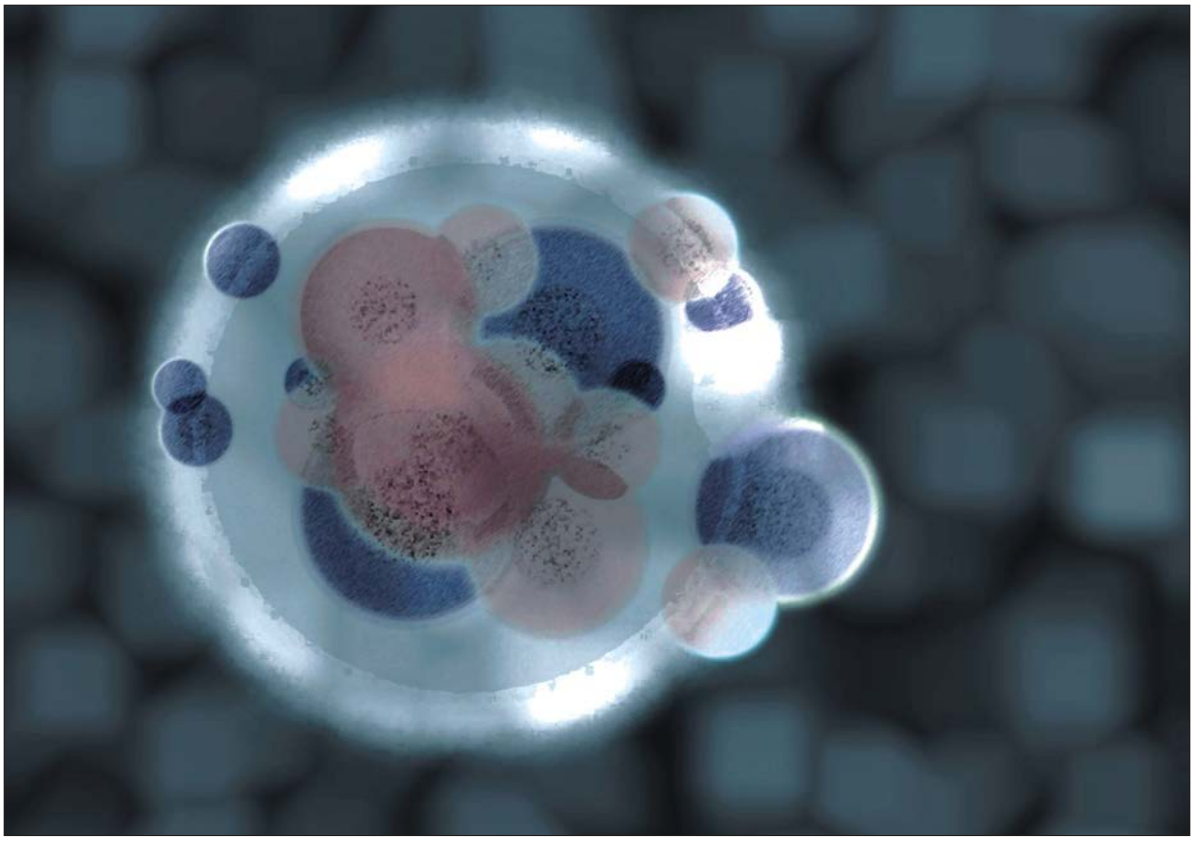

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PCR Detection and Distribution of Huanglongbing Disease and Psyllid Vectors on Citrus Varieties with Changes in Elevation in Kenya

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Abstract: The current decline in citrus production in Kenya is attributed to a great extent to Huanglongbing (HLB) (greening) disease, caused by *Candidatus* Liberibacter africanus. The control or eradication of the disease has been seriously impeded by the lack of appropriate disease indexing methods and lack of current information on the distribution of the disease and its vector. The purposes of this study were three-fold: To design a HLB detection method for Kenyan strains, to determine the the impact of variation in altitude and citrus varieties on disease distribution using both morphological and molecular techniques. A PCR detection methodology was designed to amplify the ribosomal protein (*rplJL*) gene of the *Candidatus* Liberibacter africanus strains that cause HLB disease in citrus. A 716 -720 bp fragment of the *rpL10/rpL12* rDNA was PCR amplified from HLB infected samples while samples obtained from greenhouse maintained citrus had no amplification. The PCR amplification of the *rplJL* gene provides a precise tool to detect the presence of *Candidatus* Liberibacter africanus bacteria in citrus in all environments and therefore accurate indexing for the HLB disease before and after symptoms develop on the plant. The HLB disease and its psyllid vectors were found to be most prevalent on Washington navel orange growing in altitudes above 1400 mASL and was least found in the lower midlands (800-1250 mASL). There is need to integrate stringent and early disease detection tools with the use of clean planting material to curb the spread of the disease in the country.

Key words: Citrus, PCR, *Candidatus* Liberibacter africanus, ribosomal DNA, Huanglongbing, *Trioza erytreae*, distribution in Kenya

INTRODUCTION

Huanglongbing (HLB) disease (citrus greening disease) is the most important disease affecting citrus production in Kenya, Africa, Asia and the Americas (Bové, 2006; Teixeira *et al*., 2008). This disease is caused by *Candidatus* Liberibacter africanus in Africa, *Candidatus* Liberibacter asiaticus in Asia (Jagoueix *et al*., 1994; Planet *et al*., 1995; Villechanoux *et al*., 1993; Hung *et al*., 2004) and *Candidatus* Liberibacter americanus in Brazil and America (Coletta-Filho *et al*., 2005; Teixeira *et al*., 2005a-c). Members of the *Candidatus* Liberibacter are non-cultured, gram negative, phloem limited species belonging to the alpha sub-division of the class Proteobacteria in Eubacteria (Manicom and van Vuuren, 1990; Jagoueix *et al*., 1994, 1996, 1997). This bacterium is further divided into three species; the heat-tolerant, *Candidatus* Liberibacter asiaticus which manifests disease symptoms at temperatures well above 30°C in Asia, while the heat sensitive *Candidatus* Liberibacter africanus is prevalent only in cooler areas where temperatures do not exceed 30°C in Africa (Schwartz and Green, 1972; Jagoueix *et al*., 1994; Planet *et al*., 1995; Villechanoux *et al*., 1992). The bacteria parasitizes all the important citrus species in the country including; *Citrus sinensis* Osbeck (sweet orange; Valencia orange and Washington navel orange), *C. tangarina* (tangerines) and *C. limon* (rough lemon). Two homopteran vectors are responsible for the transmission of the HLB disease among citrus. *Trioza erytrae* (Del Guercio) (Homoptera: psyllidae) transmits the disease in Africa while *Diaphorina citri* (Kuwayama) in Asia (Mclean and Oberholzer, 1965; McClean, 1974; Salibe and Cortez, 1966; Li *et al*., 2007; Urasaki *et al*., 2007). *Diaphorina citri* has also been reported to transmit the disease in Florida and Texas in the USA (French *et al*., 2001; Grafton-Cardwell *et al*., 2007; Manjunath *et al*., 2008). *Trioza erytreae* is sensitive to heat and

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temperatures of 32°C kill all stages of the insect while 27°C allows rapid development of the insect (Moran and Blowers, 1967; Catling, 1973). However, *D. Citri* is tolerant to extreme temperatures and sensitive to high rainfall and humidity (Catling, 1973; Regmi and Lama, 1988).

Though HLB is a severe and widespread disease of citrus globally, the distribution of the disease seems to be affected by the climatic requirements of the pathogen and the vector (Da Graça, 1991). The disease has been reported in Asia, south-east Asia, south and eastern Africa and the Arabian peninsular (Jagoueix *et al*., 1994; Coelho and Marques, 2002; Waithaka and Obukosia, 1980). In Africa HLB was first reported in South Africa in 1929 (Moll *et al*., 1980) and its severity has been reported in Swaziland, Zimbabwe, Ethiopia, Sudan, Kenya, Malagasy, Tanzania, Malawi, Burundi, Kenya, Somalia and Cameroon (Da Graça, 1991; Bové, 2006). In Kenya, the disease was reported in Thika, Kamiti, Kitale and in high altitude areas (above 1000 m ASL) (Schwartz, 1975). Nevertheless, the disease is found in all citrus growing areas in the highlands (Muriuki, 1989), culminating in the collapse of citriculture in some regions (Seif, 1991; Obukosia and Waithaka, 2000). Due to the lack of a successful field chemotherapy disease control technique and the long incubation period of the disease it is necessary to detect and index for the disease early so as to control its spread to other plants.

Several strategies have been developed for detection, indexing and characterization of the HLB bacteria from both infected plant tissues and from the insect vectors. This is particularly important because attempts at culturing the bacteria on nutrient media have been unsuccessful (Bové, 2006), however, recent attempts show minimal success (Sechler *et al*., 2009). Electron microscopy and cytochemistry techniques (Garnier *et al*., 1976) were among the earliest methods utilized in detection and identification of the bacteria. Monoclonal antibodies (Gao, 1988; Garnier *et al*., 1987) and DNA probes (Villechanoux *et al*., 1992; Hocquellet *et al*., 1997) have been successfully utilized for the detection of the bacteria. The PCR seems to be the most versatile method in detection of the bacteria and thereby the disease (Jagoueix *et al*., 1994; Bové, 2006). PCR methods and DNA sequencing efforts in the HLB causing proteobacteria have been concentrated within the nucleolar organizer region (16S rRNA gene, 16S/23S rRNA gene spacer region) the *rplKAJL-rpoBC* operon (Leblond-Bourget *et al*., 1996; Jagouiex *et al*., 1996, 1997; Subandiyah *et al*., 2000) and the outer membrane protein (omp) genes (Bastianel *et al*., 2005). The nucleolar organizer region exhibit concerted evolution, a phenomenon in which there is rRNA gene sequence uniformity within a species but rapid change across species boundaries (Flavell, 1986; Gerbi, 1985). The 16S rRNA has been extensively used to index and characterize the HLB bacteria (Tatineni *et al*., 2008; Das, 2004; Li *et al*., 2008, 2007; Manjunath *et al*., 2008; Kawabe *et al*., 2006; Khairulmazmi *et al*., 2008), however, due to concerted evolution this region is not effective for indexing of different strains of the bacteria in citrus (Jagoueix *et al*., 1996, 1997). In addition to the 16S rRNA, Bastianel *et al*. (2005) studied the diversity of *Candidatus* Liberibacter asiaticus based on *omp* gene sequence using a PCR-restriction fragment length polymorphism strategy. Hocquellet *et al*. (1999a) isolated *Candidatus* Liberibacter species DNA using RAPD PCR. The use of loop-mediated isothermal amplification (Okuda *et al*., 2005), cycleave isothermal and chimeric primer-initiated amplification of nucleic acids (Urasaki *et al*., 2008, Fusayasu *et al*., 2006), nested-PCR (Ding *et al*., 2005; Fang *et al*., 2004) and real time PCR (Li *et al*., 2007, 2008; Tatineni *et al*., 2008; Manjunath *et al*., 2008; Kawabe *et al*., 2006; Lan *et al*., 2004) are different modifications of the PCR that have been applied to improve the efficiency of detection and indexing of the disease in citrus trees. The ribosomal protein *rplKAJL-rpoB* gene of *Candidatus* Liberibacter species serves an important role in detection and characterization of the bacteria in citrus trees (Villechanoux *et al*., 1993; Hocquellet *et al*., 1999b; Teixeira *et al*., 2008; Okuda *et al*., 2005; Li *et al*., 2008; Urasaki *et al*., 2007, 2008; Magomere *et al*., 2009). The ribosomal proteins (*rplKAJL-rpoB*) genes, the tufB-secE-nusG genes and the intergenic regions between the proteins have variable nucleotides in different species and strains of the HLB bacteria.

The search for remedies for the control or eradication of the disease in the country has been seriously hampered by the lack of appropriate disease indexing methodology. This study tests a procedure for indexing of the HLB disease in infected citrus plants by PCR amplification of the ribosomal protein (*rplKAJL*) gene cluster of the *Candidatus* Liberibacter africanus strains that cause HLB disease in citrus varieties in Kenya. We show that PCR amplification of the *rplKAJL* gene cluster provides a precise tool to detect the presence of *Candidatus* Liberibacter africanus bacteria in citrus and therefore accurate indexing for the HLB disease in Kenya. We also present a procedure for indexing of the Kenyan strains of the bacteria by amplification of the 16S rDNA and the 16S/23S ribosomal intergenic regions. In addition, the distribution of the disease and its vector among agroecological zones in areas above 800 mASL and on agronomically important citrus varieties is analysed.

MATERIALS AND METHODS

Sampling and plant materials: In this study, Kakamega (representing the highlands >1400 mASL) and Machakos (representing the lowlands <1400 mASL) districts of Kenya were sampled (Fig. 1) and clustered according to the agro-ecological zones (AEZs) and the most important zones for citrus production were identified in the months between January 2003 and June 2004. Seven AEZs were identified in Machakos district, the AEZs were in three broad categories lower midland (LM3, LM4, LM5,) Upper Midlands (UM3, UM4,) Lower highlands (LH4 and LH5) AEZs (Table 1). In Kakamega the lower midland AEZs (LM1, LM2) and the upper midland AEZs (UM0, UM1 and UM4) (Table 1) were purposively sampled due to the higher presence of citrus orchards. Three farms were randomly sampled from each AEZ. In Machakos, four mature trees of different varieties [*Citrus sinensis* (Washington Navel and Valencia Orange), *Citrus reticulata* (tangerines) and *Citrus limon* (rough lemon)] were sampled and studied from each farm. On the other hand, only two varieties [*Citrus sinensis* (Washington Navel) and *Citrus limon* (rough lemon)] were consistently present in Kakamega orchards, therefore two samples from two varieties were sampled and studied from every farm. A total of one hundred and fourteen trees were sampled from the two districts. Leaves were picked from the trees and stored in an ice box for transporting to the laboratory where they were maintained at -20°C untill DNA extraction.

DNA extraction and partial purification: Leaf midribs (0.1 to 0.3 g) were chopped to a fine mince with a razor blade in a disposable petri-dish in extraction buffer containing 1 mL of TE buffer (10 mM Tris pH 8.0, 400 mM EDTA) plus 1% SDS and 0.25 mg of proteinase K) (Jagoueix *et al*., 1996). The homogenate was transferred to an eppendorf tube and incubated for 2 h at 65°C. The suspension was centrifuged for 15 min at 12,000x g and the supernatant mixed with 1 mL of Wizard Min Prep DNA purification resin (Promega). The resin was transferred to a mini-column and washed twice with 2 mL of 80% isopropanol. Thereafter, 50 µL of hot water (80°C) was added, incubated for one minute and centrifuged at 16,000x g for 30 sec in an eppendorf tube. This step was repeated to have a total yield of 100 µL of extract. The extract was stored at -20°C (Jagoueix *et al*., 1996).

Amplification of the *L10/L12* ribosomal protein rDNA the 16S rDNA and the 16S/23S rDNA: Two primers were used to amplify the intergenic region of ribosomal protein L10/L12 rDNA and the L10 rDNA (*rplJL* genes) of the

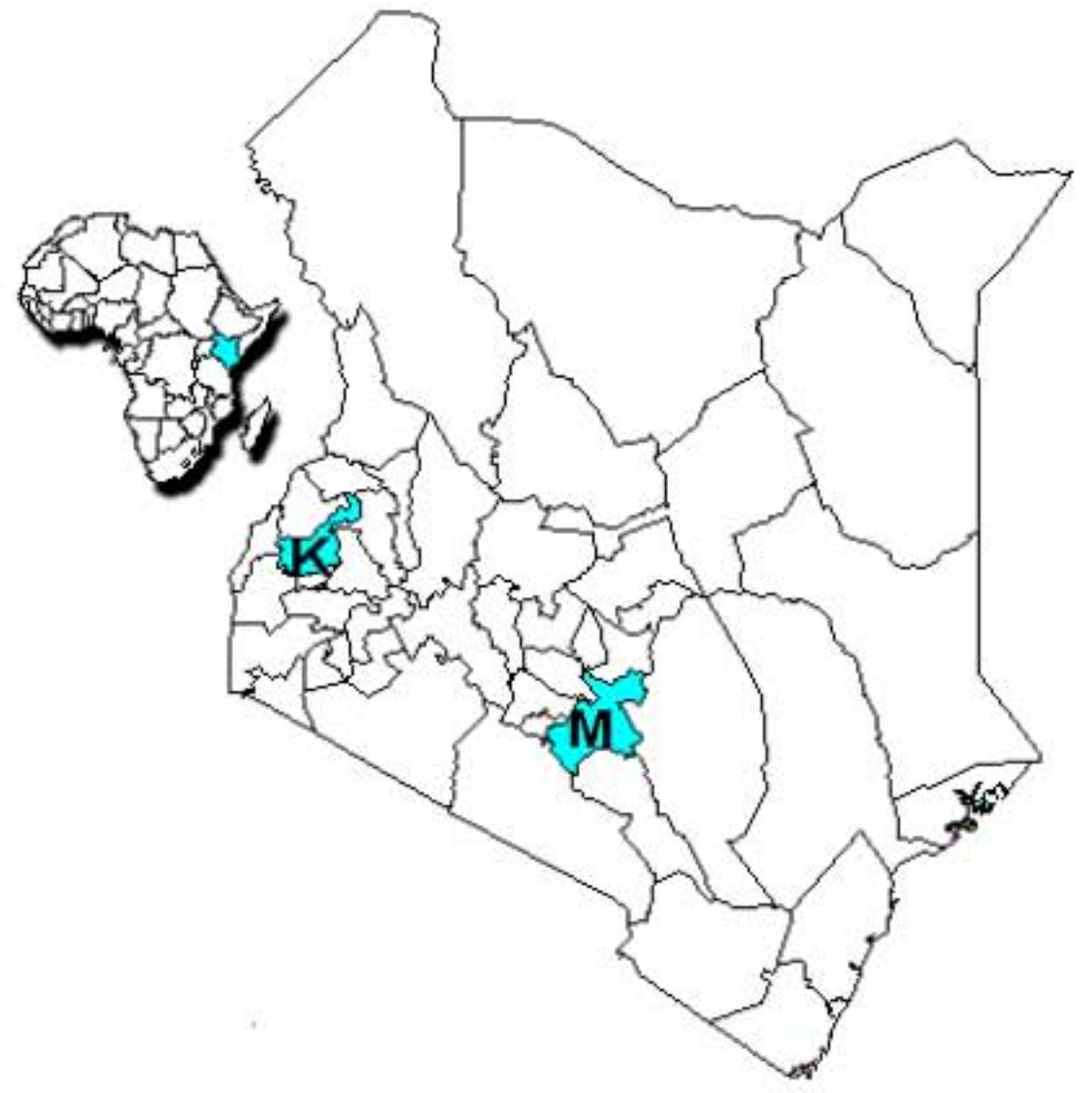

Fig. 1: Map of Kenya showing the location of the two districts sampled for the survey. Kakamega representing the highlands (>1400 mASL), Machakos representing the lowlands (<1400 mASL). Data Source: Kenya Bureau of statistics/ Cartographic Section, 1989, Kenya population census district maps, 1989. K: Kakamega sistrict and M: Machakos district

Table 1: Agro ecological zone characteristics in Machakos and Kakamega district

Agro-ecological Zone	Altitude (m)	Mean Temp (°C)	Rainfall (mm)
Machakos			
Lower Highlands (LH)	1800/1900-2200/2400	15-18	1000-1800
LH4	2200-2400	15.8-15.1	600-850
LH5	2070-2210	15.8-15.1	600-850
Upper Midlands (UM)	1300/1500-1800/1900	18.0-21	700- 1800
UM3	1400-1830	20.5-17.9	900-1050
UM4	1340-1830	20.9-17.9	700-950
Lower Midlands (LM)	800 to 1300	21.0-24	700-1800
LM3	1160-1350	22.0-20.9	750-900
LM4	1160-1280	22.0-21.3	700-850
LM5	800-1220	24.0-21.6	600-800
Kakamega district			
Upper Midlands	1300/1500-1800/1900	18.0- 21	700-2200
UM0	1500-1700	19.6-18.6	2000-2200
UM1	1500-1950	20.6-18.0	1620-1800
UM4	1500-1900	20.9-18.9	1000-1600
Lower Midlands	800 to 1500	21.0-24	700-1800
LM1	1300-1500	22.0-20.8	1650-1850
LM2	1300-1500	22.0-20.9	1550-1950

Farm management hand book of Kenya (1983)

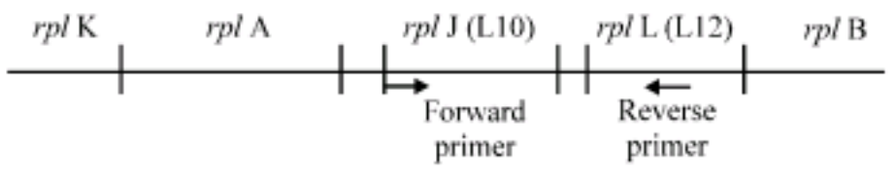

Fig. 2: Organization of the ribosomal protein genes in the *rpl*KAJL *rpo*BC operon of *Candidatus* Liberibacter africanus and the position of the forward and reverse primers utilized

bacterium. Forward primer 5 (CATCGGGAGATGAAAGTT GAATA) and reverse primer 5 (TTCCCCTGCCGCAGACG CAACA) (Fig. 2) (Magomere *et al*., 2009). Amplification was done by PCR (Hybaid Omn-E Thermal Cycler) with the reaction conditions by Jagoueix *et al*. (1996). The PCR reaction was performed in 50 µL of reaction mixture containing 1 µm of each of the primers, Gibco buffer, 200 µM of each of the four dNTP, 2 mM $MgCl_2$, 0.05% W1 detergent (Gibco BRL), 100 µg mL^{-1} BSA and 2.5 U of Taq polymerase (Gibco BRL). The reaction mix was amplified for 35 cycles with the thermal cycler at: 92°C for 20 sec (denaturation of template into single strand); 62°C for 20 sec (annealing of primers to each original strand, high temperatures are used due to the specificity of the primers) and 72°C for 45 sec (elongation and new strand synthesis). PCR products were analyzed by electrophoresis of 20 µL of each sample (Jagoueix *et al*., 1996). 16S rDNA was amplified using forward primer OI1 (5GCGCGTATGCAATACGAGCGGCA3) and reverse primerOI2c(5GCCTCGCGACTTCGCAACCCAT3)while 16S/23S rDNA was amplified using forward primer 012c (5ATGGGTTGCGAAGTCGGGAGGC3)andreverseprimer 23SI (5 CGCCCTTCTCGCGCTTGA 3) in 50 µL of reaction mixture containing 0.5 µM of each of the primers, 200 µM of each of the four dNTP, 78 mM Tris-HCL pH 8.8, 2 mM $MgCl_2$, 17 mM $(NH_4)_2SO_4$, 10 mM β mercaptoethanol, 0.05% W1 detergent (Gibco BRL), 200 µg mL^{-1} BSA and 2.5 U of Taq polymerase (Gibco BRL). This was amplified for 35 cycles with the thermocycler using the following program: 92°C for 20 sec; 62°C for 20 sec and 72°C for 45 sec (Jagoueix *et al*., 1994, 1997; Subandiyah *et al*., 2000).

Agarose gel electrophoresis: PCR products were electrophoresed on 1% agarose gels and Tris-borate buffer (1 XTBE) and stained in ethidium bromide at 0.5 µg mL^{-1}. Twenty microliter of the PCR products were mixed with 1 µL of gel loading dye and loaded into gel adjacent to a 1 Kb DNA ladder (Life Technologies). The gel was run at a voltage of 4 V cm^{-1}. The gel was photographed when exposed to ultraviolet light on a U.V. transilluminator (Magomere *et al*., 2009).

Data scoring and analysis: The symptoms of the disease (where they existed) were recorded on a visual score form. A visual score of 1 was awarded when the plant sampled had interveinal chlorosis on less than 50% of the leaves on the plant (Fig. 3a). A score of 2 was awarded to plants that had more than 50% interveinal chlorosis with blotchy mottles (Fig. 3b) and or with lopsided fruit. A score of 3 was given for trees that had small upright leathery leaves, massive fruit and leaf drop and dieback (Fig. 3b, e). Presence or absence of psyllid vectors was recorded (Fig. 3c, d). A score of 1 was awarded if psyllids were present while a 0 score was awarded when they were absent. The PCR scores were based on the presence or absence of a 716 bp DNA band on agarose gels after PCR amplification with the L10/L12 ribosomal protein DNA PCR primers. A score of 1 was given on positive amplification while a score of 0 was awarded for negative amplification. The data on scores were then statistically

Fig. 3: Huanglongbing disease symptoms (a) Primary symptoms; leaves with blotchy mottles on Valencia orange, (b) Leaves with interveinal chlorosis on Valencia orange, (c) Psyllid damage on rough lemon leaves (d) Psyllid infestation on young citrus leaves and (e) Secondary symptoms: massive leaf fall, massive fruit fall on Washington navels

analysed. Variances were partitioned (ANOVA), F-tests were done on the variances and means were compared using the tukeys test in GENSTAT 8.0 (Genstat Committee, 2005).

RESULTS

L10/L12 ribosomal protein rDNA, 16S rDNA and 16S/23S rDNA PCR amplification: The PCR reaction using the L10/L12 rDNA primers for *rplJL* genes amplified a fragment of 716 bp from HLB bacteria isolated from all diseased plant samples. The amplified fragment includes the *rplJ* protein gene, the variable intergenic region between *rplJ* and *rplL* and part of the *rplL* protein gene. The fragment was amplified from citrus diseased materials from Machakos and Kakamega districts showing the greening symptoms (Fig. 4). There was no amplification from un-diseased plants, which were propagated through tissue culture and maintained in the greenhouse. The DNA band amplified was similar from all the diseased plants in the seven AEZs (LM3, LM4, LM5, UM3, UM4, LH4 and LH5) in Machakos and the five AEZs (LM1, LM2, UM0, UM1 and UM4) in Kakamega districts. The band on 1% agarose gels was also similar from all the diseased plants from the four different varieties (Valencia, Washington navel, rough lemon, tangerine). The 16S rDNA was amplified from diseased samples from Machakos giving a DNA fragment of 1100 bp. This band was not present when disease free samples from the greenhouses were used to provide PCR template (Fig. 5). The diseased samples gave positive amplification with the L10/L12 ribosomal protein rDNA primers and the 16S/23S rDNA primers. A band of 800 bp was amplified from diseased samples from Machakos (Fig. 6), representing the 16S/23S rDNA. This band was not present when disease free samples from the greenhouses were used as the PCR template. The diseased samples also gave positive amplification with the L10/L12 ribosomal protein rDNA primers and the 16S rDNA.

PCR of the L10/L12 rDNA of the HLB bacterium detected the disease in citrus at all stages of infection: The expected PCR band was observed on all samples from

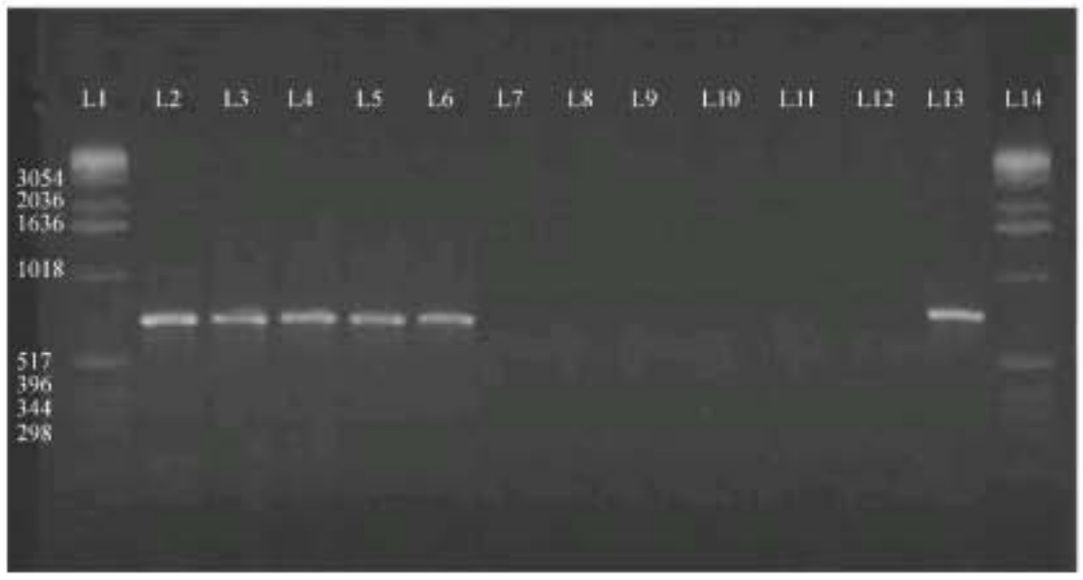

Fig. 4: 1% agarose electrophoresis gel of L10/L12 ribosomal protein rDNA. Lane12: Negative control; Lane 13: Positive control; Lanes 7-11: No. amplification from: washigton navel, Valencia, rough lemon below 1300 mASL Washington navel rough lemon above 1300 mASL respectively; Lanes 2-6: Positive 716 bp amplification from infected samples of washigton navel, Valencia, rough lemon below 1300 mASL Washington navel rough lemon above 1300 mASL respectively; Lane 1 and 14: 1 Kb DNA ladder. In this PCR reaction the bacterial L10/L12 ribosomal protein rDNA was amplified from plants that were showing symptoms associated with the disease

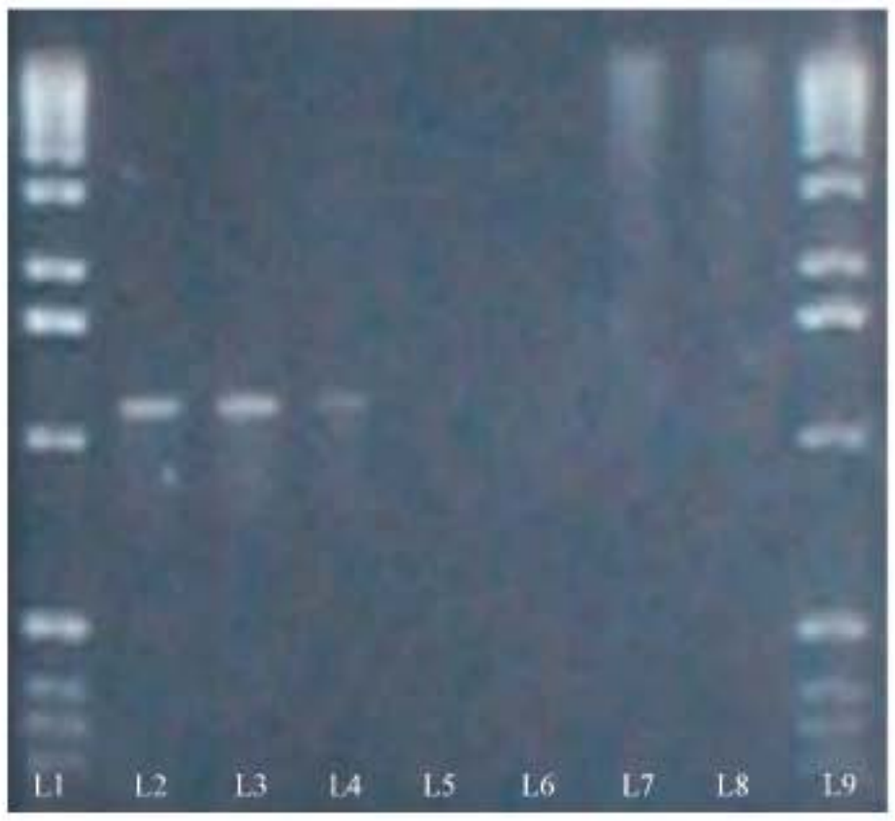

Fig. 5: 1% agarose electrophoresis gel of 16S rDNA PCR products. Lanes 5 and 6: Undiseased; Lane 2-4: Positive 1160 bp amplification from infected samples; Lane1 and 9: 1 Kb DNA ladder; Lane 7 and 8: Bacterial genomic DNA

trees that had interveinal chlorosis on less than 50% of the leaves (MPS of 1), those from trees that had more than 50% interveinal chlorosis with blotchy mottles and or with lopsided fruit (MPS of 2) and those from trees that had small upright leathery leaves, massive fruit and leaf drop and dieback (MPS of 3). Showing varsetility of the detection tool across disease development stages.

Distribution of the HLB disease across varieties: The Washington navel oranges had the highest mean

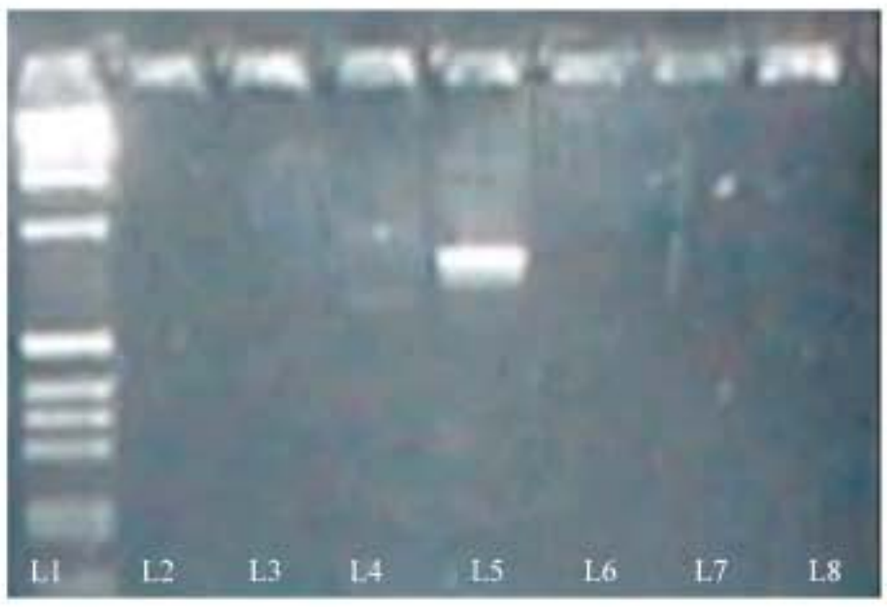

Fig. 6: 1% agarose electrophoresis gel of 16S/23S rDNA PCR products. A positive amplification band of 800 base pairs was seen in diseased samples. L1: 1 KB DNA ladder, L2: Negative control; L3: Undiseased tissue culture; L5: Diseased material; L6: Undiseased tissue culture

infection with a Mean Score (MS) of 2.62, while the tangerines showed the lowest MS of 1.57. The rough lemons and the Valencia oranges had a MS of 1.66 and 1.86, respectively (Table 2). There were significant ($p<0.05$) differences in the distribution of the HLB disease among the four citrus varieties in Machakos district. Washington navel oranges had a high MS (greater than 2) in six of the seven sampled AEZs in Machakos district, except in LM5 where it scored 1.67. Washington navel orange had the highest MS in the district, irrespective of the agro-ecological conditions and the altitude. The mean PCR scores (MPS) differed significantly ($p<0.05$) among the 4 varieties in Machakos district. The variety with the

Table 2: Mean PCR scores (MPS), mean scores (MS) of HLB disease and mean psyllid scores on citrus varieties in Machakos district

Varieties	Mean PCR score (MPS)	Mean score (MS)	Mean vector score
Rough lemon	0.429±0.0792[a]	1.667±0.164[b]	0.762±0.0727[c]
Tangerine	0.571±0.112[a]	1.571±0.232[b]	0.190±0.103
Valencia	0.571±0.0792[a]	1.857±0.164[b]	0.571±0.0727[c]
Washington navel	0.810±0.112	2.619±0.232	0.619±0.103[c]

Means in columns followed with similar letters are not different. Data are means of 84 plants, LSD (0.05). Mean comparisons were done using tukeys test

Table 3: Mean PCR scores (MPS), mean scores (MS) of HLB disease and mean psyllid scores in different AEZs in Machakos district

High and lower midlands	Mean PCR score (MPS)	Mean score (MS)	Mean vector score
LH4	0.833±0.136[a]	2.000±0.283[c]	0.833±0.125[d]
LH5	0.833±0.136[a]	2.250±0.283[c]	0.667±0.125[d]
LM3	0.500±0.136[b]	1.917±0.283[c]	0.583±0.125[d]
LM4	0.417±0.136[b]	2.000±0.283[c]	0.333±0.125[e]
LM5	0.333±0.136[b]	1.083±0.283	0.167±0.125[e]
UM3	0.583±0.136[ab]	1.917±0.283[c]	0.583±0.125[d]
UM4	0.667±0.113[ab]	2.333±0.234[c]	0.583±0.103[d]

Means in columns followed with similar letters are not different. Data are means of 84 plants, LSD (0.05). Mean comparisons were done using tukeys test

highest mean PCR scores was Washington navel, which had a score of 0.810. It was followed by Valencia, tangerine and rough lemon with mean scores of 0.571, 0.571 and 0.429, respectively (Table 2). The rough lemon had the least mean PCR scores (0.0) in LM5 and LM4, Washington navel and Valencia had the least MPS (0.0) in LM5 and LM4 respectively, while the tangerines had a mean PCR score above (0.0) in all of the AEZs in Machakos.

There were significant ($p<0.05$) differences in the distribution of the HLB disease mean scores among citrus varieties in Kakamega district. Rough lemon had the highest mean score at 2.40, while Washington navel oranges had the least mean score of 1.87 (Table 4). The rough lemon scored 3.0 in UM0 and UM1, while it had the least mean score in UM4 with 1.67. Washington navel oranges had a high mean score in UM4 (3.0), while it had a low mean score in LM2. The PCR analysis results show that the differences in the distribution of the HLB disease among the varieties in Kakamega were insignificant ($p<0.05$). However, the two varieties showed high mean PCR scores. Rough lemon had a mean PCR score of 0.667, while Washington navel had a score of 0.600 (Table 4). Washington navel had high MPS (>0.667) in all AEZs except in lower midland 1 where it scored 0.0, on the other hand rough lemon had a high MPS in all varieties except in upper midland 4.

Distribution of the HLB disease across agro-ecological zones: There were significant ($p<0.05$) differences in the distribution of HLB disease among the agro-ecological zones of Machakos district. The UM 4 AEZ had the highest mean scores of 2.33, followed by LH5, LH4, LM4, UM3, LM3 and LM5, with mean HLB disease scores of 2.25, 2.00, 2.00, 1.92, 1.92 and 1.08, respectively (Table 3). A disease trend was indicated such that the higher altitudes had higher disease mean scores than the lower altitudes in Machakos district. The PCR score analysis results showed significant ($p<0.05$) differences in the mean PCR score among the agroecological zones in Machakos district. High altitude AEZs (LH4 and LH5) had an exceptionally high mean PCR score of 0.833, while low altitude AEZs like LM4 and LM5 had low MPS of 0.417 and 0.333, respectively (Table 3). Washington navel had a maximum MPS of 1.0 in five AEZs namely LH4, LH5, LM3, LM4 and UM4. The 0.0 mean score was rare and was only found on rough lemon in LM5, Valencia in LM4 and Washington navel in LM5. These results show a trend, in which the lower highlands have large MPS, the upper midlands have average mean PCR scores and the lower midlands have low PCR mean scores.

Table 4: Mean PCR scores (MPS), mean scores (MS) of HLB disease and mean psyllid scores on citrus varieties in Kakamega district

Varieties	Mean PCR score (MPS)	Mean score (MS)	Mean vector score
Rough lemon	0.667±0.125[a]	2.40±0.194	0.867±0.125
Valencia	0.600±0.125[a]	1.87±0.194	0.533±0.125

Means in columns followed with similar letters are not different. Data are means of 30 plants, LSD (0.05). Mean comparisons were done using tukeys test

Table 5: Mean PCR scores (MPS), mean scores (MS) of HLB disease and mean psyllid scores in different AEZs in Kakamega district

Lower and upper midlands	Mean PCR score (MPS)	Mean score (MS)	Mean vector score
LM1	0.333±0.197	1.33±0.307[a]	0.667±0.197[a]
LM2	0.667±0.197[a]	1.83±0.307[a]	0.500±0.197[a]
UM0	0.833±0.197[a]	2.50±0.307[b]	0.833±0.197[a]
UM1	0.667±0.197[a]	2.67±0.307[b]	0.833±0.197[a]
UM4	0.667±0.197[a]	2.33±0.307[bc]	0.667±0.197[a]

Means in columns followed with similar letters are not different. Data are means of 30 plants, LSD (0.05). Mean comparisons were done using tukeys test

There were significant ($p<0.05$) differences in the distribution of HLB disease among the agro-ecological zones of Kakamega district. The UM1 AEZ had the highest mean scores of 2.67, followed by UM0, UM4, LM2 and LM1, with mean HLB disease scores of 2.50, 2.33, 1.83 and 1.33 respectively (Table 5). The results show that the HLB disease mean scores were higher in the UMs than in the LMs of Kakamega district. The PCR score analysis results show insignificant ($p<0.05$) differences in the mean PCR score among the agroecological zones in Kakamega district. However the general trend is that the AEZs found in the high altitudes (upper midland 0, upper midland 1 and upper midland 4) have large MPS (0.833, 0.667 and 0.667, respectively), while the AEZs in the low altitude regions (lower midland 1 and lower midland 2) have MPS of 0.333 and 0.667, respectively (Table 5). Rough

lemon has the highest mean score in upper midland 0 (1.0), while it has the lowest score in upper midland 4 (0.333).

Distribution of psyllids across varieties: The four citrus varieties differed significantly ($p<0.05$) in their mean psyllids score in Machakos district. Rough lemon had the highest mean psyllid score of 0.762 followed by Washington navel, Valencia and tangerine at a mean vector score of 0.619, 0.571 and 0.190, respectively (Table 2). The rough lemons had high mean psyllid scores, both in high and low altitude areas (>0.667). Citrus varieties in Kakamega district did not significantly ($p<0.05$) differ in their mean psyllids scores. Nevertheless, rough lemon had very high mean scores of 0.867, while the Washington navel oranges had an average mean score of 0.533 (Table 4). Rough lemon had a maximum score of (1.0) in three AEZs (UM0, UM1 and UM4), which lie within the high altitudes.

Distribution of psyllids across AEZs: There were significant ($p<0.05$) differences in the mean psyllid score among the AEZs in Machakos district. The high altitude areas in Machakos district (LH4, LH5, UM3 and UM4) had larger mean psyllid scores (0.883, 0.667, 0.583 and 0.583 respectively) while the LMs (LM3, LM4 and LM5) had lower means (0.583, 0.333 and 0.167, respectively) (Table 3). The results show a gradual decrease in the mean vector score with decrease in altitude. There were insignificant ($p<0.05$) differences in the mean psyllid score among the AEZs in Kakamega district. The agroecological zones in the lower altitudes (LM2 and LM1) had lower mean psyllid scores (0.500 and 0.667 respectively) while the agroecological zones in the higher altitudes (UM0, UM1 and UM4) had high mean scores (0.833, 0.833 and 0.667, respectively) (Table 5). The results show a gradual decrease in the mean vector score with decrease in altitude.

Correlation of the mean scores, PCR scores and vector scores: To ascertain the efficiency of the mean HLB disease PCR score, the mean HLB disease score and the mean psyllid vector scores as tools for determining the presence of the HLB disease of citrus, the results from the three tools were correlated. A correlation matrix for Machakos showed that the mean HLB disease PCR scores and the mean HLB disease visual scores were highly correlated, having a correlation coefficient of 0.703. There was low correlation between the mean psyllid vector score and the mean HLB disease visual score (0.202), similarly, the correlation between the mean psyllid vector score and the mean HLB disease PCR score was low (0.108). A correlation matrix for the Kakamega data also exhibited high correlation between the mean HLB disease scores and the mean HLB disease PCR scores (0.661). On the other hand, the correlation between the mean HLB disease visual score and the mean psyllid vector score was very low at 0.088, while that between the mean psyllid vector and the mean HLB disease PCR scores was negative (-0.045). Vector scores gave low correlation coefficients with both visual and PCR score alike, possibly due to the seasonal availability of the vector, the long incubation period of the disease and the short duration (60 min) of feeding that the vector requires to successfully infect the plant with the HLB pathogen. The correlations between the three tools were generally lower in Kakamega than in Machakos district. The regression line fitted for the PCR disease scores and the visual disease scores was highly significant ($p<0.05$) for Kakamega and Machakos data alike. This shows that the mean PCR scores could be sufficiently used in place of the mean scores to detect for the HLB disease in Machakos and Kakamega districts. These results show the versatility of the PCR tool in detection of the HLB disease.

DISCUSSION

PCR of the ribosomal proteins L10-L12 serves as a precise and reliable tool for indexing of the HLB disease in citrus varieties in Kenya.

The PCR band observed in this reaction was consistent with previous results on the greater ribosomal protein *rplKAJL-rpoB* gene of *Candidatus* Liberibacter species. The 716 bp fragment represented the *rplJL* genes of the Kenyan strains of the *Candidatus* L. africanus. Previous studies by Hocquellet *et al*. (1999b) using primer pair A2/J5 that amplify *rplAJ* genes, obtained a band of about 650 bp from plants infected with *Candidatus* L. africanus, strain Nelspruit, South Africa and a band of around 700 bp was amplified from plants infected with different strains of *Candidatus* L. asiaticus. Villechanoux *et al*. (1993) cloned a fragment (In-2.6) of the *rplKAJL-rpoBC* gene cluster which they used as a probe to detect greening BLO strains in plants infected with the Asian and African strains. Okuda *et al*. (2005) amplified the internal *nusG-rplKAJL-rpoB* gene cluster of Liberibacter isolates and determined the complete sequence of the 6.1-kb fragment which was identical among Japanese and Indonesian isolates. Ding *et al*. (2005) amplified a 400 bp product of nucleotide 39-551 of the *rplKAJL-rpoBC* of *Candidatus* L. asiaticus (GenBank sequence M94319) using nested PCR primers from symptomless leaves of wampee, eureka lemon and rough lemon, as well as from symptomatic leaves of infected

Ponkan from Yang Cun, Eureka lemon and wampee. Teixeira *et al*. (2008) used a chromosome walking procedure to obtain an extended β operon of the American liberibacter (a fragment of over 4673 bp), comprising the genes; *tufB*, *secE*, *nusG*, *rplK*, *rplA*, *rplJ*, *rplL* and *rpoB*. The *rplJ*, *rplL* genes and their corresponding proteins are considerably conserved while the intergenic regions between the ribosomal proteins (*rplKAJL-rpoB*) genes, exhibit variable nucleotides in different species and strains of the HLB bacteria thereby showing high potential for detection of specific strains of the bacteria (Magomere *et al*., 2009; Teixeira *et al*., 2008). The variation in the intergenic regions between the ribosomal proteins, differentiate both the species and strains of the bacteria. Amplification of the ribosomal protein L10-L12 DNA is precise and versatile in the detection of the bacteria and its use correlates highly with conventional morphological methods. However, most HLB morphological detection methods may lack precision especially due to the wide range of symptoms, similarity of the symptoms to nutrient deficiency and viral infection symptoms and the long incubation period of the bacteria. The PCR product of the 16S rDNA and the 16S/23S rDNA obtained were similar to those amplified previously from citrus samples showing the disease symptoms and psyllid vectors feeding on the diseased plants (Jagoueix *et al*., 1996, 1997; Subandiyah *et al*., 2000; Tatineni *et al*., 2008; Ding *et al*., 2009). These reactions were vital in confirming the presence of the HLB disease in the diseased samples due to their precision, however in Liberibacter species the 16S rDNA and the 16S/23S rDNA have limited polymorphism and are largely conserved, thus they may not have great potential in detection of different strains of the bacteria. The specificity of the *rplAJ* gene the 16S rDNA and the 16S/23S intergenic rDNA reactions renders them a vital tool in the detection of Liberibacter species in infected citrus plant leaf midribs that could harbour a diversity of microbes (47 orders of bacteria in 15 phyla) (Sagaram *et al*., 2009).

HLB disease is most prevalent on Washington navel orange growing in altitudes above 1400 mASL: In Machakos, Washington navels had the highest score (MS 2.619/MPS 0.810) whereas in Kakamega, rough lemon showed higher disease scores (MS 2.40/MPS 0.667) than Washington navel (MS 1.87/MPS 0.600). Though the rough lemon did not show HLB morphological symptoms like the other varieties in HLB disease infested areas, it had the highest infestation by psyllid vectors due to its frequent yellow flushes with a mean psyllid score of 0.762. This shows that rough lemon has the highest tolerance to the *Candidatus* L. africanus bacterial strain, which causes HLB. The results are similar to previous results (Manicom and Van Vuuren, 1990) in South Africa that show that sweet orange and mandarin were severely affected with HLB, followed by, lemon, sour orange which had moderate tolerance to *Candidatus* L. africanus strain. HLB infection of sweet orange significantly affects expression of 624 genes whose encoded proteins are associated with sugar, phytohormone and cell wall metabolism in addition to plant defence which consequently lead to phloem disruption, sucrose accumulation and plugged sieve pores (Kim *et al*., 2009). Other reports (Miyakawa, 1980; Gonzales *et al*., 1972; Fraser, 1978) found that lemon is fairly tolerant to the HLB disease to the extent that in some cases the rough lemon rootstock has been found to induce some degree of tolerance in the sweet orange scion in greenhouse trials (Kapur *et al*., 1984). The rough lemon in a HLB infected orchard, serves as a reservoir for the bacterium. Due to its frequent flashing and increased psyllid activity, an infected rough lemon could easily increase the rate of HLB infection in an orchard. Rough lemon can serve as a potential source of HLB disease tolerance genes in citrus breeding efforts. Tangerines had the least psyllid activity but had similar MPS and MS with rough lemon and Valencia orange, this can be attributed to the feeding habit of adult *T. erytreae* which prefer light green flushes not present on tangerine but can transmit the HLB pathogen in less than 60 minutes of feeding on an un-infected plant (Samways, 1987; Da Graça, 1991). In addition, the HLB pathogen has been found in the late instar nymphal stages of psyllid (Hung *et al*., 2004) which can infect darker leaves. The AEZs found in the higher altitudes had the highest levels of the HLB disease. The highest AEZ sampled in Machakos (LH5) had a HLB disease mean score of 2.3 and MPS of 2.25, while in Kakamega UM1 and UM0 had the highest MS and MPS. This is consistent with the distribution of the HLB vector (*T. erytreae*), which is found more in the UMs than the LMs. These results are similar to previous findings, which indicated that the disease was more intense in the highlands (Muriuki, 1989; Seif, 1991).

Psyllids infests rough lemon most especially in AEZs within altitudes above 1400 mASL: Psyllids (*Trioza erytreae)* were frequently encountered in AEZs where citrus plants were heavily infected with the HLB disease. Previous reports, (McClean and Oberholzer, 1965; Schwartz *et al*., 1970) show that psyllids (*Trioza erytreae*) are major vectors for the *Candidatus* L. africanus, which causes HLB disease in Africa. Therefore the distribution of these insects influences the distribution of the HLB disease. In Machakos, the mean psyllid score was highest in high altitude areas (LH4-0.833) and least in the low

altitude areas (LM5-0.167). A similar situation was seen in Kakamega where the mean psyllid score was highest in high altitude areas (UM0-0.833) and least in the low altitude areas (LM2-0.500). This suggests that the psyllid vectors are more active in the highlands than the low lands. These results are similar to the findings by Schwartz (1975), who found that *T. erytreae* thrived well in highlands where most HLB affected orchards were found (Schwartz, 1975). Since *T. erytreae* is sensitive to heat and high temperatures of 32°C (Moran and Blowers, 1967; Catling, 1969, 1972) the insect will optimally function in cool, moist upland regions more characteristic of the highlands than the low lands. This contrasts with *Diaphorina citri*, which is more resistant to extreme temperatures and is more sensitive to high rainfall and humidity (Catling, 1972; Aubert, 1987; Regmi and Lama, 1988; Xia *et al*., 1987). The psyllids seemed to prefer the young yellowish, light green flushes of rough lemon (which had a mean psyllid score of 0.867 in Kakamega and 0.762 in Machakos) than any other varieties in both districts. For instance tangerines in Machakos district had a mean psyllid score of 0.190. This could be due to the dark green colour of tangerine leaves, even on the young flashes. These results are similar to previous results (Samways, 1987) which show that lemons have frequent flushes of new growth which make them attractive to the Psylla vectors which are strongly attracted by yellow green colour of wavelength 550 nm.

CONCLUSION AND RECOMMENDATIONS

The present study shows that Huanglongbing disease is a serious disease of citrus in Machakos and Kakamega districts of Kenya and the levels of infection of the HLB disease vary among different citrus varieties and different AEZs. The detection of the disease is an important aspect of managing the disease noting that the bacteria that causes the disease is not cultured on nutrient media and it has a long incubation period in the plant. Morphological symptoms are therefore not sufficient in early detection of the disease. PCR of the *rpl* L10 – L12 protein DNA is both precise and versatile in the detection of the bacteria even during the latent phase of infection. The disease was most pronounced on Washington navel in the lowlands but the tolerance exhibited by the rough lemon is overwhelmed in the presence of high levels of disease innoculum found in the highlands. The HLB disease was more pronounced in the highlands (>1400 m ASL) and there was a decrease in the disease scores with decrease in altitude in both districts. These findings are consistent with the findings on the distribution of the HLB disease vector. The psyllid (*Trioza erytreae*) was most often encountered on citrus in the AEZs located in altitudes above 1400 m ASL. The psyllid thrives better in highlands than the lowlands. In addition, high psyllid scores were recorded on rough lemon in both districts. Psyllids preferred rough lemon due to its frequent flushing and the light yellowish colour of the young shoots. Production of clean planting material through tissue culture systems should be strengthened and insect control in orchards should be emphasized so as to rejuvenate citrus production in the highlands (Kakamega) and the lowlands (Machakos). In addition, early detection and disease diagnostics systems can be improved by regular use of the PCR indexing of samples from orchards. Adoption of these diagnostics tools for citrus HLB disease by plant inspectorate agencies and other agencies involved in citrus germplasm propagation and shipment will ensure clean citrus planting material and thereby mitigate the effects of the disease in citrus growing areas in the country.

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REFERENCES

Aubert, B., 1987. *Trioza erytreae* del guercio and *Diaphorina citri* kuwayama (Homoptera: Psyllidae), the two vectors of citrus greening disease: Biological aspects and possible control strategies. Fruits, 42: 149-162.

Bastianel, C., M. Garnier-Semancik, J. Renaudin, J.M. Bové and E. Sandrine, 2005. Diversity of *Candidatus* Liberibacter asiaticus based on *omp* gene sequence. Applied Environ. Microbiol., 71: 6473-6478.

Bové, J.M., 2006. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. J. Plant Pathol., 88: 7-37.

Catling, H.D., 1969. The bionomics of the South African citrus psylla, *Trioza erytreae* (del guercio) (Homoptera: Psyllidae): The influence of the flushing rhythm of citrus and factors, which regulate flushing. J. Entomol. Soc. Southern Afr., 32: 191-208.

Catling, H.D., 1972. Factors regulating populations of psyllid vectors of greening. Proceedings of the 5th Conference for the International Organization of Citrus Virologists, (CIOCV'1972), Tokyo, Japan, pp: 51-57.

Catling, H.D., 1973. Results of a survey for psyllid vectors of citrus psylla *Trioza erytreae* (del guercio) (Homoptera: Psyllidae). J. Entomol. Soc. Southern Afr., 36: 299-306.

Coelho, S.V.M. and A.S.A. Marques, 2002. Citrus Greening Uma bacteriose quarentenaria que representa ameaca potencial a citricultura brasileira. Ministerio da Agricultura, Pecuaria e Abastecimento. Comunicado Tecnico 58, Brasillia.

Coletta-Filho, H.D., M. Takita, M.L.P.N. Targon and M.A. Machado, 2005. Analysis of 16S rDNA sequences from citrus huanglongbing bacteria reveal a different Ca. liberibacter strain associated with citrus disease in são paulo. Plant Dis., 89: 848-852.

Da Graça, J.V., 1991. Citrus greening disease. Ann. Revi. Phytopathol., 29: 109-136.

Das, A.K., 2004. Rapid detection of *Candidatus* Liberibacter asiaticus, the bacterium associated with citrus Huanglongbing (Greening) disease using PCR. Current Sci., 87: 1163-1185.

Ding, F., G. Wang, G. Yi, Y. Zhong, J. Zeng and B. Zhou, 2005. Infection of wampee and lemon by the citrus huanglongbing pathogen (*Candidatus* Liberibacter asiaticus) in China. J. Plant Pathol., 87: 207-212.

Ding, F., X. Deng, N. Hong, Y. Zhong, G. Wang and Y. Ganjun, 2009. Phylogenetic analysis of the citrus Huanglongbing (HLB) bacterium based on the sequences of 16S rDNA and 16S/23S rDNA intergenic regions among isolates in China. Eur. J. Plant Pathol., 124: 495-503.

Fang, D., Y.G. Jun and W.G. Ping, 2004. Research on the PCR and nested-PCR detection of citrus *Huanglongbing pathogen*. Acta Hortic. Sinica, 31: 803-806.

Flavell, R.B., 1986. The structure and control of expression of ribosomal RNA genes. Oxford Surv. Plant Mol. Cell. Biol., 3: 252-274.

Fraser, L.R., 1978. Recognition and control of citrus virus diseases in Australia. Proceedings of the International Society of Citriculture, (ISC'1978), Sydney, Australia, pp: 17881-17881.

French, J.C., C.J. Kahlke and J.V. Da Graça, 2001. First record of the Asian citrus pyslla *Diaphorina citri* kuwayama (Homoptera: Psyllidae) in Texas. Subtropical Plant Sci., 53: 14-14.

Fusayasu, S., T. Sato and H. Shimizu, 2006. Detection of citrus greening disease (*Candidatus liberobacter* asiasticum) by loop-mediated isothermal amplification. Res. Bull. Plant Protec. Service Japan, 42: 81-87.

Gao, S.J., 1988. Production of monoclonal antibodies against the organism of Chinese greening disease Proceedings of the 2nd FAO-UNDP Greening Workshop, (FAO-UNDPGW'1988), Lipa, Philippines, pp: 82-83.

Garnier, M., J. Latrille and J.M. Bové, 1976. *Spiroplasma citri* and the organism associated with likubin: Comparison of their envelope systems. Proceedings of the 7th International Conference of the Organization of Citrus Virologists, (ICOCV'1976), IOCV, Riverside, pp:13-17.

Garnier, M., G. Martin-Gros and J.M. Bové, 1987. Monoclonal antibodies against the bacterial-like organism associated with citrus greening disease. Ann. Inst. Pasteur. Microbiol., 138: 639-650.

Genstat Committee, 2005. Genstat Manual. Version 8, Clarendon, Oxford, UK.

Gerbi, S.A., 1985. Evolution of Ribosomal DNA. In: Molecular Evolutionary Genetics, MacIntyre, R.J. (Ed.). Plenum Press, New York, pp: 419-517.

Gonzales, C.I., R.C. Viñas and L.A. Vergara, 1972. Observations on 110 citrus cultivars planted in an area severely infested by leaf mottling. Proceedings of the 5th International Conference of the Organization of Citrus Virologists, pp: 38-40.

Grafton-Cardwell, E.E., K.E. Godfrey, M.E. Rogers, C.C. Childers and P.A. Stansly, 2007. Asian Citrus Psyllid. University of California, Division of Agricultural and Natural Resources, Berkeley, pp: 1-9.

Hocquellet, A., J.M. Bové and M. Garnier, 1997. Production and evaluation of non-radioactive probe for the detection of the two *Candidatus liberobacter* species associated with the citrus huanglongbing (Greening). Mol. Cell. Probes, 11: 433-438.

Hocquellet, A., J.M. Bové and M. Garnier, 1999a. Isolation of DNA from the uncultured *Candidatus liberobacter* species associated with citrus huanglongbing by RAPD. Curr. Microbiol., 38: 176-182.

Hocquellet, A., P. Toorawa, J.M. Bové and M. Garnier, 1999b. Detection and identification of two *Candidatus liberobacter* species associated with the citrus huanglongbing by PCR amplification of the ribosomal protein genes of the *Beta operon*. Mol. Cell. Probes, 13: 373-379.

Hung, T.H., S.C. Hung, C.N. Chen, M.H. Hsu and H.J. Su, 2004. Detection by PCR of *Candidatus* Liberibacter asiaticus, the bacterium causing citrus huanglongbing in vector psyllids: Application to the study of vector-pathogen relationships. Plant Pathol., 53: 96-102.

Jagoueix, S., M.J. Bové and M. Garnier, 1994. The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the proteobacteria. Int. J. Syst. Bacteriol., 44: 397-386.

Jagoueix, S., M.J. Bové and M. Garnier, 1996. PCR detection of the two *Candidatus liberobacter* species associated with greening disease in citrus. Mol. Cell. Probes, 10: 43-50.

Jagoueix, S., J. M. Bové and M. Garnier, 1997. Comparison of the 16S/23S Ribosomal intergenic regions of *Candidatus liberobacter* asiaticus and *Candidatus liberobacter* africanus, the two species associated with citrus huanglongbing (Greening) disease. Int. J. Syst. Bacteriol., 20: 224-227.

Kapur, S.P., S.S. Cheema and R.S. Dhillon, 1984. Reaction of certain citrus scionic combinations to viral/mycoplasmal diseases. Rev. Plant Pathol., 65: 135-135.

Kawabe, K., N.T.N. Truc, B.T.N. Lan, L.T.T. Hong and M. Onuki, 2006. Quantification of DNA of citrus huanglongbing pathogen in diseased leaves using competitive PCR. J. General Plant Pathol., 72: 355-359.

Khairulmazmi, A., S. Kamaruzaman, H. Habibuddin, J. Kadir and S.O.S. Rastan, 2008. Occurrence and spread of *Candidatus* Liberibacter asiaticus, the causal agent of huanglongbing disease of citrus in Malaysia. Res. J. Agric. Biol. Sci., 4: 103-111.

Kim, J., U.S. Sagaram, J.K. Burns, J. Li and N. Wang, 2009. Response of sweet orange (*Citrus sinensis*) to *Candidatus* Liberibacter asiaticus Infection: Microscopy and microarray analyses. Phytopathology, 99: 50-57.

Lan, L.X., Z.S. Fang, Z.W. Jun, L. Kuan and Q.Y. Xiang *et al*., 2004. Cloning and sequencing of citrus *Huanglongbing pathogen* 16S rDNA and its detection by real-time fluorescent PCR. J. Agric. Biotechnol., 12: 80-85.

Leblond-Bourget, N., H.O. Philippe, I. Mangin and B. Decaris, 1996. 16S rRNA and 16S-to-23S internally transcribed spacer reveals the sequence inter-and intraspecific *Bifidobacterium phylogeny*. Int. J. Syst. Bacteriol., 46: 102-111.

Li, W., J.S. Hartung and L. Levy, 2007. Evaluation of DNA amplification methods for improved detection of *Candidatus* Liberibacter species associated with citrus huanglongbing. Plant Dis., 91: 51-58.

Li, W., D. Li, E. Twieg, J.S. Hartung and L. Levy, 2008. Optimized quantification of unculturable *Candidatus* Liberibacter spp. in host plants using real-time PCR. Plant Dis., 92: 854-861.

Manicom, B.Q. and S.P. Van Vuuren, 1990. Symptoms of greening disease with special emphasis on African greening. Proceedings of the 4th International Asia-Pacific Conference on Citrus Rehabilitation, (IAPCCR'1990), Chia-Mai, Thailand, pp: 127-31.

Magomere, T.O., S.D. Obukosia, E. Mutitu, C. Ngichabe, F. Olubayo and S. Shibairo, 2009. Molecular characterization of *Candidatus* Liberibacter species/strains causing huanglongbing disease of citrus in Kenya. Electronic J. Biotechnol., 12: 2-2.

Manjunath, K.L., S.E. Halbert, C. Ramadugu, S. Webb and R.F. Lee, 2008. Detection of *Candidatus* Liberibacter asiaticus in *Diaphorina citri* and its importance in the management of citrus huanglongbing in Florida. Phytopathology, 98: 387-396.

McClean, A.P.D. and P.C.J. Oberholzer, 1965. Citrus psylla, a vector of the greening disease of sweet orange. South African J. Agric. Sci., 8: 297-298.

McClean, A.P.D., 1974. The efficiency of citrus psylla, *Trioza erytreae* (del G.) as a vector of greening disease of citrus. Phytophylactica, 6: 45-54.

Miyakawa, T., 1980. Experimentally induced symptoms and host range of citrus likubin (greening disease). Ann. Phytopathol. Soc. Japan, 46: 224-230.

Moll, J.N., S.O. van Vuuren and D.L. Milne, 1980. Greening disease, the South African situation. Proceedings of the 8th International Conference of the Organization of Citrus Virologists, (ICOCV'1980), Univ. Calif., Riverside, CA., pp: 109-117.

Moran, V.C. and J.R. Blowers, 1967. On the biology of the South African citrus psylla, *Trioza erytreae* (del guercio) (Homoptera: Psyllidae). J. Entomol. Soc. Southern Afr., 30: 96-106.

Muriuki, S.J.N., 1989. The Distribution of the African Citrus Psyllid *Trioza Erytreae* Del Guercio in Kenya. National Horticultural Research Center, Kenya.

Obukosia, S.D. and K. Waithaka, 2000. Nucellar culture of *Citrus sinensis* L. and *Citrus limon* L. African Crop Sci. J., 8: 109-116.

Okuda, M., M. Matsumoto, Y. Tanaka, S. Subandiyah and T. Iwanami, 2005. Characterization of the *tuf*B-*sec*E-*nus*G-*rpl*KAJL-*rpo*B gene cluster of the citrus greening organism and detection by loop-mediated isothermal amplification. Plant Dis., 89: 705-711.

Planet, P., S. Jagoueix, J.M. Bové and M. Garnier, 1995. Detection and characterization of the African citrus greening Liberobacter by amplification, cloning and sequencing of the *rpl*KAJL-*rpo*BC operon. Curr. Microbiol., 30: 137-141.

Regmi, C. and T.K. Lama, 1988. Greening incidence and greening vector population dynamics in Pokhara. Proceedings of the 10th International Conference of the Organization Citrus Virology, (ICOCV'1988), IOCV, Riverside, pp: 238-242.

Sagaram, U.S., K.M. DeAngelis, P. Trivedi, G.L. Andersen, S. Lu and N. Wang, 2009. Bacterial diversity analysis of huanglongbing pathogen-infected citrus, using phylochip arrays and 16S rRNA gene clone library sequencing. Applied Environ. Microbiol., 75: 1566-1574.

Salibe, A.A. and R.E. Cortez, 1966. Studies on the leaf mottling disease of citrus in the Philippines. FAO. Plant Protec. Bull., 14: 141-144.

Samways, M.J., 1987. Phototactic response of *Trioza erytreae* (Del guercio) (Hemiptera: Triozidae) to yellow-coloured surfaces and an attempt at commercial suppression using yellow barriers and trap trees. Bull. Entomol. Res., 77: 91-98.

Schwartz, R.E., A.P.D. McClean and H.D. Catling, 1970. The spread of greening disease by citrus psylla in South African. Phytophylactica, 2: 45-54.

Schwartz, R.E. and G.C. Green, 1972. Heat requirements for symptom expression and inactivation of the greening pathogen. Proceedings of the 5th International Conference of the Organization Citrus Virology, (ICOCV'1972), Univ. Florida Press, Gainesville, pp: 44-51.

Schwartz, R.E., 1975. Technical report on citrus greening in Kenya. UNDP Horticultural Crops Development Project FAO of the UN KEN 71/528 pp: 14.

Sechler, A., E.L. Schuenzel, P. Cooke, S. Donnua and N. Thaveechai *et al*., 2009. Cultivation of *Candidatus* Liberibacter asiaticus, Ca. L. africanus and Ca. L. americanus associated with huanglongbing. Phytopathology, 99: 480-486.

Seif, A.A., 1991. Know and control greening disease of citrus. Information Bulleting KARI. No.5.

Subandiyah, S., T. Iwanami, S. Tsuyumu and H. Ieki, 2000. Comparison of 16S rDNA and 16S/23S intergenic region sequences among citrus greening organisms in Asia. Plant Dis., 84: 15-18.

Tatineni, S., U.S. Sagaram, S. Gowda, C.J. Robertson, W.O. Dawson, T. Iwanami and N. Wang, 2008. In planta distribution of *Candidatus* Liberibacter asiaticus as revealed by polymerase chain reaction and real-time PCR. Phytopathology, 98: 592-599.

Teixeira, D.C., A.J. Antonio, D.L. Jean, S. Eveillard, S. Colette and J.M. Bové, 2005a. First report of a Huanglongbing-like disease of citrus in São Paulo State, Brazil and association of a new Liberibacter species, *Candidatus* Liberibacter americanus, with the disease. Plant Dis., 89: 107-107.

Teixeira, D.C., D.L. Jean, S. Eveillard, E.C. Martins and W.C.J. Junior *et al*., 2005b. Citrus huanglongbing in São Paulo State, Brazil: PCR detection of the *Candidatus* Liberibacter species associated with the disease. Mol. Cell. Probes, 19: 173-179.

Teixeira, D.C., S. Colette, S. Eveillard. D.L. Jean, D.I. Paulo, A.J. Antonio and J.M. Bové, 2005c. *Candidatus* Liberibacter americanus, associated with citrus huanglongbing (Greening disease) in São Paulo State, Brazil. Int. J. Syst. Evolutionary Microbiol., 55: 1857-1862.

Teixeira, D.C., S. Eveillard, P. Sirand-Pugnet, A. Wulff, C. Saillard, A.J. Ayres and J.M. Bové, 2008. The *tuf*B-*sec*E-*nus*G-*rpl*KAJL-*rpo*B gene cluster of the liberibacters: Sequence comparisons, phylogeny and speciation. Int. J. Syst. Evolutionary Microbiol., 58: 1414-1421.

Urasaki, N., T. Uehara and S. Kawano, 2007. Highly reliable polymerase chain reaction assay for citrus huanglongbing (citrus greening disease) using the sucrose synthase gene as an internal control. Japanese J. Phytopathol., 73: 25-28.

Urasaki, N., S. Kawano, H. Mukai, T. Uemori, O. Takeda and T. Sano, 2008. Rapid and sensitive detection of *Candidatus* Liberibacter asiaticus by cycleave isothermal and chimeric primer-initiated amplification of nucleic acids. J. General Plant Pathol., 74: 151-155.

Villechanoux, S., M. Garnier, J. Renaudin and J.M. Bové, 1992. Detection of several strains of the bacterium-like organism of citrus greening disease by DNA probes. Curr. Microbiol., 24: 89-95.

Villechanoux, S., M. Garnier, F. Laigret, J. Renaudin and J.M. Bové, 1993. The genome of the non-cultured, bacterial-like organism associated with citrus greening disease contains the *nus*G-*rpl*KAJL-*rpo*BC gene cluster and the gene for a bacteriophage type DNA polymerase. Curr. Microbiol., 26: 161-166.

Waithaka, K. and S.D. Obukosia, 1988. *In vitro* ovule culture of citrus sinensis. Proceedings of the International Conference on Applied Biotechnology. (ICAB'1988), Hongkong, pp: 683-689.

Xia, Y., C. Xu and J. Chen, 1987. Population dynamics of D. citri in Fuzhou on *Murraya paniculata*. Proceedings of a Workshop on Citrus Greening Disease, (WCGD'1987), Fuzhou, China, pp: 2-2.