Survey for *Citrus tristeza virus* and Citrus Aphids in Tanzania

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ABSTRACT. Leaf samples, disease observations, and aphid incidence from 98 citrus trees, representing sweet orange, mandarin, lemon, rough lemon, and Mexican lime, were collected from the Coast, Dar-es-Salaam, Iringa, Kagera, Kigoma, Lindi, Mara, Mbeya, Mtwara, Mwanza, Ruvuma, and Tanga regions of the Tanzania mainland, and Unguja and Pemba islands of Zanzibar. The samples were tested using replicated DAS-I ELISA with Florida *Citrus tristeza virus* (CTV) antibodies (CREC28/G604). The tissues tested were either fresh or dried leaf midribs from near mature leaves. Most mature trees were observed as poorly productive with thin canopies. All mature Mexican lime seedlings that had observable stem-pitting CTV symptoms under a bark flap were positive by ELISA. Most, (97%), of the samples collected in the Bagamoyo district (Coastal region) and Kinondoni district (Dar-es-Salaam region) were CTV positive. Over all tests, 65% of samples were CTV-infected. Only one infestation of aphids was observed and a sample of these aphids was later confirmed as *Toxoptera citricida* Kirkaldy.

*Index words***.** *Citrus tristeza virus*, *Toxoptera citricida,* Brown citrus aphid, ELISA, Tanzania, East Africa

Citrus species may have been introduced into Tanzania many times during human migrations. The initial introduction and production is believed to have been concentrated along the 'Swahili coast' of Eastern Africa (10), including Malindi and Lamu (Kenya), the Zanzibar and Mafia archipelagos, and the Comoros islands. Later, citrus species may have been distributed into the low-lying adjacent coastal areas. Asian traders are most likely to have promoted the spread of citrus in the 12th century, whereas the Portuguese in the 15th century contributed to further spread of citrus into various sites (8). In 1498, Vasco da Gama collected local citrus from around the Mombasa area before reaching India (14). In the early 18th century the capital of Oman was in Zanzibar town (Unguja), so the Omani Sultans must have exchanged materials along the 'Swahili coast' of Africa.

Production of citrus in Tanzania is mainly dooryard, with a few large-scale groves at government research institutions, public schools and a limited number of commercial farms. Production of both juice and fresh citrus are inadequate to satisfy the domestic market, and fresh fruit has remained at approximately 39 metric tons from 1999-2003, with a slight increase to 40 metric tonnes in 2006 (6).

In 1981, Tanzania was reported as one of the African countries affected by *Citrus tristeza virus* (CTV) (4), and the virus was observed during a general survey carried out by Swai (11) in 1984-85. CTV has worldwide significance in decreasing fruit productivity wherever citrus is grown (1, 4). No further research has been conducted to determine the magnitude of the problem and the possible consequences to growers' economy. The current paper reports on the status of CTV in Tanzania after nearly two decades of neglect.

MATERIALS AND METHODS

Survey. A total of 48 samples were collected from August to October, 2005. The survey covered the Tanzanian mainland and Zanzibar islands (Fig. 1). The target areas were traversed through main roads. Assessment and sampling was done at 100 km intervals or more depending on the availability of citrus plants. In July, 2007, 36 samples were collected in a small area of the Coast and Dar es Salaam regions from mainly commercial nurseries. Growers were interviewed as to the plant history, age, cultivar and the utility of the crop, and after consultation, citrus plants were visually assessed for plant disease and aphid incidence. All trees inspected had a small flap of stem bark peeled back for the stem observations. Leaf samples were collected for the laboratory analyses and aphid samples, where observed, were preserved for further identification. A global positioning system, Magellan GPS-315 (Thales Navigation Inc.), was used to obtain a reference to the location altitude, latitude and longitude.

Sampling, tissue type, and storage. One fully expanded leaf, two to three leaves back from the growing shoot of the young flush from each of the four compass points of each tree were stapled together as a one sample. In one agricultural research station grove, three composite tree samples were collected; in this case one leaf was collected as before from each tree per sample, in a diagonal pattern across the grove. In all from this site three samples were collected; all were sweet orange. One sample was a composite of 10 trees, one represented eight trees and one represented five trees. All samples were stored in plastic bags at 4˚C until processing. In 2005, after each day of collecting samples, the petiole base and midrib were cut into small pieces, wrapped in tissue paper and inserted in a screw-capped vial containing silica gel. All such vials were kept at 4˚C until the laboratory tests. In 2007, the leaf samples were stored at 4˚C for the maximum of 12 h from collection, and then the midribs were cut and processed as before.

Aphid collection. All trees were inspected for aphids. Where observed, a collection of aptera and alates were placed in 80% ethanol in screw-capped vials for microscope analyses, and a sample was also sent to R. Yokomi, USDA, California, USA, for identification confirmation.

CTV ELISA. The ELISA was done as described by Garnsey and Cambra (7), where specified. Samples were ground by pestle and mortar in extraction buffer [phosphate buffered saline-Tween 20 with added polyvinyl pyrrolidone, MW 40,000, according to (7)] and placed in microfuge tubes on ice. Aliquots (100 µl) were dispensed into antibody coated ELISA plates (Barloworld Scientific Ltd, Stone-Sterilin, UK, Catalogue no. 798905). Two microplate wells were used per sample. The ELISA trapping antibody was CREC 28 (kindly provided by R. Lee, USDA-ARS-NCGRCD, Riverside, CA) diluted 1:1,000 in carbonate buffer pH 7.9. The detecting antibody was G604-10/11 (also provided by R. Lee), diluted 1:30,000 in conjugate buffer (PVP-PVP40 plus 0.2% bovine serum albumin). The conjugate was rabbit-antigoat (Sigma-Aldrich 055K4846) at 1:30,000 dilution in conjugate buffer. The substrate was ρ-nitrophenyl phosphate at 1 mg/ml in substrate buffer (10% diethanolamine, pH

9.8). Tests were done in triplicate including extensively tested CTV infected tissues, CTV negative tissues, and buffer-only controls. The antibody dilution points were standardized prior to the experiments and the control tissues were also selected during these preliminary tests. The CTV infected tissue for all tests came from a sweet orange scion grafted to an unknown rootstock situated in Dar es Salaam. Sweet orange CTV-free tissues for all the tests derived from a 1-yr-old sweet orange scion grafted to sour orange.

For the ELISA, absorbance was measured at 405 nm using a scanning multiwell spectrometer (Labsystem Multiskan Ascent, 354). Plate readings for each experiment were replicated (10 readings at 15 min intervals during the reaction). The absorbance readings were averaged to obtain the mean absorbances for each sample for a minimum of two time points around the point on each plate where the CTV-positive sample was at least twice the reading for the CTV-free control. Standard errors between duplicated sample wells were 0.05 units or less.

Data analysis and mapping the sample sites. The geographical distribution of CTV- affected sites was mapped using a GIS tool, the Arc View 3.2 (Environmental System Research Institute (12) – www.esri.com). The resultant theme layer was mapped onto the other locations of features such as prominent lakes and towns.

RESULTS

Geographical distribution of CTV in Tanzania. CTV was found in many locations in Tanzania (Fig. 1). It was detected in approximately 40% of the first survey samples and 97% of the second. The samples from the first survey were kept for 2 yr at 4^0C but with varying temperatures due to unstable electricity. The data could well be a severe underestimate of CTV presence in these trees. From both collection periods, the CTV-infected trees were mainly along the Eastern and Southern coastal regions, plus the Lake Victoria zone (North-western) part of the country, the Southern Highlands and all samples collected from Unguja and Pemba (Zanzibar archipeligo). The majority of samples were sweet orange (51 samples), mandarin (five samples), lemon (12 samples), rough lemon (six samples), and Mexican lime (10 samples).

Influence of plant age on CTV incidence. CTV was detected in the youngest seedling ($\leq$ 0.5 yr) to the oldest plant (>25 yr). Even though this was a small sample size, there were relatively more CTV-positive trees in the age range of 3-15 yr. Very few plants over 20 yr of age were sampled.

Effect of method of propagation on CTV incidence. CTV was more prevalent in grafted plants than in seedling plants. Statistical analysis using a linear regression model (Table 1) indicated a significant correlation between grafted plants and ELISA positive samples. The correlation coefficient (r) was 0.258 (significant at $p = 0.05$). CTV could not be detected in 19% of grafted plants. Table 2 shows the citrus species in the survey with an indication of propagation method and plant age.

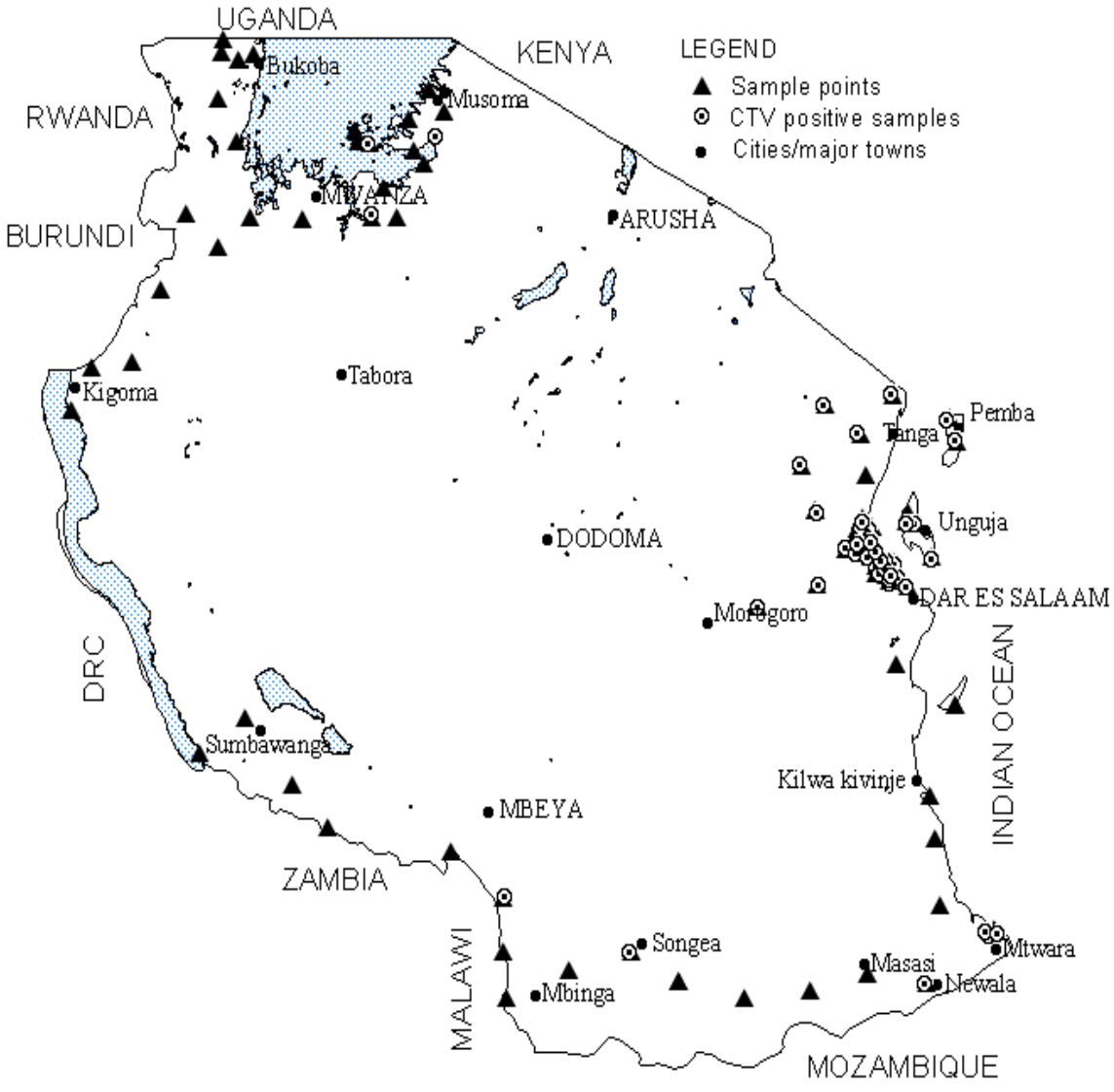

Fig. 1. Map of Tanzania showing points where citrus trees were sampled and where *Citrus tristeza virus* was detected by ELISA.

TABLE 1
STATISTICAL ANALYSES OF PARAMETERS AFFECTING *CITRUS TRISTEZA VIRUS* INCIDENCE

Test parameter	r	F-value	Probability[1]
Propagation method (seedling or grafted)	0.258	5.824	0.018
Health status in the field	0.107	0.950	0.333
Variety/type of citrus	0.171	2.480	0.119
Interaction (P x H x V)[2]	0.315	2.942	0.038

[1]significant (α=0.05). Numbers without notation letters in a column are not statistically significant
[2]P = propagation method; H = health status; V = variety

TABLE 2
AGE AND PROPAGATION METHOD FOR CITRUS SPECIES SAMPLED FOR *CITRUS TRISTEZA VIRUS* (CTV)

Citrus species	Propagation	Total no. sampled	No. CTV + (ELISA)	<1 yr	>1-10 yr	11-20 yr	>20 yr
Lemon	Seedling	6	4	0	5	1	0
	Grafted	6	6	0	4	2	0
Mexican lime	Seedling	8	4	4	3	1	0
	Grafted	6	6	0	4	2	0
Rough lemon	Seedling	6	2	0	5	1	0
	Grafted	0	0	0	0	0	0
Sweet orange	Seedling	30	13	0	26	3	1
	Grafted	21	15	18	2	1	0
Mandarin	Seedling	2	2	0	1	1	0
	Grafted	3	2	1	0	2	0

Field observations. All Mexican lime trees sampled near Bagomayo in the second survey had portions of their stem bark peeled to check for stem symptoms. Every tree had stem pits of various sizes but without gumming. Mexican lime seedlings older than 5 yr had very brittle branches; most of this was due to the longitudinal stem pits present, and seen when the bark was peeled. All Mexican lime trees also had leaf vein clearing to varying degrees in their canopy leaves. One 'lemon seedling' from the same area also had stem pits under the bark with no vein clearing in the leaves.

Fig. 2 shows stem pitting and vein clearing in a Mexican lime seedling from near Bagomoyo. Small fruit and non-uniform ripening were also observed on a few sweet orange trees at Tingeni (Muheza) and Duga, both in the Tanga region. Trees showing non-uniform ripening were grafted to unknown rootstocks and were aged 6-11 yr.

Fig. 2. Ten-year old Mexican lime seedling found to be CTV-infected during the second survey. Left panel: In the foreground, a peeled stem from this tree showing deep pitting. Right panel: The arrow indicates vein clearing or chlorosis in a secondary leaf vein of this tree.

Correlation of the field diagnosis with ELISA results. The health status of the trees as examined in the field, and the detection of CTV by ELISA, were significantly correlated. Most plants apparently in good health carried the virus. Conversely, the virus was not detected in samples collected from a few plants (<10) considered to be in poor health. Presence of CTV did not depend upon the different species and varieties being tested.

Aphid sample. One sample of aphids was collected from Bukoba (Lake zone), at S01^{0}14'246" E031^{0}39'394", 1205 meters above sea level, from a lemon seedling (approximately 6 yr old). The sample was confirmed later as being *Toxoptera citricida* Kirkaldy. This is the first report of the existence of this species in Tanzania.

DISCUSSION

Our data came from a relatively small sample number of samples, but nontheless indicate that CTV is present throughout all areas where citrus is grown in Tanzania. The majority of plants sampled (61%), were sweet orange. This suggests that sweet orange is the most common cultivar while other species, such as grapefruit, are not present. The data confirm previous reports (4, 11) and imply that CTV is endemic. CTV also co-exists with the most efficient CTV vector, *T. citricida.* The CTV incidence of approximately 65% overall, (which may well be an underestimate), is high enough to reduce the productivity of the crop. In the second round of sampling, tissues were tested very soon after collection, with better storage conditions, and CTV incidence was 96%. This is considered to better reflect the CTV incidence. Such high CTV incidence may have possibly been responsible for the major losses and decline of productivity of sweet orange production on Ukerewe island (Mwanza region) in the early 1990s, reported to the first author by several farmers from that region.

CTV seems to have high incidence along the low coastal areas near the Indian Ocean. Almost 70% of the country's gross citrus production is in these areas, and this may facilitate easy spread of the virus. Citrus has been under production in the coastal areas since the pre-colonial era and the same materials have been re-used for propagation of both rootstock (seed) and scion. There is no system to ensure good sanitation practices, hence grafting is locally done without caution in respect of spread of the virus through graft materials and, less likely, through contaminated tools.

The fruits in some of the sweet orange trees sampled in the Tanga region were relatively small. This could be due to CTV severe isolates. In Mexican lime seedlings of the second survey, the CTV indicator symptoms of stem pitting and leaf vein clearing were noted in all trees; every tree was also CTV-positive by ELISA. Similar observations were reported in South Africa (9). This suggests that severe CTV is also present, as this can reduce fruit size.

Fruits from some sweet orange trees sampled in the Tanga region also showed uneven ripening. This may indicate the presence of other infectious agents such as Huanglongbing (HLB, citrus greening) which has been recorded in Tanzania (11). These symptoms need to be investigated further.

CTV could be detected from the seedling stage (<0.5 yr) through to the oldest plant sampled (>25 yr). The highest incidence was observed in healthy-looking trees aged between 3-15 yr. Factors affecting the detection of CTV by ELISA include the latency of infection from inoculation (at grafting, or by aphids *per se*) to detection, environmental conditions (ambient temperatures and altitude), and the type and state of tissue tested.

Grafted plants showed slightly higher incidence of CTV than seedlings. Most budwood used for grafting could already be infected by the virus. This has the potential of promoting unlimited spread of the virus because most growers obtain their planting materials from nurseries whose materials are already infected. Current knowledge on the

local and long-distance spread of CTV into different regions through infected budwood suggests this (8).

CTV was detected in 29% of citrus seedlings tested. This incidence is most likely be due to aphid vectors. Although only one sample of aphids was observed and collected at one location, this was *T. citricida,* showing that the most efficient CTV vector exists in Tanzania.

The observed discrepancy between field observations and ELISA results is consistent with other studies on CTV. For some cultivars such as mandarins there may be no symptoms of CTV when a plant is infected. This is a challenge to CTV surveys where field assessments are the only options available.

Our results show that CTV is endemic to most cultivated citrus in Tanzania with higher incidence in the coastal belt. CTV is most likely being spread through grafting and infected budwood as well as *T. citricida*. Field observations, therefore, must always be backed with detection techniques such as ELISA. A more intensive survey should be undertaken in future to determine the magnitude of the problem, and evaluate other damaging pathogens which may be present. Optimally, a future survey would also involve a molecular determination of the type of CTV present and identification of possible mild CTV isolates useful for cross protection (3). A clean stock regime would first remove all deleterious pathogens from local cultivars and species through tissue culture and treatment processes. Such a program requires government support, sufficient funds to set up and maintain the facilities and staff, a critical mass of skilled personel, laboratory, screenhouse and tissue culture facilities specific for the purpose of commercial citrus production in Tanzania.

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