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SHORT COMMUNICATION

Current Distribution of Huanglongbing (citrus greening disease) in India as Diagnosed by Real-Time PCR

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Keywords
Candidatus Liberibacter asiaticus, citrus greening disease, cycle threshold (Ct), huanglongbing, real-time PCR

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Abstract

The widespread occurrence of Huanglongbing (HLB) was recorded in sixteen citrus growing states of India using the real-time quantitative PCR and the derived threshold cycle (Ct) value. All the commercially important citrus varieties of mandarin, sweet orange, lime and lemon, pummelo and Satkara were infected with '*Candidatus Liberibacter asiaticus*', the bacterium associated with HLB. Ct values positive for HLB were found in all the states except Arunachal Pradesh. The primer–probe combination HLBas-HLBr-HLBp was found specific to *Ca. L. asiaticus* and do not exhibit any cross-reactivity with other pathogenic residents of citrus.

Introduction

Huanglongbing (HLB) aka citrus greening disease is one of the most serious diseases prevalent in global citrus production including India. The disease has resulted in the decline and/or death of millions of citrus trees worldwide (Bové 2006). The causal agent, '*Candidatus Liberibacter* spp.', is a fastidious, phloem-limited and Gram-negative proteobacterium (Jagoueix et al. 1994). It is naturally transmitted through psyllid vectors (*Diaphorina citri* in Asia (Capoor et al. 1967) and America and *Trioza erytreae* in Africa) and by means of graft transmission during propagation of contaminated nursery planting materials. There are three species of this organism, '*Ca. Liberibacter africanus*' is found in African countries, '*Ca. Liberibacter americanus*' found only in Brazil, and '*Ca. Liberibacter asiaticus* (Las)' being the most prevalent in Asia, North and South America (Gottwald 2010). *Ca. Liberibacter* infects almost all citrus species and their hybrids, causing HLB, for which no cure is available to date and control measures are limited to vector management. Thus, prevention and early diagnosis of the bacterium inhabiting the host can help in plant health management and delimit the spread and devastation of HLB. Currently, real-time quantitative PCR is the preferred detection method for *Ca. Liberibacter* species (Li et al. 2006). Compared with conventional PCR, real-time PCR offers both sensitive and rapid detection of these bacteria. Real-time PCR is reported to enhance the sensitivity for *Liberibacter* detection by 100–1000 times relative to conventional PCR (Teixeira et al. 2008).

Citrus alone contributes 10% of total fruit production and is largest crop cultivated after mango and banana in India (Anonymous 2010). HLB has accounted for citrus decline in India for decades (Das 2009). However, no systematic studies have been carried out to find the distribution of this disease in India. Here, we diagnosed HLB in various commercially cultivated citrus species collected from different citrus growing states of the country using real-time PCR system.

Materials and Methods

Citrus samples were collected by number of surveys from 2007 through 2012 in 16 states of India, namely Punjab and Rajasthan in North-West India, Madhya Pradesh and Maharashtra in central India, Andhra

Pradesh, Karnataka and Tamil Nadu in Southern India, West Bengal and Sikkim in Eastern India, and the seven sister states in North-Eastern (NE) India (Arunachal Pradesh, Assam, Meghalaya, Manipur, Mizoram, Nagaland and Tripura) (Fig. 1). Commercially important citrus cultivars like sweet orange (Mosambi, Sathgudi, Valencia), mandarin (Nagpur, Kinnow, Coorg, Khasi, Darjeeling), acid lime (Kaghzi, Jayadevi) and lemon (Assam, Lisbon, Elachi), Pummelo, Satkara and rootstock species (Rough lemon, Rangpur lime) were surveyed (Table 1). Different kinds of symptoms characteristic of HLB were observed *viz*., yellow shoot, leaf 'blotchy' mottle, vein yellowing of leaves and stylar end greening of infected fruits (Fig. 2). Symptomatic leaves with stems intact were collected in zip-lock bags and brought to Plant Pathology Lab, National Research Centre for Citrus, Nagpur for HLB testing.

DNA was extracted from 150 mg of crushed leaf midribs using DNeasy Plant mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's directions. Real-time PCR was carried out with primers and probe (TaqMan) for amplification of 16S rDNA of '*Ca*. Las' as described by Li et al. (2006). The final concentration of PCR reagents constituted was as follows: 250 nM each of the primers HLBas and HLBr, 150 nM of probe, HLBp and 1 × TaqMan Universal PCR master mix (Applied Biosystems, Foster City, Calif., USA). All primers and probes were procured from Integrated DNA Technologies, USA. The real-time PCR amplifications were performed with ABI 7300 (Applied Biosystems) machine in a 20 μl reaction mixture. The standard amplification protocol was 95°C for 15 min followed by 40 cycles at 94°C for 15 s and 58°C for 60 s. All reactions were performed in triplicate with positive, healthy and water controls as

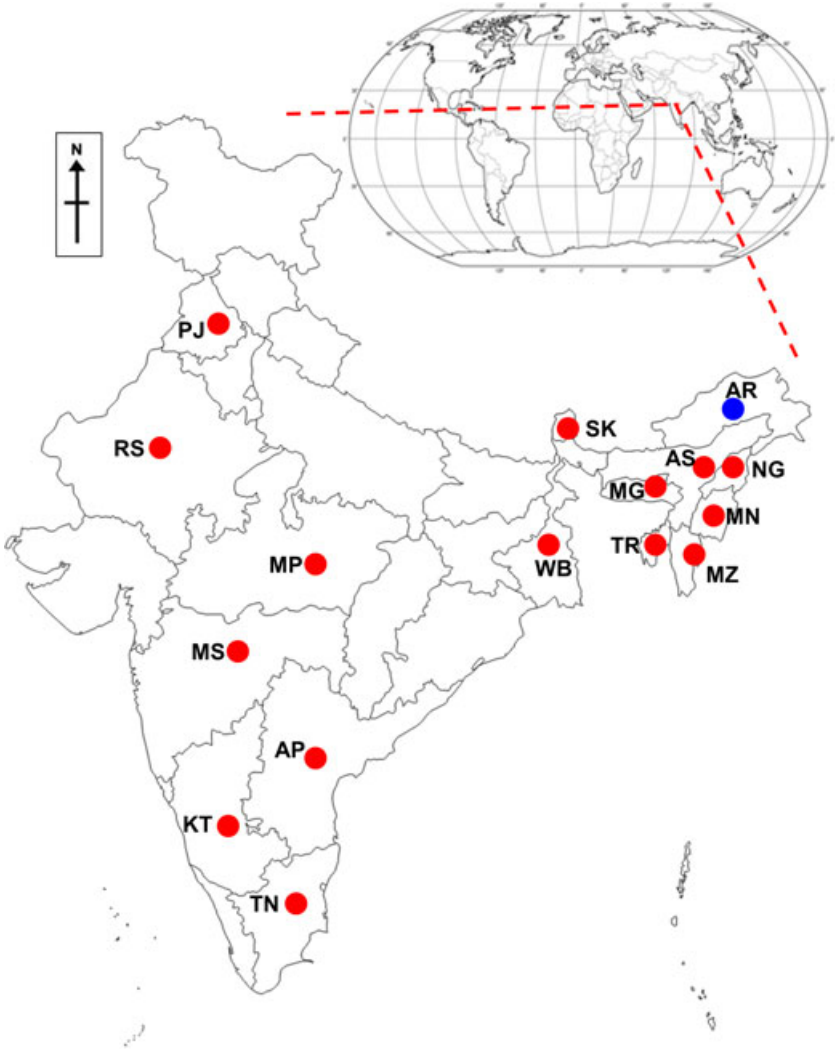

Fig. 1 The surveyed citrus growing states of India. PJ, Punjab; RS, Rajasthan; MP, Madhya Pradesh, MS, Maharashtra; AP, Andhra Pradesh, KT, Karnataka; TN, Tamil Nadu; SK, Sikkim; MG, Meghalaya; TR, Tripura; AR, Arunachal Pradesh; AS, Assam; NG, Nagaland; MN, Manipur; MZ, Mizoram and WB, West Bengal. (●) HLB-infected states and (●), putative infection.

Table 1 Detection of *Ca. Liberibacter asiaticus* by real-time TaqMan PCR assays in different citrus cultivars grown in various states of India

Locality/Dist/State	Host		Ct Value* ± SD
	Citrus cultivar	Botanical name	
Bonala, Kadapa, Andhra Pradesh	Sathgudi	*Citrus sinensis*	26.17 ± 0.29
Mukundapuram, Anantapur, Andhra Pradesh	Sathgudi	*C. sinensis*	23.83 ± 0.26
Maheboobnagar, Maheboobnagar, Andhra Pradesh	Sathgudi	*C. sinensis*	25.13 ± 0.28
Basar, West Siang, Arunachal Pradesh	Khasi mandarin	*C. reticulata*	36.94 ± 0.34
Along, West Siang, Arunachal Pradesh	Khasi mandarin	*C. reticulata*	Undetected
Pasighat, East Siang, Arunachal Pradesh	Valencia	*C. sinensis*	34.08 ± 0.48
Kahikuchi, Kamrup, Assam	Assam lemon	*C. limon*	23.58 ± 0.34
Sonapur, Kamrup, Assam	Gol nimbu	*C. jambhiri*	22.61 ± 0.26
Chettalli, Kodagu, Karnataka	Coorg mandarin	*C. reticulata*	19.06 ± 0.17
Chettalli, Kodagu, Karnataka	Pummelo	*C. grandis*	19.87 ± 0.15
Osera, Chhindwara, Madhya Pradesh	Nagpur mandarin	*C. reticulata*	26.17 ± 0.25
Zirola, Chhindwara, Madhya Pradesh	Mosambi	*C. sinensis*	27.09 ± 0.28
Kalmeshwar, Nagpur, Maharashtra	Nagpur mandarin	*C. reticulata*	17.32 ± 0.19
Ladgaon, Aurangabad, Maharashtra	Mosambi	*C. sinensis*	19.21 ± 0.21
Katol, Nagpur, Maharashtra	Acid lime, Kaghzi	*C. aurantifolia*	22.72 ± 0.26
Amravati Road, Nagpur, Maharashtra	Rangpur lime	*C. limonia*	22.37 ± 0.24
Amravati Road, Nagpur, Maharashtra	Pumello	*C. grandis*	30.85 ± 0.35
Chandurbazar, Amravati, Maharashtra	Nagpur mandarin	*C. reticulata*	25.84 ± 0.12
Rahuri, Ahmednagar, Maharashtra	Rough lemon	*C. jambhiri*	21.08 ± 0.23
Thangal, Tamenglong, Manipur	Khasi mandarin	*C. reticulata*	20.07 ± 0.18
Cheihruphi, Jaintia Hills, Meghalaya	Khasi mandarin	*C. reticulata*	25.24 ± 0.26
Umnsing, Ri-Bhoi, Meghalaya	Khasi mandarin	*C. reticulata*	24.10 ± 0.22
Umnsing, Ri-Bhoi, Meghalaya	Mosambi	*C. sinensis*	20.19 ± 0.25
Vaipuanpho, Mamit, Mizoram	Khasi mandarin	*C. reticulata*	30.20 ± 0.29
Medziphema, Dimapur, Nagaland	Lisbon lemon	*C. limon*	23.01 ± 0.26
Abhor, Fazilka, Punjab	Linda Valencia	*C. sinensis*	31.01 ± 0.32
Abhor, Fazilka, Punjab	Kinnow mandarin	'King' *(C. nobilis) x* 'Willow Leaf' *(C. deliciosa)* mandarin	21.70 ± 0.24
Sri Ganganagar, Sri Ganganagar, Rajasthan	Kinnow mandarin	-do-	21.38 ± 0.18
Jhalrapatan, Jhalawar, Rajasthan	Mosambi	*C. sinensis*	27.46 ± 0.34
Singtom, East Sikkim, Sikkim	Mosambi	*C. sinensis*	24.75 ± 0.26
Singtom, East Sikkim, Sikkim	Khasi mandarin	*C. reticulata*	23.49 ± 0.21
Coimbatore, Coimbatore, Tamil Nadu	Lemon	*C. limon*	22.51 ± 0.20
Periyakullum, Theni, Tamil Nadu	Acid lime, Jayadevi	*C. aurantifolia*	24.35 ± 0.21
Vanghmun, North Tripura, Tripura	Khasi mandarin	*C. reticulata*	21.33 ± 0.30
Behliangchhip, North Tripura, Tripura	Satkara	*C. macroptera*	26.70 ± 0.28
Teliamura, West Tripura, Tripura	Elachi Nimboo	*C. limon*	30.22 ± 0.35
Kalimpong, Darjeeling, West Bengal	Darjeeling mandarin	*C. reticulata*	29.75 ± 0.32
Mirik, Darjeeling, West Bengal	Darjeeling mandarin	*C. reticulata*	26.75 ± 0.24
Controls			
Positive control (HLB - inoculated Mosambi sweet orange plant maintained in an insect-proof screen house)			24.64 ± 0.28
Healthy control (Shoot-tip grafting derived Nagpur mandarin plant)			Undetected
Water (sterile) control			Undetected
Phytophthora nicotianae Isolate NRCPh-56 DNA			Undetected
Xanthomonas citri subsp. *citri* (pathotype A) Isolate Xac-6 DNA			Undetected

*Ct (Cycle threshold) values of real-time PCR are means ± standard deviation (SD). Ct ≤ 32, positive, Ct 32–36, putative positive, Ct > 36, negative.

well as other citrus pathogen (*Phytophthora, Xanthomonas*) – DNA, and the mean value of the threshold cycle (Ct) was presented with standard deviation.

In real-time PCR, Ct value was monitored for each sample; the higher the Ct value, the lower the concentration of the DNA of interest in the sample. If

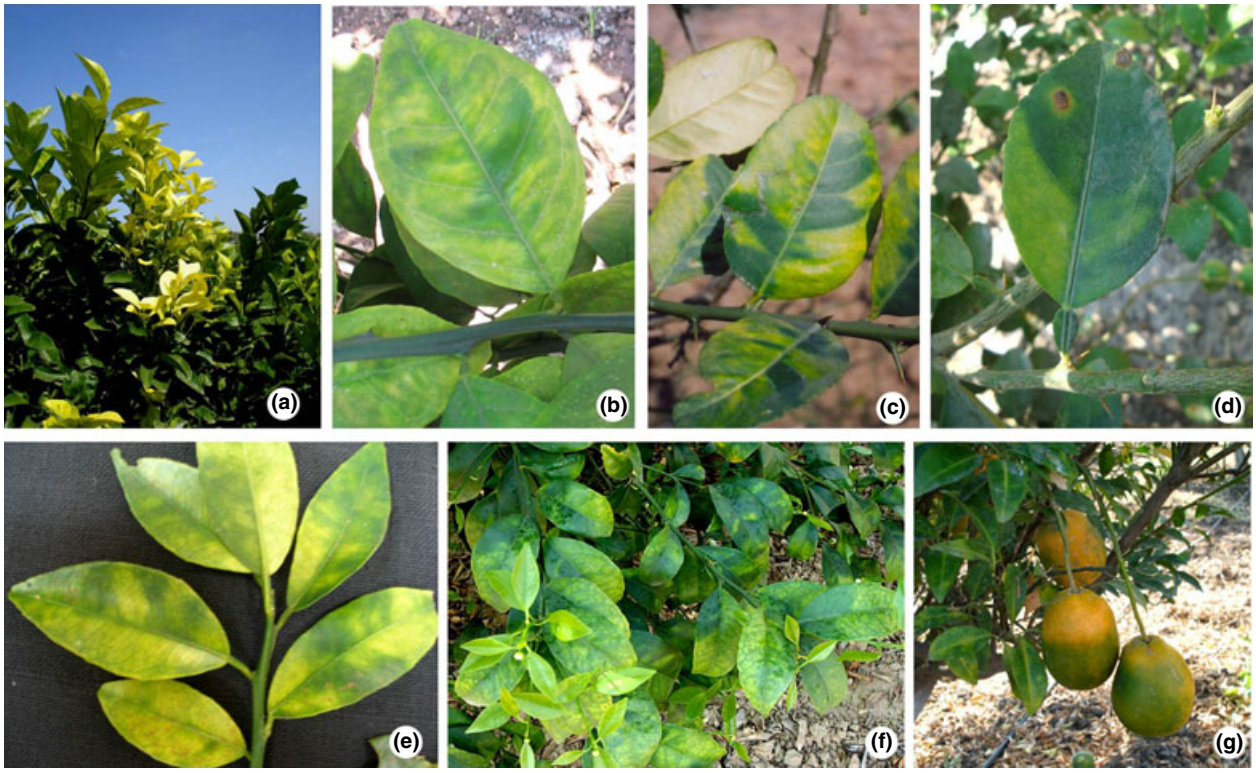

Fig. 2 Symptoms associated with Huanglongbing in diverse citrus cultivars grown in different states of India. Appearance of 'yellow shoot' in Mosambi sweet orange in Maharashtra (a), Classic 'blotchy mottle' leaf symptom in Sathgudi sweet orange in Andhra Pradesh (b), Assam lemon in Assam (c), acid lime in Tamil Nadu (canker lesions are also seen) (d), Khasi mandarin in Meghalaya (e), and Kinnow mandarin in Punjab (f), Leaf vein yellowing and stylar end greening of Nagpur mandarin fruits in Maharashtra (g).

sample DNA contained '*Candidatus* Las' DNA, amplicon was created in the PCR and the amount was reflected in the Ct value. To test for '*Ca*. Las' infection, a Ct of 32 or less was considered positive for detection (Gottwald et al. 2012). However, Ct values between ~32 and 36 are considered a 'grey' area in which it is difficult to say with certainty that the sample is positive, negative or in the early stages of developing HLB. (Windham et al. 2011). Hence, the range between 32 and 36 Ct value was considered as 'putative' positive. A value above 36 was considered negative for HLB infection under present experimental conditions.

Results and Discussion

This study represents the first systematic survey of HLB in India using real-time PCR. HLB was confirmed previously by indexing on indicator hosts (Bhagavati 1993) and conventional PCR method (Das 2004). HLB was detected in all surveyed states and in all samples of commercial citrus (Table 1). The lone exceptions were in the state of Arunachal Pradesh where two of three samples were negative, and one sample was a putative positive (Fig. 1 and Table 1). Additional samples need to be tested to confirm the occurrence of HLB in this state, though the disease and its insect vector, *D. citri* was reported previously from this region (Bhagavati 1993). Furthermore, citrus orchards of Arunachal Pradesh (Basar and Along, in particular) had green and healthier growth compared to other parts of NE India, which indicated low prevalence of HLB. The real-time PCR assays with HLBas-HLBr-HLBp primer–probe combination yielded negative results in healthy controls, sterile water control and with DNA obtained from *Phytophthora nicotianae* (Isolate NRCPh-56) and *Xanthomonas citri* subsp. *citri* (pathotype A, Isolate Xac-6) (Table 1) demonstrating specificity of the primer–probe combination in detecting '*Ca*. Las' and do not exhibit any cross-reactivity with other pathogenic residents of citrus.

Huanglongbing affects all citrus cultivars, regardless of root stock and there is no resistance within the major commercial citrus cultivars (Folimonova et al. 2009), and thus even if their symptoms are less severe, infested trees remain sources for infection of

others. A well-coordinated, area-wide, integrated management tactic comprising of the use of HLB-free nursery stock, reduction of the inoculum by the removal of symptomatic trees and suppression of the psyllid vector would go a long way to ensure continued viability and productivity of the Indian citrus industry.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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