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Survey and Detection of *Candidatus Liberibacter asiaticus* Causing Huanglongbing (HLB) Disease of Citrus in Manipur

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A B S T R A C T

Huanglongbing (HLB), also known as the "greening disease" is a serious disease affecting major citrus growing groves in India as well as in the world. The presumptive causal agent is a fastidious, gram negative, α- subdivision of the proteobacteria named *Candidatus Liberibacter asiaticus* (CLas) transmitted by Asian citrus psylla (*Diaphorina citri*). Different symptoms varying from yellowing of leaves, whole branch (called yellow shoot disease in China),drying of twigs, yellow patches in newly emerged flushes, irregular patches of yellowing on leaves (blotchy mottle), rabbit ear like appearance of leave and mineral deficiency like symptoms (leave showing symmetrical pattern of chlorosis on both sides of mid rib) were mainly observed on different citrus species during the survey conducted at various citrus growing pockets of Manipur in the present study. The leaves of citrus tree exhibiting the symptoms akin to mineral deficiency and rabbit ear like appearance were tested positive for HLB infection. Out of total 40 samples tested from different parts of Manipur by normal conventional PCR using specific primer targeting partial 16S rDNA, 19 samples were tested positive (47.50% infection) indicating high prevalence of HLB in different parts of Manipur. Highest percent infection of HLB was observed in Kachai Village, Ukhrul district, Manipur (72.72%) followed by Noney region of Tamenglong District (50%of tested samples were positive) and lowest incidence was recorded from Imphal West and East districts of Manipur (33.33%of tested samples were positive). These findings indicated widespread prevalence of HLB in different citrus growing pockets of Manipur.

Introduction

Huanglongbing (HLB) is a bacterial disease of citrus showing diverse symptoms ranging from yellowing of leaves, branches, drying of twigs, yellow patches on newly emerged flushes, irregular patches of yellowing on leaves (blotchy mottle), mineral deficiency like symptoms (leaves showing symmetrical pattern of chlorosis on both sides of mid rib), rabbit ear like appearance of leave etc. HLB has different names in different countries viz. vein phloem degeneration or phloem necrosis (Indonesia), Likubin (Taiwan), dieback

(India), mottle leaf (Philippines) and greening (South Africa). Chinese name is the worldwide accepted one (Moreno *et al.,* 1996). The disease has been reported as most destructive and devastating in India where more than 60 million trees have estimated to be wiped out due to this disease (Das *et al.,* 2004).

The presumptive causal organisms of HLB are fastidious, phloem limited, gram-ve, alpha protobacterium which is classified as genus *Candidatus Liberibacter* (Bove, 2006). There are three forms of HLB pathogen: Asiatic form known as *Candidatus Liberibacter asiaticus* (CLas), African form known as *Candidatus Liberibacter africanus* (CLaf) and American form known as *Candidatus Liberibacter americanus* (CLam) (Jagouex *et al.,* 1994, Gannier *et al.,* 1984). A sub-species (CLaf sub sp. carpensis) was recorded from cape chestnut (*Calodendrum carpense*) in South Africa. Mixed infections of three forms of HLB may sometimes occur (Bove 2006). HLB is transmitted by two phloem-feeding insect vectors, the Asian citrus psyllid, *Diaphorina citri* and the African citrus psyllidor two-spotted citrus psyllid, *Triozaerytreae* (Bove, 2006). CLas bacterium has a wide host range and can infect, although not necessarily cause disease, on most rutaceous species and some solanaceous species (Halbert and Manjunath, 2004). The Asiatic and American types are transmitted by Asian citrus psylla (*Diaphorina citri*) and African type by *Tryozaerytreae*. The CLaf is heat sensitive and does not cause symptoms at temperatures greater than 25–30° C. The CLas primarily distributed in Asia, is heat tolerant and able to cause symptoms even at temperatures greater than 35°C and is considered to be the most destructive one. The CLam appears heat sensitive similar to that of the CLaf. The HLB has emerged as one of the most important diseases in citrus plantation of different parts of the world. Disease has been reported from different citrus growing belts of India including North East India (Das *et al.,* 2004; Ghosh *et al.,* 2015). A few samples of CLas collected from different states of NE India and characterized at molecular level except for Manipur (Ghosh *et al.,* 2015). Although based on symptoms, the disease is quite prevalent in different citrus orchards of Manipur, the systematic investigation of survey and detection has not been undertaken. The present study was therefore undertaken to study the prevalence of CLas infection in different citrus groves of Manipur.

Materials and Methods

Survey for huanglongbing disease at different citrus growing groves of Manipur

Systematic surveys were conducted at different citrus growing groves of Manipur. The districts covered were Imphal West (NaoremLeikai and Chajing), Imphal East (Thongju, Khabam and Top Awangleikai), Tamenglong (Noney) and Ukhrul (Kachai) during 2017 to 2018. During the surveys, symptom expression on different citrus species was recorded. Symptomatic samples suspected to have HLB infection were collected and immediately store din cool box to avoid the degradation of DNA. The collected samples were brought to laboratory and processed either immediately or stored in the DNA later solution at -20°C or -80°C for future use.

Detection of CLas bacterium associated with huanglongbing disease of citrus in Manipur

Total DNA was extracted from the mid rib, petiole of the collected samples using Plant DNeasy mini kit (Qiagen, Germany). DNA was isolated following two validated protocols viz., DNeasy Plant Mini Kit (Qiagen, Germany) or CTAB extraction protocol.

CTAB method gave good yield of DNA but low quality as compared to QiagenDNeasy Plant Mini Kit. DNA isolation using DNeasy Plant Mini was done following the manufacturer protocol using column for DNA isolation. 100 mg of samples were used for DNA extraction. The quality and quantity of the isolated DNA was determined by taking OD value at 260nm and 280nm using a NanoVuePlus Spectrophotometer (JENWAY Genova Nano, United Kingdom).

PCR amplification

Amplification was performed in Thermal cycler (Applied Biosystems) through conventional PCR using primers set LASF/R (Fujikawa and Iwanami, 2012) targeting partial 6SrDNA (most conserved region of the CLas genome). The reaction mixture was prepared for 25µl volume using 5 µl of 10x buffers, 0.5 µl of dNTPs (10mM) and 2.0 µl $MgCl_2$(25mM), 2.0 µl of forward and reverse primers (10 µM), 0.3 µl Taq polymerase (5 units/µl, Genei^{TM}), DNA template of 5 µl (100-200 ng/µl) and remaining volume was make up with nuclease free water. The thermal cycling conditions were: initial denaturation of 94°C for 4 min, followed by 30 cycles of 94°C for 45s, annealing temperature of 56°C for 45s, 72°C for 1min and final extension at 72°C for 10 min. The amplification product was analyzed at 1% agarose gel containing ethidium bromide in Tris acetate EDTA buffer. The amplicons were checked through UV illumination in gel documentation system.

Results and Discussion

Survey of huanglongbing disease at different citrus growing pockets of Manipur

Most the samples were collected during the cooler season of the year as concentration of CLas bacterium was expected to be high during these periods. During the survey, diverse symptoms varying from yellowing of leaves, branches, rabbit ear like appearance of leaves, shoot with yellow patches, irregular mottling on leaf lamina (blotchy mottle), mineral deficiencies like symptoms (regular pattern of yellowing or vein yellowing or clearing on leaf lamina) were observed. Most interestingly leaves with mineral deficiency like symptoms and rabbit ear like appearance were found positive for CLas.

The irregular blotching called mottling and chlorosis are the characteristics symptoms of HLB (Baranwal, 2004). These findings indicated that CLas isolates prevalent in Manipur exhibit different symptoms under field conditions. All the citrus species collected from different surveyed areas were susceptible to HLB as reported earlier (Garnier and Bove, 1993). The different symptoms as observed under field conditions in the present study might be due to the prevalence of diverse strains/haplotypes of CLas bacterium and susceptibility patterns of the citrus genotypes (Tsai *et al.*, 2008).

Detection of CLas bacterium associated with huanglongbing disease of citrus in Manipur

Detection of CLas bacterium was by PCR employing the primer targeting partial 16S rDNA (most conserved region of CLas bacterium genome). The expected amplicon of approx. 500bp observed on the agarose gel was observed in the citrus samples having CLas infection. Out of the 37 samples collected from different locations of citrus growing pockets of Manipur, 19 samples were tested positive (54.16 % percent infection). The details of the samples, symptoms expression at field level, host plant are given in Table 1.

Table.1 Survey and detection of CLas bacterium associated at different locations of Manipur

Sl. No	Locations	District	Host	Symptoms	HLB infection
1	Thongju	Imphal East	Acid Lime	Drying of twigs	-ve
2.	Thongju	Imphal East	Mandarin	Yellowing and mineral deficiency like symptoms on leaves	-ve
3	Top AwangLeikai	Imphal East	Acid lime	Irregular yellow patches on leaves (blotchy mottling)	-ve
4.	Top AwangLeikai	Imphal East	Acid lime	Yellowing and mottling of leaves	-ve
5.	NaoremLeikai	Imphal West	Mandarine	Yellowing of leaves, drying of twigs.	
6	NaoremLeikai	Imphal West	Acid lime	Irregular yellow patches on leaves (blotchy mottling)	-ve
7.	Chajing1	Imphal West	Assam Lemon	Yellowing and mottling of leaves	+ve
8.	Chajing2	Imphal West	Acid Lime	Blotchy mottle and mineral deficiency like symptoms on leaves	-ve
9.	Chajing3	Imphal West	Assam Lemon	Irregular patches of yellowing of leaves	+ve
10.	Chajing4	Imphal West	Pumello	Irregular patches of yellowing of plants	-ve
11.	Chajing5	Imphal West	Mandarin	Mineral deficiency like symptoms on leaves	-ve
12.	Chajing6	Imphal West	Acid lime	Yellowing of leaves and mineral deficiency like symptoms	+ve
13.	Chajing7	Imphal West	Sweet orange	Mineral deficiency like symptoms on leaves	-ve
14.	Chajing8	Imphal West	Acid Lime	Yellowing and mottling of leaves	+ve
15.	Khabam	Imphal East	Acid Lime	Blotchy mottle on leaves	+ve
16.	Khabam	Imphal East	Acid Lime	Yellowing and Mineral deficiency like symptoms	-ve
17.	NaoremLeikai	Imphal West	Acid lime	Irregular patches of yellowing leaves	-ve
18.	NaoremLeikai	Imphal West	Sweet orange	Mineral deficiency like symptoms on leaves	-ve
19.	NaoremLeikai	Imphal West	Acid Lime	Irregular patches of yellow on leaves and irregular chlorosis	-ve

20.	Naorem Leikai1	Imphal West	Acid Lime	Mineral deficiency like symptoms on leaves	**+ve**
21.	Naorem Leikai2	Imphal West	Acid Lime	Yellowing, mottling of leaves	**+ve**
22.	Noney-1	Tamenglong district	Sweet orange	Mineral deficiency like symptoms, yellowing of leaves	**+ve**
23.	Noney-2	Tamenglong district	Pumello	Yellow patches on the surface of leaves	**-ve**
24.	Noney-3	Tamenglong district	Mandarin	Yellowing of leaves	**-ve**
25.	Noney-4	Tamenglong district	Mandarin	Red rust like symptoms on the leaf lamina	**-ve**
26.	Noney-5	Tamenglong district	Acid Lime	Yellowing of leaves and mineral deficiency like symptoms	**+ve**
27.	Noney-6	Tamenglong district	Assam Lemon	Intervienalchlorosis on the leave	**+ve**
28.	Noney-7	Tamenglong district	Pumello	Yellowing of whole tree	**-ve**
29.	Noney-8	Tamenglong district	Pumello	Mineral deficiency like symptoms	**+ve**
30.	Kachai(K-1)	Ukhrul	Kachai Lemon	Rabbit ear like appearance of leave and yellowing of leaves	**+ve**
31.	Kachai(K-2)	Ukhrul	Kachai Lemon	Irregular Chlorosis, mineral deficiency like symptoms on leaves	**+ve**
32.	Kachai(K-3)	Ukhrul	Kachai Lemon	Yellowing of whole leaves	**-ve**
33.	Kachai(K-4)	Ukhrul	Kachai Lemon	Mottling, Yellowing, Mineral deficiency like symptoms on leaves	**+ve**
34.	Kachai(K-5)	Ukhrul	Kachai Lemon	Mottling and yellowing of leaves	**+ve**
35.	Kachai(K-6)	Ukhrul	Kachai Lemon	Mottling and yellowing of leaves	**+ve**
36.	Kachai(K-7)	Ukhrul	Kachai Lemon	Yellowing of whole leaves	**-ve**
37.	Kachai (K-8)	Ukhrul	KachaiLemon	Mottling, yellowing of whole leaves	**+ve**
38.	Kachai (K-8)	Ukhrul	Kachai Lemon	Yellowing of whole leaves	**+ve**
39.	Kachai (K-8)	Ukhrul	Kachai Lemon	Irregular chlorosis, mineral deficiency like symptoms on leaves	**+ve**
40.	Kachai (K-8)	Ukhrul	Kachai Lemon	Yellowing of whole leaves	**-ve**

Table.2 Prevalence of CLas bacterium based on 16S rDNA

Sl.no.	Region/districts	Total no. of samples collected	No. of sample tested positive for HLB	Incidence of HLB (%) *
1.	Imphal West	15	5	33.33
2.	Imphal East	6	2	33.33
3.	Tamenglong	8	4	50.00
4.	Ukhrul	11	8	72.72
	Total	**40**	**19**	**47.50**

*PCR based detection

The percent infection of HLB was highest at Kachai village of Ukhrul (72.72%) followed by Noney in Tamenglong district (50%). The lowest per cent infection was recorded from the samples collected from Imphal West and Imphal East districts with 33.33% infection (Table 2). An incidence of 5-30% in Sikkim and 5-8.3% on Khasi mandarin was earlier reported from NE India (Das *et al.*, 2007). Ghosh *et al.*, (2015) reported a HLB incidence of 32% in different areas surveyed in North East except Manipur. Present study showed high prevalence of HLB in Manipur and reported susceptibility of all citrus species to CLas. Present study also reported the applicability of PCR based detection in routine indexing of citrus for HLB infection. Future studies on characterization of HLB haplotypes will throw more light on the CLas population prevalent in Manipur

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