaV) was reported to be associated with citrus concave gum disease (Navarro et al., 2018) and citrus virus A (CiVA) with citrus impietratura (Beris et al., 2021). Understanding the aetiology of these diseases can provide valuable information to apply in management strategies. The implementation of the Citrus Improvement Scheme (CIS) of South Africa provided an effective control strategy for graft transmissible diseases through the supply of pathogen-free propagation material to the industry. Confirmation that plant material is pathogen-free is achieved through biological indexing and PCRs. The development of detection assays for diseases with an unknown aetiology can be difficult as sequence information of the causal agent is required.

CiVA was detected in South African citrus with the use of HTS (Bester et al., 2021). Subsequently, in this study, a survey was conducted to determine the distribution of CiVA in South African citrus production regions. The genetic diversity between CiVA isolates from different production regions and citrus types were investigated. Citrus impietratura was first reported in South Africa in 1976 on grapefruit (Moll et al., 1976), therefore the association of CiVA with citrus impietratura was investigated. A real-time RT-PCR was also developed for implementation in the South African CIS to simultaneously detect CiVA and CCGaV.

Typical citrus impietratura symptoms were observed on grapefruit (*C. paradisi* Macf.) cv. 'Marsh' trees in 2018 and subsequently confirmed CiVA positive. A correlation between the presence of CiVA and symptom expression was observed as asymptomatic trees from the same orchard were CiVA negative. These results and observations most likely suggest an association of CiVA with citrus impietratura on 'Marsh' grapefruit. This was observed for a small number of samples. No symptoms were observed the following year in the same orchard, corresponding to historic reports that citrus impietratura symptoms are not observed every year (Cartia & Catara, 1972; Cartia & Catara, 1974; Scaramuzzi et al., 1968).

CiVA was also detected in symptomatic and asymptomatic sweet orange (*C. sinensis* (L.) Osb.) cv. 'Delta' Valencia trees. Historical reports describe citrus impietratura fruit rind symptoms on sweet oranges (Cartia & Catara, 1974) similar to those observed on the 'Delta' Valencia fruit rind. However, no gumming in the albedo and abnormal fruit drop, typically associated with citrus impietratura (Moreno, 2000), was observed for the CiVA positive trees. Previous reports also indicate that

environmental conditions have an influence on symptom expression (Bar-Joseph, 1976; Scaramuzzi et al., 1968; Van Vuuren et al., 1984). CiVA positive trees with un-affected fruit were possibly influenced by environmental conditions and symptom expression may appear in the next season. Sweet orange indicator seedlings displayed leaf flecking when inoculated with CiVA positive material, which has been a characteristic symptom associated with citrus impietratura during biological indexing (Bar-Joseph & Loebenstein, 1970; Roistacher, 1991; Vogel & Bové, 1974). These results are suggestive that the fruit rind symptoms observed on 'Delta' Valencia is possibly citrus impietratura and that CiVA is most likely associated with the symptom expression on the fruit rind.

Phylogenetic analysis and nucleotide identity matrices indicated that genetic diversity was associated with CiVA isolates from the different citrus types and not with the different production regions for the 'Delta' Valencia isolates. This suggests that the genetic diversity observed between CiVA isolates was primarily due to different sources of infection. The isolates from citrus characterized in this study did not differ significantly from an isolate previously characterized from pear in South Africa, suggesting that the host plant might have minimal influence on the genetic diversity of the virus.

The most conserved gene region of the CiVA was identified as the movement protein (MP), followed by the RNA-dependent RNA polymerase (RdRp) gene region. The nucleocapsid protein (NP) gene region was the most diverse between CiVA isolates with the lowest nucleotide identity percentages between isolates with different origins.

A limited number of isolates for grapefruit and clementine species were obtained and is seen as a limitation in this study. These isolates were also only representative of one citrus production region, Nkwaleni and Hoedspruit, respectively. More representative isolates from each citrus species should be included in future analyses. CiVA detected in citrus species and cultivars, other than those sampled for this study, should be sequenced and included in phylogenetic analysis and nucleotide identity matrices. This would also indicate whether CiVA can be transmitted in the field or if budwood was supplied with different infection sources.

The distribution of CiVA in South Africa was determined to be most likely spread through infected plant propagation material and not natural spread. The absence of a glycoprotein gene region in the CiVA genome supports the finding that natural spread did not occur as the glycoprotein is what usually interacts with the arthropod vector (Hulswit et al., 2021; Kormelink et al., 2021). Therefore, the spread of CiVA can effectively be controlled through the supply of CiVA free plant propagation material available within the CIS. Understanding the distribution and genetic diversity of CiVA provides a better understanding of the disease and the control strategies that need to be implemented.

A real-time RT-PCR detection assay was developed to simultaneously detect CiVA and CCGaV and was implemented within the CIS to ensure the release of CiVA free plant propagation material, effectively controlling the spread of CiVA. Optimization of the detection assay included comparing two cDNA synthesis methods and two primer pairs during amplification efficiency, limit of detection and specificity experiments. The use of a dual-priming oligo (DPO) as a reverse primer, increased specificity. The importance of detection assay optimization was demonstrated in this study as the cDNA synthesis method and choice of primer pair had an influence on each of the optimization experiments. The detection of CCGaV was validation on a single positive control sample.. Once CCGaV is detected in SA, all validation experiments performed in this study should be repeated for CCGaV. The detection assay can be further improved to a quantitation assay that can be applied to determine the spatial distribution and measure the seasonal variation of CiVA and CCGaV after detection.

CiVA was detected throughout the plant in the four tissue types sampled, confirming the systemic infection of CiVA. Lower Ct values were consistently associated with green bark and leaf midribs and it is therefore recommended that these tissue types be used for total RNA extraction and downstream RT-PCR analyses. Determining tissue specificity of CiVA and optimization of the real-time RT-PCR detection assay led to the establishment of a standard operating procedure (SOP) for the detection of CiVA and CCGaV in South African citrus.

Previously, concave gum and citrus impietratura detection was reliant on biological indexing. This is a time-consuming process and the leaf flecking/oak leaf symptoms on indicator seedlings are transient and can easily be missed (Navarro et al., 2018; Roistacher, 1991). The implementation of the detection assay will therefore limit the number of tests needed to be conducted to ensure the release of CiVA free budwood to the citrus industry.

A limitation of this study was the sample size of the ‘Marsh’ grapefruits as an association between symptom expression and the presence of CiVA was only observed in ten samples. A larger sample size needs to be analysed to confirm the association, therefore a large-scale survey in the Nkwaleni region of the Kwazulu-Natal province need to be conducted when symptoms appear. The association of CiVA with citrus impietratura-like symptoms observed on ‘Delta’ Valencia and the role that CiVA plays in the aetiology of the disease need to be investigated further. Long term trials with various citrus types, inoculated with plant material containing only CiVA, need to be conducted to establish disease association. Further, the variation of symptom expression on different citrus types needs to be determined as well as the impact of environmental conditions on the development of symptoms. Unfortunately, environmental conditions in the six citrus production regions were not documented, although the grower in the Hoedspruit region associated cooler night temperatures with symptom

development. Environmental conditions during symptom development should therefore be documented in future.

Maintaining the health of the citrus industry in South Africa is an important function of the CIS and the research of citrus diseases is an integral part of providing the CIS with the necessary information and tools required for virus detection, allowing the optimal performance of the South African CIS. In this study, a broader understanding of CiVA and its possible association with citrus impietratura on grapefruit and similar symptoms on the fruit rind of 'Delta' Valencia sweet oranges were achieved. Further, a detection assay was developed for the simultaneous detection of two citrus-infecting coguviruses to ensure the release of pathogen-free budwood to the citrus industry of South Africa.

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