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Identification of tolerance in Rangpur citrus against huanglongbing

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Abstract

Rangpur citrus group was tested during the present study for the identification of tolerance against huanglongbing. Control of huanglongbing (HLB) disease is necessary around the world to avoid the complete destruction of citrus. Rangpur accessions were graft challenged with huanglongbing positive budwood to study the resistance and symptom expression. Vein yellowing symptoms appeared on Rangpur poona nucellar and tuningmeng nucellar while vein yellowing and blotchy mottle symptoms appeared only on tuningmeng nucellar. Kirumakki nucellar and knorr nucellar expressed no symptoms. Being a drought-tolerant rootstock with less HLB symptom expression, Rangpur rootstock cultivars especially kirumakki nucellar and knorr nucellar may be used to test the production and survival of plant with compatible scion varieties.

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Introduction

Citrus greening or huanglongbing (HLB) is the most destructive disease of citrus (Hall *et al.*, 2012). It has caused a substantial loss to the citrus industry all around the world. *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). The Asian citrus psyllid (ACP) (*Diaphorina citri* Kuwayama) can transmit both Las and Lam. The most abundant bacterium species among huanglongbing infected trees is Las (Jagoueix *et al.,* 1994; Bove, 2006) that can also be transmitted by grafting (Lin, 1956). A very high ACP population exists in the citrus orchards of Pakistan for more than 100 years (Yaqub *et al.,* 2017).

Citrus greening symptoms on leaves, branches, and fruits have been observed on various rootstock and scion varieties of citrus including Rangpur lime. It is suggested that the blotchy mottle symptom on leaves is the most important diagnostic tool for HLB (Gomez, 2008). Some trifoliate oranges and citrus relatives do not express symptoms even after infection (Miyakawa, 1980). Sweet orange is a good indicator of HLB symptoms and more susceptible as compared to kinnow (Aslam *et al.,* 2017a). In mandarins, Citrus sunki, Kinnow, Parson's Special and Sun Chu Sha also exhibit blochy mottle symptom of HLB (Aslam *et al.*, 2017b).

All commercial varieties of citrus are affected by HLB. Identification of tolerant germplasm is required to know. Efforts are going on to control HLB by producing healthy plants under greenhouse conditions, pruning of diseased parts of the plant, uprooting, biological and chemical control by antibiotics, thermotherapy and vector control by chemicals (Yaqub *et al.,* 2019 a&b). The best way to control HLB would be the Identification of tolerant germplasm. However, no resistant trees or scion-rootstock combinations have been identified yet (Ghosh *et al.*, 2018).

Rootstocks are an important component of citrus production. Use of a good rootstock influences lifespan and productivity of a citrus orchard (Castle, 2010). The main reason for the extensive use of rootstock in citrus is their potential to induce resistance to diseases. With the discovery of HLB in Florida in 2005 (Halbert, 2005), the importance of rootstock as a solution to sustain citrus production and reduce the negative impacts of this noxious disease has increased all over the world. Some trifoliate used as rootstocks influenced the tree to remain productive under high pressure of HLB (Albrecht and Bowman, 2012; Bowman *et al.*, 2016; Ampatzidisa *et al.*, 2019).

In the present study, Rangpur group of citrus was analysed for the identification of tolerance against HLB. The Rangpur is of horticultural importance principally as a rootstock. Common names for this fruit include Rangpur in India, Canton lemon in South China, Hime lemon in Japan, Cravo lemon in Brazil, and mandarin-lime in the United States. Rangpurs are highly acid and can be used as a substitute for commercial limes. In the United States, it is used as an ornamental or potted plant. Rangpur is considered as the mandarin-like fruits for the reason of clear and countless resemblance with mandarin. The Rangpur and rough lemon are also very close in similarities and some differences (Citrus variety collection, UCR).

The availability of water for irrigation is a global issue. Rangpur is drought-resistant rootstock. The objective of the current study was to identify resistance in Rangpur group of rootstock varieties against HLB and introduce it to Pakistan.

Materials and methods

Rearing of Rangpur citrus

Exotic seeds of Rangpur citrus (Table 1) were sown for the identification of tolerance against huanglongbing. The seed was obtained from the USDA National Clonal Germplasm Repository for Citrus and Dates. Seedlings were raised in the *Diaphorina citri* kuwayama free environment in the

greenhouse Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. Canal water was used for irrigation throughout the raising period whenever required. Macro and micronutrients were also applied with irrigation water once in a month throughout the experiment period to make up the deficiency and to avoid the confusion of nutrient deficiency symptoms with the HLB disease.

Establishment of Rangpur citrus germplasm field

Germplasm field was established in the square number 32, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. Total 4 accessions of survived Rangpur citrus were transplanted in the field. Healthy controls of all accessions were kept in the insect-free greenhouse. Layout in the field was done by keeping plants to plant and row to row distance at 10 ft each. For the field, both drip and flood irrigation methods were used depending upon the availability of canal water. Macro and micronutrients were applied in the field at one-month interval throughout the experiment period. The field was never ploughed after germplasm transplantation to prevent root damage. Pruning and training of the germplasm were done whenever required to make the shape of the trees. Pruining scissor were sterilized with 10% bleach and washed with distilled water every time after each plant pruning or training to avoid contamination.

Graft challenge of the germplasm and evaluation of symptom expression

Germplasm was graft inoculated after six months of transplanting in the field (Fig.1). All plants were PCR tested and found HLB free at the time of field transplantation. Conventional PCR was performed by using 16s rDNA primer OI1/OI2c (Jagoueix *et al.*, 1996) and rplKAJL-rpoBC operon primers A2/J5 (Hocquellet *et al.*, 1999) for the test. The graft wood was taken from PCR tested HLB positive C*itrus sinensis* culivar succari plants (Fig. 2). Graftwood was taken from highly symptomatic branches of these trees. Field transplanted germplasm was graft challenged using T-grafting method. Each plant was grafted with three bud sticks on different branches i.e. one bud stick on one branch. 4-5 buds were present on each bud stick. After inoculation, the inoculated area was covered with the polythene sheet. After sixteen weeks of graft challenge of the germplasm in the field, data collection was started for symptom appearance. Every plant in the field was monitored. Common symptoms of HLB including blotchy mottling, vein corking and vein yellowing were observed on the leaves of plants for the detection of HLB in the inoculated plants.

Molecular studies and sampling for identification of tolerance against HLB in citrus germplasm

The molecular studies for the detection of *Candidatus* Liberibacter asiaticus in the healthy controls (uninoculated plants maintained in the greenhouse), and in the target after graft challenge was conducted by the PCR technique. Mature leaves not attacked by any other pest or injured by any cultural practice were collected after 12 months of graft inoculation. Efforts were made to collect those leaves which had symptoms as much as appeared on the accessions and collected within an area of 12 inches of graft inoculation. Leaves were collected, placed in zip lock bags, labeled properly, put in icebox immediately, transported to the laboratory and stored in the refrigerator at 4°C until DNA extraction. Collected samples were further processed on the same day and not stored for a long time.

Plant's DNA extraction and conventional PCR

The DNA was isolated from leaf midribs and petioles of treated and healthy plants by CTAB method as described by Yaqub *et al.* (2017). Singleplex and duplex conventional PCR were performed using 16S rDNA primer OI1/OI2c (5´GCGCGTATGCAATACGAGCGGCA3´ / 5´GCCTCGCGACTTCGC AACCCAT3´) and A2/J5: (5´TATAAAGGTTGACCTTTCGAGTTT3´/5´ACAAAA GCAGAAATAGCACGAACAA3´) for ribosomal protein gene for the detection of HLB pathogen in inoculated 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 the 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 was: 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's cytochrome oxidase (COX) gene was used as a positive internal control to assess the quality of the DNA extracts. The sequence of the 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´ and cox 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 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 seconds and annealing at 58 °C for 20 seconds 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) 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 and discussion

HLB symptom expression in citrus germplasm

A total of four accessions of Rangpur citrus were survived (Table 1) and transplanted in the field for the identification of tolerance against huanglongbing.

Table 1. Rangpur Citrus germplasm reared for the identification of tolerance against huanglongbing.

Sr.No.	Cultivars sown	Cultivars survived	Binomial	Accession No.
1	Kirumakki Nucellar	Kirumakki Nucellar	*Citrus limonia* Osbeck	RCRC 4131
2	Knorr Nucellar	Knorr Nucellar	*Citrus limonia*	RCRC 4132
3	Rangpur Poona nucellar	Rangpur Poona nucellar	*Citrus limonia*	RCRC 4135
4	Tuningmeng Nucellar	Tuningmeng Nucellar	*Citrus limonia*	RCRC 4139
5	Lima Criolla Brasilia nucellar	-	*Citrus limonia*	RCRC 4133
6	Srirampur Nucellar	-	*Citrus limonia*	RCRC 4137

Table 2. Rangpur cultivars expressing huanglongbing symptoms**.**

Sr.No.	Cultivar	Blotchy mottle	Vein Yellowing	Vein corking	Leaf yellowing	Leaf pale green
1	Kirumakki Nucellar	-	-	-	-	-
2	Knorr nucellar	-	-	-	-	-
3	Rangpur Poona nucellar	-	yes	-	-	-
4	Tuningmeng Nucellar	yes	yes	-	-	-

After graft challenge of the germplasm in the field, data was collected for the expression of blotchy mottling, vein corking, vein yellowing, leaf yellowing and pale green leaf symptoms on the experimental plants. Out of 4 accessions, 2 accessions expressed HLB symptoms whereas, 2 accessions expressed no observable symptoms (Table 2). Development of HLB symptoms is the result of molecular, cellular and physiological changes in host plants as a defense response to stop the spread of pathogens in plant

tissues (Donnell *et al.*, 2003). There may be many reasons of non-appearance of symptoms, i.e. deciduous nature of the plant, uneven distribution of the HLB pathogen *Candidatus* Liberibacter asiaticus, less or no reproduction of the pathogen in an accession, the slow movement of the pathogen in the sap of a particular accession, low titre in an accession or latency. The expression of vein yellowing symptom was highest while blotchy mottle symptom was expressed only in tuningmeng nucellar (Fig. 3).

Fig. 1. HLB graft challenge on Rangpur in the field.

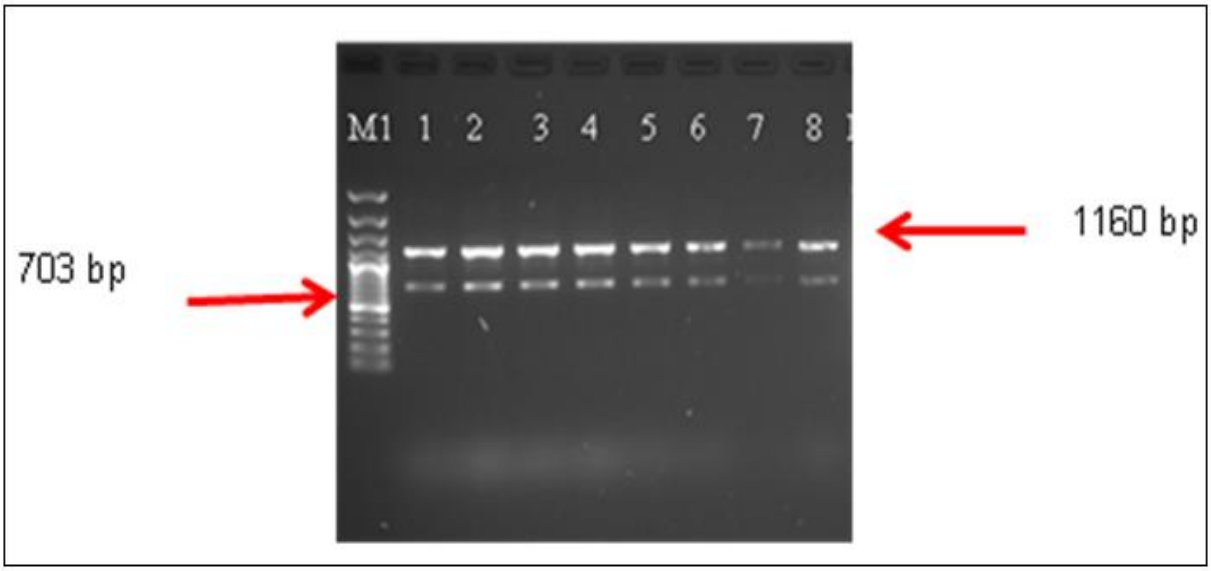

Fig. 2. Multiplex PCR product of HLB positive graft wood used for germplasm inoculation: Lane M1, 100bp plus DNA size marker; Lane 1-8, samples from graft wood source trees used for inoculating the germplasm.

Molecular studies of graft inoculated germplasm for identification of tolerance

Germplasm samples before transplantation and graft inoculation in the field were tested by using OI1/OI2c and A2/J5 primers for HLB negativity. No amplification observed by electrophoresis on 1.2% agarose gel of DNA in all the samples confirmed that they were healthy (Fig. 4). Graft wood was taken for inoculation of the germplasm from PCR tested positive sweet orange (*Citrus sinensis*) plants for

confirmation of the presence of *Candidatus* liberibacter asiaticus. As a result of multiplex conventional PCR, two bands of 1160bp and 703bp by electrophoresis on 1.2% agarose gel using OI1/OI2c and A2/J5 primers confirmed the presence of *Candidatus* liberibacter asiaticus in the source trees used to obtain grafts in the identification of tolerance for HLB study (Fig. 2).

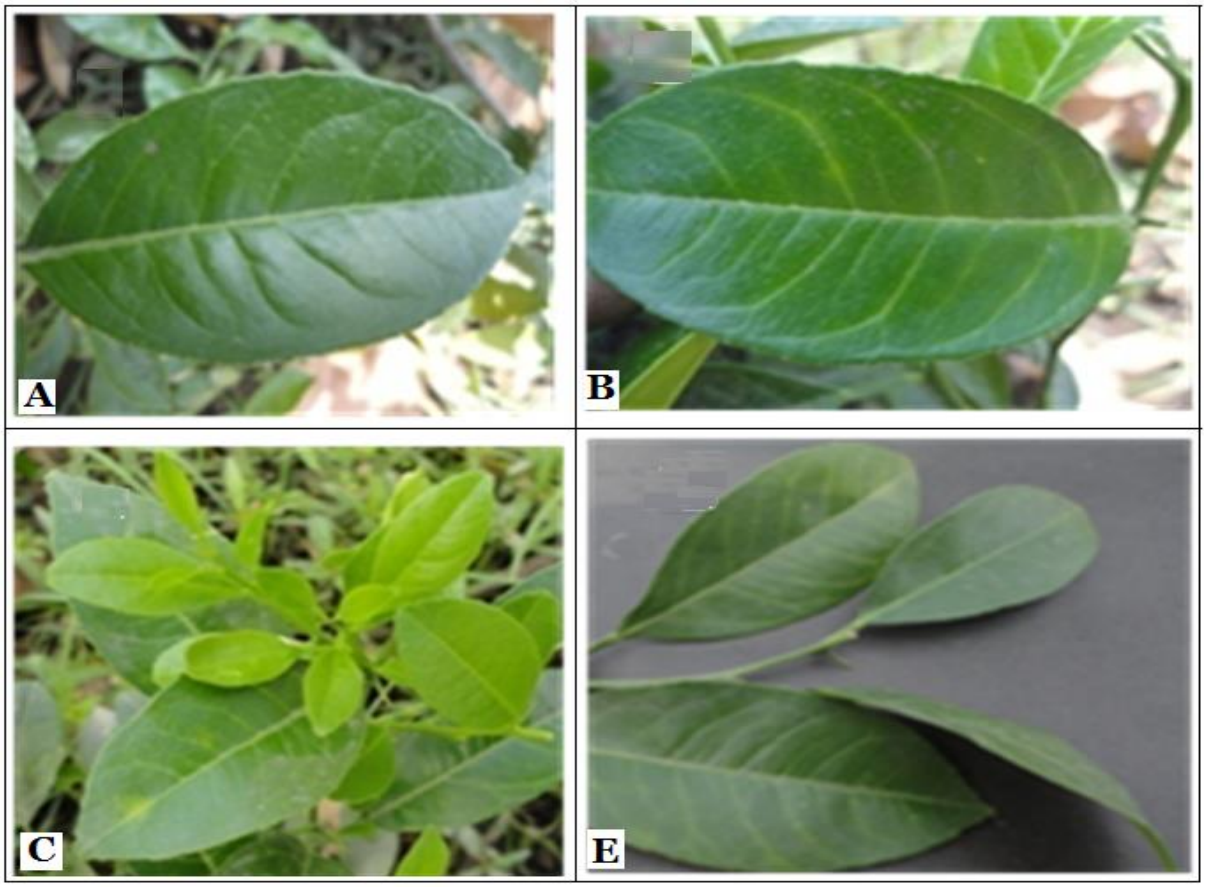

Fig. 3. Vein yellow symptom in leaves: A, Tuningmeng nucellar; B, Tuningming nucellar; E, Rangpur Poona nucellar; C, blotchy mottle symptom in leaves of Tuningming nucellar.

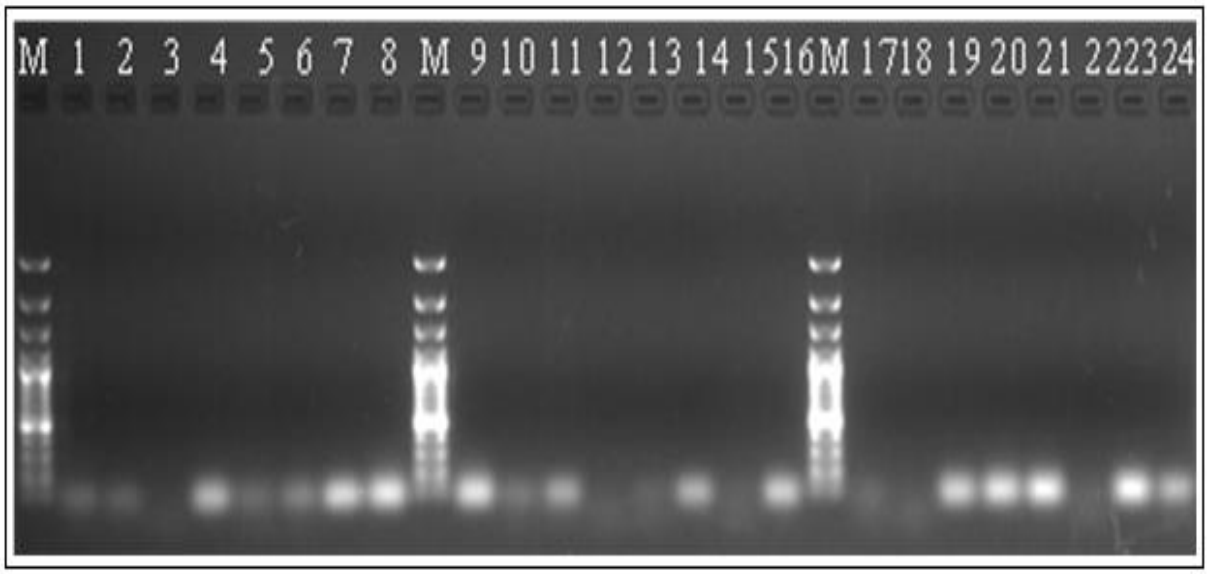

Fig. 4. Agarose gel electrophoresis of PCR product of healthy germplasm: Lane M, 100bp plus DNA ladder; Lane 1 to 24, PCR product of germplasm samples before graft inoculation.

Multiplex conventional PCR of experimental graft challenged Rangpur cultivars produced no band on agarose gel (Fig. 5). Taqman based real-time PCR for all of the four genotypes belonging to Rangpur group amplified expressing cycle threshold (ct) values between 26.44 to 27.75 and 20 for positive control DNA from succari sweet orange (Fig. 6). Graph of ct values for kirumakki nucellar, Rangpur poona nucellar, knorr nucellar, tunungmeng nucellar and positive control is shown in Fig. 7. Despite being HLB positive, some accessions of the citrus germplasm graft challenged did not express any symptoms.

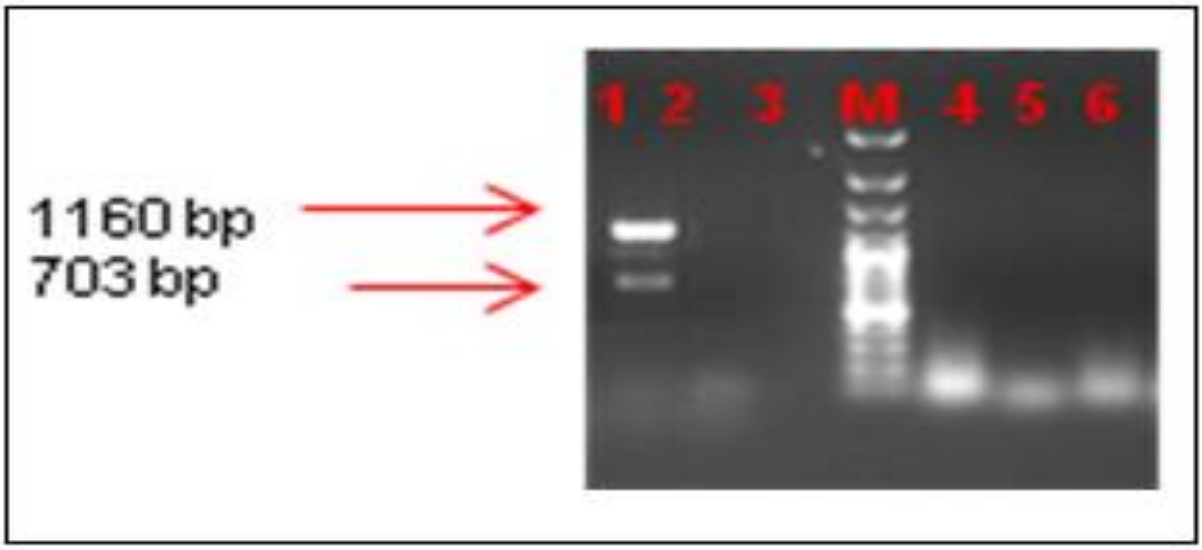

Fig. 5. Electrophoresis of PCR product of inoculated germplasm on 1.2% agarose gel of DNA amplified with OI1/OI2c and A2/J5 primers: Lane M, 100 bp plus DNA size marker (NEB); Lane1, positive control; Lane2, no template or negative control; Lane3, kirumaki nucellar; Lane4, knorr nucellar; Lane5, Rangpur poona nucellar; Lane6, tuningmeng nucellar.

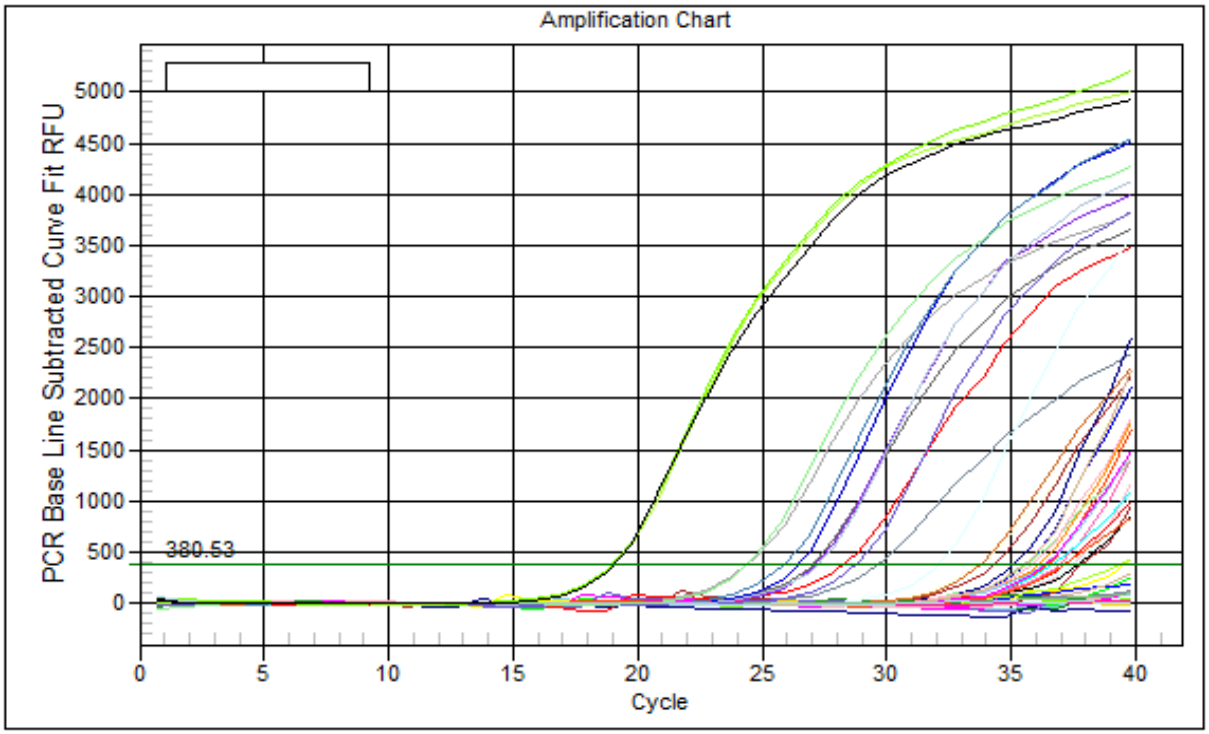

Fig. 6. *Candidatus* Liberibacter asiaticus 16S rDNA amplification plot showing cycle threshold values for Las detection in Rangpur group of citrus by taqman qPCR.

It is assumed from this observation that, there may be less formation and deposition of callose and pp2 that result in regular sap flow and ultimately less or no expression of HLB symptoms. Some trifoliate rootstocks keep the tree to remain productive under high pressure of HLB (Albrecht and Bowman, 2012; Bowman *et al.*, 2016; Ampatzidisa *et al.*, 2019), rangpur rootstock exhibit the same properties despite being detected HL Bpositive by qPCR. As irrigation water shortfall is a great challenge for agriculture, drought-tolerant rootstocks along with HLB resistance is needed. Being a drought-tolerant rootstock with less HLB symptom expression, Rangpur rootstock cultivars especially kirumakki nucellar and knorr nucellar may be used to test the results with compatible scion varieties.

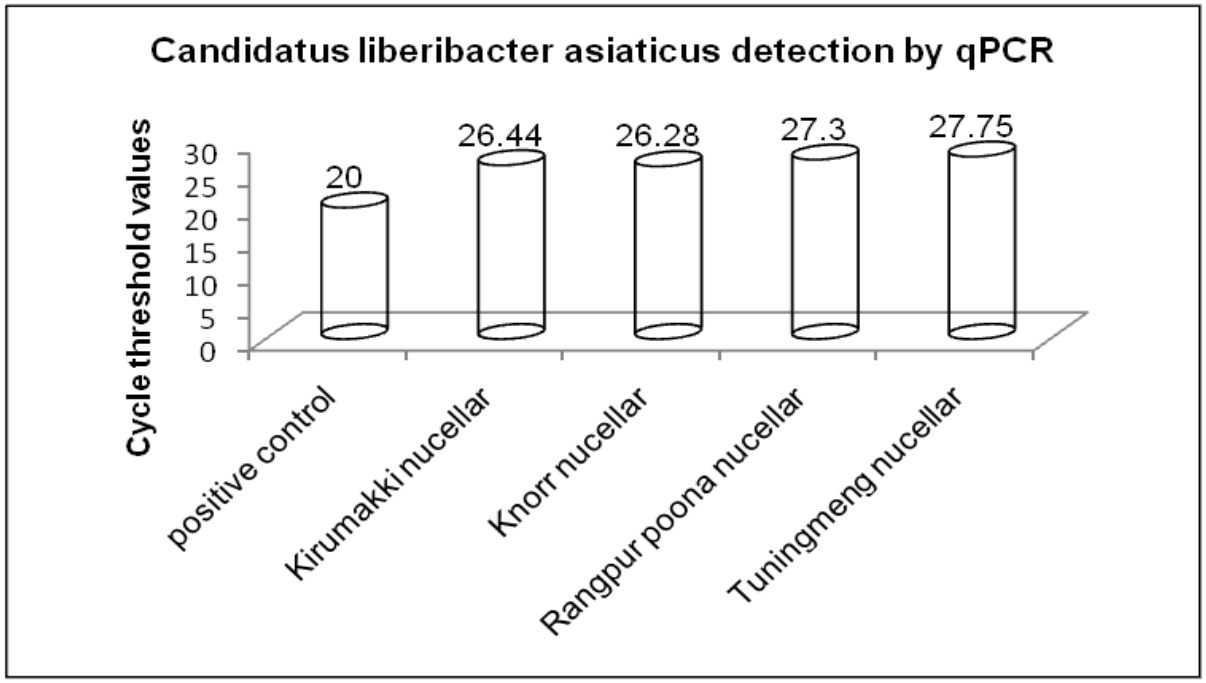

Fig. 7. Cycle threshold values for *Candidatus* Liberibacter asiaticus detection in Rangpur group of citrus by taqman qPCR for identification of tolerance.

Conclusion

The present study was an effort to assess the identification of tolerance for HLB in the Rangpur group of citrus germplasm. From 4 accessions, the expression of vein yellowing symptom was produced in 50% cultivars while blotchy mottle symptom was produced only in 25% of all types. 50% cultivars expressed no symptom of HLB confusing the growers as if they are resistant to HLB.

By real-time qPCR detection of *Candidatus* Liberibacter asiaticus, the results were considered negative if Ct values were observed up to or above 36.9 (Hoffman *et al.*, 2013), or no amplification (NA). *Citrus limonia* cultivars kirumakki nucellar and knorr nucellar may be used to test the production and survival of plants with compatible scion varieties in water-stressed areas.

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