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DETECTION OF TRISTEZA VIRUS AND FASTIDIOUS BACTERIA IN CITRUS GERMPLASM

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ABSTRACT

Citrus tristeza virus (CTV), citrus fastidious bacteria (*Liberibacter asiaticum*; the causal agent of citrus greening disease), and *Xylella fastidiosa* (causal agent of citrus variegated chlorosis disease) are the most devastating systemic pathogens occur in Asian countries. Transmissions of pathogens occur through infected bud woods, cuttings and insect vectors. Therefore, experiments were conducted to identify healthy plants to establish a disease-free mother plant orchard of citrus for the production of healthy planting materials. Plant samples were collected from citrus plants in different locations of Sri Lanka. The CTV and *Xylella fastidiosa* were detected by enzyme linked immunosorbent assay (ELISA) and *Liberibacter asiaticum* was detected by polymerase chain reaction using the specific primers derived from the sequences of the cloned DNA fragment of greening fastidious bacterium (GFB). Out of 628 samples subjected for ELISA, 95 % were positive for CTV. *Xylella fastidiosa* and *Liberibacter asiaticum* were rarely found in the tested samples. Germplasm of orange and pummello, which were free from all three pathogens, were grafted with healthy rough lemon root stocks and established in a insect proof net house for the production of healthy planting material.

KEYWORDS: Citrus, Tristeza virus, Fastidious bacteria

INTRODUCTION

Citrus is one of the important fruit crops grown in Sri Lanka and mainly cultivated in low country intermediate zone and the dry zone of the country including Monaragala, Hambantota, part of Rathnapura, Matale, Kurunegala Districts and Jaffna Peninsula (Medagoda and Jayawardena, 1997). There are some fungal, bacterial and viral diseases affecting the citrus cultivation in Sri Lanka. Among them, citrus tristeza virus (CTV) is reported as one of the most severe systemic pathogens of citrus cultivation in Sri Lanka. Besides CTV, the citrus fastidious bacteria (*Liberibacter asiaticum*), the causal agent of citrus greening disease and *Xylella fastidiosa*, the causal agent of citrus variegated chlorosis disease, are the most devastating systemic pathogens occur in other Asian countries resulting in severe losses to the citrus industry (Timmer *et al.*, 2003), by shortening the lifespan of the infected trees.

The CTV and *X. fastidiosa* can be diagnosed by the enzyme linked immunosorbent assay (ELISA) (Beretta *et al.*, 1992). However, it is not easy

to detect fastidious bacterium *Liberibacter asiaticum*, by ELISA as low concentration and uneven distribution of these bacteria in its natural hosts.

The citrus greening bacterium is found in most of the tropical countries in Asia (Woo-Nanag and Jan Bay, 2003). It is a non-culturable fastidious bacterium that inhabits the phloem of its hosts. A standard protocol using a DNA probe has been developed and successfully applied in detection of citrus greening fastidious bacteria (GFB) for several years (Hung *et al.*, 1999).

Fungal diseases can be controlled by chemical methods. However, systemic pathogens such as CTV and fastidious bacteria-affected trees must be removed and replaced with healthy plants or tolerant varieties or rootstocks to prevent further spread of the diseases. The objective of this study was to identify of healthy plants to establish a systemic disease-free mother plant orchard of citrus for the production of healthy planting materials.

MATERIALS AND METHODS

Sample collection

A field survey was conducted in the Department of Agriculture orchards, private farms, nurseries and farmers fields in the citrus growing districts of Sri Lanka, namely Monaragala, Kandy, Badulla, Kurunegala, Polonnaruwa, Anuradhapura, Rathnapura and Hambanthota, to identify healthy and resistant/tolerant citrus germplasm/cultivars against CTV and fastidious bacteria *i.e. Xylella fastidiosa* and *Liberibacter asiaticum*. Leaf samples of citrus spp. were collected from locations of these districts (Table 1). Third and fourth leaves from apical buds of 3-5 years old fruit bearing asymptomatic citrus plants were selected for this study. In contrast, leaves were collected from a few plants, which showed CTV symptoms to detect and confirm the presence of CTV in symptomatic plants.

Serological detection of the CTV and *Xylella fastidiosa*

Midribs of the leaves were used to detect variegated chlorosis disease, as *Xylella fastidiosa* is limited to the xylem system of citrus plants. Collected citrus leaf samples were subjected to serological detection at Horticultural Crops Research and Development Institute (HORDI), Gannoruwa, Sri Lanka. Indirect double antibody sandwich ELISA (DAS ELISA) was performed with citrus leaf samples for detection of CTV and *X. fastidiosa* using the protocol of Agdia Inc. Ltd; U.S.A. A leaf roller grinder was used to extract the plant sap by grinding 0.5 g leaf sample with 5 ml of sample extraction buffer. A 100 µl plant sap was placed in the antibody coated ELISA-well with two replicates. Positive control and negative control purchased from Agdia Inc. Ltd, USA were also included in the ELISA plate. The monoclonal antibodies to ascertain

the presence of CTV and polyclonal antibodies for *X. fastidiosa* were used. Final color reaction was recorded and the quantitative measurement of the CTV and *X. fastidiosa* were obtained using ELISA Micro Plate Reader at 405 nm and 492 nm, respectively.

Detection of citrus greening disease caused by *Liberibacter asiaticum*

A rapid and sensitive method developed by Hung *et al.* (1999), based on polymerase chain reaction (PCR), was used in this study for the diagnosis of citrus greening disease. *Liberibacter asiaticum* was diagnosed by PCR using specific primers (forward primer – 794888 GFB and reverse primer – 794889) derived from the sequences of the cloned DNA fragment of greening fastidious bacterium. The primer pair composed of the forward primer 5'-CACCGAAGATATGGACAACA-3' and the reverse primer 5'-GAGGTTCTTGTGGTTTTTCTG-3' (Perkin Elmer, Norwalk. CT, USA). One set of the primer pair, which generates a 226 bp GFB-specific fragment from total DNA templates extracted from plants, was subjected to PCR. Presence of DNA was confirmed by agarose gel electrophoresis based on the UV spectrophotometry. Nucleic acid extracted from Pummelo (*Citrus maxima*) plant, which was previously identified using PCR as GFB infected (Prof. Su, University of Taiwan), was used as positive control.

Extraction of DNA from plant samples

Mid ribs of leaves and twigs of collected citrus plant samples were used to extract DNA. Samples were chopped and 0.5 g were frozen in liquid nitrogen and ground to a fine powder with a motor and pestle. Then 3 ml of DNA-extraction buffer and 0.3 ml sarkosyl (10 %) was added and mixed with the tissue powder. The suspension was transferred to a 1.5 ml Eppendorf tube and incubated at 55°C for 1 hr. Then the tubes were centrifuged at 3018*x*g for 5 min. The supernatant was saved and 100 µl of 5M NaCl and 100 µl of CTAB/NaCl (10 % CTAB in 0.7 M NaCl) were added and incubated at 65°C for 10 min. Equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added to the supernatant, mixed thoroughly and spinned at 10145*x*g for 5 min using angle rotor. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) were added to the saved supernatant and mixed thoroughly and spinned at 12074*x*g for 5 min. The supernatant was saved and 0.6 ml of cold isopropanol was added to precipitate the nucleic acid. Again it was spinned at 12074*x*g for 30 min. The pellets were washed with 100 µl of 70 % ethanol to remove the residual CTAB and spinned at 12074*x*g for 10 min. The pellets were briefly dried and re-suspended in 50 µl of TE buffer (1mM TRIS, pH 8.0, 0.1 mM EDTA, pH 8.0) and stored at -20°C (Hung *et al.*, 1999).

Polymerase chain reaction (PCR)

After the conformation and quantification of DNA, the PCR was performed using 15 µl of reaction mixture containing 10 mM Tris-HCL (pH 8.4), 50 mM KCl, 1.5 mM of $MgCl_2$, 0.8 mM of each dATP, dTTP, dCTP and DGTP, 1 unit of Taq DNA polymerase (SIGMA), 0.6 µl of 50 ng/µl forward primer (794888 GFB), 0.6 µl of 50 ng/µl reverse primer (794889 GFB), 3 µl of 274.21 µg/ml of template of the nucleic acid preparation. The thermal cycle conditions were one cycle at 94°C 1min, 56°C 1 min, 72°C 2 min, 30 cycles at 94°C 1min, 56°C 1 min, 72°C 2 min, then followed by a 72°C extension for 10 min. Reactions were carried out in a DNA Thermal cycler (DNA Thermal Cycler 480, Perkin Elmer).

Analysis of PCR products by electrophoresis

The PCR products were analyzed by gel electrophoresis using 1.4 % agarose in TBE buffer (Tris–acetate, pH 8.0, 1mM EDTA). The electrophoresis was carried out for 1 hr using high voltage (130V) and gel was stained with Ethidium Bromide (0.5 ng/ml) and visualized under BIO-RAD Gel documentation apparatus.

RESULTS AND DISCUSSION

The CTV was found in all citrus cultivations even in most of the apparently healthy looking, fruit bearing plants of orange (*Citrus sinensis* L), mandarin (*C. reticulate* L) and pummello (*C. maxima* L) (Table 1). Out of 628 apparently healthy looking citrus plants tested, only 31 were identified as free from CTV, indicating that over 95 % of productive citrus plants were infected with CTV. The ELISA readings (test wavelength 405 nm) of the healthy citrus plants ranged from 0.190 - 0.359, whereas mean values of positive and negative control were 2.475 and 0.355, respectively. In most of the sampling locations, no plants were identified as CTV-free. However, 5 out of 210 samples tested in wet zone (2.4 %), 6 out of 88 in intermediate zone (6.8 %), and 20 out of 330 in dry zone 20 (6.1 %) were free from CTV. This indicates that the disease incidence is comparatively low in dry and intermediate zones when compared to the wet zone. Further, 16 out of 156 (10.3 %) orange plants (variety – Bibile Sweet) tested in Nikaweratiya area (DL_{1B} agro-ecological zone) were found as CTV-free. This may be due to the low aphid population in dry zone area, isolated locations, and farmers in this area used their own grafted plants for planting. Tristeza disease of citrus, caused by citrus tristeza virus (CTV), a closterovirus, occurs in most citrus-producing areas of the world and is the most economically important viral disease of citrus (Agrios, 1997; Timmer *et al.*, 2003). There are different types of isolates of CTV and these isolates varied markedly in ability to induce symptoms and severity, which were generally consistent with field observations of these isolates. It is

suggested that yellowing of seedlings, decline in grafted sweet orange (when sour orange used as stock), stem pitting in sweet orange, and stem pitting in grapefruit are independent expressions of CTV pathogenicity and can occur in various combinations (Garnsey *et al.*, 2007; Timmer *et al.*, 2003). The virus is disseminated *via* infected bud woods and spread naturally by aphid vectors and hence, it is widespread at present in most of the citrus cultivations in Sri Lanka.

Table 1. Detection of CTV and *Xylella fastidiosa* (Xf) in citrus samples collected from various citrus growing areas by DAS ELISA technique

Sampling area	*No. of tested samples*	*Host (Cultivar)*	*No. of CTV free plants*	*No. of Xf free plants*
Galabadde	14	Orange, Mandarine	0	12
Opanayake	22	Orange, Grapefruits	0	21
Karapinche	10	Orange	0	10
Kalatuwana	10	Orange, Mandarine	0	10
Nikaweratiya	156	Orange, Grapefruits	16	156
Rahangala	24	Orange	0	22
Pussallawa	16	Mandarin, lime	0	16
Weerapana	04	Orange, Grapefruits	1	04
Bataatha	22	Orange, Mandarine	0	21
Polonnaruwa	22	Orange, Mandarine	0	22
Mahailluppallama	22	Orange, Mandarine	1	19
SCS farms	21	Orange	0	21
Ulpothagama	12	Orange	2	12
DTC farm-Bibile	14	Orange	0	14
Kothmale	15	Orange, Grapefruits	0	15
ISTI/Gannoruwa	05	Calamensis	0	05
HORDI/ISTI	30	Orange, Pummelo, Mandarine	4 0	28
Neelabamma	13	Orange	0	13
PGRC	01	Pummelo	0	01
Rambukkana	01	Orange	0	01
HORDI Unit II	09	Pummelo, Orange, Mandarine	0	08
Mahawa	35	Orange	2	35
Poddiwela	16	Orange, Mandarine	0	16
Matthaka	05	Orange	0	04
Mawanella	88	Orange, Pummelo	3	88
Ranorawa	18	Orange	1	18
Deraniyagala	23	Orange	1	23

Citrus variegated chlorosis has become a serious and widespread disease in some part of the world, mainly south America (Timmer *et al.*, 2003). However, variegated chlorosis caused by *X. fastidiosa* was rarely detected in citrus cultivation in Sri Lanka (Table 1). Out of 628 citrus plants tested, only 13 were infected with *X. fastidiosa*, indicating that over 98 % of productive citrus plants are free from this pathogen. A few of the mother plants in government farms in the country (2 in Rahangala, 3 in Mahailluppallama, and 3 in HORDI) were found as infected with *X. fastidiosa*.

However, 5 out of 210 samples (2.4 %) tested in wet zone, 3 out of 88 samples (3.4 %) tested in intermediate zone, and 5 out of 330 samples (1.5 %) tested in the dry zone were identified as infected with this pathogen. The results suggest that the disease incidence of variegated chlorosis is almost the same in the three ecological zones. The quantity of DNA of the citrus samples varied from 106.6 to 708.4 µg/ml. The GFB infection was detected in only one pummelo plant (+control), but all the other tested plants, which were free from CTV and *X. fastidiosa* were also free from GFB (Figure 1).

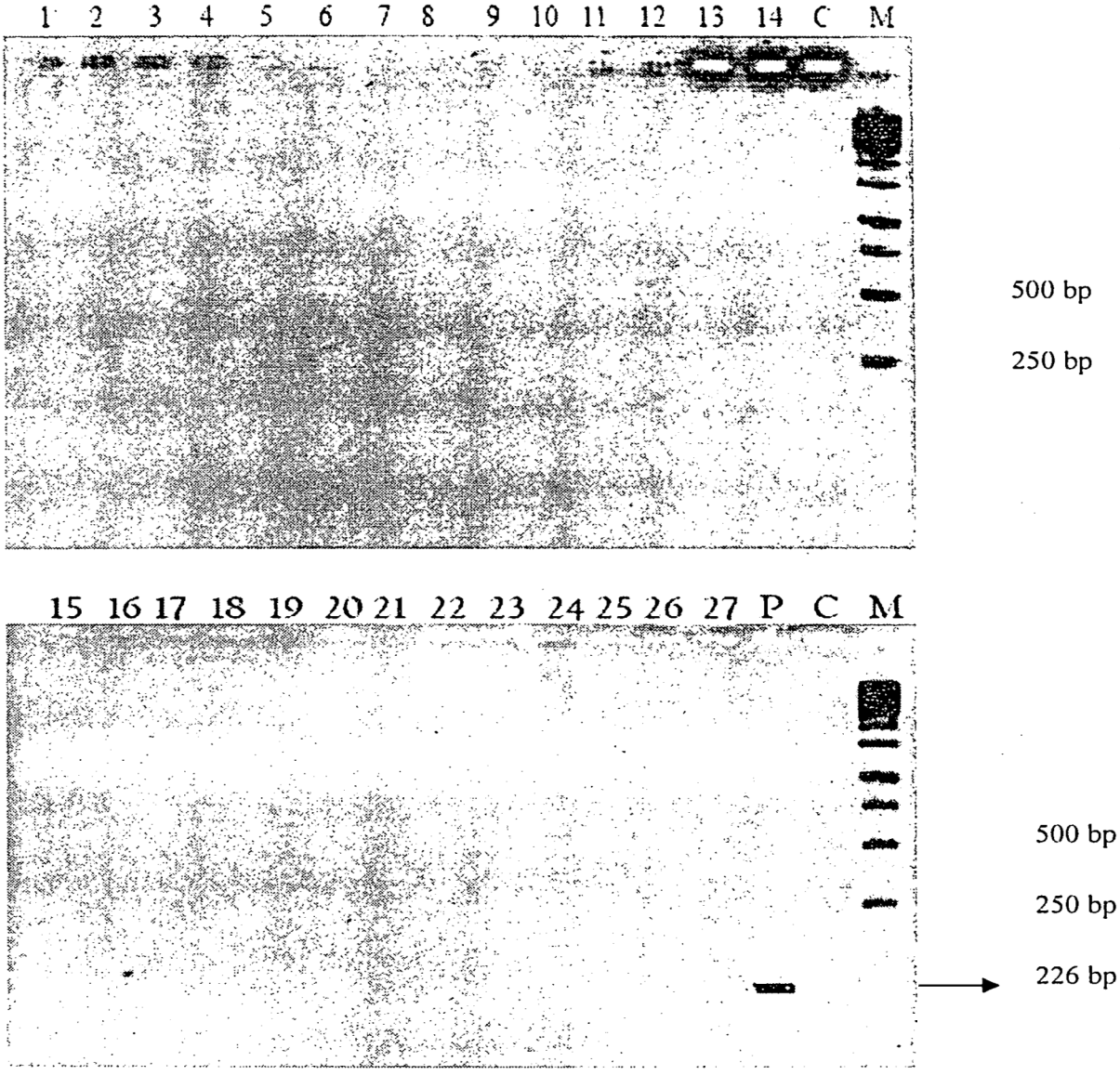

Figure 1. Results of the PCR amplification for detection of GFB in citrus plants. The GFB-specific product of 226-bp (arrowed) fragment found in sample P (+ control) collected from pummelo plant at PGRC. The 1000 bp DNA ladders (M) were included as markers and C was negative control.

Hung *et al.* (1999) reported that citrus greening has been widespread in all citrus growing areas in Asia and caused great damage to the citrus industry by shortening the lifespan of trees. Dassanayake *et al.* (2004) reported that some plants of lime, orange, grape fruit and pummelo in local orchards are infected with GFB.

Xylella fastidiosa was rarely found even in citrus plants, which showed yellowing symptoms on leaves. The GFB was found only in one plant, which was previously identified as infected by Prof. Su, University of Taiwan (personal communications) and Dassanayake *et al.* (2004). It revealed that variegated chlorosis disease caused by *Xylella fastidiosa* and greening disease caused by *Liberibacter asiaticum* were not prevalent in citrus cultivation in Sri Lanka.

Systemic diseases of citrus are easily transmitted through grafting (Timmer *et al.*, 2003). Grafted plants are produced in farms using their own mother plants and most of the mother plants, except a few are infected especially, with CTV. Therefore, most of the grafted plants are planted in the field with CTV infection. The basic control strategies for most graft-transmissible systemic diseases of citrus are to prevent infection where possible. Programmes to obtain and promote propagation of pathogen-free bud-woods for new plantings are effective on delaying build up of virus concentration in plants and minimized yield loss.

CONCLUSIONS

Among the tested citrus germplasm in this study, over ninety five per cent citrus plants were found as infected with CTV. *Xylella fastidiosa* and *Liberibacter asiaticum* were rarely found. Twenty one orange and six pummello germplasm were identified as samples free from CTV, *Xylella fastidiosa* and *Liberibacter asiaticum.*

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