INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 17, No. 2, p. 72-82, 2020

RESEARCH PAPER OPEN ACCESS

Thermotherapy of citrus budwood to control *Candidatus* Liberibacter asiaticus transmission for huanglongbing management

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Key words: Budwood, Citrus, Disease, Greenhouse, Natural vector.

http://dx.doi.org/10.12692/ijb/17.2.72-82 Article published on August 18, 2020

Abstract

Citrus greening or huanglongbing (HLB) is the most destructive disease of the citrus. Citrus trees infected by phloem resident *Candidatus* liberibacter asiaticus, the causal organism of huanglongbing either by its natural host *Diaphorina citri* kuwayama (Hemiptera: Psyllidae) or during budding/grafting process are demanding to cure in the field, greenhouse and nurseries by cultural practices. Climate has a serious effect on the evolution of liberibacter species associated with citrus huanglongbing. Thermotherapy of citrus budwood was conducted in the current study for cleaning the huanglongbing positive budwood to control *Candidatus* Liberibacter asiaticus transmission for HLB management. Budwood was obtained from PCR tested HLB positive sweet orange trees and subjected to temperature treatment ranging from 56 °C to 68 °C. Temperature treated budwood was then grafted on *citrus jambhiri* lush rootstocks. Real-time PCR assays revealed that thermotherapy at 68 °C is effective to inhibit the transmission of *Candidatus* Liberibacter asiaticus from budwood to rootstock.

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Introduction

Citrus greening the huanglongbing (HLB) is a challenge for citrus industry around the world to maintain a productive crop (Bove, 2006). Citrus fruits are outstanding source of vitamin C that produces immunity against diseases in human body. Citrus trees infected with HLB produce unsalable fruit with unsweat taste and small size as it abscise prematurely (Baldwin *et al*., 2010). HLB can affect all species and hybrids of Citrus. It is caused by three species of phloem resident α- proteobacteria i.e. *Candidatus* Liberibacter asiaticus (Las), *Candidatus* Liberibacter americanus (Lam) and *Candidatus* Liberibacter africanus (Laf) (Jaguiex *et al.,* 1994; Texeira *et al*., 2005). The natural vectors for the transmission of this disease in the field are the psyllid species *Diaphorina citri* Kuwayama (Hemiptera: Sternorryncha: Liviidae) for *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter americanus in Asia and Americas and *Trioza erytreae* (del Guercio) (Hemiptera: Sternorryncha: Triozidae) for *Candidatus* Liberibacter africanus in Africa (Graca and Korsten, 2004). Huanglongbing is also transmitted through budding grafting in the propagating nurseries (Lin, 1956; Aslam *et al.,* 2017a). The Asian form, *Candidatus* Liberibacter asiaticus is heat tolerant and can show symptoms above 30°C whereas the African form *Candidatus* Liberibacter africanus is heat sensitive (Garnier *et al.,* 2000). *Candidatus* Liberibacter asiaticus show symptoms in both cool and warm (22 to 32°C) environment.

In the beginning of HLB symptoms development, leaves of citrus plants exhibit yellow and blotchy mottle style followed by the development of chlorosis; trees express stunted growth; and fruit become lopsided and inedible and drop pre-maturely.

In 2 to 3 years trees move towards decline, leading to the eventual death of the tree in 5 to 8 years (Baldwin *et al*., 2010; Halbert and Manjunath, 2004). Populations of D. citri have been known to present in Asia for more than hundred years (Hussain and Nath, 1927; Yaqub *et al*., 2017).

Control of this disease is very difficult because *D. citri* has a broad host range within the Aurantioideae and may even use Rutaceae species outside the orange subfamily as a food source (Westbrook *et al*., 2011). All three HLB-causing Liberibacter species are uncultured. Unculture able nature of these bacteria is increasing the difficulty in designing an effective management strategy of HLB. However, significant progress towards understanding this disease complex has been made since last decade.

Various techniques have been explored to save HLB affected citrus including antibiotics (Zhang *et. al*., 2014; Yaqub *et al*., 2019). Previous research indicated that heat therapy could be used to control HLB caused by Las. Yellow shoot diseased citrus budwood treated with hot water and with water vapour saturated hot air in different treatments revealed hot air temperature of 48°C to 58°C best for getting virus free propagation material (Lin, 1964). Greening symptoms disappear by placing HLB affected plants at 38°C or 40°C in a heat treatment chamber for a duration of three weeks (Nariani *et al.,* 1975). In other studies, temperature treatments i.e. 40, 42 and 45°C for two to ten days were given to citrus trees for Las control. As a result, Las titer significantly decreased and reached to a level that is not detectable. Thermal treatments of continuous 40 to 42°C for 48 hours were found effective to eliminate Las from potted plants (Doud *et al.,* 2012; Hoffman *et al.,* 2013). Solar thermotherapy of HLB infected citrus trees for 3 to 6 weeks using portable plastic enclosure in the field resulted in the decrease of Las titer but not complete elimination (Doud *et al*., 2017).

In Pakistan, temperature reaches up to 50°C at day time in summers that is the natural source of thermotherapy for HLB infected trees in the field; despite exposure to high temperature Las population do not decline in the field. In this way plant life may be increased little bit but still that is not the solution. To save the precious scion and rootstocks there should be some strategies. For this purpose, present work was started with the objective to kill the bacterium from the infected budwood and prevent its

transmission to the rootstock. For this purpose high temperature treatments were applied to the HLB infected budwood for testing which temperature treatment is better for eliminating bacteria from the budwood. Las titer was measured using Taqman based quatitative real time PCR (qPCR).

Materials and methods

Rough lemon (*Citrus jambhiri* Lush.) was used as an indicator plant. These seedlings were raised in the insect free screenhouse and confirmed huanglngbing negative by conventional PCR before grafting.

Selection, preparation and temperature treatment of budwood

Budwood was taken from HLB positive sweet orange (*Citrus sinensis*) cultivar succari plants for temperature treatment. Positivity of sweet orange plants before acquisition of grafts for thermotherapy was confirmed by conventional PCR using Las specific 16s rDNA primers OI1/OI2C and *rpl*KAJL-*rpo*BC operon primers A2/J5 for amplification (Table1). As a result of multiplex conventional PCR, bands of 1160bp and 703bp for OI1/OI2c and A2/J5 primers respectively (Hocquellet *et al.,* 1999) confirmed the presence of *Candidatus* liberibacter asiaticus in the trees used in the temperature treatment experiment for HLB management study (Fig.1).

Budwood containing approximately 4-6 buds per stick was prepared for the treatment and washed with distilled water to remove dirt or any other material on the surface. The PCR tested HLB positive grafts were treated with different doses of temperature for 15 minutes (Table 2) to clean them from Las and grafted on to the indicator plants. Each treatment replicated five times having two treated grafts on each indicator plant. For thermotherapy, five bud sticks were kept in the 50 mL falcon tubes, sealed with parafilm and immersed in water bath for specified time and temperature per treatment. Grafts treated with different doses of temperature grafted on to the indicator plant immediately after heat treatment in parallel with control (untreated) buds. Grafting success was confirmed by graft survival of healthy control. Grafts survival and death data along with the grafts sprouting and indicator plant's death was recorded upto 30 weeks after temperature treatment (Fig. 2A, 2B & 2C).

DNA extraction and PCR

Leaf samples were collected for DNA extraction and PCR analysis from indicator plants to test the effect of treatments on budwood for the elimination of Las. DNA for conventional PCR was extracted after 30 weeks of treatment; whereas, for quantitative real time PCR it was extracted after 48 weeks.

The DNA was isolated from leaf midribs 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

Singleplex and multiplex 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. A total volume of 25 µL was used in the PCR reaction mix. Amplification was carried out in a peqSTAR 96 universal gradient thermal cycler 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 (Li *et al.*, 2006). Sequences of primer-probe sets are given in Table 1. 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 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 sec and annealing at 58 °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 temperature treated indicator plants

Graft survival data was collected up to thirty weeks after treatment as up to 90% of treated grafts had died till then. Up to twelve weeks after treatment, 73.3% grafts died of all treated grafts, only 5% sprouted and 21.66% remained green and alive while, up to thirty weeks after treatment, 90% grafts died of all treated grafts, 3.33% sprouted and 10% remained green and alive. 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 thirty weeks.

If we see the individual treatments upto twelve weeks, 40% grafts in positive control (T1), 70% in 56 °C (T2) and 20 % in 59 °C (T3) remained alive.

Table 1. 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

All grafts in treatments i.e. 62°C (T4), 65°C (T5), and 68°C (T6) died before 12 weeks. Up to 30 weeks after treatment only 10% grafts in positive control (T1), 30% in 56°C (T2) and 20% in 59°C (T3) remained alive whereas100% grafts in 62°C (T4), 65°C (T5) and 68°C (T6) died (Fig. 3A & 3B).

Most of the indicator plants grafted by untreated budwood expressed blotchy mottle and vein yellowing symptoms after thirty weeks of grafting. Leaf texture in all treatments was rough. Moderate leaf drop was also observed in all treatments except positive control (Fig. 2C). 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. At day for some time in the field temperature reached up to 50°C but plants not cleaned of HLB due to that

temperature so we use higher temperature in our study. Graft survival data shows that 62 °C to 68°C are not favorable for graft survival. Most of the other scientists used different temperature ranges and durations. Other workers (Lin, 1964; Nariani *et al.,* 1975; Huang, 1978; Doud *et al.,* 2012; Hoffman *et al.*, 2013) also used temperature treatments for huanglongbing control but their method of treatment was different and they also used low treatment 30°C to 58°C but we used high temperature 56°C to 68°C and we obtain negative result at high temperature but this treatment showed bad effect on graft survival.

Table 2. Temperatures and duration of treatment for thermotherapy of HLB positive citrus budwood.

Sr.No.	Treatment	Replication	Temperature (degree Celsius)	Duration (Minutes)
1	Healthy control/negative control	5	Without temperature	0
2	T1/ Positive control	5	Without temperature	0
3	T2	5	56°C	15
4	T3	5	59°C	15
5	T4	5	62°C	15
6	T5	5	65°C	15
7	T6	5	68°C	15

Conventional PCR

Samples were taken from the indicator plants because we want to test the transmission from grafts if the treatments do 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. DNA from died plants was not extracted.

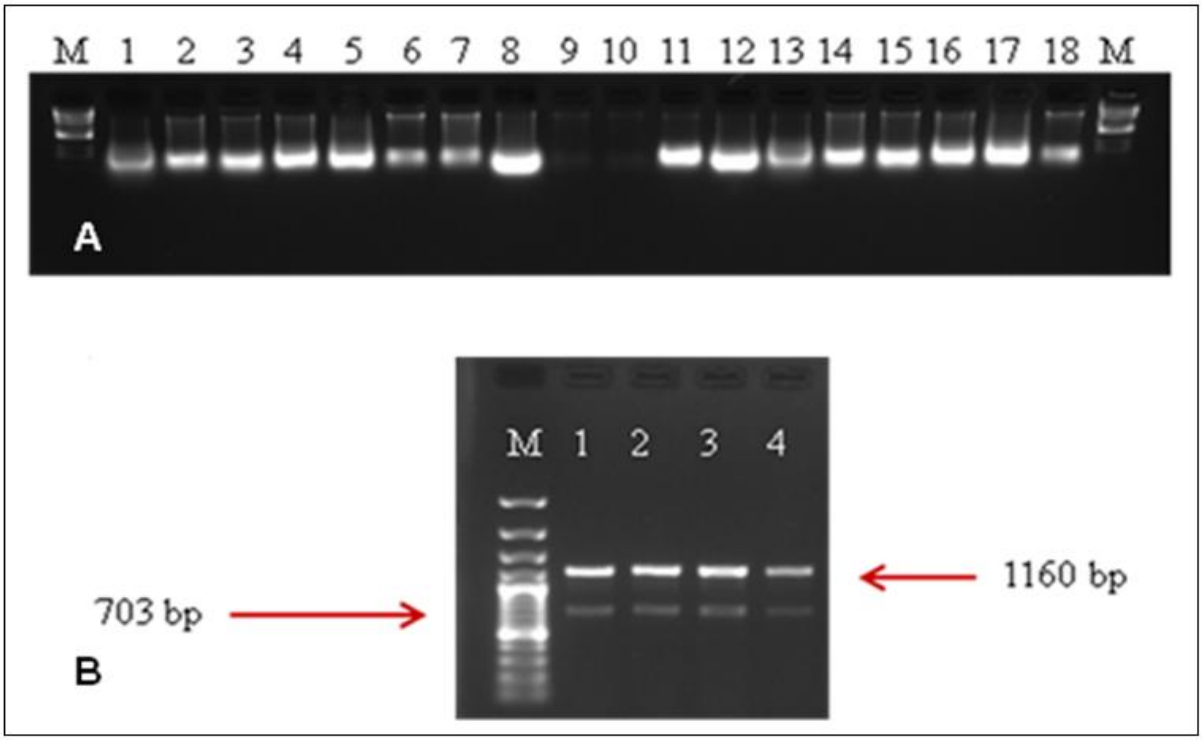

Fig. 1. Gel electrophoresis for DNA quantification and PCR product for HLB positive samples before temperature treatment: **A,** Gel **e**lectrophoresis for DNA on 1.2% agarose gel. Lane M= Lambda DNA (NEB), Lane 1-18 genomic DNA of indicator plants grafted with temperature treated grafts; **B,** Electrophoresis of PCR product for HLB positive samples before temperature treatment on 1.2% agarose gel of DNA amplified with OI1/OI2c and A2/J5 primers. Lane M=100bp plus DNA size marker, Lane 1-4 samples from source trees of grafts used for temperature treatment.

Because the plant death may be due to media, irrigation etc that needs further experiments. Singleplex conventional PCR using OI1/OI2c primers produced amplicons of 1160bp specific for Las 16SrDNA in the product of treatments: T1P4, T1P5, T2P1, T3P4, T4P2 and T4P4 as shown in lane 3, 4, 5, 6, 9 and 10 of Fig. 4 respectively. No amplicon was observed in rest of the treatments by conventional PCR. From conventional PCR results it is concluded that 56°C, 59°C and 62°C temperature is not effective for cleaning HLB pathogen from graft. For further testing q PCR was also performed. In different studies, 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 (Doud *et al.*, 2012).

Fig. 2. Rough lemon (*Citrus jambhiri* Lush) indicator plants used as a rootstock for thermotherapied HLB infected grafts: A, Indicator plants having grafts after temperature treatment in the controlled environment of the greenhouse; B, Sprouted graft; C, Indicator plants with symptomatic leaves having blotchy mottle, leaf yellowing and leaf drop symptoms after inoculation of HLB bacterium from the thermotherapied grafts not succeeded in Las elimination.

Thermotherapy is environment friendly, does not require a regulatory permit, is suitable for both organic and conventional farming, and can be quickly adopted by growers (Doud *et al.,* 2017). Our work is different from the previous studies in a way that temperature was used to treat HLB infected trees either in the fields or to the potted plants (Doud *et al.*, 2012; Hoffman *et al.*, 2013) but here in the current study we used infected grafts to eliminate the HLB causing bacteria.

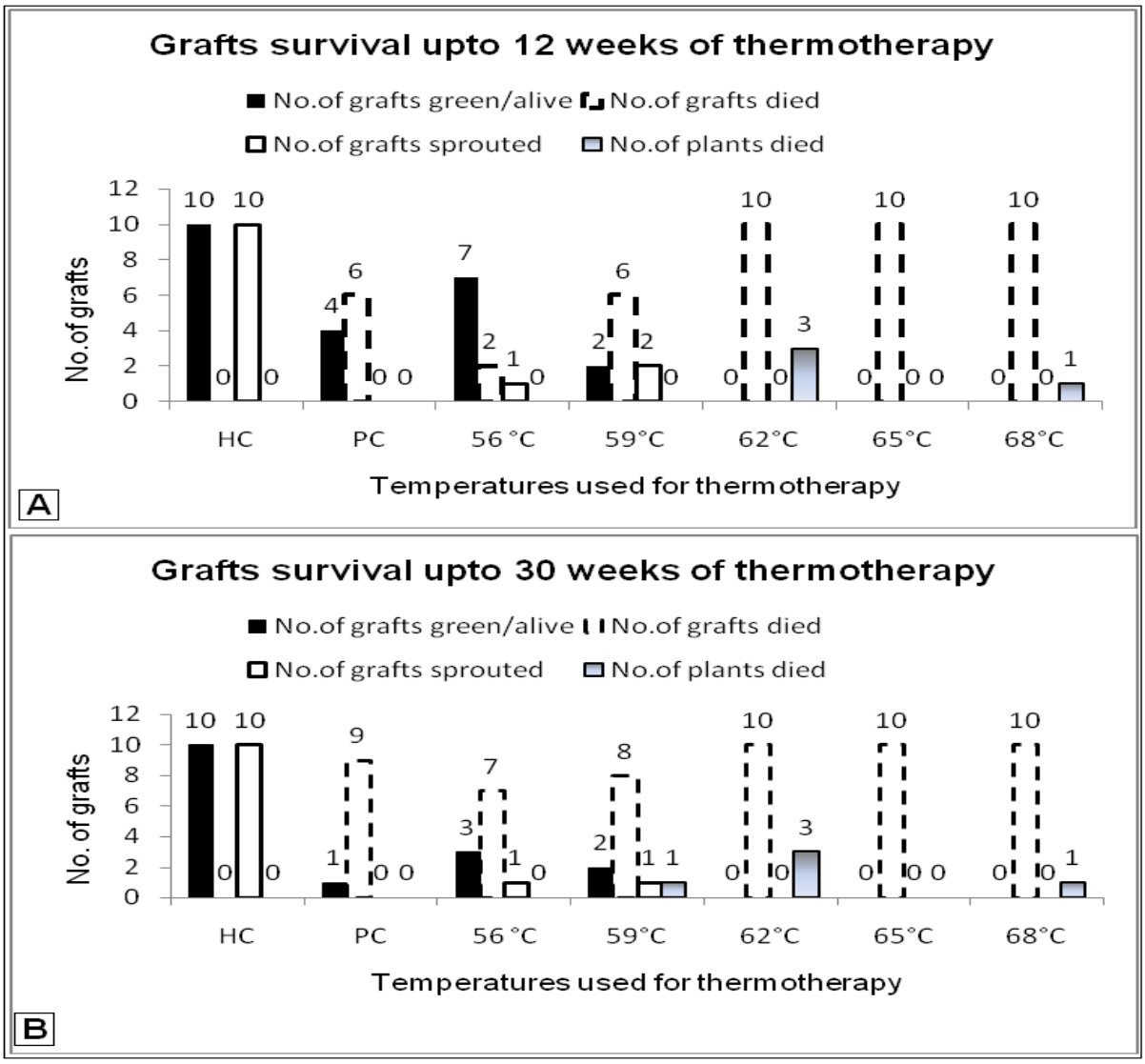

Fig. 3. Graft survival after temperature treatment of HLB affected budwood: **A,** Graft survival up to 12 weeks after thermotherapy; **B,** Graft survival up to 30 weeks after thermotherapy.

Real time PCR for temperature treatment indicator plants

In multiplex real time PCR, Taqman primer-probe sets HLBas, HLBp, HLBr (HLBas fpr) and Coxf, Coxp and Coxr (Coxfpr) (Li *et al.*, 2006) were used for detection of *Candidatus* Liberibacter asiaticus and internal control plant cytochrome oxidase (Cox) gene to assess the quality of DNA in the samples of temperature treatment experiment. HLBasfpr probe was labeled with FAM and Coxfpr with CY3. Statplus 2009 software was used for the data analysis. We consider the results positive for HLB pathogen if Ct values were observed up to or less than 36.9. We considered the results negative for HLB pathogen if Ct values were observed above 36.9 or no amplification (NA) in the treatments. Lowest mean cycle threshold (Ct) value (26.39) for the pathogen was observed in the positive control treatment.

Highest mean Ct value (35.21) was observed at 65°C temperature while no amplification was observed in healthy control and 68°C treatments (Fig. 5). No amplification results indicate that there was no detectable titre in the non-amplified treatments. At day for some time in the field, temperature reached up to 50°C in Pakistan but plants not cleaned of HLB due to that temperature so we used higher temperature in our study. Up to 65°C HLB pathogen did not eliminate from the grafts as revealed by real time qPCR results.

As no amplification was resulted at 68°C temperature, it is concluded that at this temperature bacteria can be eliminated from the infected grafts for further transmission during budding grafting.

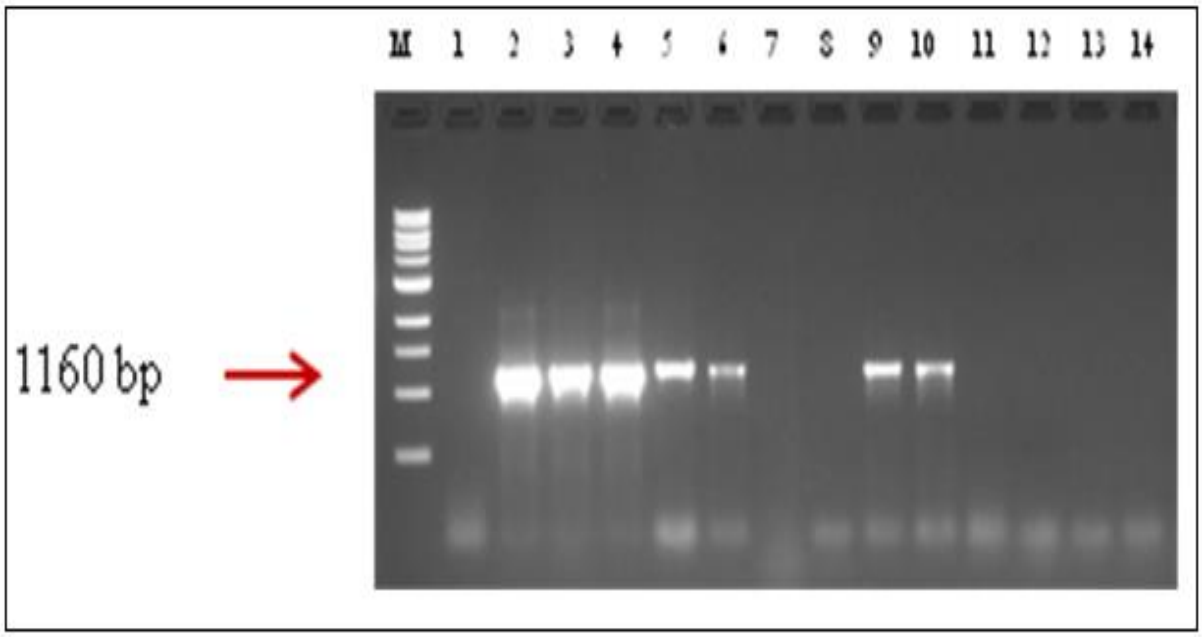

Fig. 4. Conventional PCR of temperature treatment experiment. Electrophoresis on 1.2% agarose gel of DNA amplified with OI1/OI2c. Lane M= 1kb DNA Ladder (NEB), Lane 1= negative control, Lane2= positive control, Lane 3-14= Temperature treatment indicator plants samples.

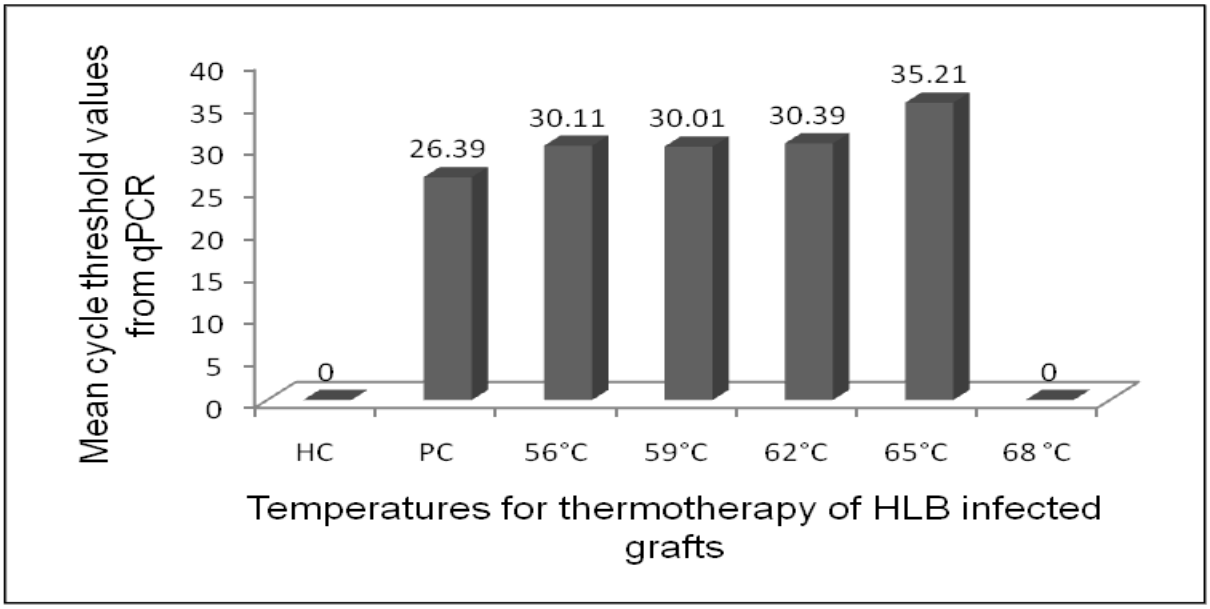

Fig. 5. Mean Ct values for thermotherapy of citrus budwood to eliminate *Candidatus* Liberibacter asiaticus from HLB affected grafts.

It can be further suggested that if 68 °C temperature is achieved in the field trees in and outside of the canopy by some means, Las can be controlled and plants life and fruit productivity may be enhanced.

Conclusion

The current study was conducted to test the effect of temperature to clean HLB infected budwood from Liberibacter. From conventional PCR results, it is concluded that 56°C, 59°C and 62°C temperature is not effective for cleaning HLB pathogen from the graft. Further testing by q PCR revealed the highest mean Ct value (35.21) at 65°C temperature while no amplification was observed in healthy control and 68°C treatment. We used higher temperatures in our study because the temperature in the field reaches above 45 °C in the fields of Pakistan but that temperature does not control HLB due to temperature difference in and outside the canopy.

At the day for some time in the field, the temperature reached up to 50°C but plants not cleaned of HLB due to that temperature. As no amplification has resulted at 68°C temperature, it is concluded that at this temperature bacteria can be eliminated from the infected grafts for further transmission during budding grafting. It can be further suggested that if 68 °C temperature is achieved in the field trees in and outside of the canopy by some means, Las can be controlled and plant life and fruit productivity may be enhanced. Graft survival data shows that 62 °C to 68°C is not favorable for graft survival, there is a need to work further on graft survival.

Acknowledgments

This study was supported financially by Higher Education Commission (HEC) of Pakistan and Pakistan-U.S. Science and Technology Cooperation Program for the Project "Management of greening by producing healthy plants, monitoring vectors and identification of tolerance". We are thankful to Dr. Mikeal L. Roose and Clair Thomas Federici (Department of Botany and Plant Sciences, UCR, USA) for their help in the molecular studies and review of this manuscript. We also acknowledge the management of the Centre of Agricultural Biochemistry and Biotechnology (CABB) UAF, for providing the facility for initial molecular study.

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