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Citrus budwood treatment with antibiotic and temperature to control *Candidatus Liberibacter asiaticus* transmission for huanglongbing management

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Abstract

Citrus trees infected by the *Candidatus* liberibacter asiaticus the causal organism of huanglongbing (HLB) either by its natural host *Diaphorina citri* kuwayama (Hemiptera: Psyllidae) or during budding/grafting process are difficult to treat in the field and green house and nurseries by cultural practices. In the controlled conditions of growth room, sweet orange budwood infected with *Candidatus* Liberibacter asiaticus were treated with tetracycline and temperature simultaneously in this study to eliminate the bacterium with the objective that the pathogen elimination from the budwood could be comparatively more effective. Tetracycline doses used were 250 ppm, 500 ppm, 750 ppm, 1000 ppm and 1250 ppm while a temperature was kept constant at 50 °C for all doses of tetracycline. Budwood source trees were confirmed for HLB bacterium existence by conventional PCR using 16S rDNA primers OI1/OI2C and rplKAJL-rpoBC operon primers A2/J5. Budsticks treated with the above mentioned doses of antibiotics were grafted on rough lemon plants. Real time PCR analysis results of plants grafted with treated budwood revealed no amplification in healthy control as well as 1000ppm tetracycline + 50°C treatment indicating no detectable titer in the said treatment. These results provide a hope in the darkness to save the citrus industry of the world.

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Introduction

Among biotic stresses to citrus industry, huanglongbing (HLB) is a high risk all around the world. Citrus trees affected by HLB produce unmarketable fruit with bitter taste and small size as it abscise prematurely and mostly die within 5 to 8 years (Baldwin *et al.*, 2010). Most of the scion varieties of citrus are susceptible to HLB. Citrus mandarins including: Kinnow, Sun Chu Sha, *Citrus sunki* and Parson's special also found positive for *Candidatus* Liberibacter asiaticus by Taqman based qPCR (Aslam *et al.*, 2017). Citrus HLB is caused by 2µm long Gram negative phloem limited bacterium called as *Candidatus* Liberibacter (Murray and Schleifer, 1994; Bove, 2006). The three recognized types of this bacterium are: *Candidatus* Liberibacter asiaticus, *Candidatus* Liberibacter africanus (Da Graca, 1991) and *Candidatus* Liberibacter americanus (Teixeira *et al.*, 2005). HLB causing bacteria are transmitted from diseased citrus plants to healthy plants by an insect known as citrus psyllid. *Diaphorina citri* (*D. citri*) Kuwayama (Hemiptera: Sternorryncha: Lividae) also known as Asian citrus psyllid (ACP) and *Trioza erytreae* (*T. erytreae*) (Del Guercio) (Hemiptera: Sternorrynca: Triozidae) also known as African citrus psyllid are reported to cause HLB (Aubert, 1987). HLB is a communicable disease as citrus cultivars are mostly propagated through budding/grafting. Millions of trees have been uprooted round the world to reduce the source of inoculums for natural vector of HLB pathogen (Hung *et al.*, 2000).

Spray and injections of different antibiotics at different doses for different time periods have been applied to eliminate the HLB bacterium since 1971 but complete cure for HLB could not be achieved (Nariani *et al.,* 1971; Zhang *et al.*, 2012). Heating of diseased budwood by water saturated hot air at 45°C for duration of six hours or 47°C for a time period of four hours has been practiced that suppressed the greening pathogen. Greening symptoms also found to disappear by placing HLB affected plants at 38°C or 40°C in a heat treatment chamber for duration of three weeks (Nariani *et al.,* 1975).

Citrus is the most important fruit crop of Pakistan. Citrus industry of Pakistan contributes for 19.8% GDP and provides employment to about 42% labor. Pakistan ranks at 14th position in the world with 1907.4 thousand tons per hectare of total citrus fruit production according to Food and Agriculture Organization of the United Nations (FAOSTAT, 2017). According to data compiled by the Pakistan Fruit and Vegetable exporters, importers and merchants Association (PFVA), $ 222 million have fetched from 370000 tons kinnow export in 2017-18 season. In our suspicion, HLB is one of the causes for Pakistan to take less production of citrus per hectare from its orchards as compared to other citrus growing countries. ACP is known to cause damage to citrus in Punjab region of Pakistan since 1927, so the presence of ACP in citrus orchards of Pakistan has been a more than hundred year story (Hussain and Nath, 1927; Yaqub *et al.*, 2017). Control of ACP is not so easy because of its alternate host plants. HLB has been diagnosed in ACP and citrus plants at molecular level in KPK and Punjab province of Pakistan (Chohan *et al.*, 2007; Yaqub *et al.*, 2017).

The present study was conducted with the objective of elimination of *Candidatus* Liberibacter asiaticus to prevent its transmission from HLB infected budwood by the use of antibiotics and heat treatment.

Material and methods

Selection and preparation of budwood for antibiotic + temperature treatment

Antibiotic with temperature was used in this experiment. Rough lemon (*Citrus jambhiri* Lush.) was used as an indicator plant. These plants were raised in the insect free screenhouse, and confirmed HLB negative before grafting. Budwood was treated with different doses of tetracycline antibiotic along with temperature and grafted on the indicator plant. Sweet orange (*Citrus sinensis*), cultivar succari trees were selected for budwood source and confirmed for HLB bacterium existence by conventional PCR before treatment by using 16s rDNA primers OI1/OI2C (Jagoueix *et al.*, 1996) and rplKAJL-rpoBC operon primers A2/J5 (Hocquellet *et al.,* 1999). Conventional

PCR amplification results showed 1160bp and 703bp bands for OI1/OI2C and A2/J5 respectively in HLB bacterium positive plant samples (Fig. 1).

Budwood containing approximately 4-6 buds per stick were prepared and washed with distilled water to remove dirt or any other material on the surface. Different concentrations of tetracycline solution as mentioned in Table 1 were prepared. Five bud sticks with leaves were kept in antibiotic solution and placed in preheated incubation oven in case of every treatment. Temperature was kept 50°C for all the treatments. Budsticks were T- grafted on rough lemon rootstocks immediately after heat treatment in parallel with control (untreated) buds.

After one week of grafting, plants and graft survival data collection was started. Leaves were sampled for DNA extraction and PCR analysis from indicator plants to test the effect of treatments on budwood for the elimination of HLB bacterium.

DNA extraction and PCR

The DNA was isolated from leaf midribs and petioles of treated and healthy indicator plants by CTAB method (2% CTAB, 1% Lauroyl sarcosine, 100 m*M* Tris HCl, 1.4 m*M* NaCl and 20 m*M* EDTA) as described by Yaqub *et al.* (2017). DNA used for qPCR was further purified by ZYMO RESEARCH DNA cleaning kit (ZR-96 DNA Clean & Concentrator™-5. Catalog No. D 4024) following manufacturer's instructions. Samples were taken from the indicator plants because we want to test the transmission from grafts if the treatment does not kill the bacteria. There were chances if samples be taken from the grafts may gave false positive results. Died bacteria in the grafts can give result by PCR.

Conventional PCR

Conventional PCR was performed using 16S rDNA primer OI1/OI2C and ribosomal protein gene specific for the detection of *Candidatus* Liberibacter asiaticus primer A2/J5 in treated and healthy samples (Table 2). A total volume of 25 µL was used in the PCR reaction mix. Amplification was carried out in a peqSTAR 96 universal gradient thermocycler with the following thermal profile: one cycle for initial denaturation at 94°C for 2 min; 35 cycles at 94°C for 30 sec, 58°C for 1 min and 72°C for 1 min; one cycle for final extension at 72°C for 10 min. The PCR products were analyzed by gel electrophoresis using 1% agarose in 0.5X TBE buffer.

Real-time PCR

Quantitative TaqMan PCR was conducted using 16S rDNA based TaqMan primer-probe set specific to *Candidatus* Liberibacter asiaticus. Plant cytochrome oxidase (COX) gene was used as a positive internal control to assess the quality of the DNA extracts. Sequences of primer-probe sets are given in Table 2.

The qPCR assays were performed using a Bio Rad iQ5 real time thermal cycler. For all qPCR reactions 25 µL volume was used. Thermal cycling conditions for qPCR were: initial denaturation at 95 ^{O}C for 3 min followed by 40 cycles of denaturation at 95 ^{O}C for 10 sec and annealing at 58 ^{O}C for 20 sec as described by Aslam *et al.* (2017 a &b).

Statistical analysis

iQ5 Optical System software version 2.1 was used for data analysis conditions including baseline and threshold.

Results and discussion

Evaluation of antibiotic + temperature treatment indicator plants

Data for graft survival collected up to twenty eight weeks after treatment because more than 90% of treated grafts had died till then. Up to twelve weeks after treatment, 74% grafts died of all treated grafts, 13.75% sprouted and 24% remained green and alive while, up to twenty eight weeks after treatment, 97% grafts died of all treated grafts, 13.75% sprouted and 13.75% remained green and alive (Fig. 2A & 2B).

In case of healthy controls, in which healthy grafts grafted on indicator plants, 100% grafts sprouted and remained alive. Moreover, no plant and graft died in healthy controls upto twenty eight weeks.

Table 1. Doses of Antibiotic + temperature for HLB infected bud wood treatment.

Sr.No.	Treatment	Replication	Dose	Duration (Minutes)
1	T1(Positive control)	5	Without water, tetracycline and temperature	0
2	T2 (water control)	5	Water + exposed to 50°C temperature	15
3	T3	5	250ppm tetracycline+ 50°C	15
4	T4	5	500 ppm tetracycline +50°C	15
5	T5	5	750 ppm tetracycline + 50°C	15
6	T6	5	1000 ppm tetracycline +50°C	15
7	T7	5	1250 ppm tetracycline +50°C	15

All indicator plants grafted by untreated budwood expressed typical blotchy mottle and vein yellowing symptoms after twenty eight weeks of grafting. Leaf texture in all treatments was rough. Severe leaf drop was also observed in all treatments except positive control (Fig. 3). Grafts may not survived because the above graft part of the rootstock was not pruned with the objective that more leaves remain available for symptom appearance and DNA extraction. Moreover, budsticks bearing 4-5 leaves on the grafts were treated with the objective to transpire with the antibiotic solution running in the whole graft to clean the budwood from HLB bacterium. Very high rate of grafts death in all of the treatments may be due to antibiotic phytotoxicity as described by Zhang *et al.* (2014, 2015).

Table 2. The primer/probe sequence for cPCR/qPCR studies.

Primer/Probe	Sequences	Target DNA	Orientation	Region of amplification	Reference
OI1	GCG CGT ATG CAA TAC GAG CGG CA	Las	Forward	16s ribosomal RNA	Jagoueix *et al.*, 1996
OI2c	GCC TCG CGA CTT CGC AAC CCA T	Las	Reverse	16s ribosomal RNA	Jagoueix *et al.*, 1996
A2	TAT AAA GGT TGA CCT TTC GAG TTT	Las	Forward	rplKAJL-rpoBC(β operon)	Hocquellet *et al.*,1999
J5	ACA AAA GCA GAA ATA GCA CGA ACA A	Las	Reverse	rplKAJL-rpoBC(β operon)	Hocquellet *et al.*,1999
HLB as f	GTC GAG CGC GTA TGC AAT AC	Las	Forward	16s ribosomal RNA	Li *et al.*, 2006
HLB as r	TGC GTT ATC CCG TAG AAA AAG GTA G	Las	Reverse	16s ribosomal RNA	Li *et al.*, 2006
cox f	GTA TGC CAC GTC GCA TTC CAG A	*Citrus* cox-1 gene	Forward	*Citrus* cox-1 gene	Li *et al.*, 2006
cox r	GCC AAA ACT GCT AAG GGC ATT C	*Citrus* cox-1 gene	Reverse	*Citrus* cox-1 gene	Li *et al.*, 2006
HLB as p	FAM- AGA CGG GTG AGT AAC GCG-BHQ1	Las probe	Forward	16s ribosomal RNA	Li *et al.*, 2006
cox p	JOE-ATC CAG ATG CTT ACG CTG G-BHQ2	*Citrus* cox-1 gene probe	Forward	*Citrus* cox-1 gene	Li *et al.*, 2006

Conventional PCR

As a result of multiplex conventional PCR two bands of 1160bp and 703bp by using OI1/OI2c and A2/J5 primers respectively confirmed the presence of *Candidatus* Liberibacter asiaticus in the sweet orange grafts source plant samples used for the thermo antibiotic treatment experiment for HLB management study (Fig.1).

When DNA from indicator plants was tested by singleplex conventional PCR using OI1/OI2c primers, amplicons of 1160bp were obtained in the product of positive controls, water controls, 250ppm with 50°C treatment and 500ppm with 50°C treatment. No amplicon was observed in rest of the treatments by conventional PCR (Fig. 4). From conventional PCR results it is concluded that 250ppm and 500 ppm doses of tetracycline along with 50°C temperature are not effective for cleaning HLB pathogen from graft while, 750 ppm and rest of doses used in the current study are effective against HLB bacterium elimination.

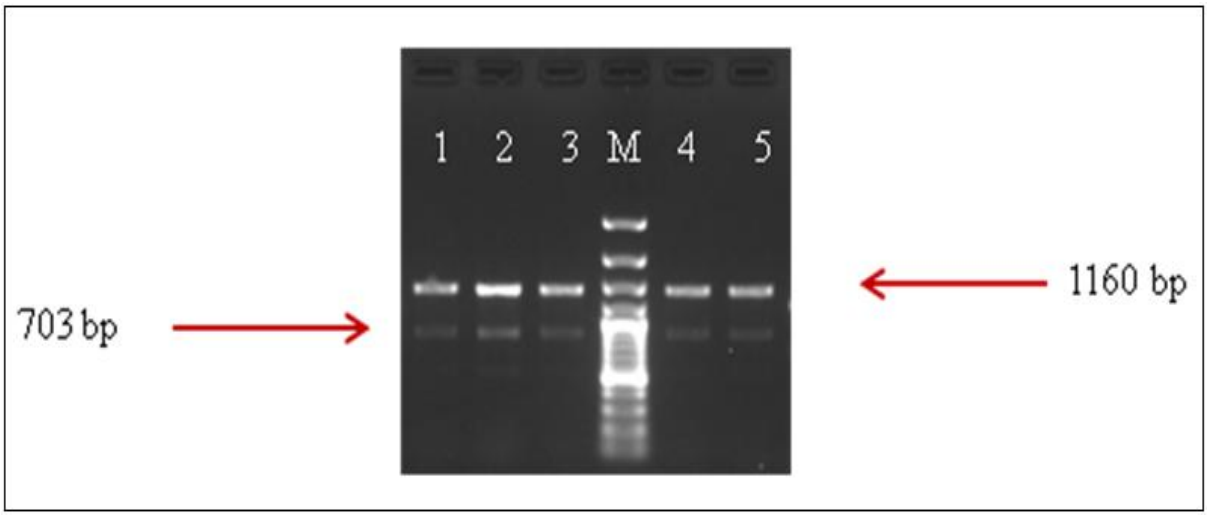

Fig. 1. Gel electrophoresis of HLB positive graft before antibiotic+temperature treatment. Electrophoresis on 1.2% agarose gel of DNA amplified with OI1/OI2c and A2/J5 primers. Lane M=100bp plus DNA size marker, Lane 1-5 samples from graft wood used for antibiotic+temperature treatment.

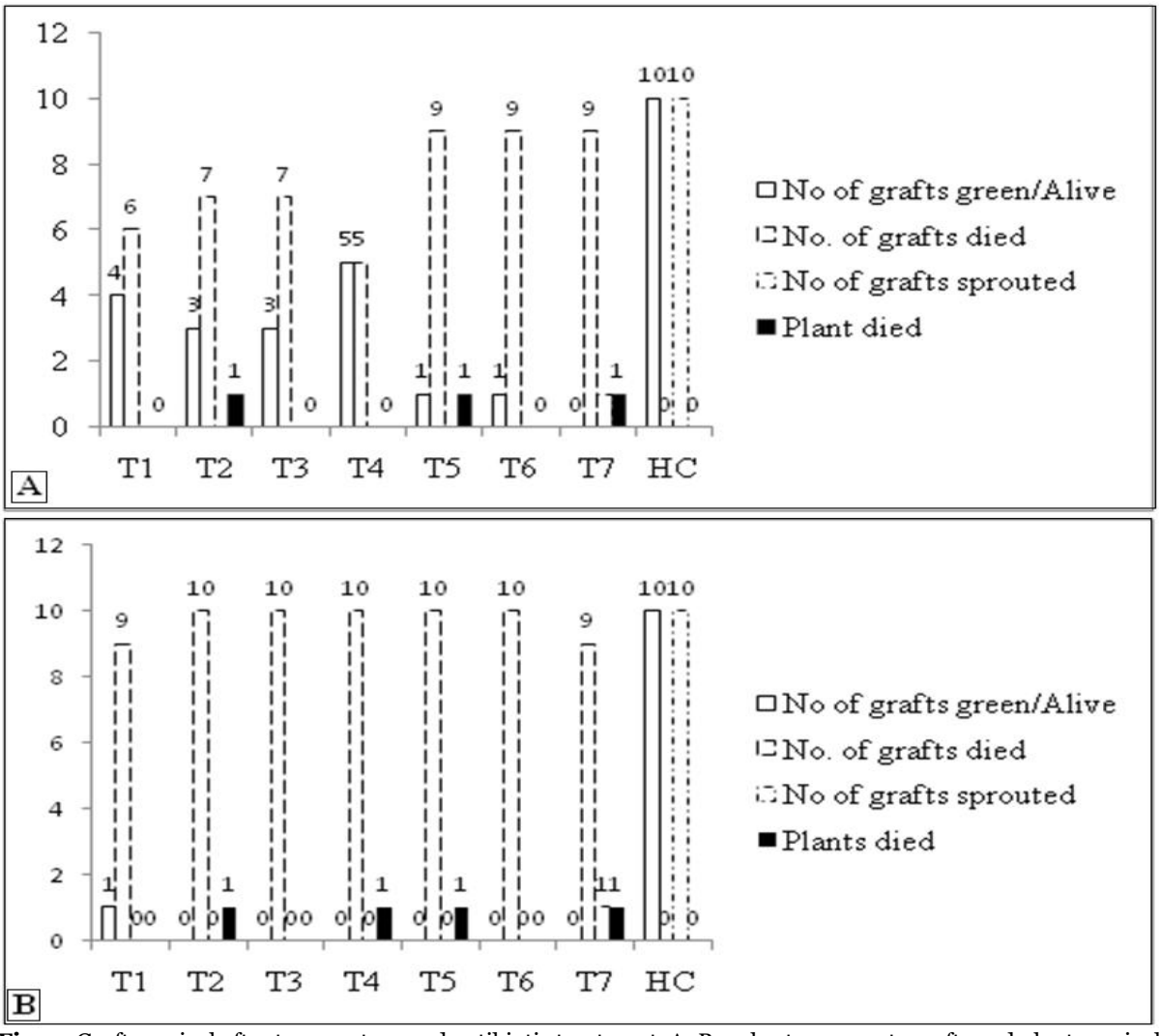

Fig. 2. Graft survival after temperature and antibiotic treatment. A: Bar chart represents grafts and plant survival data after treatment up to 12 weeks. B: Bar chart represents grafts and plant survival data after treatment up to 28 weeks.

In various studies on tetracyclines spray or shoot or trunk injection at 500 ppm dose, temporary recovery from huanglongbing with reappearance of HLB symptoms has been recorded (Nariani *et al.,* 1971; Nariani *et al.,* 1975). Injection of 1000µg/ml tetracycline in combination with 1000µg/ml $ZnSO_4$ resulted in 92 % control of HLB (Kapur *et al*., 1996). Our work was different from the previous studies because they injected or sprayed HLB infected plants with different doses of antibiotics while we used thermoantibiotic technique for HLB infected budwood to eliminate the bacteria by immersion in the tetracycline solutions.

Fig. 3. Tetracycline and temperature treated indicator plants (A to F): A= Indicator plant grafted with HLB positive graft without antibiotic, water, and temperature treatment. Twisted leaves with blotchy mottle symptom. Less leaf drop; B= Indicator plant grafted with HLB positive graft treated with distilled water only and subjected to 50 °C temperature. Severe leaf drop with new spouts; C = Indicator plant grafted with HLB positive graft treated with 250 ppm tetracycline solution and subjected to 50 °C temperature. Twisted leaves with blotchy mottle symptom are present on indicator plant.; D = Indicator plant with severe leaf drop having graft treated in 500 ppm tetracycline solution and 50 °C temperature; E = plant indicating severe leaf drop having very few mottled leaf with graft treated in 750 ppm tetracycline solution and temperature; F = Indicator plant having graft treated with1000 ppm tetracycline solution and temperature. A plenty of leaves present on indicator plant with slight twist in the leaves indicating no transmission of HLB causing bacterium from graft to indicator plant.

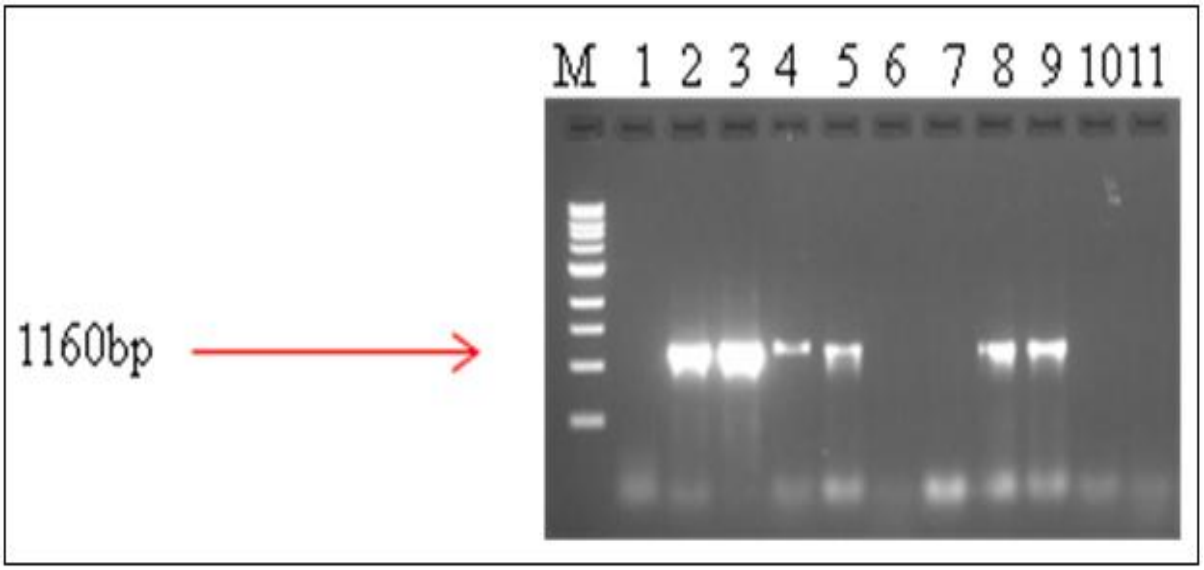

Fig. 4. Conventional PCR of tetracycline+temperature treatment plants: Electrophoresis on 1.2% agarose gel of DNA amplified with OI1/OI2c. M= 1kb DNA size marker (NEB), Lane 1= Negative control i.e., without template DNA, Lane2 and 3= Positive controls, Lane 4 ans5= water controls, Lane 6 ans7= healthy controls, lane 8= 250ppm tetracycline + temperature, lane 9 = 500ppm tetracycline + temperature, lane 10= 750ppm tetracycline + temperature and lane 11= 1000ppm tetracycline + temperature.

Real time PCR for antibiotic + temperature treatment indicator plants

In multiplex real time PCR, fluorogenic PCR assay primer-probe sets HLBas, HLBp, HLBr (HLBas fpr) and Coxf, Coxp and Coxr (Coxfpr) were used for detection of *Candidatus* Liberibacter asiaticus and internal control plant cytochrome oxidase (Cox) gene to assess the quality of DNA in the DNA samples of tetracycline + temperature treatment experiment. We considered the results positive for HLB pathogen if Ct values were 36.9 or less.

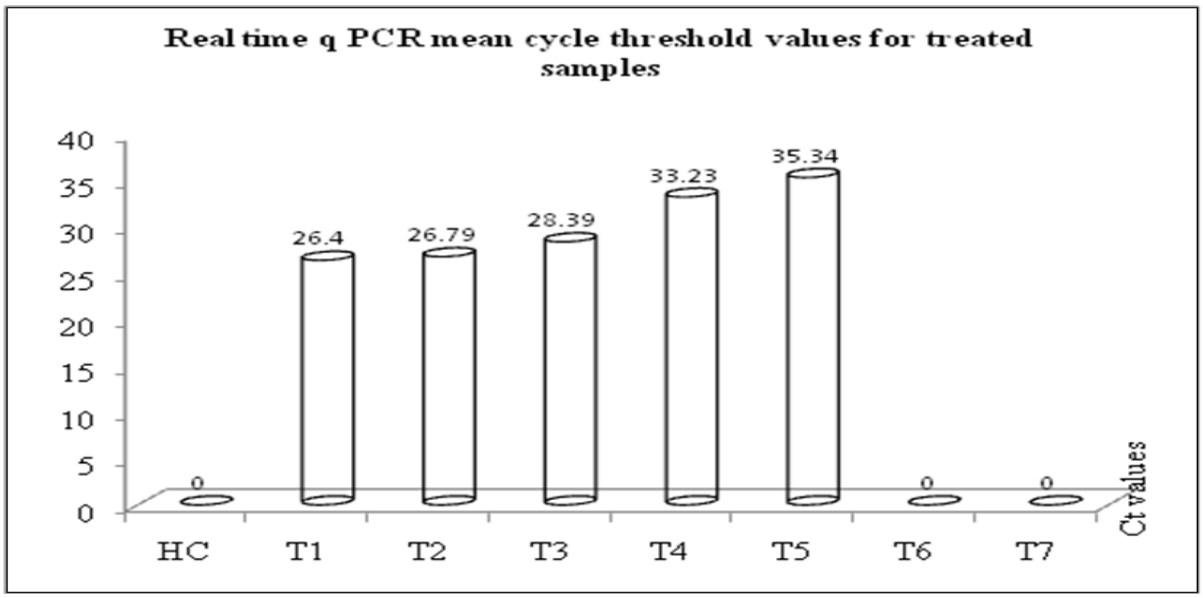

Fig. 5. Mean cycle threshold values from real time PCR analysis for tetracycline antibiotic and temperature treated samples to eliminate *Candidatus* Liberibacter asiaticus from HLB affected budwood. HC= healthy control, T1= positive controls, T2= water control treatment, T3=250ppm, T4= 500ppm, T5=750ppm, T6=1000ppm and T7=1250ppm.

The results were considered negative for HLB pathogen above 36.9 Ct value or no amplification (NA) in the treatments according to Hoffman *et al.* (2013). No amplification results indicate no detectable titer in the sample. Lowest mean cycle threshold (Ct) value of 26.17 for the pathogen was observed in the positive controls treatment.

Highest mean Ct value of 35.34 was observed for 750ppm+ 50°C treatment while no amplification was observed in healthy controls, 1000ppm+ 50°C and 1250ppm+ 50°C treatments (Fig. 5). No amplification results indicate that there was no detectable titer in the non-amplified treatments. Similar dose of 1000ppm tetracycline with different method and same results were presented by Kapur *et al.* (1996) and Zhang *et al.* (2012). Kapur *et al.* (1996) injected tetracycline with $ZnSO_4$ and. Zhang *et al.* (2012) used tetracycline without temperature whereas we used tetracycline with temperature. In different studies by Doud *et al.* (2012), temperature treatments ranging from 40 to 45°C for two to ten days given to citrus trees for *Candidatus* Liberibacter asiaticus control resulted in undetectable titer of Las.

Conclusion

It is concluded from real time PCR analysis results that, tetracycline at 1000 ppm and 50 °C temperature applied for 15 minutes were found potent to stop the transmission of *candidatus* Liberibacter asiaticus through budding/grafting. There is need to find more antibiotics and temperature ranges for different time periods that could also be better for the elimination of HLB bacterium and greater survival rate of grafts without causing phytotoxicity. Cleaning of budwood with antibiotic, temperature and some other means can be a hope for survival of the citrus industry if all the citrus plants in a grove become infected with *Candidatus* Liberibacter bacterium.

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