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Antibiotic treatment of citrus budwood for the management of huanglongbing disease

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Abstract

Control of huanglongbing (HLB) disease is necessary around the world to avoid complete destruction of citrus. Antibiotics were used by many scientists in the form of sprays and trunk injections to control HLB, but the control was not effective for longer time and disease symptoms appeared again after some time because of uneven distribution of pathogen in the host plant. Tetracycline and penicillin antibiotics were used in this study to eliminate *Candidatus* Liberibacter bacterium from sweet orangebudwood with the objective that the pathogen elimination from the budwood could be comparatively more effective. Tetracycline doses used were 250ppm, 500ppm, 750ppm, 1000ppm and 1250ppm. Penicillin doses were 25ppm, 50ppm, 75ppm, 100ppm and 125ppm. Kinnow mandarin (*Citrus reticulata* Blanco) nurtured in screen house were used as indicator plant. These plants were confirmedHLB negative before grafting. Budwood was obtained from HLB positive plants confirmed 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 the indicator plants. Tetracycline at 1000 ppm with mean Ct value of 38.40 and penicillin antibiotic at 75ppm dose with mean Ct value of 38.61 were found effective to eliminate *Candidatus* Liberibacter asiaticus from budwood to stop further spread of the disease through budding/grafting.

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Introduction

Citrus greening or huanglongbing (HLB) is the most ruinous disease of citrus (Hall *et al.*, 2012). *Candidatus* Liberibacter, a Gram negative bacterium, is the causal organism of HLB (Li *et al.,* 2012). There are three isolate types of the bacterium: *Candidatus* Liberibacter asiaticus (Las), *Candidatus* Liberibacter africanus (Laf) (Da Graca, 1991) and *Candidatus* Liberibacter americanus (Lam) (Teixeira *et al.,* 2005). There are two species ofinsect vector;*Diaphorina citri* Kuwayama (Hemiptera: Sternorryncha: Lividae) and *Trioza erytreae* Del Guercio (Hemiptera: Sternorrynca: Triozidae) reported in case of citrus greening (Aubert, 1978). The Asian citrus psyllid (ACP) (*Diaphorina citri* Kuwayama) can transmit both *Candidatus* Liberibacter asiaticus (Las) and *Candidatus* Liberibacter americanus (Lam). The most abundant bacterium species among huanglongbing infected trees is Las (Jagoueix *et al.,* 1994; Teixeira *et al.,* 2005; Bove, 2006) that can also be transmitted by grafting (Lin, 1956). Husain and Nath (1927) described severe damage caused by populations of ACP at Sargodha from 1915 to 1920. At present, ACP population is very high in the citrus orchards of Pakistan that has been present in Pakistan (Indo-Pak subcontinent) for 100 years (Yaqub *et al.,* 2017). In Pakistan, the occurrence of HLB was reported on the basis of disease symptoms in the citrus collection of Peshawar experimental station(Cochran, 1976). Gene expression data analysis results in response of HLB proved that sweet orange is a good indicator of HLB symptoms and more susceptible as compared to kinnow (Aslam *et al.,* 2017a).

Diagnosis of disease leading to the detection of causal agent is basic step for the management of disease. Huanglongbing was diagnosed first time by conventional PCR in 2007 from leaf and insect samples of Khyber Pakhtoon Khawa citrus orchards (formerly NWFP) of Pakistan (Chohan *et al.,* 2007). In four genotypes of citrus belonging to mandarin group including: *Citrus sunki*, Kinnow, Parson's Special and Sun Chu Sha;*Candidatus* Liberibacter asiaticus was detected by Taqman based real time qPCR for the development of HLB management plansin Pakistan (Aslam *et al.*, 2017b).

Different strategies have been adopted to control HLB including: biological control of vector, eliminating vector population by the use of pesticides and removal of diseased trees to reduce inoculum source. Disease control by thermotherapy and antibiotics is also being practiced. Spray of three tetracyclines: auromycin, ledermycin and achromycin at 500 ppm dose in the glasshouse and on field plants for eight weeks resulted in a temporary recovery from huanglongbing, but later the greening symptoms reappeared (Nariani *et al.,*1971; Nariani *et al.,*1975). Good results were achieved by injecting tetracycline HCl (1000µg/mL) in combination with $ZnSO_4$ (Kapur *et al.,* 1996). In South Africa 140% increase in production has been recorded by the use of tetracycline injections. Liberibacter infected periwinkle cuttings treated with 50 µg/mL penicillin G sodium presented HLB negative results by both qRT PCR and nested PCR (Zhang *et al.,* 2010). Penicillin and streptomycin combination has been found to be effective for the elimination of *Candidatus* Liberibacter asiaticus bacterium in infected citrus. Injection to six year old diseased trees with 100 mL of 5.0g/tree penicillin was also found to be effective (Zhang *et al.,* 2012).

The objective of present study wasthe elimination of *Candidatus* Liberibacter asiaticus from HLB infected budwood by the use of antibiotics for HLB management to save the citrus industry of the world.

Materials and methods

Antibiotic treatment of citrus budwood

Budwood was collected from Liberibacter positive Succari sweet orange (*Citrus sinensis* (L.) Osbeck) trees with HLB symptomatic leaves. Conventional PCR was performed to confirm the presence of Las in the budwood source trees before antibiotic treatment. 16S rDNA primers OI1/OI2C (Jagoueix *et al.,* 1996) and rplKAJL-rpoBC operon (β operon) primers A2/J5 (Hocquellet *et al.,* 1999) were used for bacterial DNA amplification. Citrus stems containing 4-6 buds per stick prepared for antibiotic treatment. Budsticks

were submersed in the antibiotic solutions for 4 h for treatment. Budsticks treated with different doses of tetracycline (Table 1) and penicillin (Table 2) antibiotics were grafted on Kinnow indicator plants immediately after treatment. Five plants for each dose of antibiotictreated budstickwere grafted. Two budsticks were grafted on each replicate plant. For controls, HLB positive budwood without antibiotic treatment, positive budwood dipped only in water and healthy budwood taken from PCR confirmed Liberibacter negative plants were also grafted on indicator plants.

Leaves from indicator plants were sampled for DNA extraction to test the effect of tetracycline and penicillin treatment. DNA for conventional PCR was extracted after 24 weeks of treatment; whereas, for quantitative real time PCR it was extracted after 48 weeks.

Plant's DNA extraction and conventional PCR

TheDNA was isolated from leaf midribs and petioles of treated and healthy indicator plants by cetyltrimethylammonium bromide (2% CTAB, 1% Lauroyl sarcosine, 100m*M* Tris HCl, 1.4m*M* NaCl and 20m*M* EDTA) method 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. D4024) followingmanufacturer'sinstructions.

Singleplex and duplex conventional PCR were performed using 16S rDNA primer OI1/OI2c (5´GCGCGTATGCAATACGAGCGGCA3´ / 5´GCCTCGCGACTTCGC AACCCAT3´) and A2/J5:(5´TATAAAGGTTGACCTTTCGAGTTT3´/5´A CAAAAGCAGAAATAGCACGAACAA3´) for ribosomal protein gene were used for the detection of Liberibacter 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 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 from Li *et al.* (2006). Primer - probe sequence specific to *Candidatus* Liberibacter asiaticus were: HLB asf, 5´GTC GAG CGC GTA TGC AAT AC 3´; HLB asr, 5´TGC GTT ATC CCG TAG AAA AAG GTA G3´ and HLB asp,FAM- AGA CGG GTG AGT AAC GCG-BHQ1.Plant cytochrome oxidase (COX) gene was used as a positive internal control to assess the quality of the DNA extracts. Sequence of primer-probe set was: cox f, 5´GTA TGC CAC GTC GCA TTC CAG A3´; cox r, 5´GCC AAA ACT GCT AAG GGC ATT C3´ andcox p,JOE-ATC CAG ATG CTT ACG CTG G-BHQ2. 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). We considered the results positive for HLB pathogen if Ct values were 36.9 or less. 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 that there was no detectable titre in the sample.

Statistical analysis

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

Results

Evaluation of antibiotic treated grafts

After 13 weeks following tetracycline treatment graft survival was 40 % in without antibiotic or water treatment, 250ppm and 1000ppm treatments; 20% in infected grafts only dipped in distilled water; 60% in 500ppm, 50% in 750ppm and 1250ppm treatments;

whereas, 100% in healthy controls. No graft was sprouted in without antibiotic or water and infected grafts only dipped in distilled water treatment. In 250ppm, 500ppm and 750ppmtreatments; 10%, 20% and 30% grafts were sprouted respectively. In 1000ppm and 1250ppm no graft sprout was observed. In healthy control 100% grafts were sprouted (Fig. 1A).

Table 1. Tetracycline doses for treatment of HLB positive budwood.

Treatment	Plant replicates grafted	Tetracycline doses
Healthy control (HC)	5	Nil
HLB positive control	5	Without antibiotic or water treatment
Water control	5	Grafts immersed in distilled water
T1	5	250 ppm
T2	5	500 ppm
T3	5	750 ppm
T4	5	1000 ppm
T5	5	1250 ppm

After 29 weeks of tetracycline treatment, graft survival was 10% in without antibiotic or water treated grafts, infected grafts immersed only in distilled water, 250ppm, 500ppm, and 750ppmtreatments while 20% in 1000ppm treatment.

Table 2. Penicillin doses for treatment of HLB positive budwood.

Treatment	Plant replicates grafted	Penicillin doses
Healthy control (HC)	5	Nil
HLB positive control	5	Without antibiotic or water treatment
Water control	5	Grafts dipped in distilled water
T1	5	25 ppm
T2	5	50 ppm
T3	5	75 ppm
T4	5	100 ppm
T5	5	125 ppm

In 1250ppm treatment no graft was remained alive. In healthy control 100% grafts were survived. As far as graft sprouting is concerned in tetracycline treatment following 29 weeks, no graft sprout was observed in without antibiotic or water,infected grafts only dipped in distilled water, 1000ppm and 1250ppm treatments. In 250ppm, 500ppm and 750ppm treatments 10 % grafts were sprouted whereas, in healthy controls 100% grafts were sprouted (Fig. 1B).More than 30% of grafts remained alive in all the treatments up to 13weeks after penicillin treatment whereas 100% grafts remained alive in case of healthy plants. Grafts were sprouted only in 75 ppm dose of penicillin; whereas 100% spouted in healthy control (Fig.2A).

Twenty nine weeks following penicillin treatment, only 10% grafts remained alive in without antibiotic or water immersed budsticks, infected grafts only dipped in distilled water, 25ppm, 50ppm and 100ppm treatment; whereas 100% grafts died in 75ppm and 125ppm treatments.In healthy controls, 100% grafts remained alive (Fig.2B).

Conventional PCR for testing effect of tetracycline and penicillin antibiotics

When DNA from leaves on the receptor plants of tetracycline treated budwood was tested by multiplex conventional PCR using OI1/OI2c and A2/J5 primers, amplicons of 1160bp and 703bp were obtained in PCR product from indicator plants grafted with HLB positive budwood without water immersion and antibiotic treatment as shown in lane 3-7; PCR products from indicator plants grafted with HLB positive budwood immersed only in distilled water as shown in lane 8, 9 and 10 and PCR products from 250 ppm tetracycline treatment indicator plant samples as shown in lane 11-14 of Fig.3. No amplicon was observed in the rest of the treatments by conventional PCR. From conventional PCR results it is concluded that dose of tetracycline at 250ppm is not effective for eliminating HLB pathogen from citrus budwood.

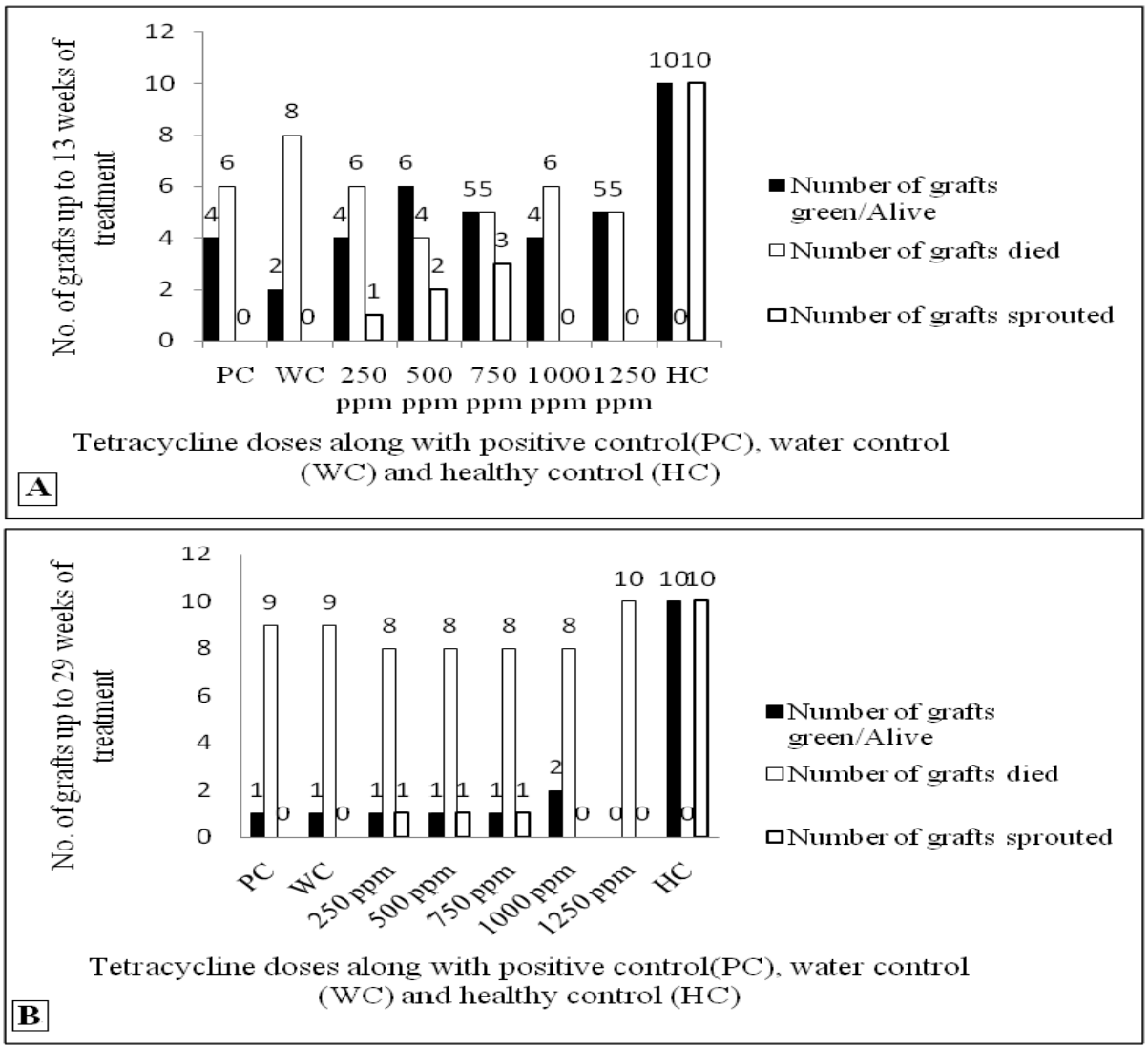

Fig. 1. Graft survival after tetracycline treatment of HLB affected budwood: **A,** Graft survival up to 13th week after tetracycline treatment; **B,** Graft survival up to 29th week after tetracycline treatment.

In the same way when DNA from penicillin treated budwood indicator plants was tested by duplex PCR using OI1/OI2c and A2/J5 primers, amplicons of 1160bp and 703bp were obtained in the products of positive control indicator plants as shown in lane 1, 2, 3, 4, 5; HLB positive budwood immersed only in distilled water as shown in lane 6, 7, 8; budwood treated with 25ppm penicillin as shown in lane 9, 10, 11, 12 and 50 ppm as shown in lane 17 of Fig.4. No amplicon was observed in the rest of the treatments.

From these results it is concluded that 25ppm and 50ppm dose of penicillin is not effective for cleaning HLB pathogen from graft.

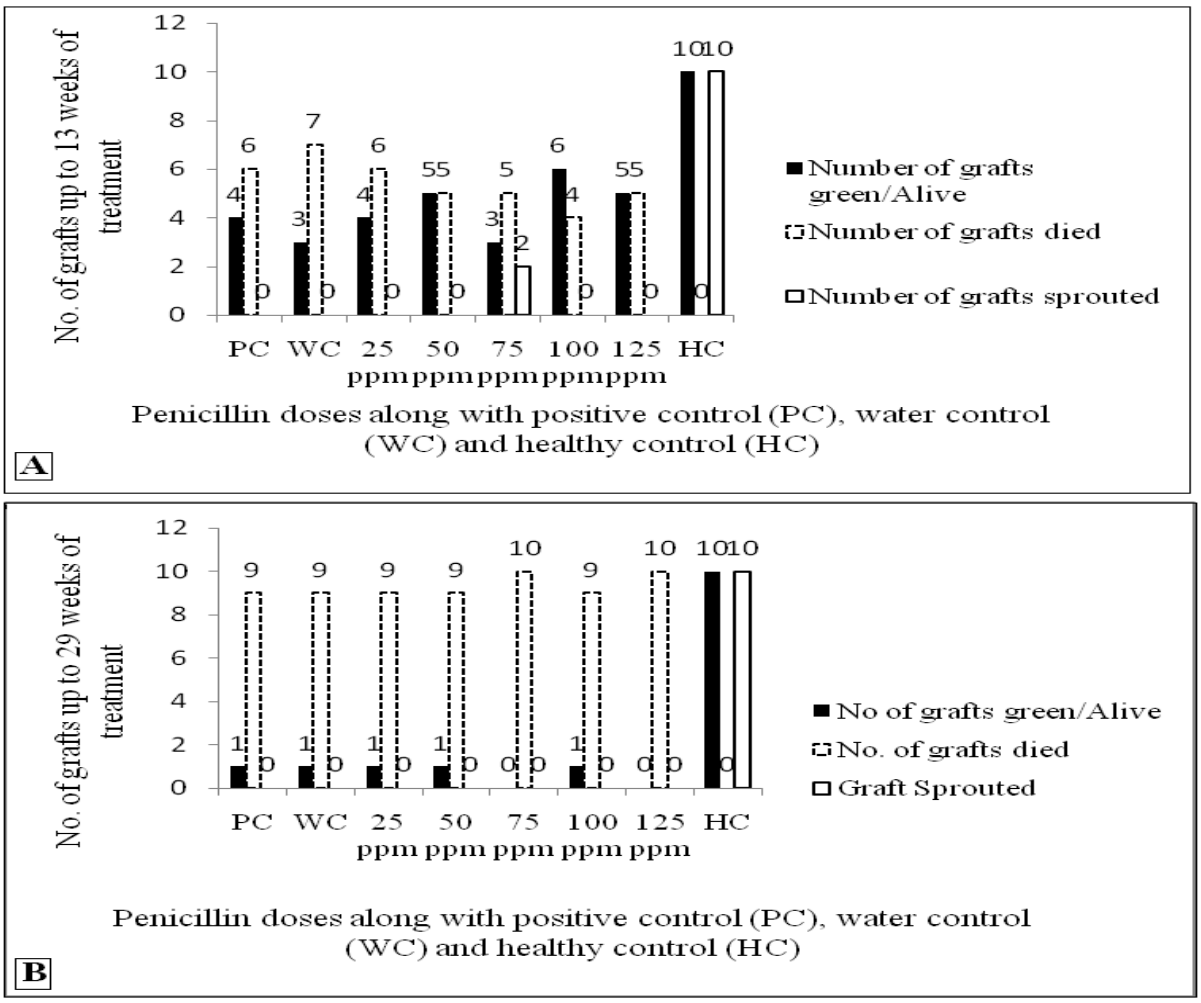

Fig. 2. Graft survival after penicillin treatment of HLB affected budwood:**A,** Graft survival up to 13 weeks after penicillin treatment; **B,** Graft survival up to 29 weeks after penicillin treatment.

Real time PCR for tetracycline and penicillin treatment indicator plants

In multiplex real time PCR, Taqman primer-probe sets HLBas fpr and 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 antibiotic treatment experiment. Highly significant differences were obtained in Ct values between tetracycline treatments (F= 22.42, P= 0.0001). Lowest mean cycle threshold (Ct) value (22.44) for the pathogen was observed in the positive control treatment. Highest mean Ct value (38.40) was observed in 1000ppm tetracycline treated samples while no amplification was observed in healthy control and 1250 ppm treatments (Fig.5A).

In case of penicillin treatment, also highly significant differences were obtained in Ct values (F=818.07, P=0.0001). Lowest mean cycle threshold (Ct) value (22.77) for the pathogen was observed in the positive control treatment. Highest mean Ct value (38.61) was observed in 75ppm penicillin treated samples while no amplification was observed in healthy control and 125ppm treatments (Fig.5B).

Discussion

Effect of antibiotics on grafts

In case of positive control and water control treatments, most of the grafts died because no antibiotic treatment was applied and presumably due to the accumulation of starch in response of HLB disease there was more plugging in the phloem sieve tubes, as a result movement of sap did not occur and grafts died. Bacteria were alive in positive controls and transmitted to the indicator plants through grafts

resulting in very high titer of HLB bacterium in the indicator plants as obtained from qPCR. In case of tetracycline doses at250ppm, 500ppm, 750ppm, 1000ppm and 1250ppm; 50% buds survived up to 13 weeks after treatment. When data recorded 29 weeks after treatment, very few grafts were alive in each of the treatments but in case of 1250ppm treatment, there was no graft alive that may be due to antibiotic phytotoxicity as described by Zhang *et al.* (2014; 2015).

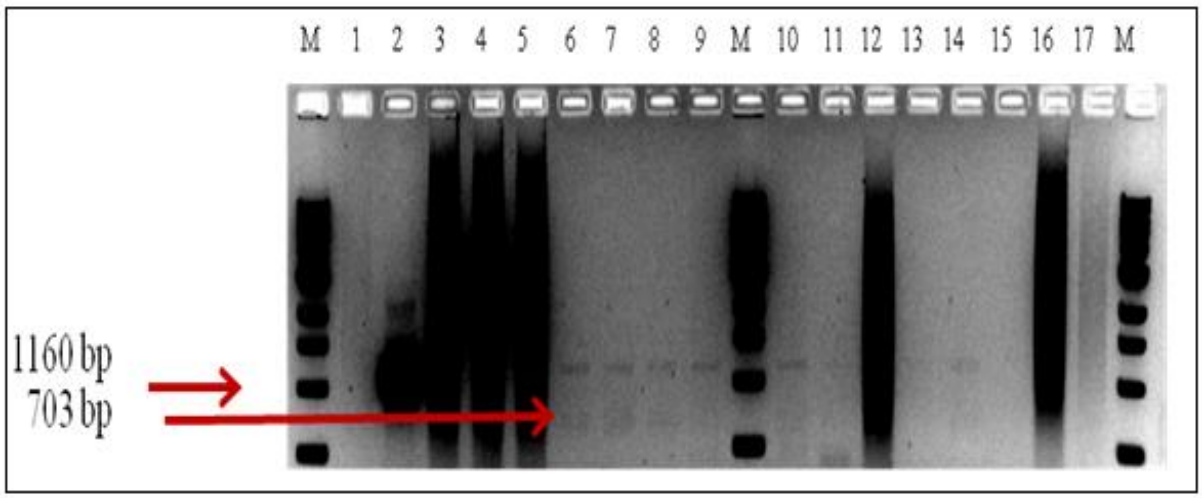

Fig. 3. Conventional PCR of tetracycline treatment experiment:Electrophoresis on 1.2% agarose gel of DNA amplified with OI1/OI2c and A2/J5 primers. Lanes indicated by M= 1kb DNA size marker (NEB);lane 1= healthy control;lane2= positive control;lane 3-7= PCR product from indicator plants grafted with HLB positive budwood without water immersion and antibiotic treatment; lane 8, 9 and 10 = PCR products from indicator plants grafted with HLB positive budwood immersed only in distilled water; lane 11-14 =PCR products from 250 ppm tetracycline treatment indicator plant samples; lane 15 = PCR products from 1000 ppm tetracycline treatment indicator plant samples; lane 16 = 500 ppm tetracycline treatment indicator plant sample and lane 17= 750 ppm tetracycline treatment indicator plant sample.

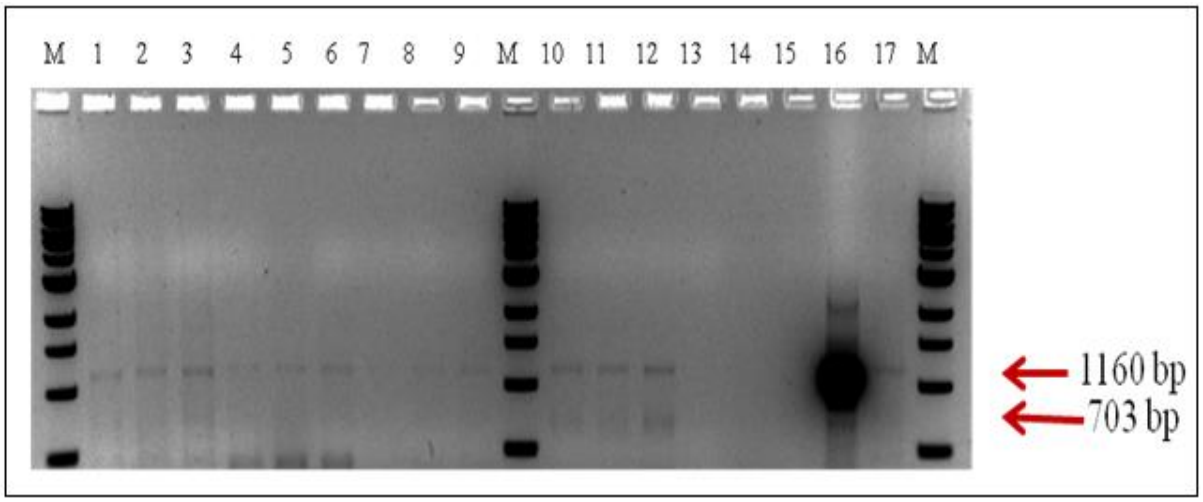

Fig. 4. Conventional PCR of penicillin treatment experiment:Electrophoresis on 1.2% agarose gel of DNA amplified with OI1/OI2c and A2/J5 primers. Lane M= 1kb DNA size marker (NEB); lane 1-5= PCR product from indicator plants grafted with HLB positive budwood without water immersion and antibiotic treatment; lane 6-8 = PCR product from indicator plants grafted with HLB positive budwood immersed only in distilled water; lane 9-12 = PCR product from indicator plants grafted with 25ppm penicillin treated budwood; lane16= positive control and lane17 = PCR product from indicator plants grafted with 50 ppm penicillin treated budwood.

Significance levels of treatments from real time PCR analysis

By using qPCR highly significant differences were obtained between treatments. Highest mean Ct value of 38.40 was observed in 1000ppm tetracycline treated samples. In a study on tetracyclines spray at 500 ppm dose, temporary recovery from huanglongbing was observed;but later the greening symptoms appeared again. In another experiment promising results were obtained when tetracycline was used for shoot or trunk injection (Nariani *et al.,* 1971; Nariani *et al.,*1975).

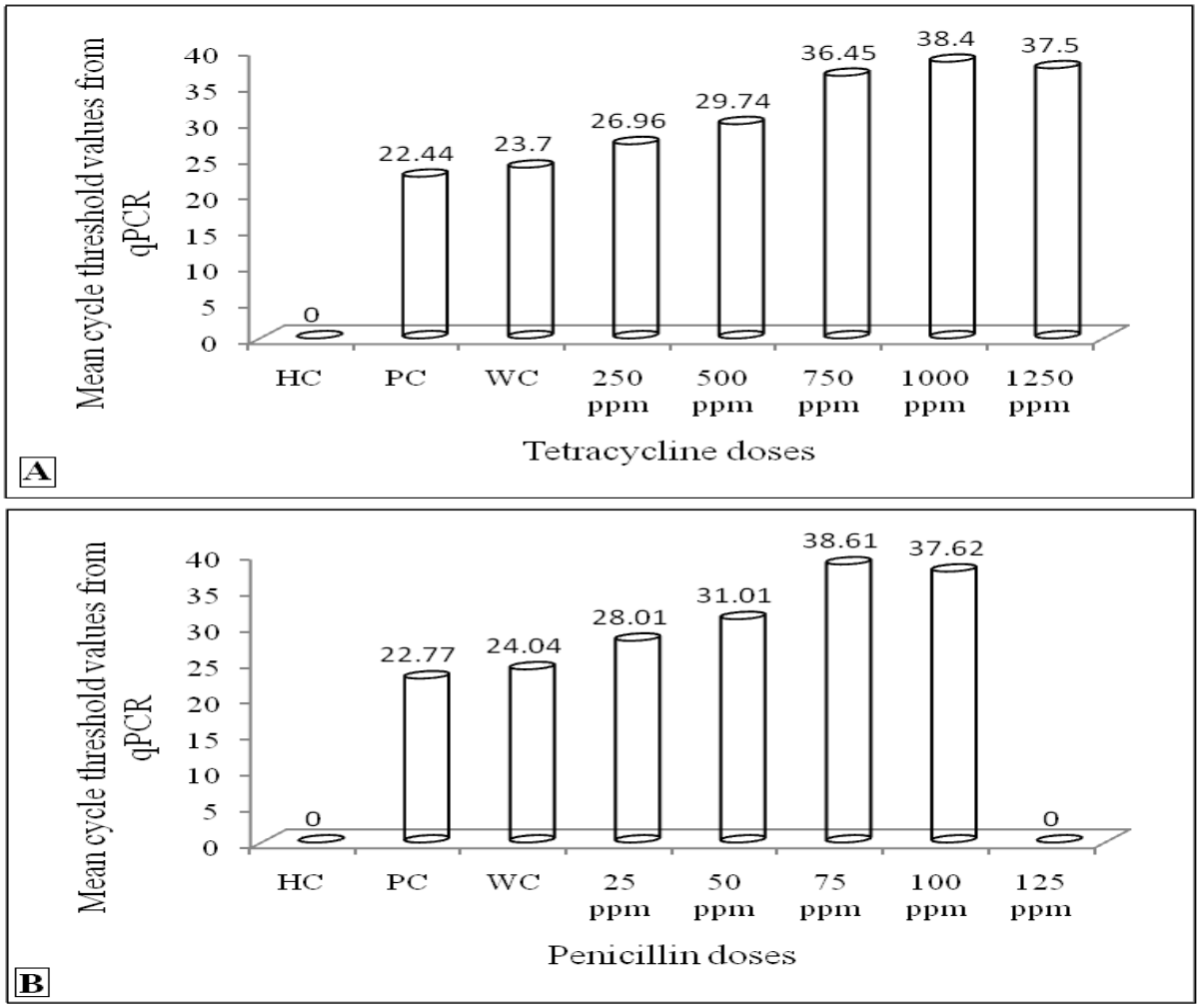

Fig. 5. Mean Ct values for tetracycline and penicillin antibiotics to eliminate *Candidatus* Liberibacter asiaticus from HLB affected budwood: **A,** Mean cycle threshold values for different doses of tetracycline antibiotic; **B,** Mean cycle threshold values for different doses of penicillin antibiotic.

In the above experiments, tetracycline treatment led to recovery of plants from greening symptoms only in the greenhouse grown plants but not in the field grown plants. Kapur *et al.* (1996) used tetracycline HCl 1000µg/ml after combining with $ZnSO_4$ 1000µg/ml and achieved the highest control (92 %) by injecting tetracycline HCl in combination with $ZnSO_4$.

Our work was different from the previous studies; because they injected or sprayed HLB infected plants with different doses of antibiotics while we used HLB infected budwood to eliminate the bacteria byimmersion in the tetracycline and penicillin solutions. Zhang *et al.* (2012) presented similar results.

Novelty of work and results

In our results, 75ppm penicillin dose eliminated *Candidatus* Liberibacter asiaticus from the citrus

budwood whereas, Zhang *et al.* (2010) reported that diseased periwinkle cuttings showed negative results by PCR at 50 µg/mL dose of penicillin G sodium. In another experiment, an Avo-Ject syringe injector used to inject 100 ml of 5.0 g/tree penicillin, but we used HLB positive citrus cuttings (budsticks) for the treatment because cleaning of cuttings was thought to be easier and efficient than whole tree. Bacteria transmission efficiency was found higher if the titer in the material used for inoculation purpose was abundant but the less number of scion were survived; and if there was less titer, then survival percentage of scion was higher as also described by Zhang *et al.,* 2012.

Cuttings were used under the hypothesis that we could kill bacteria from this small part with modest effort but in case of big tree as if a tree is infected with the HLB pathogen in the field then it is very difficult to control this disease due to its uneven distribution in the host. By using spray or injection technique, only bacteria in those plant parts which receive antibiotic are likely to be killed.

If the antibiotic does not reach all bacteria then some will remain in the plant and its population in the host will again increase. New flushes from those parts can serve as inoculums for the natural insect vector of the disease as well as with the passage of time bacterial titer will be increased again in the plant and symptoms will reappear as proved by Nariani *et al.,* 1975.

Conclusion

Tetracycline at 1000 ppm and penicillin antibiotic at 75ppm dose were found to be effective to eliminate Liberibacter bacterium from budwood to stop the further spread of the disease through budding/grafting. There is need to find more antibiotics that could also be better for the elimination of HLB bacterium and greater survival rate of grafts without causing phytotoxicity. The treatment of budwood with antibiotic can be a hope for survival of the citrus industryif all the citrus plants in agrove become infected with *Candidatus* Liberibacter bacterium.

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