

Incidence and distribution of *Citrus tristeza virus* in citrus cultivars in Ibadan, southwest Nigeria

K.T. Kareem, B.O. Odu¹, V.C. Umeh, V.A. Chikaleke, K.E. Oke, O. Arogundade, A.O. Adediji^{2*} and O.O. Odedara³

National Horticultural Research Institute, Ibadan, Oyo State, Nigeria. ¹Department of Plant Science and Crop Protection, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. ²Department of Crop Protection and Environmental Biology, University of Ibadan, Oyo State, Nigeria. ³Department of Microbiology, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. *E-mail: adedapo.adediji@yahoo.com

Abstract

A survey was conducted on a 33 year old orchard to determine the incidence and distribution of *Citrus tristeza virus* (CTV), genus *Closterovirus* in different citrus cultivars at National Horticultural Research Institute (NIHORT), Ibadan, Nigeria. Compound enzyme-linked immunosorbent assay (Compound ELISA) was used to detect the presence of CTV in the citrus leaf samples. The results revealed 100% incidence in all the varieties tested. Of all the sweet oranges (*Citrus sinensis*) budded on Cleopatra mandarin rootstock, Washington Navel had the least plant survival of 2 out of the 12 planted with a canopy spread of 7.91 m and fruit yield of 7.00 kg per tree. It also had the highest mean ELISA reading with an absorbance ($A_{405\text{nm}}$) of 3.4780 while Bende had the least titre of 3.2158 with plant survival of 12 out of the 12 trees planted and fruit yield of 57.70 kg per tree. No significant difference was observed in Agege variety of sweet orange budded on different rootstock types and their ELISA values ranged from 3.283 to 3.384. Among the seedling trees, White grape recorded the highest mean ELISA value of 3.4698 while the average ELISA values of the other seedling trees were not statistically different from each other with their titres ranging between 3.2712 and 3.3615. The results establish the pandemic status of CTV in NIHORT orchard. The use of resistant cultivars and cross protection are recommended as effective, economical and environment-friendly means of preventing the incidence and spread of CTV.

Key words: *Citrus sinensis*, *Citrus tristeza virus*, ELISA, absorbance, rootstock, Nigeria

Introduction

Citrus is believed to have originated in Southeastern Asia, extending from Eastern Arabian to Philippines and from the Himalayas South to Indonesia or Australia (Davies and Albrigo, 1994). Citrus fruit contains appreciable amount of ascorbic acid, fair amount of vitamin A and B, as well as minerals, such as calcium, phosphorus and iron which add to its nutritive properties (Niaz *et al.*, 2004). Graft-transmissible virus and viral diseases are some of the major factors limiting citrus production throughout the world (Roistacher, 1991). *Citrus tristeza virus* (CTV), genus *Closterovirus* (Martelli *et al.*, 2005), is the causal agent of Tristeza, one of the most economically important viral diseases of citrus. The virus has a host range restricted to most species of the family Rutaceae (Roistacher, 1991) and can be disseminated long distances by movement of virus-infected plant material and locally by several aphid species in semi-persistent mode (Lee and Bar-Joseph, 2000). CTV is widely distributed in all citrus growing areas of the world and has caused the death of millions of trees in Brazil, Spain and Argentina (Cambra *et al.*, 2000).

CTV is a single-stranded RNA *Closterovirus* with flexuous rod-shaped particles measuring approximately 12 x 2,000 nm. It has many strains which are complicated, vary greatly and range from mild isolates to severe stem pitting isolates (Roberts *et al.*, 2001). It infects all species, cultivars and hybrids of citrus, regardless of rootstock, inducing mild or masking conditions to severe symptoms. Several techniques have been developed for the detection and differentiation of CTV isolates. Indexing on a

standard host range (Garnsey *et al.*, 2005) detects and categorizes the different biological types of CTV, but it is laborious and expensive for large-scale tests. Serological techniques have been used since the 1970s for detecting CTV (Bar-Joseph *et al.*, 1979; Garnsey *et al.*, 1993; Moreno *et al.*, 2008). Enzyme-linked immunosorbent assay (ELISA) and direct tissue blot immunoassay (DTBIA) are the most common techniques because of their reliability, rapidity and relatively low cost (Saponari *et al.*, 2008). Previous studies have shown the existence of the virus in Nigeria (Atiri, 1985; Varma and Atiri, 1993; Adediji, 2012). Therefore, the objective of this study was to determine the incidence and distribution of CTV through ELISA at the commercial orchard of the National Horticultural Research Institute (NIHORT), Ibadan, Nigeria with a view to develop sustainable intervention to prevent its spread.

Materials and methods

Location: Field survey was carried out at citrus orchard established in 1977 at National Horticultural Research Institute (NIHORT), Ibadan, Nigeria (latitude 7°54' N; longitude 3°54' E and 213m above sea level) in August, 2010. The varieties were arranged in a randomized complete block design and the spacing was 7 x 7 m. Budwood produced from the orchard are for commercial purpose and serve as source of planting materials for citrus establishment in other orchards. The orchard is also one of the biggest citrus plantations in Nigeria and fruits obtained are for sale to the general public.

Collection of samples: Symptomology was the main criterion in the collection of leaf samples from the infected/suspected to be infected trees (Roistacher, 1991). Four leaf samples each, were collected from the different cultivars of *Citrus sinensis* budded on different rootstocks and from rootstocks grown as seedling trees. The samples were kept in sterilized polythene bags and stored in a refrigerator at 4°C until analysed. For the sweet oranges, there were a total of 120 plants consisting of the following varieties; Bende, Etinan, Hamlin, Lue-Gim-Gong, Meran, Parson Brown, Pineapple, Umudike, Valencia and Washington Navel. Each variety was replicated 12 times. For the seedling trees, there were seven varieties consisting of Calamondin, Cleopatra mandarin, Rangpur lime, Rough lemon, Sour orange, Swingle citrumelo and White grape.

Detection of CTV: The presence of CTV was determined by using compound enzyme-linked immunosorbent assay (compound ELISA) obtained from Agdia, Elkhart, Indiana, USA. Leaf samples were ground in extraction buffer using pestle and mortar. The extracts were decanted into eppendorf tubes and were allowed to settle. 100 µL of prepared sample was loaded into the sample wells. 100 µL of the positive control was dispensed into the positive control wells while 100 µL of sample extraction buffer was dispensed into the buffer wells. Plates were incubated for 2h in humid box at room temperature. After incubation, plates were washed 7 times with 1X phosphate-buffered saline with Tween 20 (PBST). 100 µL of enzyme conjugate prepared at 1:200 was dispensed into each well and plates were incubated for 2 h in humid box at room temperature. Plates were washed 8 times with 1X PBST. 100 µL of P-nitrophenyl phosphate (PNP) substrate was dispensed into each well and plates were incubated in humid box for 60 minutes. Wells were measured spectrophotometrically at 405 nm with ELISA plate reader. Samples were considered positive if the A_{405} nm value was greater than twice that of the negative control.

$$\text{Disease incidence} = \frac{\text{Number of ELISA positive trees}}{\text{Number of trees examined}} \times 100$$

Determination of plant survival, growth and yield parameters:

The number of plant survival was determined by counting the number of stands that remain on the plot. Plant height was obtained by measuring the height of the plant with a measuring tape while the canopy spread was measured by placing a ruler edge on the stem of the tree and the measurement taken horizontally along the tree branches. Fruit yield was evaluated by weighing the total number of fruits produced by each variety for the year in consideration.

Statistical analysis: All the data obtained were subjected to analysis of variance (ANOVA). Comparison of means was done using Duncan Multiple Range Test (DMRT) at 5% level of probability.

Results

Results obtained on the incidence of CTV in different varieties of *C. sinensis* budded on Cleopatra mandarin showed that all the samples collected tested positive to the virus and of all the varieties, Washington Navel had the highest virus titre (3.4780) while Bende had the least titre (3.2158). The titres of CTV in Etinan, Hamlin, Meran, Parson Brown and Valencia were not

significantly different from each other with mean ELISA values ranging between 3.3353 and 3.4138. The absorbance values ($A_{405\text{nm}}$) of Lue-Gim-Gong, Pineapple and Umudike were 3.3128, 3.2940 and 3.3155, respectively (Table 1). All the sweet orange trees in the orchard had the symptom of stem scaling irrespective of the concentration of CTV in them. Fig. 1 shows stem scaling and stem pitting in Washington Navel while Fig. 2 shows yellow leaves representing another symptom of CTV in Bende variety.

There was no significant difference between the ELISA value of the positive control and those of Agege budded on different rootstocks. More so, 100% incidence was recorded for all the rootstock combinations with Agege (Table 2). The titre of CTV in rootstock seedling trees revealed that White grape had the highest mean ELISA value of 3.4698 while the average CTV titres of the other rootstock seedling trees were not statistically different from each other and their means ranged from 3.2712 to 3.3615. One hundred percent incidence was recorded for all the seedling trees (Table 3).

The variety Umudike recorded the highest plant height (6.82 m) followed by Etinan (6.25 m) and Parson Brown (6.19 m) (Table 4). The least plant height was recorded by Bende (5.03 m). Bende, Lue-Gim-Gong and Valencia retained all their 12 stands that were initially planted 33 years ago. However, Washington Navel recorded the least number of stands surviving with only

Table 1. Incidence and distribution of CTV in varieties of *C. sinensis* budded on Cleopatra mandarin

Variety	Total sample	Number infected with CTV	Disease Incidence (%)	ELISA value ($A_{405\text{nm}}$)
Bende	2	2	100	3.2158cd
Etinan	3	3	100	3.3740ab
Hamlin	4	4	100	3.3353ab
Lue-Gim-Gong	5	5	100	3.3128bcd
Meran	4	4	100	3.4100ab
Parson Brown	6	6	100	3.3738ab
Pineapple	3	3	100	3.2940bcd
Umudike	3	3	100	3.3155bcd
Valencia	5	5	100	3.4138ab
Washington Navel	1	1	100	3.4780a
Positive control	-	-	-	3.1668d
Buffer	-	-	-	0.3356e

Means followed by the same letters in a column are not significantly different from each other at $P=0.05$. Means separated by Duncan Multiple Range Test (DMRT).

Table 2. Incidence and distribution of CTV in *C. sinensis* (Agege) grafted on different rootstocks

Rootstock type	Total sample	Number infected with CTV	Incidence (%)	ELISA value ($A_{405\text{nm}}$)
Cleopatra mandarin	6	6	100	3.384a
Rangpur lime	5	5	100	3.283a
Rough lemon	5	5	100	3.359a
Positive control	-	-	-	3.325a
Buffer	-	-	-	0.336b

Means followed by the same letters in a column are not significantly different from each other at $P=0.05$. Means separated by Duncan Multiple Range Test (DMRT).



Fig. 1. Stem scaling on a tree of Washington Navel variety

Table 3. Incidence and distribution of CTV in rootstock seedling trees

Rootstock type	Total sample	Number infected with CTV	Incidence (%)	ELISA value ($A_{405\text{nm}}$)
Calamondin	1	1	100	3.2826b
Cleopatra mandarin	5	5	100	3.3062b
Rangpur lime	1	1	100	3.3615b
Rough lemon	4	4	100	3.2712b
Sour orange	4	4	100	3.3328b
Swingle citrumelo	1	1	100	3.2759b
White grape	5	5	100	3.4698a
Positive control	-	-	-	3.1660c
Buffer	-	-	-	0.3834d

Means followed by the same letter in a column are not significantly different according to DMRT at 5% level of probability

Table 4. Performance of sweet orange varieties in NIHORT orchard (33 years old)

Varieties	Plant survival out of 12 planted	Plant height (m)	Canopy spread (m)	Fruit yield (kg/tree)
Bende	12.00a	5.03e	6.69c	57.70e
Etinan	10.00b	6.25b	6.30d	168.30a
Hamlin	9.00c	5.77bc	7.29b	76.20d
Lue-Gim-Gong	12.00a	5.43d	5.90e	80.46d
Meran	8.00d	5.79bc	6.38d	156.15a
Parson Brown	9.00c	6.19b	7.27b	62.25e
Pineapple	10.00b	5.68c	6.62c	96.64c
Umudike	10.00b	6.82a	7.78a	121.90b
Valencia	12.00a	5.61c	6.81c	72.85d
Washington Navel	2.00e	6.00b	7.91a	7.00f

In each column, means followed by the same letter are not significantly different according to DMRT at 5% level of probability.

two trees. The highest fruit yield (kg per tree) for 2010 was recorded by Etinan (168.3), followed by Meran (156.15) and then Umudike (121.90). Washington Navel recorded the lowest fruit yield for that year.

Discussion

Findings of the investigation revealed that all the citrus trees were infected with CTV regardless of scion-rootstock combination. The



Fig. 2. Leaf yellow of *Citrus tristeza virus* on Bende variety

CTV ELISA tests on the citrus samples were positive including the trees that did not show visible symptoms. This corroborates the report of Citrus Clonal Protection Program (2002) which stated that the presence of CTV in a tree should be determined by a pathogen detection method such as ELISA rather than by symptom expression. Also, results indicated that CTV was present in varying concentrations in all the leaves of the *Citrus* cultivars used in this study. Reports have shown that leaf portion was considered to be good sample for the ELISA test (Iftikhar *et al.*, 2009). Furthermore, Anfoka *et al.* (2005) considered that the midribs and petioles were the best tissues for CTV detection through DAS-ELISA.

One hundred percent incidence was recorded in all the sweet orange varieties including the rootstock types. Ruiz-García *et al.* (2005) reported that in their experiment with 68 known-positive trees, 100% were positive by immunoprinting-ELISA whereas, 67 (98.5%) were positive by DAS-ELISA. The high incidence of CTV in all the leaf samples based on the ELISA tests showed that CTV is an important viral disease in Nigeria. The age of the orchard could also be a factor in the high incidence of the virus. This is in line with the findings of Dodds and Gumpf (1991) who reported a high incidence of CTV-infected trees in parts of central California. Age of the tree, prevalent strain and the nature of the virus are important in the diagnosis of CTV (Gafny *et al.*, 1995).

Washington Navel, which had the highest virus titre, also had the highest growth parameters. Therefore, the presence of CTV in a tree should be determined by a pathogen detection method such as ELISA rather than by symptom expression (Citrus Clonal Protection Program, 2002). However, the fruit yield of Washington Navel was very low compared to the other sweet orange varieties. Lee and Bar-Joseph (2000) reported that CTV reduces growth of trees accompanied by a decline in fruit yield, fruit size and quality in severe cases. More so, all the Agege varieties budded on different rootstocks and the seedling trees were positive to CTV irrespective of the varieties. This conforms to the report of Iftikhar *et al.* (2009) and Atta *et al.* (2012) who reported that CTV infects all species, cultivars and hybrids of citrus, regardless of rootstock while also inducing mild or masking conditions to severe symptoms.

Further work is needed to determine the distribution of CTV in citrus-growing regions of Nigeria and also to identify the different strains occurring in various *Citrus* species and varieties. Breeding for resistant cultivars and cross protection are suggested as means of preventing the spread of CTV (Stewart, 2006) to other areas where it is not found since the orchard is the source of budwood to commercial nursery stocks.

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