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Application of chemicals and nutrients differs in bacterial titre of *Candidatus* Liberibacter asiaticus on infected *Citrus sinensis* (L.) Osbeck

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Abstract
Huanglongbing (HLB), or citrus greening disease, is significant havoc to the citrus cultivation that influences citrus industry decline in India and elsewhere. HLB is a systemic, fastidious, phloem inhabited alpha-proteobacterial disease caused by '*Candidatus* Liberibacter asiaticus' (*C*Las). Currently, there is no established cure for this emerging but century-old disease. The present study discussed change in cycle threshold (Ct) values of *C*Las bacterium using qPCR diagnostic method upon applying antibiotics and micronutrients to HLB infected sweet orange cv. Sathgudi plants. Five antibiotics and three micronutrients were selected and applied as a foliar spray in 3 different spraying periods. Results demonstrated that ampicillin sodium (Ct=25.41) and rifampicin (Ct=23.62) showed a significant effect on the suppression of bacterial titre compared to other selected antibiotics at 90 days after the first spray. However, there was no significant effect on the bacterial population regarding its titre and symptom expression when any of the selected micronutrients were applied in any of the spraying periods. Hence, the reported antibiotics could help in the management of an infected HLB citrus orchard at the expense of minimizing total yield loss when they are judiciously used.

Keywords: HLB, qPCR, antibiotics, micronutrients, bacterial titre, Ct value

Introduction
Global citrus production is constantly being altered by various economic, biological, and environmental factors. Biotic and abiotic factors are the major threats that affect the vitality of the world's citrus industry. Citrus greening (syn., *Huanglongbing*) is currently the most destructive disease of Citrus. It has been rapidly spreading worldwide, resulting in a decline in the production and profitability of the citrus industry (Gottwald, 2010) [7]. To an estimate, more than 100 million infected trees have been destroyed with this cause in Asia (Belasque *et al*. 2010) [2]. HLB has been known in East Asia for over a century and is currently widespread in most citrus areas of Asia, Africa, and the Americas. In India, it was first inadvertently described in 1927 when the authors were reporting the damage due to citrus psyllid, *Diaphorina citri* Kuwayama infestation (Hussain and Nath, 1927) [15]; later, the experimental proof on the presence of HLB in India came in 1967 when the successful transmission of HLB agent was obtained by its insect vector (Capoor *et al*. 1967) [5]. The associating agent of HLB disease is *Candidatus* Liberibacter, later renamed as Liberibacter, phloem restricted gram-negative α-proteobacteria (Jagoueix *et al*. 1994) [16]. All commercial citrus varieties currently available are susceptible to HLB, so the citrus industries in affected areas have suffered a decline in both production and profit (Wang and Trivedi, 2013) [22]. To recuperate from disease, many integrated management programs consist of psyllid control with insecticides, removal and destruction of HLB-infected trees, nursery certification for the propagation of pathogen-free budwood sources and nursery trees has been practised across the global citrus orchards. Since HLB is a systemic disease, eliminating the bacterium from standing citrus trees, including roots and branches, is essential for adequate control. Unfortunately, the condition has no cure, and management of HLB is complex due to its habitat in the host, and no resistant citrus germplasm has been found. Genetic solutions will take several years in the future (Duan *et al*. 2009) [6]; meanwhile, the disease spreads and increase at a devastating rate (Gottwald, 2010) [11]. Citrus researchers and producers seek possible ways to overcome the effect of the disease.

Foliar sprays of various nutrients can extend the vigor and productivity of HLB-infected trees (Giles, 2011) [7]. It can also be achieved by the practical application of antibiotics. and vector control. Studies have shown that antibiotic application to the infected citrus trees provides a temporary remission of symptoms (Gottwald *et al*. 2007) [10]. However, there are fewer reports examining change in bacterial titre with immediate response to antibiotic and nutrient application. Therefore, the present communication was aimed and keeping the above in view; we attempted to evaluate various nutrients and antibiotics for their efficacy on change in bacterial titre of HLB in a greening infected standing citrus trees (determine Ct values with the help of qRT-PCR) and its symptom expression and the results are reported in this paper.

Materials and Methods

Plant material and research area

Individual sweet orange *Citrus sinensis* (L.) Osbeck var. Sathgudi trees were selected randomly based on putative symptoms of HLB (Gopal *et al*. 2010) [9] and tagged (n=54). All trees chosen for the experiment were confirmed to be HLB-positive using the conventional PCR assay with HLB-specific primers. The treatment trial was conducted in a 10-year-old sweet orange field block in Citrus Research Station, Tirupati province.

Treatment specification of micronutrients and antibiotics on bacterial population of HLB (*C*Las)

The present regime consisted of nine treatments(T) *viz.*, T_1: Di-potassium hydrogen phosphate (K_2HPO_4) @5g/l; T_2: Manganese Sulphate ($MnSO_4$) @2g/l; T_3: Copper Sulphate ($CuSO_4$) @3g/l; T_4: Penicillin G-Potassium @500ppm; T_5: Ampicillin Sodium(Amp) @500ppm; T_6: Oxytetracycline hydrochloride @500ppm; T_7: Chloramphenicol @500ppm; T_8: Rifampicin @500ppm; T_9: Control (water alone) replicated thrice. Treatment application was made every month in three repetitions. Individual treatments were applied with the help of a battery-type knapsack sprayer until runoff to ensure complete coverage of foliage. All the antibiotics and micronutrients were purchased from Himedia biosciences for this study. Solutions were freshly prepared before application. Water was used as a solvent for dissolving all the chemicals in this study except Chloramphenicol, which was dissolved in 1 ml of ethanol and then adjusted the final volume with water. From the time of treatment application, at every 15 days, citrus leaves were collected and processed for DNA extraction to determine bacterial population by using qPCR. Visual scoring was recorded at 30 days after each spray schedule by using the disease index scale given by Kranz (1988) [17], Bowen (2004) [4], and Gopal *et al*. (2010) [9]; scoring had done to estimate the disease severity with the formula given below.

$$\text{Disease severity} = \frac{\text{sum of the symptomatic plant and their corresponding rating}}{\text{total number of sampled plants x highest rate}} \times 100$$

Disease index scale

0 = no symptom 0%
1 = mild (blotchy mottling symptoms) 1- 25%
2 = moderate (yellowing symptoms) 26-50%
3 = severe (blotchy mottling, midrib yellowing, and twigs dieback symptoms) 51-75%
4 = very severe 76-100%.

Citrus genomic DNA extraction and PCR detection

Total DNA (from midribs of HLB-like symptomatic and healthy leaves) extracted through Sodium Sulphite-Tris EDTA (SS-Tris EDTA) method (Gopal *et al*. 2004) [8] was subjected to PCR assay with primer pair HLB-F: 5' TGGGTGGTTTACCATTCAGTG3' and HLB-R: 5'CGCGACTTCGCAACCCATTG 3' based on 16S rDNA region (Harakava *et al*. 2000) [13]. The PCR amplification program included 95 °C for 2 min followed by 35 cycles of 95 °C for 30s, 58 °C for 30s and 72 °C for 1 min with a final extension of 72 °C for 10 min. Expected amplicon size ~ 451bp was observed in all the symptomatic samples but not in healthy/asymptomatic samples. This confirms the HLB infection in selected plants.

Preparation of standard curve for quantification

For absolute quantification, the standard curve method is used for determining the concentration of unknown samples from their Ct values. In this, cloning the target sequence into a known vector is the best procedure for knowing the exact copy number of the target genome.

Cloning and sequencing of HLB 16S rDNA genome

Total genomic DNA isolated from an HLB infected plants tissue was used to amplify the amplicon size of 451bp, representing the 16S rDNA gene region. The PCR reaction mixture and conditions were similar as previously described above. The PCR amplified product was separated in 1% Agarose gel, and the product was purified using a Promega Gel Extraction Kit. The purified product was later cloned in a pGEM®T vector before transforming it into JM109 competent cells. Plasmid DNA was isolated from a recombinant clone and sequence verified (Eurofins Genomics India, Pvt. Ltd, Bangalore). Sequence analysis was carried out using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov) to determine the sequence similarity between cloned product and NCBI database entries.

SYBR Green real-time PCR

SYBR Green real-time PCR was performed to determine HLB load in different tissue samples. The amplification was then carried out in a Rotor-gene Q real-time thermocycler (Qiagen Hilden, Germany) in 10 µl reaction mixture containing 2x Quant fast Probe PCR Master Mix, 1 µM of both reverse and forward primer (same as used for conventional PCR), and up to 100 ng of DNA template. The thermal cycle profile was 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 45s. The baseline was automatically determined by Rotor gene Q series software 2.3.1. The software automatically decided the threshold with the aid of two standards (having a known copy number with a specific Ct value) from another run. Subsequently, a post melt curve analysis was run as default according to the Rotor-gene Q real-time PCR system user manual.

Quantification of HLB genomes in plant tissues and data analysis

A serial dilution of plasmid DNA carrying the HLB 16S rDNA sequence was prepared (10^{-1} to 10^{-5} copies per reaction) using Qiagen supplied sterile distilled water as a diluent. The real-time PCR protocol was the same as

described above. HLB titre in different tissue samples was measured using a standard curve method. A standard curve was then made using Ct values obtained from serially diluted plasmid DNA with a known copy number. Plasmid copy number was calculated using formula *viz.,* Number of copies/µl = (X ng/µl DNA/[plasmid length in bp x 660 x10^9]) x 6.022 x 10^{23}. Each sample was run in qPCR, and its Ct value was taken to calculate the bacterial copy number. The copy number of HLB in the tissue of the tested sample was calculated by extrapolating the Ct values of the samples into the standard curve. To calculate the bacterial copy number accurately, all tested DNA samples were diluted to equal concentration. The data of chemical treatments were analyzed by Duncan's multiple range test at $p<0.05$.

Results

Detection of HLB by conventional PCR in sweet orange

The addition of sodium sulphite in Tris-EDTA yields suitable quality DNA that appeared to be promising for detecting the HLB disease by PCR. The results of PCR amplification with primers specific to HLB revealed successful amplification of 451bp product of 16S rDNA region of HLB in mild symptomatic tested leaf samples but not in the healthy control sample, which indicated the specificity of primer pair. So, all labelled trees which are desired for treatment application were tested PCR+ to HLB at the onset of the treatment trial (Fig 1a-1d).

Cloning and sequencing

The transformed JM109 cells yield clear white colonies on LB medium representing successful cloning (Figure not enclosed). The DNA nucleotide sequence of *C*Las sweet orange isolate has been deposited in the NCBI database under accession number MK584159, respectively.

Optimization of SYBR Green real-time PCR assay for measuring HLB titre in a leaf sample

To determine optimal annealing temperatures, qPCR experiments were conducted by using total DNA containing the 16S rDNA gene sequence from Las. Annealing temperatures were set from 58 ^{0}C and 62 ^{0}C for 30 sec and 45 sec (figure not enclosed) in that order. The best amplification plots with the least Ct values were obtained at 60 ^{0}C for 45 sec. HLB primers (document in materials section) used for both SYBR green real-time PCR and conventional PCR gives an amplicon with a melt curve peak at 86.5 °C (Figure 2a&b).

Development of Standard curve for HLB

A 10-fold dilution series of plasmid DNA containing HLB 16S rDNA partial sequence was used to generate an HLB standard curve. A linear relationship was observed between input plasmid DNA and Ct value with a regression coefficient (r^2) value of 0.99, slope (M) value of -3.364, and its regression equation from the cycle is Y= 11.088-0.297X, where Y is the estimated log concentration of templates and X is the qPCR Ct values (Figure 2c). This suggests the sensitivity and efficiency with minimum variation in the PCR kinetics. According to the Qiagen manual, if the slope is about –3.322 that PCR has an efficiency of 1. The dilution series of the target template copy number varied from 3.50E+08 (for Ct value 8.18) to 3.50E+04 (for Ct value 21.94). The detection limit for serial dilutions made in buffer was 10 copies (35 ng or 0.035 µg), and the upper detection limit of the real-time PCR was up to 10^{10} copies (0.35 µg) of the cloned plasmid pGEM®t DNA per reaction.

Effect of antibiotics and plant nutrients on Las

Concerning per cent disease severity, there was no significant difference among all plants selected for treatment imposition, and its severity ranged from 75.0 to 95.83. The disease severity ranged between 87.50 to 100.00%, without any significant difference observed among the nine treatments. At 30 days after 1st spray, there was an overall increase in the disease severity irrespective of the treatments. At 30 days after 2nd spray, ampicillin sodium (T5) and rifampicin (T8) recorded minimum disease severity of 58.33 and 62.50 respectively and both were statistically superior and on par with each other and maximum percent of disease severity was observed in control, T9 (91.67). The same trend was observed even at 30 days after the 3rd spray with per cent disease severity of T5 (20.83) and T8 (25.00), which were significantly differed with other treatments.

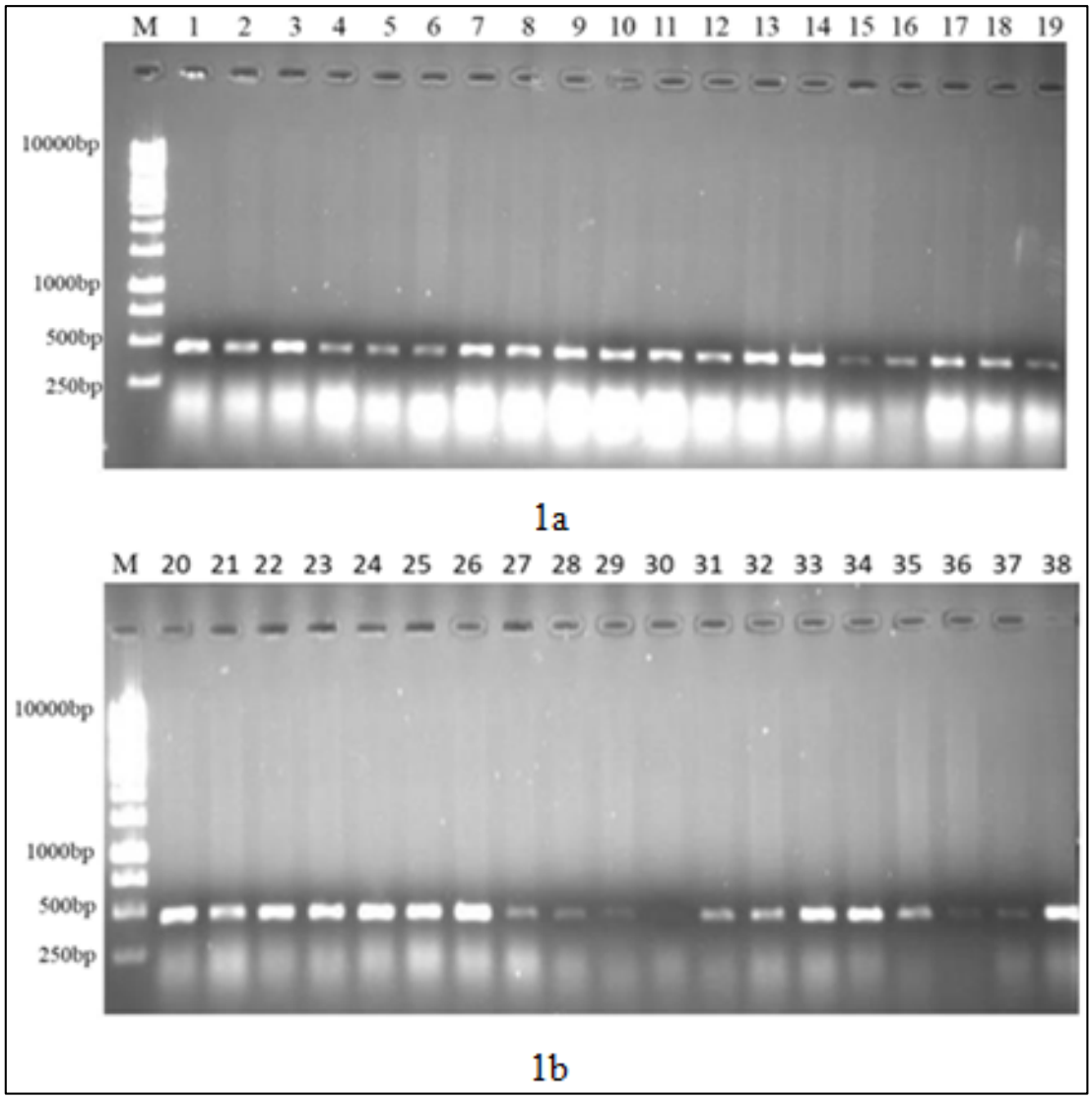
In this study, we observed the change in symptom expression in contrast to the change in day temperature. In contrast, the maximum percentage of disease severity was observed in T9 (54.17). But, there was an overall reduction in the disease severity in all nine treatments compared to other day intervals, which might be due to the presence of young foliage at the time of visual assessment (Table. 1). During January and early February, the visual observation noticed apparent HLB-like symptoms *viz.,* mottling, green islands, yellow twigs, upright twigs, and yellow midrib.

Samples collected at every fortnight intervals up to 90 days after initial spray subjected to qPCR analysis were showed positive to HLB and melt curve (around 86.3 ^{0}C) results revealed there was no ambiguity in amplification of all qPCR plots (Figure 3a&b - 9a&b). DMRT results showed significant differences in Las titres (represent in Ct values) between chemical treatments. Treatments of antibiotics and micronutrients were listed in materials. The Las bacterial populations were effectively reduced in T5 and T8. The Las titre of T5 was decreased from the Ct mean value of 21.45 to 25.41 after 90 days of initial treatment/30 days after 3rd spray. And the Las titre of T8 was decreased from 21.80 to 23.62 respectively (Table. 2). No significant differences were observed in the other day's interval. Results indicated that more or less there is no/mild change in Las titre of control (untreated trees), and its mean Ct value was recorded in a range from 21.93 to 20.96. Except ampicillin sodium and rifampicin, all treatments showed a positive difference in mean Ct value between before spray (0 days) and 90 days after the initial spray, which resembles an increase in Las titre. In addition to Ampicillin and rifampicin, Chloramphenicol alone recorded a negative difference in its mean Ct value between before spray (20.25) and 90 days of 1st spray (20.79).

Table 1: Estimation of disease severity based on visual scoring

Treatments	Before spray	30 DAS		
		1st spray	2nd spray	3rd spray
T_1	75.00 (64.99) [1]	91.67 (79.99)	70.83 [bc 2] (57.39)	45.83 [cde] (42.57)
T_2	95.83 (83.09)	100.00 (90.00)	79.17 [cde] (63.07)	50.00 [de] (44.98)
T_3	87.50 (77.41)	95.83 (83.09)	75.00 [cd] (59.98)	41.67 [bcd] (40.16)
T_4	95.83 (83.09)	95.83 (83.09)	83.33 [de] (66.17)	37.50 [bc] (37.57)
T_5	83.33 (70.50)	100.00 (90.00)	58.33 [a] (49.81)	20.83 [a] (26.89)
T_6	87.50 (73.08)	91.67 (76.18)	83.33 [de] (66.17)	33.33 [b] (35.16)
T_7	95.83 (83.09)	100.00 (90.00)	79.17 [cde] (63.58)	41.67 [bcd] (40.16)
T_8	79.17 (68.08)	91.67 (79.99)	62.50 [ab] (52.22)	25.00 [a] (29.99)
T_9	79.17 (68.08)	87.50 (73.08)	91.67 [e] (76.18)	54.17 [e] (47.39)
CD(P=0.05)	NS	NS	11.308	8.101
S.Em±	10.828	7.081	3.74	2.679
CV	25.14	14.808	10.512	12.109

1 Figures in parenthesis are arcsin transformed values. 2 Means within a column with different letters are significantly different, and means followed by the same letters are not significantly different according to DMRT test at $p \leq 0.05$ levels. CV: coefficient of variation; CD: critical difference; S.Em: standard error of the mean; NS: non-significant



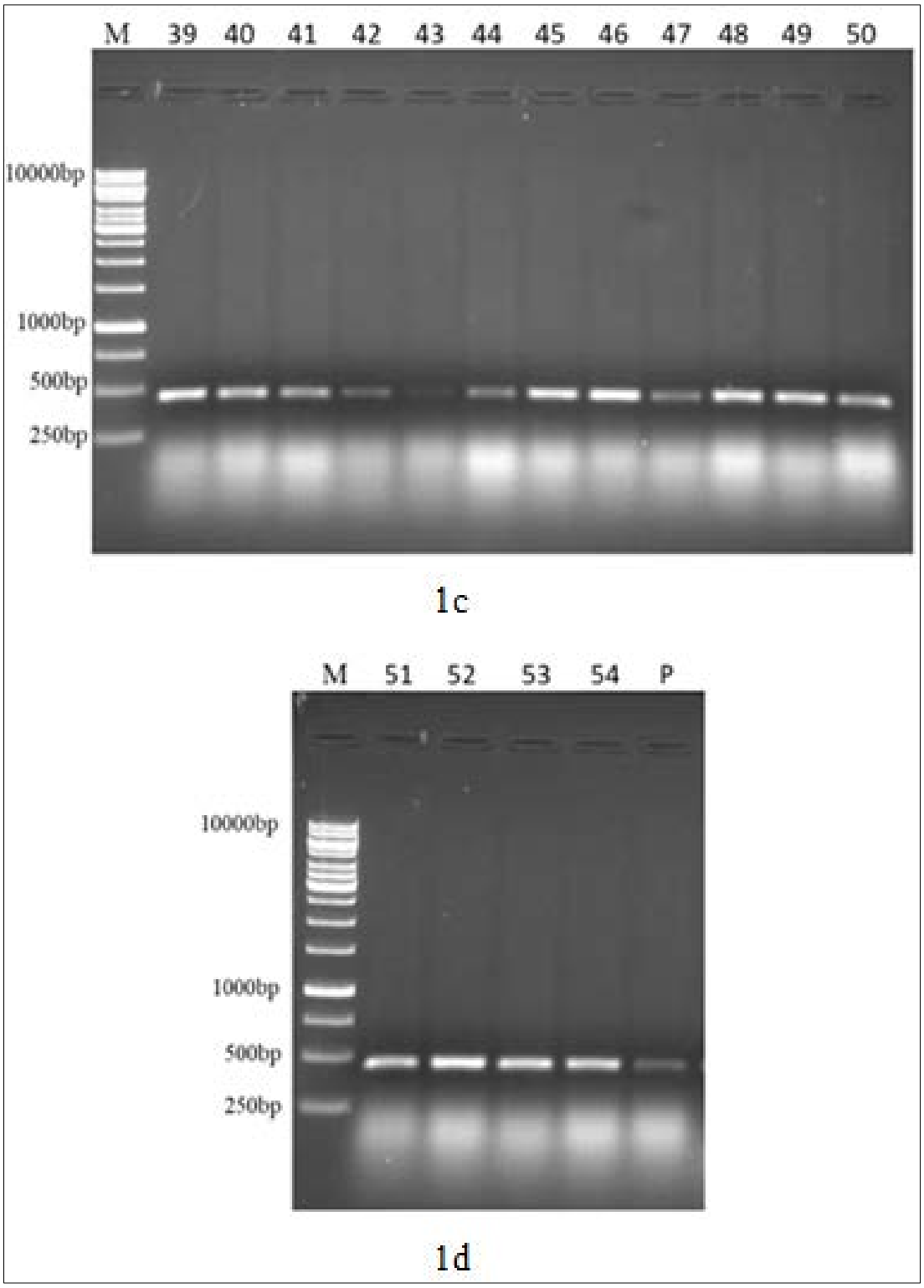
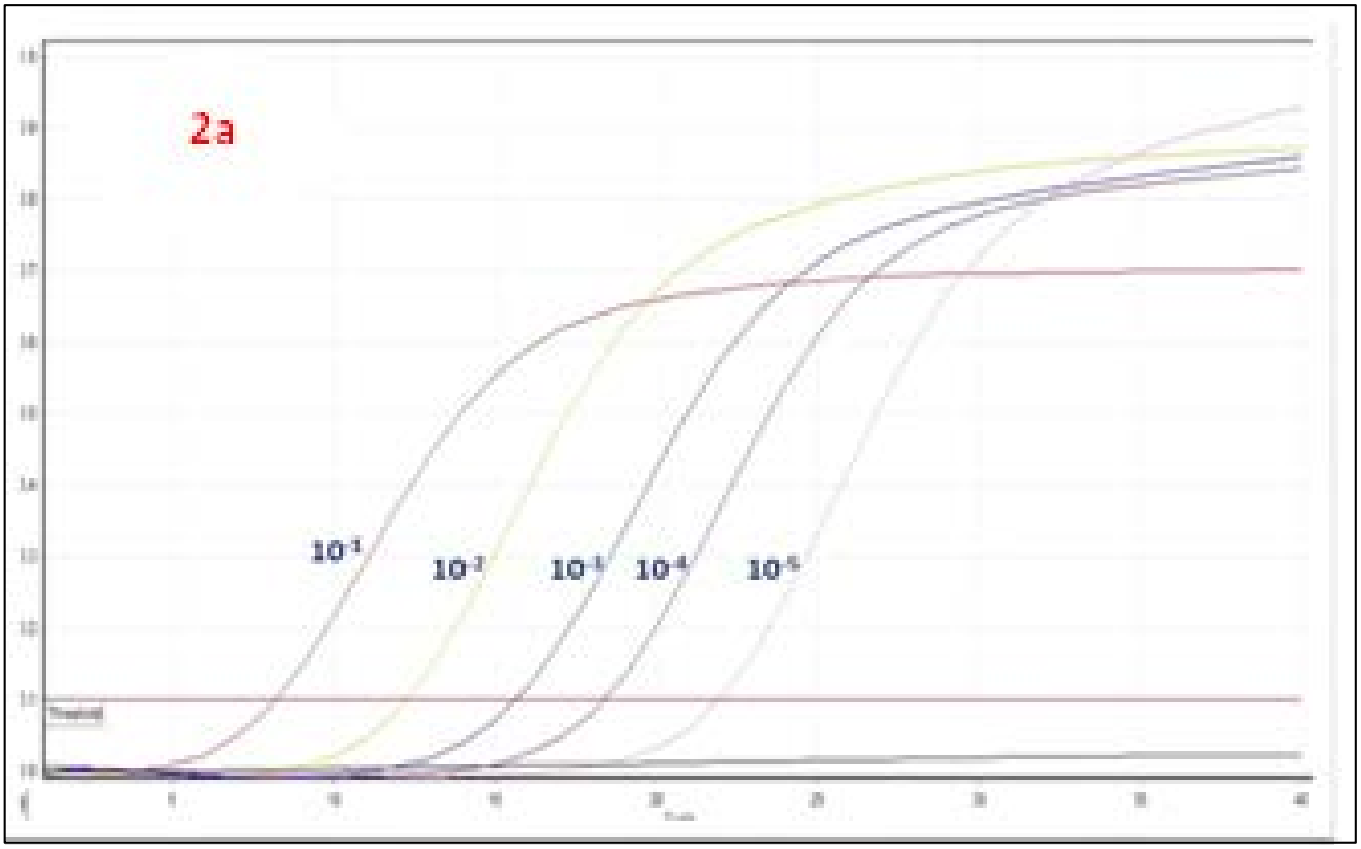
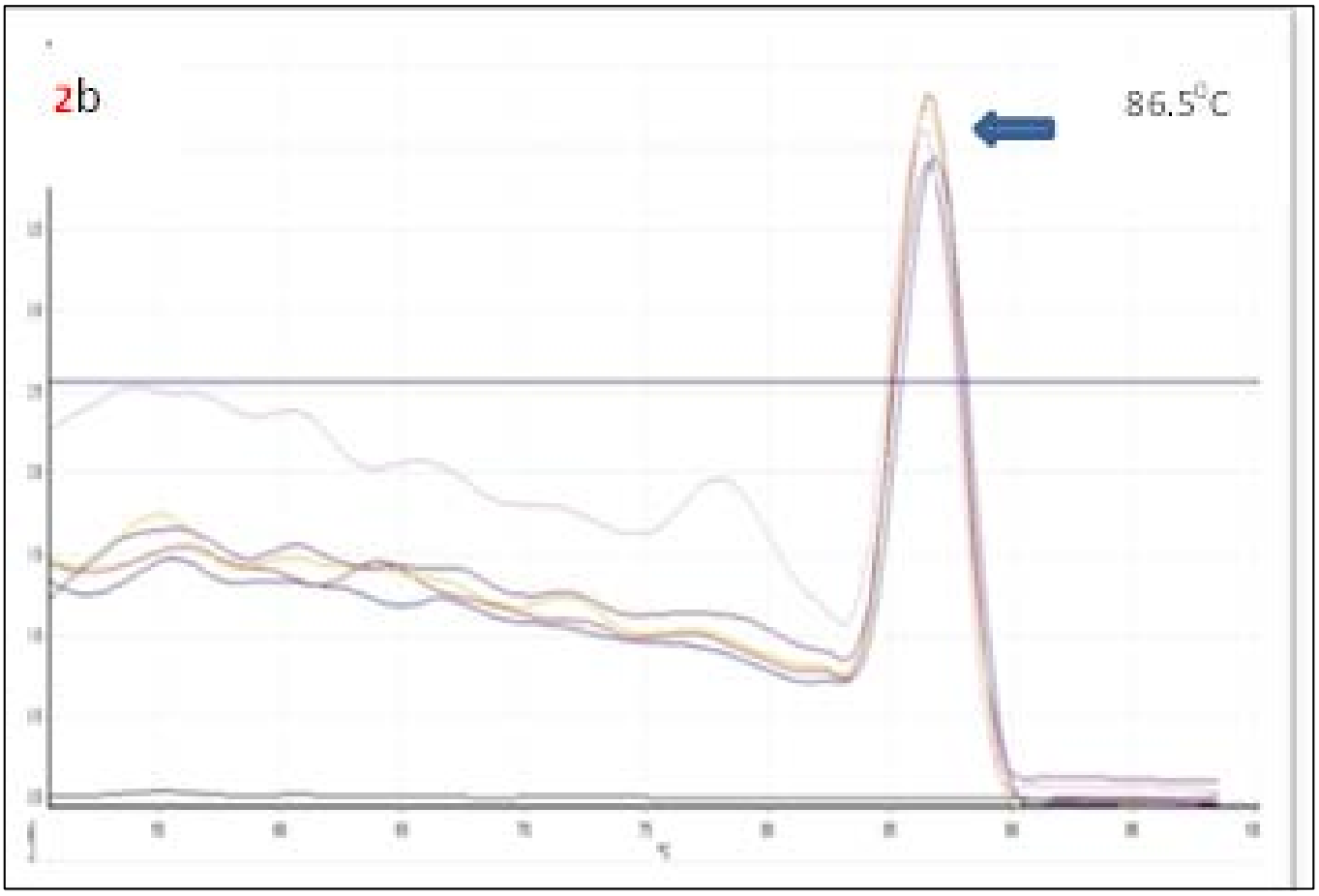


Fig 1a-1d: PCR Detection of HLB in greening-like symptomatic leaf samples of sweet orange Sathgudi plants from the block of a treatment trial. Lane M: 1Kb DNA ladder; Lane 1 to 54: HLB infected samples showing amplicon size of 451bp; Lane P: Positive control


2a
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}


2b
86.5°C

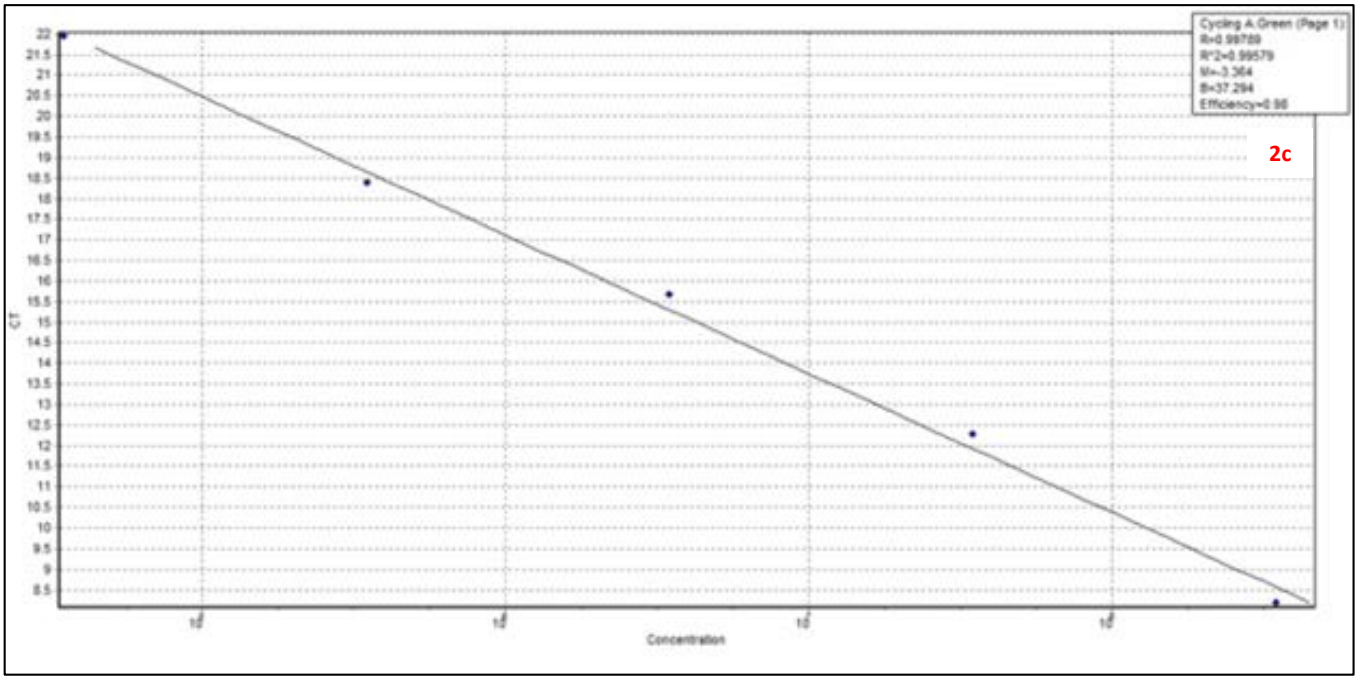
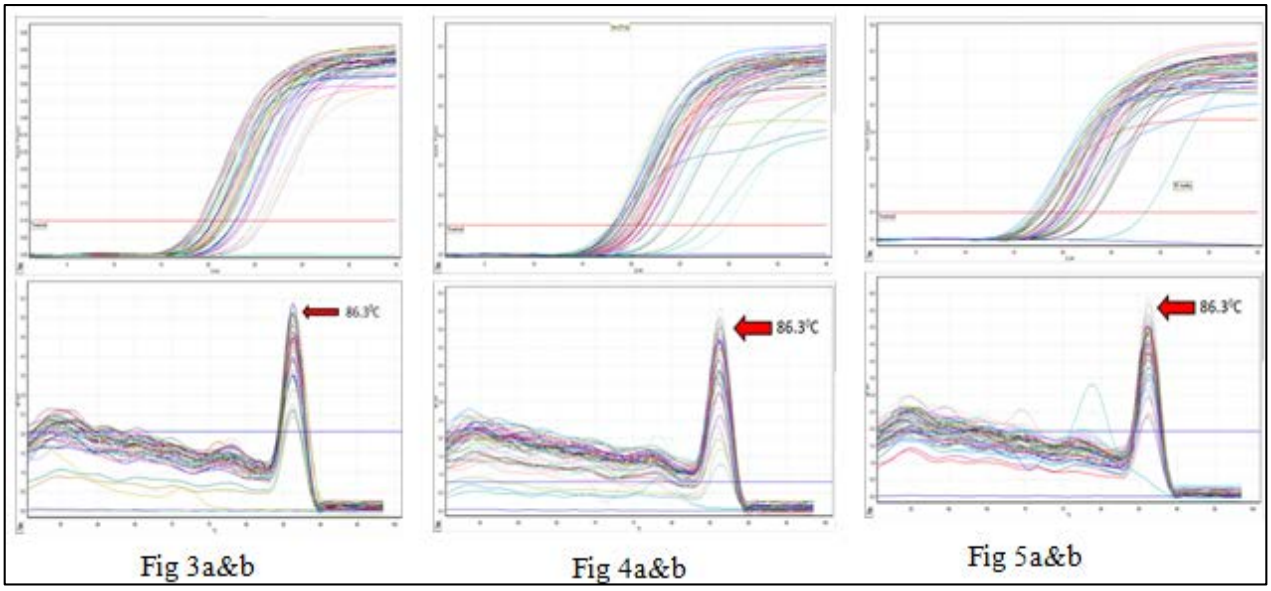
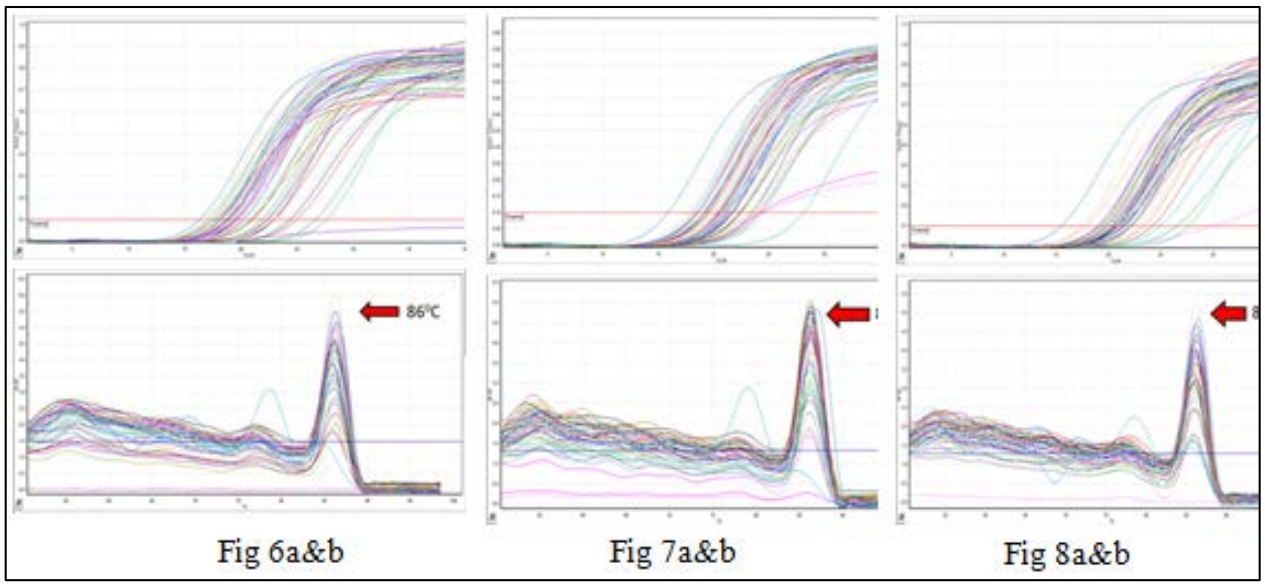


Fig 2 a-c: Standard curve of log concentrations of plasmid DNA serial dilution samples against Ct values





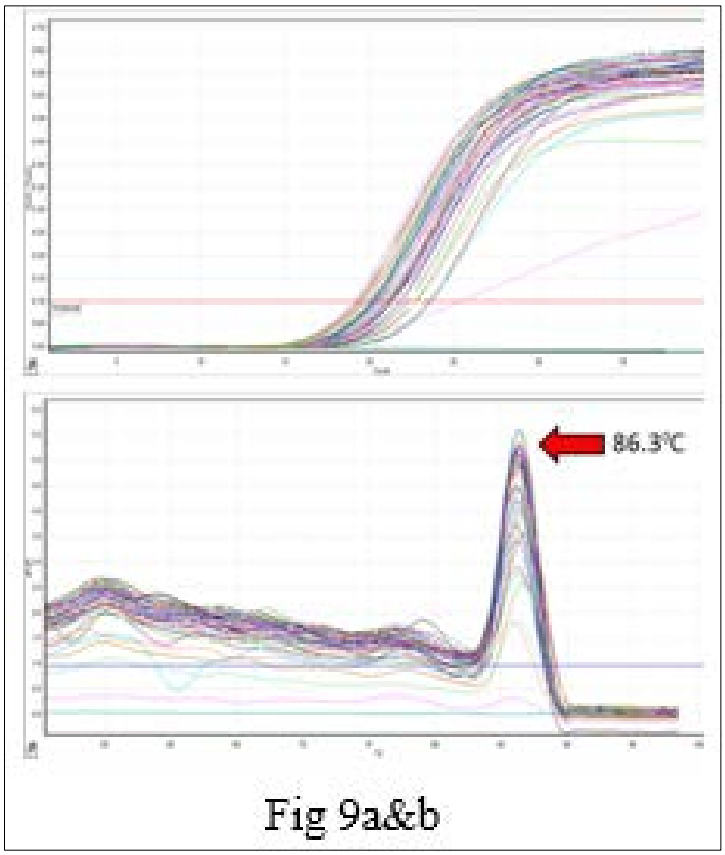


Fig 3 a&b-9a&b. 3a to 9a: Amplification plots of 0 days (before spray); 15 DAS/30 DAS (first spray); 15 DAS/30 DAS (second spray); 15 DAS/30 DAS (third spray). 3b to 9b Melt curve plots of 0 days (before spray); 15 DAS/30 DAS (first spray); 15 DAS/30 DAS (second spray); 15 DAS/30 DAS (third spray) DAS: Days after Spray

Table 2: Effect of different treatments on the bacterial population of citrus greening

Treatments	Mean Ct values at different days						
	0	**15**	**30**	**45**	**60**	**75**	**90**
T_1	21.91	21.92	20.30	19.89	21.47	21.19	20.88[b] *
T_2	21.52	19.85	19.45	20.00	21.01	20.83	20.64[b]
T_3	21.26	20.27	19.33	19.26	20.38	19.98	20.67[b]
T_4	22.11	20.37	19.53	20.85	20.75	20.45	20.71[b]
T_5	21.45	19.71	19.27	19.98	21.38	22.66	25.41[a]
T_6	21.89	19.49	19.79	19.38	20.38	20.76	20.63[b]
T_7	20.25	18.88	18.29	18.21	19.76	20.63	20.79[b]
T_8	21.80	20.57	19.65	22.57	21.36	22.41	23.62[a]
T_9	21.93	21.02	19.13	20.62	21.99	21.62	20.96[b]
CD(P=0.05)	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	1.805
S.Em±	0.803	1.043	0.771	0.99	0.726	0.692	0.603
CV	6.446	8.932	6.875	8.543	6.002	5.659	4.836

* Means within a column with different letters are significantly different and means followed by the same letters are not significantly different according to DMRT test at $p \leq 0.05$ levels.

Discussion

HLB is one of the major threats to Indian citriculture and causes heavy loss by its likely infection. The disease is difficult to control due to a lack of HLB-resistant citrus cultivars and effective control methods (Bove, 2006 and Gottwald *et al*. 2007) [10]. In >50 years, about 40 antibiotics have been screened to control bacterial diseases of fruit trees. Of all screened antibiotics, around 25% have been found more effective and commercialized. The use of streptomycin or tetracycline antibiotics alone or the combination of both is commonly applied to fruit trees worldwide to manage bacterial diseases (Zhang *et al*. 2014) [26].

To revive the citrus industry from HLB, citrus orchards have been treated with codicil nutrients in parallel with effective antibiotics to maintain a satiable yield for some period. Many studies have documented the effect of micronutrients and antibiotics and their different application methods, such as foliar sprays, root drench, soil application and trunk injections, against the response of HLB infected citrus trees (Puttamuk *et al*. 2014; Zhang *et al*. 2011, 12) [19]. To date, the practical method of application remains to be determined for citrus trees. So we followed the foliar spray method for chemical application which is a more effortless and convenient method for broad-spectrum application.

At the stage of the treatment trial, most of the selected trees attain mild symptoms, putative symptoms, and symptomless stature, and there is no study done on the nutrient status of plants chosen before treatment application. A nutrient

imbalance is often present in plant tissues of HLB-affected trees. This stage of infection was selected based on the statement that the nutrient and antibiotic application maintain the productivity and health of HLB-infected trees.

Zhang and his co-workers (2014) [26] evaluated 31 antibiotics through a graft-based chemotherapy method for their effectiveness against Las and their phytotoxicity to Citrus. They pooled antibiotics into three groups based on their results. In which six compounds included under highly effective antibiotics (Ampicillin, Penicillin G potassium, Rifampicin, Carbenicillin, Cefalexin, and Sulfadimethoxine) and nine were in partly effective (Chloramphenicol, Validoxylamine A, Hygromycin B, Kanamycin sulphate, Spectinomycin dihydrochloride pentadrate, Rifamycin sodium, Rifaximin, Sulfathiazole sodium, and Sulmethoxazole) at different concentrations. In this paper, five antibiotics belonging to three different antibiotic classes *viz.*, Ampicillin & Penicillin G potassium(beta-lactam class); Rifampicin (Ansamycin class); Chloramphenicol (tetracycline class) [based on the best research findings of Zhang *et al*. 2014] [26] and Oxytetracycline (tetracycline class), a most popular antibiotic were selected.

Our results indicating that the treatments ampicillin sodium and rifampicin gave the best results at 500ppm compared to all other treatments at 90 days after first spray application, which supports findings of Zhang *et al*. 2014 [26] where they reported Ampicillin @100ppm and Rifampicin @50ppm are categorized as highly effective in eliminating or suppressing *C*Las. Ampicillin inhibits bacterial growth by inactivating enzymes located in the bacterial cell membrane, known as penicillin-binding proteins, which are involved in cell wall synthesis (Spratt and Cromie, 1988) [21] and are reported as the most effective chemical compound against the citrus Las bacterium with the lowest phytotoxicity to Citrus (Zhang *et al*. 2012, 2013, 2014) [27-28]. Yang *et al*. (2016) [24] examined the effect of different application methods in combination with other antibiotics along with thermotherapy against Las bacterial titres. They found a significant reduction of Las in Ampicillin and actidione+validoxylamine (Act+VA) treatments applied by the bark paint method combined with thermotherapy. In another instance, Las bacterium could not detect in Ampicillin treated graft-inoculated plants with Las-infected scions at a high concentration of 1000ppm (Zhang *et al*. 2013) [27]. Antibacterial activity of rifampicin results from the inhibition of bacterial DNA-dependent RNA synthesis; it shows a bacteriostatic effect against gram-negative bacteria (Sachin *et al*. 2007) [19].

Puttamuk *et al*. (2014) [19] Zhang *et al*. (2012 and 2014) delineate tetracycline and penicillin effectively suppressed the Las population, which contradicts the present study. Moreover, tetracycline is bacteriostatic and needs a repeated application for better effect (Abdullah *et al*. 2009) [1]. However, these are broad-spectrum antibiotics and age-old recommended compounds against many bacterial diseases. So, there might be a development of antibiotic resistance by bacterium against these two compounds. In addition to these results of Oxytetracycline, we observed moderate phytotoxic symptoms in all replications due to Oxytetracycline at 500ppm. At 100ppm, Zhang and his co-workers observed complete failure of living scions due to phytotoxicity. Zhang *et al*. 2014 [26] noticed maximum loss of living scions due to high phytotoxicity at 100ppm, made to exclude from statistical analyses, though it effectively reduces Las titter. So, Oxytetracycline concentration is critical as it shows a severe phytotoxic effect on Citrus.

Chloramphenicol at 30ppm was recorded as a partly influential antibiotic group in the findings of Zhang *et al*. 2014 [26]. So we increased its concentration to 500ppm and observed satisfactory results, as the only antibiotic showed a negative difference in its Ct value next to Ampicillin and Rifampicin.

The present study's data revealed no effect of copper sulphate and manganese sulphate on the suppression of bacterial population, and similar findings were reported from their studies of Gottwald *et al*. 2012 [12]. After two seasons of three applications of Mn, Cu & Zn, no significant differences were observed in the dynamics of bacterial titre between treated and non-treated plants. Due to the phytotoxic nature of $CuSO_4$, defoliation was observed in treated sweet orange plants. There was no impact of Di-potassium hydrogen phosphate (DPHP) on reduction of Las population which are in opposite to findings of Hu and his co-workers, where they recorded a positive impact on fruit yield and quality by reducing Las population and imparts induced resistance by trunk injections (Hu *et al*. 2017). With the results of our present study, we agree the statement of Yang *et al*. 2015 [23] and Gottwald *et al*. 2012 [12] that the foliar spray method doesn't give proper delivery of chemicals due to the thickness of the leaf cuticle, indicating as a barrier and Enhanced nutrition programs (ENPs) did not reduce disease progression in the experiments, trees continued to express systemic HLB symptoms. All these nutritional programs and antibiotic treatments are meant to minimise the deleterious effects of HLB Rather, and chemical control is considered an effective short-term strategy to combat this disease (Yang *et al*. 2018) [25]. The assumption for the use of micronutrient treatments is that HLB-affected trees could be maintained in a productive state for years, thus prolonging commercial viability.

Conclusion

In conclusion, periodical application of antibiotics could be a good measure in controlling HLB. Antibiotics such as Ampicillin and Rifampicin sprayed at 500 ppm in our study showed promising results in lowering the bacterial titre. However, the application of nutrient compounds such as K_2HPO_4, $MnSO_4$, and $CuSO_4$ did not influence the Las titre. To maintain the health of HLB-infected citrus trees with satiable yield, this study has been an attempt to know the impact of antibiotics and nutrients on the difference in bacterial titre and on the health of Citrus (HLB+) when applied in a monthly periodical. Detailed investigation on the effectiveness period of antibiotics and combinations at various concentrations could offer better results. Further research on the correlation between chemical treatments and the age of citrus trees draws a reliable approach to HLB management. To our knowledge, in the Southern part of India, this is the first study conducted on using qPCR to determine the cycle threshold (Ct) differences of *C*Las bacterium in antibiotic-treated citrus plants on an immediate application.

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References

1. Abdullah TL, Shokrollah H, Sijam K, Abdullah SNA. Control of Huanglongbing (HLB) disease with reference to its occurrence in Malaysia. African Journal of Biotechnology. 2009;8(17):4007-4015.
2. Belasque Jr J, Bassanezi RB, Yamamoto PT, Ayres AJ, Tachibana A, Violante AR, *et al*. Lessons from huanglongbing management in Sao Paulo State, Brazil. Journal of Plant Pathology 2010;92:285-302.
3. Bove JM. Huanglongbing: A destructive, newly-emerging, century-old disease of Citrus. Journal of Plant Pathology. 2006;88:7-37.
4. Bowen KL. Plant Pathology, Concepts and Laboratory Exercises. CRC Press, New York, 2004, 576.
5. Capoor SP, Rao DG, Viswanath SM. *Diaphorina citri* Kuwayama, a vector of the greening disease of Citrus in India. Indian Journal of Agricultural Sciences. 1967;37:572-576.
6. Duan YP, Zhou LJ, Hall DG, Li WB, Doddapaneni H, *et al.* Complete genome sequence of citrus huanglongbing bacterium, *Candidatus* Liberibacter asiaticus" obtained through Metagenomics. Molecular Plant-Microbe Interactions. 2009;22:1011-1020.
7. Giles F. Daring to be different. Fla. Grow. 2011;104(6):8-10.
8. Gopal K, Gopi V, Khayum Ahammed S, Narayana N, Prasad Babu G, Reddy BC. Molecular Diagnosis and Management of Greening Disease of Sweet orange in Andhra Pradesh, India Paper presented in XVI Conference of the International Organization of Citrus Virologists held at Monterrey, Mexico from, 2004 November 7-13th, 79.
9. Gopal K, Gopi V, Kalyani L, Sreelatha M, Sreenivasulu B. Symptom-based diagnosis of Huanglongbing (citrus greening) disease by PCR in sweet orange (*Citrus sinensis* Osbeck) and Acid lime (*Citrus aurantifolia* Swingle). Archives of Phytopathology and Plant Protection. 2010;43(9):863-870.
10. Gottwald TR, da Graca JV, Bassanezi RB. Citrus huanglongbing: the pathogen, its epidemiology, and impact. Plant Health Progress, 2007.
11. Gottwald TR. Current epidemiological understanding of Citrus Huanglongbing. Annual Review Phytopathology. 2010;48:119-139.
12. Gottwald TR, Graham JH, Irey MS, McCollum TG, Wood BW. Inconsequential effect of nutritional treatments on huanglongbing control, fruit quality, bacterial titre and disease progress. Crop Protection. 2012;36:73-82.
13. Harakava R, Morais LJ, Ochasou J, Manjuanth KL, Febres VJ, Lee, RF. In Proc 14th Conf IOCV, IOCV Riverside, California, 2000, 195-199.
14. Hu J, Jiang J, Wang N. Control of citrus huanglongbing (HLB) via trunk injection of plant activators and antibiotics. Phytopathology. 2017;108:186–195.
15. Husain MA, Nath D. The citrus psylla (*Diaphorina citri* Kuw.) [Psyllidae: Homoptera]. Mem. Dept. Agric. India, Entomologica Series. 1927;10:1-27.
16. Jagoueix S, Bove JM, Garnier M. The phloem limited bacterium of the citrus greening disease of Citrus is a member of the subdivision of the Proteobacteria. International Journal of Systematic Bacteriology. 1994;44:379-386.
17. Kranz J. Measuring plant disease. In: Rotem, J.K.A.J. (Ed.), Experimental Techniques in Plant Disease Epidemiology. Springer Verlag, Berlin, Heidelberg, New York, 1988, 35-50.
18. Li J, Pang Z, Duan S, Lee D, Kolbasov V, Wang N. The in planta effective concentration of Oxytetracycline against *Candidatus* Liberibacter asiaticus for suppression of citrus huanglongbing. Phytopathology. 2019;109:2046 2054.
19. Puttamuk T, Zhang S, Duan Y, Jantasorn A, Thaveechai N. Effect of chemical treatments on 'Candidatus Libeibacter asiaticus' infected pomelo (*Citrus maxima*). Crop Protection. 2014;65:114-121.
20. Sachin BS, Sharma SC, Sethi S, Tasduq SA, Tikoo MK. Herbal modulation of drug bioavailability: Enhancement of rifampicin levels in plasma by herbal products and a flavonoid glycoside derived from *Cuminum cyminum*. Phytotherapy Research. 2007;21:157-163.
21. Spratt B, Cromie K. Penicillin-binding proteins of gram-negative bacteria. Reviews of Infectious Diseases. 1988;10:699–711.
22. Wang N, Trivedi P. Citrus Huanglongbing: A Newly Relevant Disease Presents Unprecedented Challenges. Phytopathology Review. 2013;103(7):652-665.
23. Yang C, Powell CA, Duan Y, shatters R, Zhang M. Antimicrobial nanoemulsion formulation with improved penetration of foliar spray through citrus leaf cuticles to control citrus huanglongbing. Plos One. 2015;10(7):e0133826.
24. Yang C, Powell CA, Duan Y, Shatters RG, Lin Y, Zhang M. Mitigating citrus huanglongbing via effective application of antimicrobial compounds and thermotherapy. Crop Protection. 2016;84:150-158.
25. Yang C, Zhong Y, Powell CA, Doud MS, Duan Y, Huang Y, Zhang M. Antimicrobial Compounds Effective against *Candidatus* Liberibacter asiaticus Discovered via Graft-based Assay in Citrus. Scientific Reports. 2018;8:17288
26. Zhang M, Guo Y, Powell CA, Doud MS, Yang C. Effective antibiotics against '*Candidatus* Liberibacter asiaticus' in HLB-affected citrus plants identified via graft-based evaluation. PLoS ONE. 2014;9(11):e111032.
27. Zhang M, Powell CA, Guo Y, Doud MS, Duan Y. A graft-based chemotherapy method for screening effective molecules and rescuing huanglongbing-affected citrus plants. Phytopathology. 2012;102:567-574.
28. Zhang M, Powell CA, Lesley B, Zhou H, Duan Y. Deciphering the Bacterial Microbiome of Citrus Plants in Response to '*Candidatus* Liberibacter asiaticus'-Infection and Antibiotic Treatments. PLoS ONE. 2013;8(11):e76331.
29. Zhang M, Powell CA, Zhou L, Stover Ed, Duan Y. Chemical compounds effective against the citrus huanglongbing bacterium 'Candidatus Liberibacter asiaticus' in Plant. Journal of Bacteriology. 2011;101(9):1097-1103.