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Further Investigations on *Citrus tristeza virus* (CTV) in Sudan Using Molecular Techniques

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Authors' contributions

This work was carried out in collaboration between all authors. Author MYAA designed the study, wrote the protocol and the first draft of the manuscript. Authors AIAI and SME managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The natural occurrence of *Citrus tristeza clostero virus* (CTV) in the main citrus producing regions of Sudan was further substantiated by using a nested RT-PCR approach. Two-thirds of the test samples collected from mandarin, sweet orange, grapefruit, and lime trees reacted positively and a fragment size of 132 bp using an internal and external primer sets were obtained. Cloning and sequencing of these fragments further confirmed the identity of the Sudanese isolates of CTV. The sequence analyses of the first group showed 97% nucleotide identity to a CTV strain from California causing severe stem pitting symptoms (AF01623). The second group was identical to the reference sequence (DSMZ PV-0332 from Israel) and showed 99% identity to CTV strains T30 and T36 from Florida (AF260651 and U 16304). Whereas the third group showed 94% identity to CTV strain T318A from Spain that causes severe stem pitting in sweet orange. The three

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different sequences obtained from Sudan revealed a lower nucleotide identity among them (about 97%) indicating that at least three different CTV-strains might be present in citrus growing areas of Sudan.

Keywords: CTV; Closterovirus; nested RT-PCR; cloning; sequencing; citrus diseases; Sudan.

1. INTRODUCTION

Citrus was introduced to Sudan at the turn of the 20th century where it was established at the beginning in some of the government orchards in the northern States. Sudan with all its vast area, wide range of soils, diverse climatic conditions and ample water resources lies within the citrus belt and hence the potentialities for citrus production in this country should be enormous.

The presence of *Citrus tristeza closterovirus* (CTV) in Sudanese citrus orchards has long been suspected by some local scientists as well as visiting scientists. The results indicated the importance of tristeza, as it has been prevalent naturally in all the surveyed areas infecting orange, mandarin, grapefruit and lime [1]. The virus infection is responsible for quick decline, death of citrus trees and severe yield losses [2,3].

CTV infection causes a wide range of symptoms in citrus hosts, depending on host species, scion-rootstock combination, virus isolates and the environmental conditions [4].

CTV has a single-stranded, positive sense RNA molecule of about 19,296 in flexuous nucleotides encapsidated filamentous particles about 2000 nm long [5]. Several CTV strains have been described that differ in their biological characteristics, particularly in the symptoms elicited in various citrus hosts [6,7]. Some strains are responsible for quick decline of trees grafted onto sour orange while others may also induce stem pitting of the branches of sweet orange or grapefruit. These are the most economically damaging strains. Other strains that cause symptoms only on Mexican lime indicator plants are referred to as mild strains [8, 9]. The virus is phloem-limited and is transmitted in nature in a semi-persistent manner by several aphid species, mainly *Toxoptera citricida* and *Aphis gossypii* and by grafting infected budwood [1].

The disease is widely distributed in almost all citrus growing areas of the world. It has been reported from Africa, Asia, Europe, the Mediterranean region, North Central and South Americas, Australia and Pacific Islands [10,11, 12,13,14,15,3,7,16,17,1].

Several techniques have been developed to diagnose, detect and establish the identity of CTV, some of which are time consuming, expensive and often imprecise such as biological indexing [18,19]. However, the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) based on monoclonal antibodies (MCA) is considered as the most useful technique for routine plant virus diagnosis [20,21, 22]. Other immunochemical techniques, such as direct tissue blot immunoassay (DTBIA) also provide results that similar to ELISA [10,23]. Recent advances in molecular biology and biotechnology are being applied to the development of rapid, specific and very sensitive tools for the detection of plant pathogens even under the conditions of low pathogens titre such as DNA probe hybridization [24], Double-stranded RNA (dsRNA) analysis [25,26], restriction fragment length polymorphism (RFLP) analysis [27,28] and different methods of reverse transcription polymerase chain reaction (PCR) such as immunocapture (IC)-RT-PCR-based technology [29,30], bi-directional reverse transcription polymerase chain reaction [31,7], Co-operational amplification (Co-PCR) [32] and Reverse transcription and polymerase chain reaction (RT-PCR) [33,34,30,17]. RT-PCR is being used increasingly for detection, typing and cloning RNA viruses [35,15,16]. The DNA synthesized by this method was highly specific and could be cloned readily [36].

The objective of this paper was to further characterize and confirm the presence of *Citrus tristeza virus* (CTV) in Sudan using molecular techniques.

2. MATERIALS AND METHODS

2.1 Plant Materials

Two field surveys were conducted during the years 2004-2005 in the main citrus growing areas of Khartoum, River Nile and Northern

states for collection of CTV samples. The surveys covered six locations (El Gureir, Shendi, El Faki Hashim, Shambat, and Tumaniat. A total of 57 samples were collected, 46 of them showed typical symptoms of CTV. The completely remaining 11 samples were randomly collected from the vicinity of the suspected diseased trees. The samples consisted of leaf materials and young shoots from new flushes at the four quadrants of the selected citrus trees.

2.2 Molecular Diagnosis

2.2.1 Nucleic acid extraction

Total RNA extracts from leaves and bark were used as templates for RT-PCR. Invisorb ® Spin Plant RNA Mini kit (Invitek, Germany) was used following the manufacturer's protocol. Starting from 100 mg plant material, RNA was eluted in 50mlof elution buffer supplied by manufacturer. RNA extracts were quantified by a NanoDrop (ND-1000 Spectrophotometer) then stored in small aliquots at -80℃.

2.2.2 Primers

Four oligonucleotide primers were used, two external [CTV PEX 1(5'TAAACAACACACACTCTAAGG-3') and PEX2 (5'-CATCTGATTGAAGTGGAC-3')] and two internal primers [PIN1 (5'GGTTCACGCA ACGTTAAGCCTCACTT-3') and PIN2 (5' TATCACTAGACAATAACCGGATGGGTA-3')].
The external primers were involved in a basic RT-PCR, while the internal primers were used in a nested RT-PCR approach. The external primer set and the internal primer set were used to amplify a fragment of 241bp and 132bp, respectively. These primers for CTV detection were designed from conserved 3'UTR region, allowing the amplification of all isolates tested [33].

2.2.3 Complementary DNA synthesis (cDNA synthesis)

cDNA was synthesized using total nucleic acid extracted from citrus leaves and bark as templates. The RNA of the CTV was reverse transcribed into cDNA using a reverse transcriptase enzyme (M-MMLV Reverse Transcriptase) in the presence of the CTV PEX-2 reverse primer. The cDNA was then shortly centrifuged and stored in a freezer at-20℃.

2.2.4 Nested RT-PCR

The RT-PCR and nested RT-PCR were carried out separately. The amplifications were performed with Taq DNA polymerase in a Robocycler Gradient 96 (Stratagene). The PCR cycle profile was one cycle at 94ºC for 2 min followed by 40 cycles for RT-PCR and 30 cycles for nested RT-PCR at 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min, with a final extension step at 72ºC for 10 min. The amplified DNA fragments were subjected to electrophoresis in a 2% agarose gel and visualized under 312 nm UV-light. The positive control was RNA extracted from CTV infected citrus leaves kindly provided by Dr. Stephan Winter (DSMZ, Braunschweig, Germany).

2.2.5 Cloning

The PCR products were first purified using Nucleospin ® Extract II kit (Macherey- Nagel), ligated into the pGEM ® -T Easy Vector System I. The plasmid DNA was then transformed into a competent Escherichia coli (JM 109). The blue-white screening test was done and finally the plasmids were purified for restriction analysis using Nucleospin® Plasmid kit (Macherey-Negal) following the manufacturer's instructions.

2.2.6 Sequencing and sequence analysis

The positive plasmid DNA samples with the correct insert size were sequenced by cycle sequencing with vector specific primers and analyzed on automated sequencer (Abiprism^{TM}, Applera Deutchland GMbH). Sequences were then checked using BioEdit 7.4 program [37]. Multiple alignments of sequences were performed using the program Clustal X [38].

3. RESULTS

3.1 Molecular Diagnosis

3.1.1 Nucleic acid extraction

The total RNA preparations from leaves and twigs bark of infected citrus trees were found intact with more RNA being extracted from twigs bark than from leaves as might be inferred from intensity of bands in the gel (Fig. 1).

3.1.2 Nested RT-PCR

The nested RT-PCR was able to amplify fragments corresponding in size to 132 bp were obtained from 39 out of 57 test samples, after reverse transcription of the total RNA into a cDNA using M-MMLV Reverse Transcriptase enzyme in presence of the reverse primer (Fig. 2). The positive CTV samples represented 68.4% from the total number of samples under test.

3.1.3 Cloning

The positive CTV-PCR products samples were successfully cloned giving white colonies with the recombinant plasmid vector (Fig. 3) and then confirmed using the colony PCR (Fig. 4). On purification, the plasmid DNA concentration was found in the range of 56.6-98.8 ng/µl. The restriction analysis of the plasmid DNA using EcoR1 enzyme yielded fragment sizes of 3015bp and 132 bp (Fig. 5).

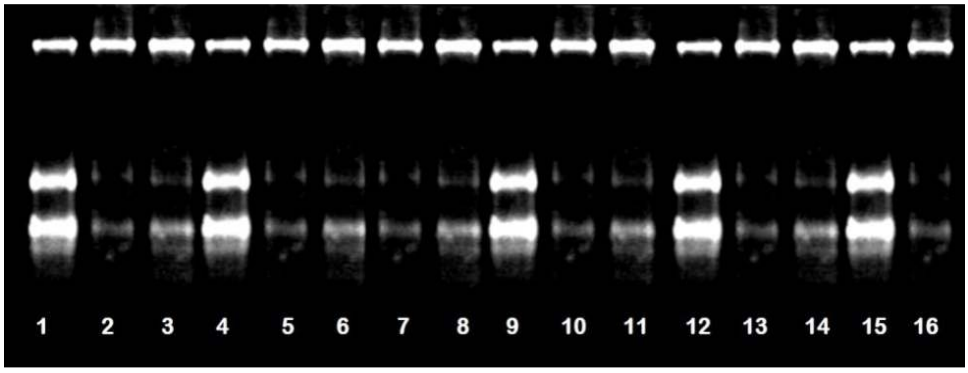

Fig. 1. Gel electrophoresis (1% agarose) of total RNA extracted from fresh samples collected from suspected CTV-infected citrus trees. Lanes 1, 4, 9, 12 represent bark tissue preparations, and the remaining lanes represent leaf tissue preparations

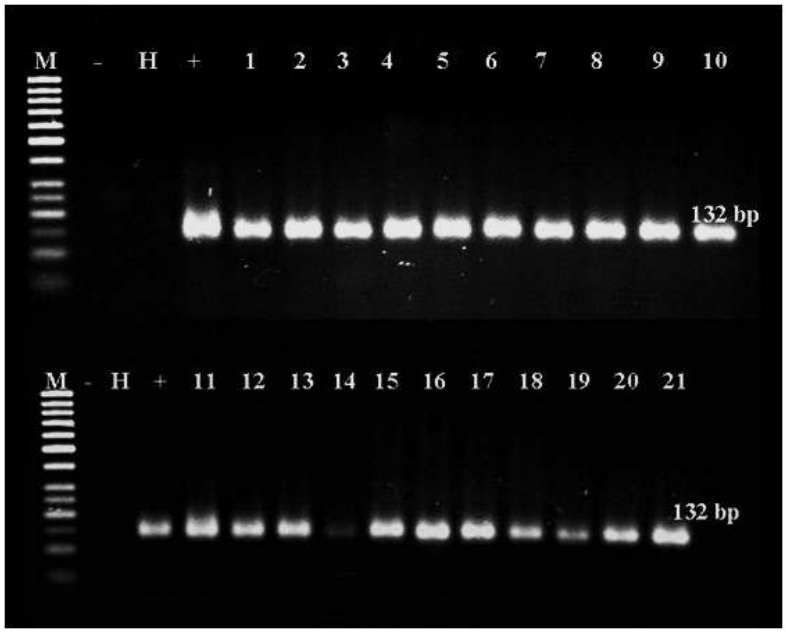

Fig. 2. Gel electrophoresis (2% agarose) of nested RT-PCR products amplified from leaf and bark extracts of *Citrus tristeza virus* (CTV)-suspected citrus trees using CTV-PEX and CTV-PIN primer sets. Lane M, 50 bp DNA ladder; -, water; +, positive control from CTV-infected tree; H, extract of healthy citrus plant; 1-21, tested samples. 132 bp positions represent CTV-specific bands on the gel

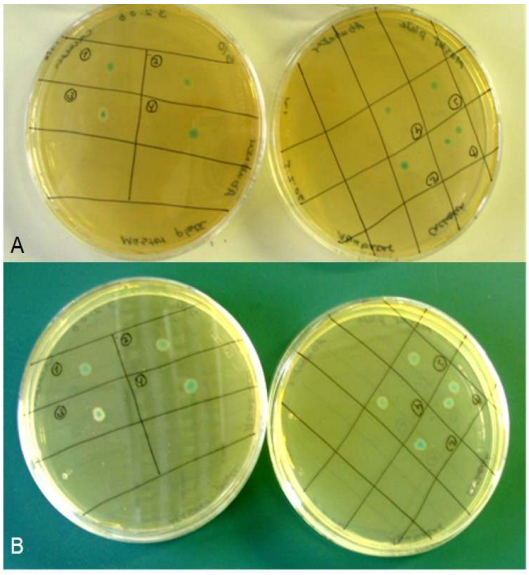

Fig. 3. Different LB/Ampicillin/ IPTG/X-Gal master plates showing the development of *Escherichia coli* colonies of the bacterial cells (JM 109) after colony PCR, selection and incubation over night at 37℃. Each sample represented by one master plate with 4-5 colonies

A. Blue colonies bear empty plasmid DNA
B. White colonies contain the integrated insert

3.1.4 Sequence analysis

Sequencing of nested RT-PCR products amplified from the Sudanese citrus samples confirmed their positive CTV identity based on blast search of NCBI database. The multiple sequence alignments of Sudanese CTV isolates sequences with other selected CTV isolates previously reported using ClustalX program, revealed the presence of three nucleotide sequence groups depending on the maximum sequence identity (Fig. 6). The analysis of the first sequence group showed 97% nucleotide sequence identity to (AF01623), a CTV strain from California, causing severe stem pitting symptoms. The second group was identical to the reference sequence DSMZ PV-0332 from Israel and showed 99% identity with CTV strains T30 (AF260651), T36 (U 16304) from Florida and with CTV-Qaha strain (AY340974) from Egypt. While the third group showed 94% identity to CTV strain T318A from Spain that causes severe stem pitting in sweet orange. The three different sequences obtained from Sudan revealed a lower nucleotide identity among them (about 96%) indicating at least three different CTV-strains to be present in citrus orchards in Sudan.

4. DISCUSSION

Citrus tristeza Closterovirus was detected as an emerging disease of citrus orchards for the first time in Sudan based on visual external symptoms on foliage and fruits and on characteristic internal symptoms on bark and wood at the bud union region of affected citrus trees [1]. The occurrence of CTV was confirmed by DAS ELIASA and direct tissue blot immunoassay (DTBIA) serological tests. It was naturally prevalent in all the surveyed areas infecting grapefruit, orange, lime and mandarin. The natural occurrence of *Citrus tristeza Closterovirus* (CTV) in the main citrus producing regions of Sudan was further substantiated by using a nested RT-PCR approach, followed by cloning and sequencing of the PCR products. The main advantages of the nested RT-PCR are the high sensitivity obtained without risk of contamination and the possibility of using external primers with the low annealing temperature and internal primers with the highest, in contrast with previously described protocols [33]. This procedure saves time in establishing the optimal primers ratio because external primers do not interfere in the second amplification. Therefore, it has been successfully applied to the detection and characterization of *Citrus tristeza Closterovirus* isolates in plant tissues.

The accurate and reliable molecular techniques used in the present study not only successfully detected the virus but also established the identity of at least three different CTV sequences resembling CTV strains from California, Florida and Spain causing severe stem pitting symptoms. The presence of the complex severe strains is not surprising since most of our mother trees are old lines that have been introduced without knowing their health status. Thus, the introduced plant material may be contaminated with several citrus pathogens similar to those prevalent in the source of origins such as, Egypt, Kenya, Palestine, South Africa and USA [39,40].

The 79 bp nucleotide sequence that was obtained from amplicons using PIN and PEX primer sets in a nested RT-PCR approach which were amplified from the CTV non translated genome region (NTR), successfully established the identity of the Sudanese CTV strains prevalent in the country, although, very short for convenient genome comparison and characterization, Therefore, the nucleotide

sequence of the coat protein (CP) gene of different Sudanese CTV-isolates or the complete sequencing of the genome have to be determined in order to compare perfectly our Sudanese isolates with those distributed worldwide in any further work.

The common practice of grafting of different *Citrus* spp. on sour orange root stock could be one of the main reasons for CTV occurrence and distribution. Sour orange has been one of the most popular root stocks world-wide and it is known to be susceptible to various graft-transmitted pathogens including CTV [2,41]. Therefore, it is essential that quarantine regulations should be strictly enforced to prevent introduction of CTV through: a) exchange of and movement of infected nursery plants and bud-wood b) awareness of citrus growers, farming community, researchers, extensionists on CTV problem and its prevention, is badly needed c) eradication and immediate disposal of inoculum source by removing old citrus trees d) rootstocks other than sour orange should be tested for future plantation. Moreover, infestation of insects regardless of CTV vector, on citrus nurseries and trees should carefully be monitored and controlled through insecticidal sprays.

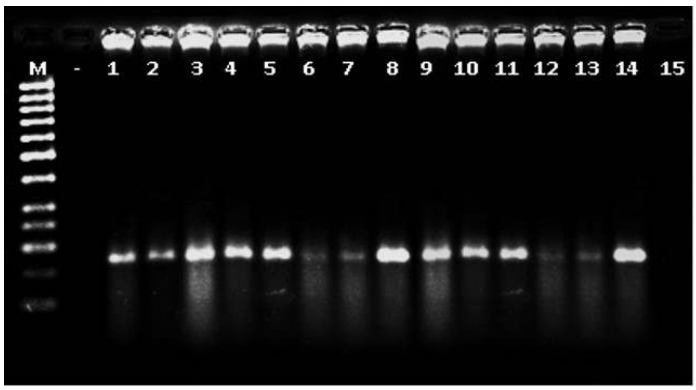

Fig. 4. 2% agarose gel electrophoresis of colony PCR products. Lane M, 50 bp DNA ladder; -, water; 1-14, tested clones; each 5 clones represent one sample. Positive clones with correct inserts were recognized by the appearance of CTV-specific bands on the gel at 132 bp position

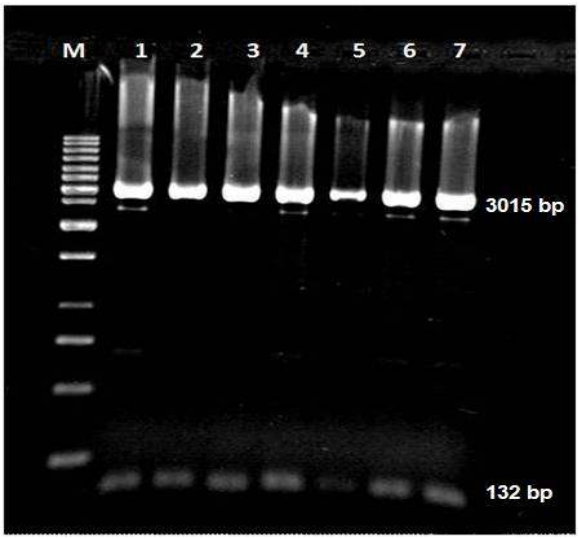

Fig. 5. 1% agarose gel restriction analysis of CTV recombinant DNA plasmid using EcoRI after overnight incubation at 37℃. Lane M, 1Kb ladder; l ane 1-7, Sudanese clones, each two clones represent one sample except clone 5 which represents a single sample. Positive clones carrying an insert of the correct size were indicated by the appearance of two bands, 3015 bp (plasmid) and CTV–specific bands on the gel at 132 bp positions

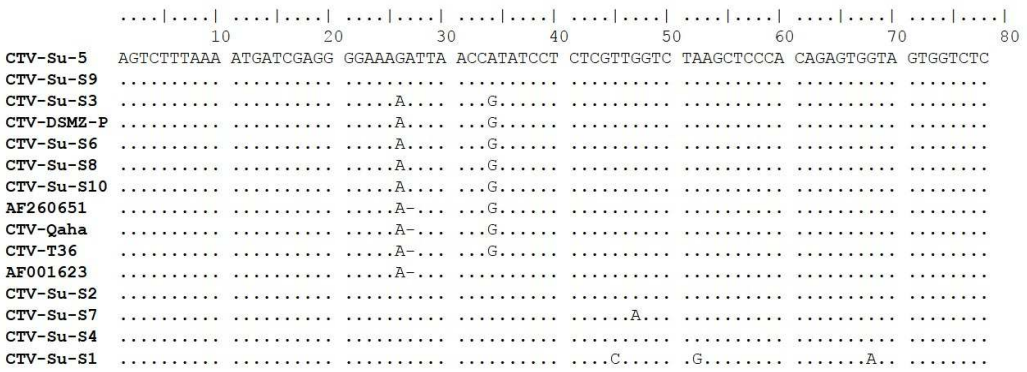

Fig. 6. Sequence alignment of the Sudanese CTV isolates with other CTV stains from NCBI database

5. CONCLUSION

This finding establishes beyond doubt the identity of the Sudanese strains of CTV and represents the first authentication of the natural occurrence of CTV in our commercial citrus orchards in Sudan. It appeared that RT-PCR is adequately sensitive, reliable and rapid technique and can be used successfully for the detection and diagnosis of CTV in citrus materials intended for use in disease management and budwood certification programs.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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