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Status of *Phytophthora* and Huanglongbing Diseases of *Citrus* Causing Decline in Coorg Mandarin in India

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Abstract: Coorg mandarin is a famous ecotype of mandarin grown in multi-tier cropping system in coffee and pepper plantations in Coorg region of India. The roving survey indicated disease incidence of huanglongbing (HLB) and *Phytophthora* ranging from 35%-64.2% and 22.5%-35.6% in different places of Coorg region. A total of 177 *Phytophthora* infected (plant roots 59, soil 118), and 576 greening or HLB infected Coorg mandarin samples were collected from 184 Coorg mandarin orchards, of these 111 *Phytophthora* isolates were isolated and characterized in this study on the basis of colony growth patterns and morphological structures. Based on the virulence, only 52 of 111 *Phytophthora* isolates were amplified by PCR using universal internal transcriber spacer (ITS) primers, cloned and sequenced. The sequences analysis of *Phytophthora* isolates revealed more than 97% nucleotide (nt) similarity within themselves, except eight isolates of *P. palmivora* and four isolates of *P. nicotianae* had varied nt identity (70.6% to 97.4%) with other *Phytophthora* species compared. Similarly, 523 of 576 Coorg mandarin samples were confirmed the presence of HLB infection by PCR using 16S rRNA gene specific universal primers. The representative five of 523 Coorg mandarin isolates showed nt identity ranged from 94.4% to 95.3% with *Candidatus Liberibacter asiaticus* (AB008366) isolate infecting citrus in Asia and one isolate of HLB shared nt identity of 96.9% with *Ca. L. asiaticus* (KJ944269).

Key words: *Phytophthora*, citrus greening, gummosis, RPARH medium, Asian psyllid.

1. Introduction

Citrus is considered as one of the most important tropical fruit crop in India. It plays a vital role in the fruit economy of the country, next to mango and banana. India ranks sixth among top *Citrus* producing countries of the world. In India, *Citrus* is primarily grown in Assam, Andhra Pradesh, Maharashtra, Punjab, Karnataka, Uttaranchal, Bihar, Orissa and Gujarat. Andhra Pradesh occupies first place in *Citrus* production by producing 1,805.64 tonnes, constituting 24.19% share of total production in India [1].

The *Citrus* cultivation in India is hampered due to non-availability of disease-free planting material, bud wood transmissible diseases, general neglect, scarcity of water and poor management practices [2]. Beside this, *Citrus* spp. are prone to be attacked by more than 150 diseases and disorders caused by fungal, bacterial, viral and few non-viral pathogens right from nursery level to harvesting stage resulting in considerable yield losses [3]. The most important commercial *Citrus* in India is the mandarin orange, followed by the sweet oranges and acid limes. Coorg mandarin is a famous ecotype of mandarin grown in Kodagu, Hassan and Chikmagalur of districts of Karnataka (India) as a component of the multi-tier cropping system with coffee and pepper plantations for more than 150 years with an area of 24,000 ha area. Coorg oranges are regarded as man-made hybrids of mandarins (*Citrus reticulata*). Greenish-yellow in colour, they have a tight skin and a sweet-sour taste, unlike Nagpur oranges, which are known to have loose skin and sweet taste. Coorg oranges are said to have longer shelf life compared to other varieties [4]. This cropping system proved efficient with higher land use efficiency. The excess shade and sub optimal

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management practices affect the growth and fruiting of the trees.

In recent years, the Coorg mandarin cultivation area has come down to less than 2,000 ha. The region was, in fact, known as an orange belt till the *Phytophthora* rot and citrus greening disease took a heavy toil of crop [5]. The yield of single plant has come down to around 10 kg, which was once more than 50 kg. Diseases related to *Phytophthora* and greening or huanglongbing (HLB) disease are difficult to estimate as both cause decline of trees [6-8]. The most important *Phytophthora* sp. reported to cause diseases in Coorg mandarin include *P. palmivora*, *P. parasitica* Dastur (*P. nicotianae*) and *P. citrophthora* [9]. These *Phytophthora* species have been shown to cause some serious soil borne diseases of *Citrus*, including damping off of seedlings in the seedbed, root and crown rot in nurseries, foot rot and brown rot of fruits. The second important disease, which severely hampered the production of Coorg mandarin, is citrus greening disease. *Candidatus Liberibacter asiaticus* is the most predominant species in the region, which is vectored by Asian psyllid (*Diaphorina citri* Kuwayama) [10]. Differences in aggressiveness, competitiveness and response to fungicides varied among these two pathogens. Therefore, the aim of the present study was to characterize the *Phytophthora* and HLB disease pathogens associated with Coorg mandarin decline and also to know the status of disease on Coorg mandarin.

2. Material and Methods

2.1 Survey, Collection of Disease Samples and Disease Assessment

The roving survey was employed during 2015-2016 in 184 different farmers' fields in Kodagu and Hassan districts (Karnataka) in India (Fig. 1), to estimate the

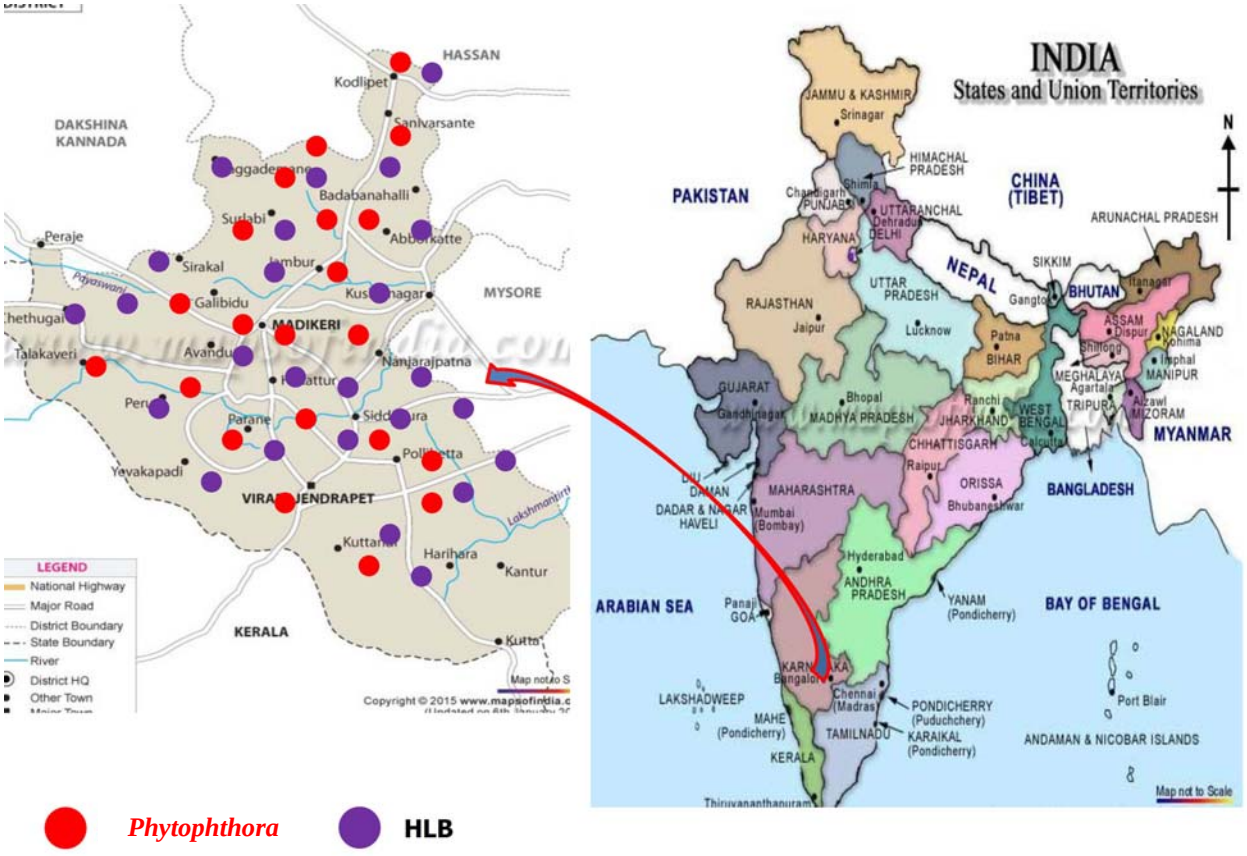

Fig. 1 Survey conducted in different places of Coorg region in Karnataka.

incidence of *Phytophthora* and HLB disease on Coorg mandarin (plants raised from true seeds and grafted on Rangpur lime rootstock).

The disease incidence was estimated in each field by visual examination of plants by making two diagonal transects across the field in the form of an "X" (50 plants along each diagonal). In each orchard, 100 trees per orchard were rated for foot rot and gummosis lesion severity on a scale of 0 (no lesion) to 5 (entire trunk covered with lesion and gummosis). The incidence was determined as the proportion of plants showing symptoms of *Phytophthora* (root rot, discoloration of the bark surface, discoloration of the underlying tissues, dieback, dried the whole part of the plant and exudation of gum from infected tissues) and HLB (mottling and yellowing) expressed as a percentage of the total number of plants assessed. The rhizosphere soil and infected root and plant parts were also collected from surveyed areas to study occurrence and distribution of *Phytophthora* species. Similarly, HLB disease incidence was rated by using three-point scale with 0 representing no symptom observed on plant canopy, 1 = mild (from 1% to 30% of the canopy), 2 = moderate (from 31% to 50% of the canopy), 3 = severe (more than 50% of the canopy). Percentage of disease incidence of *Phytophthora* and HLB was calculated based on the number of observed symptomatic plants over the total number of the assessed plants. The percentage of disease incidence (PDI) was estimated using Eq. (1).

$$\text{PDI (\%)} = \frac{\text{Total infected } \textit{Citrus} \text{ trees}}{\text{Total number of trees observed}} \times 100 \quad (1)$$

Disease severity index (DSI) was then calculated as Eq. (2):

$$\text{DSI (\%)} = \frac{X_1 + X_2 + \ldots + X_n}{Y \times \text{maximum rating scale}} \times 100 \quad (2)$$

where, X = sum score of disease severity of each *Citrus* plant; Y = total number of plant assessed.

2.2 Collection of Disease Samples of Phytophthora and HLB

A total of 177 *Phytophthora* infected plant roots (59), soil (118) and 576 greening or HLB infected Coorg mandarin samples were collected from 184 Coorg mandarin orchards, showing typical symptoms of *Phytophthora* infection, greening, yellowing and mottling in different places of Coorg and Hassan, and one sample from each location without any symptoms on Coorg mandarin plants was also collected.

2.3 Isolation of Phytophthora Species from Plant Parts and Soil

2.3.1 Coorg Mandarin Infected Plant Parts

Phytophthora infected Coorg mandarin plant parts (twigs, trunk and gummosis part) were collected from different orchards in Coorg region. The collected samples which may have been contaminated with different pathogens were removed by surface sterilization using standard protocol [11]. Then samples were aseptically placed on specific media amended with pimaricin, ampicillin, rifamficin, pentachloronitrobenzene (PCNB) and hymexazol (RPARH) medium.

2.3.2 Soil from the Rhizosphere of Infected and Healthy Plants

The soil sample collected from rhizosphere (1 gm) of infected and healthy plants were thoroughly mixed in 10 mL of sterile distilled water, and 1 mL each of soil suspensions was transferred into specific containing Petriplates and incubated at 25 ± 2 °C for 48 h. Later, the media on Petriplate was washed with sterile distilled water to observe growth of *Phytophthora* culture. The colony resembling *Phytophthora* were picked and transferred to specific media. Stock cultures were maintained on V8 agar. The pure culture of isolated *Phytophthora* isolates was used for morphological studies (colony growth, sporangia, oogonia, antheridia, chlamydospores, hyphal swellings and aggregations with species descriptions in the literature using light microscope (Nikon Eclipse 50i) at 400× magnification. The images were imported to ImageJ (National Institutes

of Health, USA) and analyzed by setting the scale at 3.6 pixels/μm. Radial growth was calculated by placing a mycelial plug on fresh (RPARH) media, and incubating for three days at room temperature under continuous fluorescent light. Three plates per isolate were prepared. Colony pattern was characterized according to morphology for 7-day-old colonies [12]. Mycelial growth was classified as reduced or appressed, scanty-fluffy or densely-fluffy following the descriptions described by Erwin and Ribeiro [12].

Pathogenicity test: To confirm pathogenicity of casual agent, the un-infected healthy three-month-old seedlings of Coorg mandarins were inoculated with sporangial suspension at a concentration of 1×10^6 conidia/mL by making the wounded (pin prick method) at crown region of the plant. The inoculated Coorg mandarin plants were kept under controlled condition to monitor the expression of wilting or rotting, gummosis and lesions symptoms. Subsequently, pathogen was re-isolated from the diseased plant showing gummosis and lesions development symptoms on crown region of the plant, confirming Koch's postulates. The test was repeated twice.

2.4 Molecular Characterization

2.4.1 Fungal Genomic DNA Isolation

The pure culture of the fungus was grown on potato dextrose broth at 25 ± 2 ºC for 7 d. The fungal mycelium was harvested by filtration through Whatman No.1 filter paper and washed with sterile distilled water and dried. Two grams of dried mycelium were used for total genomic DNA isolation by following modified protocol of CTAB method [13]. The quality of the genomic DNA was checked on 0.8% agarose gel and stored at -20 °C till further use. To confirm identity of the pathogen, Total genomic DNA was amplified by PCR using universal internal transcriber spacer region primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [14]. The DNA amplification was performed with 35 cycles of denaturation for 1 min at 94 °C, primer annealing for 45 s at 55 °C for *Phytophthora* and primer extension for 90 s at 72 °C, with an initial denaturation at 94 °C for 3 min and a final extension for 10 min at 72 °C. The PCR reactions were carried out in a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler. All amplifications were performed in volumes of 25 μL PCR mix containing 2 μL DNA templates, 1.5 U Taq DNA polymerase, 25 mM $MgCl_2$, 2 mM dNTPs and 20 pmol of each primer. PCR products were electrophoresed (1 h at 80 V) in 0.8% agarose gels in Tris-Borate-EDTA buffer, pH 8. Gels were stained with ethidium bromide (10 mg/mL) and viewed in a gel documentation system (Alpha Innotech, USA).

2.4.2 HLB Disease

The total nucleic acid was isolated from Coorg mandarin samples (576 HLB infected) by using CTAB method [13]. Finally, DNA pellets were dried under vacuum, resuspended in 50 μL of TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0) and maintained at -20 °C until being used. The presence of HLB in 540 samples was confirmed by PCR using 16S rRNA gene specific universal primers [15]. The DNA amplification was performed with 35 cycles as described above, except primer annealing for 45 s at 56 °C for HLB. The amplified nested PCR products HLB (1.2 kbp) and *Phytophthora* (550 bp) was purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Hilder, Germany) and cloned into pTZ57R/T cloning vector according to manufacturer's directions (MBI Fermentas, Germany). The transformation was performed using *Escherichia coli* (DH5α) cells. The transformed plasmid was isolated using Qiagen plasmid miniprep kit (Qiagen, Hilder, Germany) and sequenced using automated DNA sequencing facility at Eurofins Genomics India Pvt. Ltd., Bangalore, India.

2.4.3 Sequence Analysis

The sequences of HLB (1.2 kbp) and *Phytophthora* (550 bp) were subjected to NCBI BLAST to search

for similar sequences in the database. The related sequences obtained from the database belonging to different *Liberobacter* and *Phytophthora* species infecting diverse hosts were used for analysis (Tables 1a and 1b). Sequences were aligned using MUCSLE method implemented in SEAVIEW program [16]. The nucleotide (nt) sequence identity matrixes for the HLB and *Phytophthora* were generated using BioEdit sequence alignment editor (version 5.0.9) [17]. The phylogenetic tree was constructed by neighbor joining method using MEGA 6.0.1 version software [18] with 1,000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously.

Table 1a *Phytophthora* species sequence accession numbers obtained from GenBank database used in the analyses.

Phytophthora species	Accession number	*Phytophthora* species	Accession number
P. nicotianae-NRCPh-1	HM807369	*P. palmivora*-NRCPh-15	JF792543
P. nicotianae-NRCPh-2	JF792525	*P. palmivora*-NRCPh-20	JF792544
P. nicotianae-NRCPh-3	JF792526	*P. palmivora*-NRCPh-21	JF792545
P. nicotianae-NRCPh-4	HM807370	*P. palmivora*-NRCPh-23	JF792546
P. nicotianae-NRCPh-5	JF792527	*P. palmivora*-NRCPh-27	JF792547
P. nicotianae-NRCPh-6	HM807371	*P. palmivora*-NRCPh-29	JF792548
P. nicotianae-NRCPh-7	HM807372	*P. palmivora*-NRCPh-79	JX965378
P. nicotianae-NRCPh-9	JF792528	*P. palmivora*-NRCPh-94	JN559844
P. nicotianae-NRCPh-1	JF792529	*P. palmivora*-NRCPh-100	KF010299
P. nicotianae-NRCPh-17	JF792530	*P. palmivora*	KR920754
P. nicotianae-NRCPh-18b	JF792531	*P. palmivora*	JX198553
P. nicotianae-NRCPh-19	JF792532	*P. palmivora*	KR920757
P. nicotianae-NRCPh-22	JF792533	*P. palmivora*	JX198562
P. nicotianae-NRCPh-24	JF792534	*P. palmivora*	KP183963
P. nicotianae-NRCPh-31	JF792535	*P. palmivora*	KR920758
P. nicotianae-NRCPh-37	JF792536	*P. palmivora*	HQ237479
P. nicotianae-NRCPh-41	JF792537	*P. palmivora*	KR920760
P. nicotianae-NRCPh-52	JF792538	*P. palmivora*	JF792548
P. nicotianae-NRCPh-56	JF792539	*P. palmivora*	KU877819
P. nicotianae-NRCPh-58	JX965375	*P. palmivora*	AB669142
P. nicotianae-NRCPh-61	JX965375	*P. palmivora*	AF266780
P.nicotianae-NRCPh-66	JX965375	*P. citrophthora*-NRCPh-97	JN559845
P. nicotianae-NRCPh-70	JX965375	*P. citrophthora*	GU993889
P.nicotianae-NRCPh-71	JX965376	*P. citrophthora*	HQ643208
P. nicotianae-NRCPh-76	JX965377	*P.citrophthora*	HQ643207
P. nicotianae-NRCPh-81	JX965379	*P. citrophthora*	HQ261535
P. nicotianae-NRCPh-89	JN559843	*P. citrophthora*	AF266785
P. nicotianae-NRCPh-98	JN559846	*P. capsici*	AF266787
P. nicotianae-NRCPh-99	JX965380	*P. cinnamomi*	AF266764
P. nicotianae	KJ549640	*P. insolita*	AF271222
P. nicotianae	GU111669	*P. inundata*	AY659717
P. nicotianae	KJ549641	*P. ipomoeae*	AY770742
P. nicotianae	KJ494919		
P. nicotianae	KJ494917		
P. nicotianae	KJ494914		
P. nicotianae	KJ494913		
P. nicotianae	KJ494911		
P. nicotianae	AF266776		

Table 1b *Ca. Liberibacter* species sequence accession numbers obtained from GenBank database used in the analyses.

Ca. Liberibacter species	Accession number	*Ca. L.* species	Accession number
Ca. L. asiaticus-MPW1456	AB558580	*Ca. L. asiaticus*-H22Y	JQ867419
Ca. L. asiaticus	AB480072	*Ca. L. asiaticus*	EU224393
Ca. L. asiaticus	DQ471900	*Ca. L. asiaticus*-F11Dade	EU130552
Ca. L. asiaticus-GFB-Selangor	EU224393	*Ca. L. asiaticus*-H36YPENINSULAR	JQ867409
Ca. L. asiaticus	EU130552	*Ca. L. asiaticus* strain ACL	KC551941
Ca. L. asiaticus	DQ157275	*Ca. L. asiaticus* clone 33a	JX430434
Ca. L. asiaticus	DQ157273	*Ca. L. asiaticus*	AB555706
Ca. L. asiaticus	DQ303210	*Ca. L. asiaticus*	DQ157274
Ca. L. asiaticus	AY919311	*Ca. L. asiaticus*	JQ867445
L. Phytophthora Okinawa-7	AB008366	*Ca. L. asiaticus*	KU761591
Ca. L. asiaticus	KJ944269	*Ca. L. asiaticus*-H23Y	JQ867420
Ca. L. asiaticus	KJ944267	*Ca. L. asiaticus*-H16Y	JQ867418
Ca. L. asiaticus	KJ944262	*Ca. L. asiaticus*-H15QR 1	JQ867447
Ca. L. asiaticus-TNal-9	KC800958	*Ca. L. asiaticus*-H31Y	JQ867425
Ca. L. asiaticus-TNal-8	KC800957	*Ca. L. asiaticus*-Polibetta	FJ827779
Ca. L. asiaticus-TNmal-2	KC800951	*Ca. L. asiaticus*-Guangxi-GL-19-CHN	EU921616
Ca. L. asiaticus isolate Naga-1	KC119094	*Ca. L. asiaticus*-Guangxi-GL-7-CHN	EU921614
Ca. L. asiaticus-H13QR	JQ867445	*Ca. L. asiaticus*-Guangxi-STY partial sequence	DQ432000
Ca. L. asiaticus-CAHLB2012	JX455745	*Ca. L. asiaticus*-Guangdong-PG	DQ431999
Ca. L. asiaticus-Umiam-2	JX284240	*Ca. L. americanus*	AY742824
Ca. L. asiaticus-gj1	JN049632	*Ca. L. americanus*-SaoPaulo-40	EU921625
Ca. L. asiaticus	EU265646	*Ca. L. americanus*-SaoPaulo-275	EU921623
Ca. L. asiaticus-MH	JQ900232	*Ca. L. americanus*-SaoPaulo-PW-49	EU921624
Ca. L. asiaticus-MDL1394-DR002-8	FJ821713	*Ca. L. americanus*-LJZ-5110	FJ263689
Ca. L. asiaticus isolate MDL1391-DR002-5	FJ821710	*Ca. L. americanus*-LJZ-5025	FJ263695
Ca. L. asiaticus	EU224394	*Ca. L. americanus*-LJZ-5020	FJ263694
Ca. L. asiaticus-F17PalmBeach	EU130554	*Ca. L. americanus*-LJZ-5130	FJ263692
Ca. L.-Kinnow	LN835770	*Ca. L. americanus*-LJZ-5128	FJ263691
Ca. L. asiaticus	KM224448	*Ca. L. americanus*-LJZ-5135	FJ263693
Ca. L. asiaticus clone C2	KM224447	*Ca. L. americanus*-LJZ-5115 1	FJ263690
Ca. L. asiaticus-Mmnd-1	KC800962	*Ca. L. europaeus*	JX244259
Ca. L. asiaticus-TNal-7	KC800956	*Ca. L. europaeus*-Psy6	JX244258
Ca. L. asiaticus-TNal-5	KC800954	*Ca. L. africanus*	KX990287
Ca. L. asiaticus-ACL-Katol-3	KC551941	*Ca. L. africanus*	KX990288
Ca. L. asiaticus-H20QR	JQ867450	*Ca. L. africanus*	KY000562
Ca. L. asiaticus-H18QR	JQ867448	*Ca. L. africanus*	KY000560
Ca. L. asiaticus-H12QR	JQ867444	*Ca. L. psyllaurous*-Tx15	EU812556
Ca. L. asiaticus-11QR	JQ867443	*Ca. L. psyllaurous*-Tom100	EU812558
Ca. L. asiaticus-Florida-8	EU921617	*Ca. L. psyllaurous*-PRR1	EU812559
Ca. L. asiaticus-Jiangxi-GC	DQ432003	*Ca. L. solanacearum*	EU935004
Ca. L. asiaticus-Chongqing-ZG	DQ432004		
Ca. L. asiaticus-H34Y	JQ867427		
Ca. L. asiaticus	KJ944265		
Ca. L. asiaticus-H2C	JQ867437		
Ca. L. asiaticus-H35C	JQ867434		
Ca. L. asiaticus-AL-Kahikuchi	KC551939		

3. Results

3.1 Survey

3.1.1 HLB Disease

A systematic random survey, conducted in 184 orchards of Kodagu district, revealed that the disease incidence of HLB was more severe in most of the Coorg mandarin plants. The disease incidence ranged from 35% to 64.2% on Coorg mandarin plants raised from true seeds and grafted plants growing in commercial groves in the Kodagu district, Karnataka state of India (Table 2). No Coorg mandarin plants (raised from true seeds or budded) displayed a consistently low or high level HLB incidence in every grove sampled. Further, it was also observed that the occurrence of yellowing was more in budded plants after six years of planting than the plant raise from true seeds. The infected plants showed symptoms of sparse foliage, very short twigs with narrow leaves characterized with yellowish colored leaves similar to zinc deficiency. Mottled leaves and fruits with aborted seeds provided good indication of greening disease in all surveyed areas, both in true seeds and grafted Coorg mandarin plants (Fig. 2). The severity of disease increased proportionally in orchards wherein true seed plants were more than 10 years old and budded plants were more than six years in age (Table 2).

3.1.2 *Phytophthora* Disease

A systematic random survey for incidence and severity of *Phytophthora* on Coorg mandarin plants revealed the occurrence of disease in all surveyed orchards with different magnitude of infection (Table 3). Incidence of the disease was not found to be

Table 2 Citrus greening bacteria infecting Coorg mandarin samples was collected from Kodagu and Hassan.

Place	No. of orchards	Age group (years)	No. of samples collected	Type of symptoms	Greening (HLB)		No. of samples PCR positive for greening
					Av. PDI (%)	DSI (%)	
Madikeri	48	8-15	165	Only yellowing, yellowing and mottling	61.56	4.1	152
Somawarpet	70	5-15	230	Only yellowing, yellowing and mottling	64.20	3.1	206
Virajpet	58	8-15	161	Only yellowing, yellowing and mottling	57.96	3.6	146
Belur (Hassan)	8	4-5	20	Yellowing and mottling	35.00	2.0	19
Total	184		576				523

Fig. 2 Coorg mandarin plant showing yellowing, mottling and complete yellowing and greening symptoms under natural conditions.

Table 3 ***Phytophthora*** **infecting Coorg mandarin samples was collected from Kodagu and Hassan.**

Place	No. of orchards	Age group (years)	No. of samples collected	No. of samples collected	Type of symptoms	*Phytophthora*		No. of samples positive to *Phytophthora*
			Root	Soil		Av. PDI (%)	DSI (%)	
Madikeri	48	8-15	10	23	Foot rot, wilting, gummosis	35.62	2.5	23
Somwarapet	70	5-15	20	52	Lesions in collar region, foot rot, wilting, gummosis	35.34	3.2	45
Virajpet	58	8-15	25	33	Lesions in collar region, foot rot, wilting, gummosis	31.42	2.57	34
Belur (Hassan)	8	4-5	4	10	Foot rot, wilting	22.50	1.0	9
Total	184		59	118				111

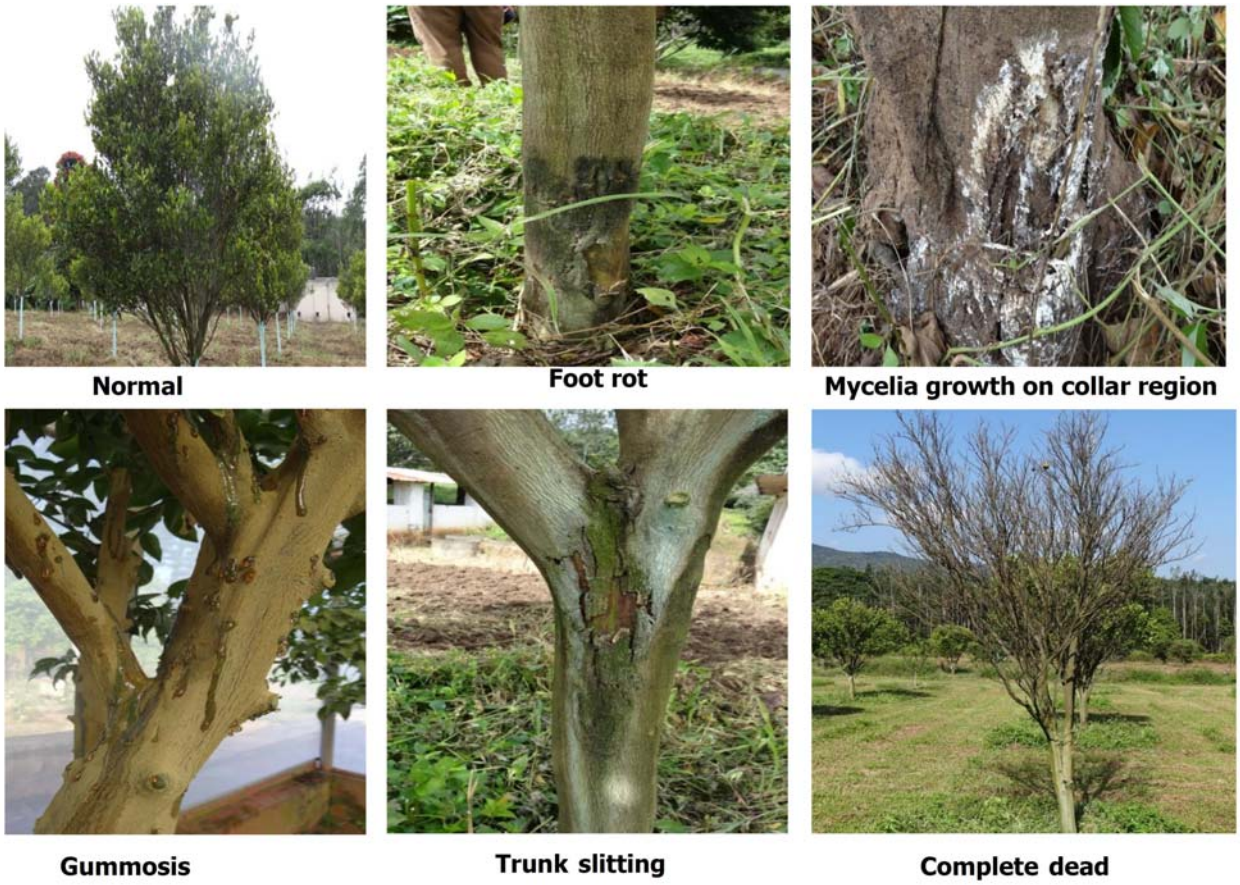

Fig. 3 **Coorg mandarin plant showing (a) foot rot, (b) gummosis, (c) collar rot and (d) trunk slit.**

affected by seasons, which could be attributed to the limited fluctuations in ambient temperatures and relative humidity. In post-monsoon season, the main symptoms observed in different orchards were rotting of the rootlets, leading to disintegration of the cortical tissue on feeder roots, with a white thread-like stele (inner tissue of the fibrous root). During summer season, heavy gum oozing was observed on higher branches irrespective of age of the plants. As the disease progressed, bark shrinks, cracks, shredding in lengthwise direction exposing black discolored internal tissue. Under severe infection, plants blossom heavily and die after fruit mature (Fig. 3).

Foot rot infection was recorded especially in

budded plants wherein the bud union lied close to the soil line promoting buildup of *Phytophthora* populations and increased risk of foot rot infection. Crown rot resulted from bark infection below the soil line when susceptible rootstocks were used. Incidence of *Phytophthora* disease was similar in true seeds and grafted Coorg mandarin plants in all surveyed areas. The highest incidence of *Phytophthora* disease was recorded in Madikeri (35.62%) with DSI rating 2.5, followed by Somwarapet and the lowest in Hassan (22.5%) with DSI rating 1 (Table 3).

3.2 Morphological Characterization

Out of 177 *Phytophthora* isolates, 111 were characterized from 33 places of Kodagu district. Among these, 21 isolates were isolated from symptomatic plant roots (*P. palmivora* and *P. nicotianae*). Fifty-eight (58) isolates belonging to *P. palmivora*, were isolated from 60.89% of soil samples and thirty-two (32) isolates belonging to *P. nicotianae*, were isolated from 41.55% of soil samples, respectively (Fig. 4).

The pathogen was isolated on specific media, the pure cultures of *Phytophthora* isolates were maintained in V8 slants for further use. On the basis of colony growth patterns coupled with microscopic features of morphological structures, the isolates were first grouped in five distinct morphotypes as described by Erwin and Ribeiro [12] (data not shown). Later, the isolates were finalized to two morphotypes, which were identified as *P. palmivora* and *P. nicotianae*.

3.2.1 *Phytophthora palmivora*

P. palmivora produced papillate sporangia with large chlamydospores, which could be easily distinguished from *P. citrophthora* and *P. nicotianae* by the caducous sporangia. Abundant sporangia were observed 5 d after inoculation and were typically papillate with short pedicels. Chlamydospores were spherical to ellipsoid structures with a thick cell wall (4 μm) and a diameter of 35-45 μm. Most of the isolates were heterothallic. Antheridia were amphigynous and spherical or oval and oogonia were smooth, spherical and 15-64 μm in diameter.

3.2.2 *Phytophthora nicotianae*

Tufted colony morphology and an arachnoid branching mycelium were the typical characteristic of *P. nicotianae*. Sporangia were more regular, symmetrical with a single apex, non-caducous, ellipsoid or ovoid or pyriform to spherical sporangia with usually a single papillum [12] and it could be easily

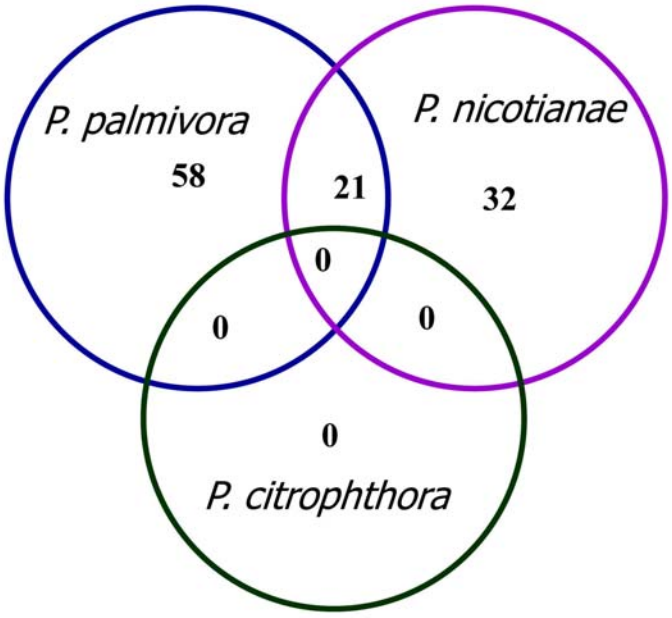

Fig. 4 Percentage of samples showing infection of different *Phytophthora* species.

differentiated from the caducous sporangia of *P. palmivora.*

3.3 Molecular Characterization

3.3.1 *Phytophthora*

Out of 111 *Phytophthora* isolates, 52 representative isolates were chosen from the morphological groups for molecular analysis. The total genomic DNA of 52 *Phytophthora* isolates was amplified by PCR using universal ITS region specific primers. The expected PCR amplicon size of 550 bp was amplified (data not shown), which corresponds to ITS region of the fungi. The amplified PCR product was cloned and sequenced. The sequence analysis showed that 30 of 52 *Phytophthora* isolates belonging to *P. palmivora* with nt identity more than 98% (data not shown) remaining 10 isolates (FG7, FG8, FG9, FG10, FG11, FG12, FG30, FG34, FG38, FG41) with varied nt identity (70.6%-97.4%) were selected for further analysis. Similarly, eight isolates belong to *P. nicotianae*, showed nt identity more than 97% among themselves (data not shown) of these four isolates (FG16, FG17, FG18, FG20) were selected with varied nt identity (91.6%-100%) for further analysis. The sequences of 10 representative *P. palmivora* and four *P. nicotianae* isolates were available in database under following accession number (Table 4).

The comparative ITS region analysis between 10 representative *P. palmivora* and four *P. nicotianae* isolates infecting Coorg mandarin showed less than 80% nt identity. Further, the ITS sequences of *P. palmivora* and *P. nicotianae* isolates infecting Coorg mandarin were compared with other *Citrus* infecting species, viz., *P. palmivora* (21 isolates), *P. nicotianae* (37 isolates), *P. citrophthora* (six isolates) and *Phytophthora* spp. (five isolates) infecting different crops available in the database (Table 1a). The analysis showed that all 10 representative *P. palmivora* isolates (FG7, FG8, FG9, FG10, FG11, FG12, FG30, FG34, FG38 and FG41) isolated from Coorg mandarin shared nt identity range from 87.6% to 100% with *P. palmivora* isolates, < 75% nt identity with *P.nicotianae* isolates and < 70% nt identity with *P.citrophthora* isolates available in the NCBI database infecting *Citrus* in India. Similarly, *P. nicotiana*e isolates (FG16, FG17, FG18 and FG20) were analyzed. The analysis showed that *P. nicotianae* isolates shared the nt identity ranging from 92.4% to 100% with *P. nicotianae* isolates infecting *Citrus*, less than 75% nt identity with *P. palmivora* and *P. citrophthora* isolates infecting *Citrus*, respectively. The phylogenetic tree based on comparison of ITS analysis of 10 representative *P. palmivora* and four *P. nicotianae* isolates infecting Coorg mandarin with other *Citrus* infecting *P. palmivora* (21), *P. nicotianae* (37), *P. citrophthora* (six) and other *Phytophthora* spp. (five). The analysis showed that all *P. palmivora*, *P. nicotianae* isolates infecting Coorg mandarin clustered with respective isolates of *P. palmivora* and *P. nicotianae* infecting *Citrus* in India (Fig. 5).

3.3.2 HLB Disease

Total 576 infected leaf samples of Coorg mandarin were collected from 184 Coorg mandarin orchards, showing typical symptoms of greening, yellowing and mottling. The total DNA isolated from 576 samples of Coorg mandarin was amplified by PCR using 16S rRNA gene specific primers. And 523 of 576 samples were confirmed the presence of HLB infection, with expected PCR amplicon size of 1.2 bp being amplified (data not shown) from all samples. The sequencing of representative samples showed a match with previously identified citrus greening bacteria. Therefore, only six (CGB1, CGB1a, CGB2 CGB3 CGB4 and CGB5) out of 523 samples were selected for cloning and sequencing, based on symptom severity and location. The 16S rRNA gene sequence analysis showed that six HLB Coorg mandarin isolates shared nt identity ranging from 94.3% to 98.9% among themselves. Further, HLB Coorg mandarin isolates 16S rRNA gene was compared with other *Ca. L. asiaticus* (65 isolates), *Ca. L. americanus* (11 isolates), *Ca. L. africanus* (four isolates), *Ca. L. psyllaurous* (three

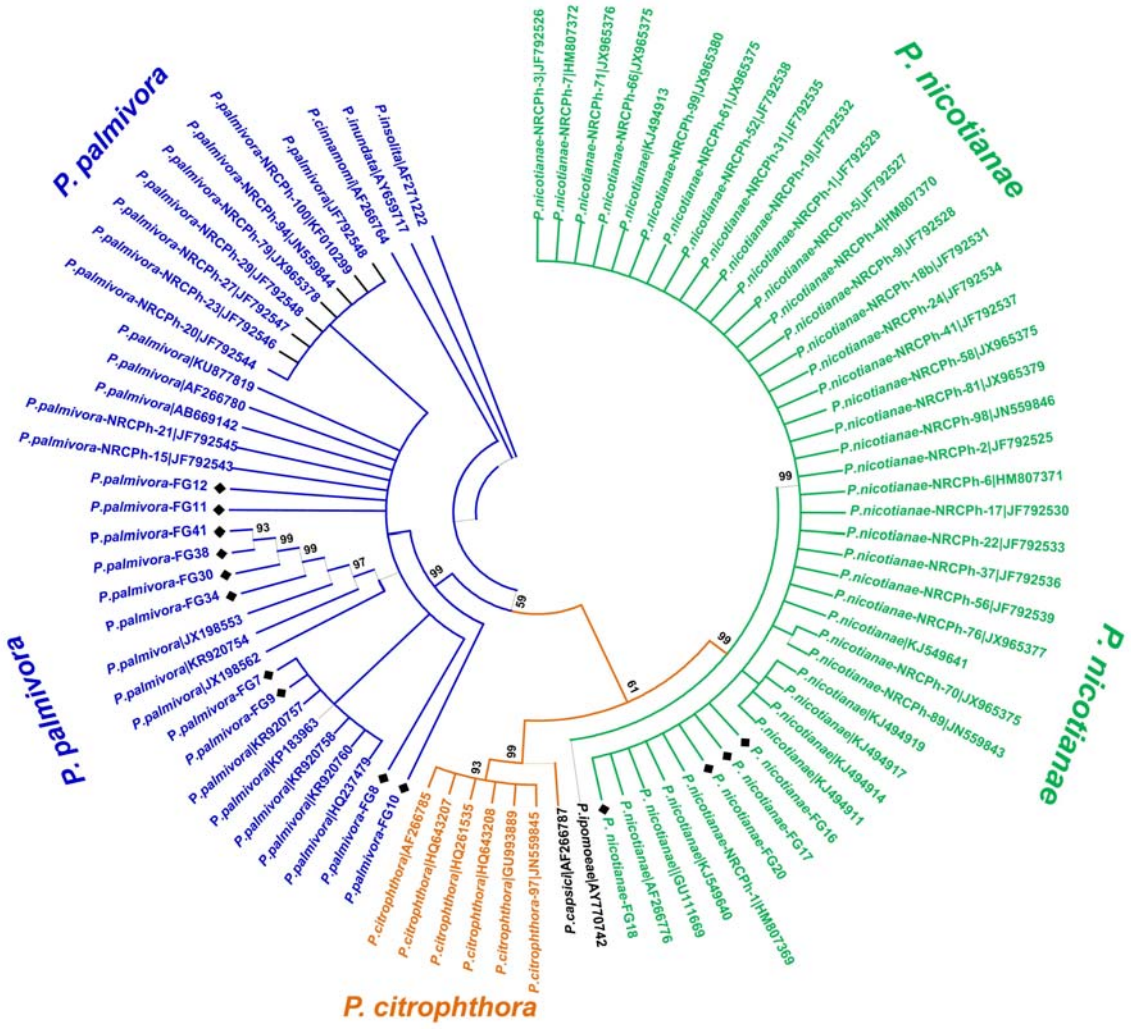

Fig. 5 Phylogenetic analysis of *Phytophthora* species infecting Coorg mandarin using rDNA ITS using neighbor-joining algorithm. Horizontal distances are proportional to sequence distances; vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1,000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches.

isolates), *Ca. L. solanacearum* (one isolate) retrieved from GenBank database. The analysis showed that three HLB Coorg mandarin isolates (CGB1, CGB1a, CGB3) shared nt identity ranging from 94.4% to 95.3% with *Ca. L. asiaticus* (AB008366) isolate infecting *Citrus* in Asia, one HLB isolate (CGB2) shared nt identity of 96.9% with *Ca. L. asiaticus* (KJ944269). While two HLB isolates (CGB4, CGB5) shared nt identity of 96% and 92.7% with *Ca. L. asiaticus* (EU265646, FJ821710) and *Ca. L. asiaticus* (EU130552) infecting *Citrus* crop, respectively (Table 1b). The nt identity of six (CGB1, CGB1a, CGB2 CGB3 CGB4 and CGB5) HLB isolates is ranged from 63.6% to 73.5% with *Ca. L. americanus* isolates infecting *Citrus* and periwinkle crop, 73.1% to 75.6% with *Ca. L. africanus* isolates infecting *Clausena anisata*, *Teclea gerrardii* wild *Citrus*, 72.5% to 74% *Ca. L. psyllaurous*, *Ca. L. solanacearum* infecting tomato and potato, 70.0% to 71.6% with *Ca. L. europaeus* infecting *Cytisus scoparius*, respectively.

The phylogenetic tree based on comparison of 16S rRNA gene sequence of HLB Coorg mandarin isolates characterized in this study with sequences of *Ca. L.*

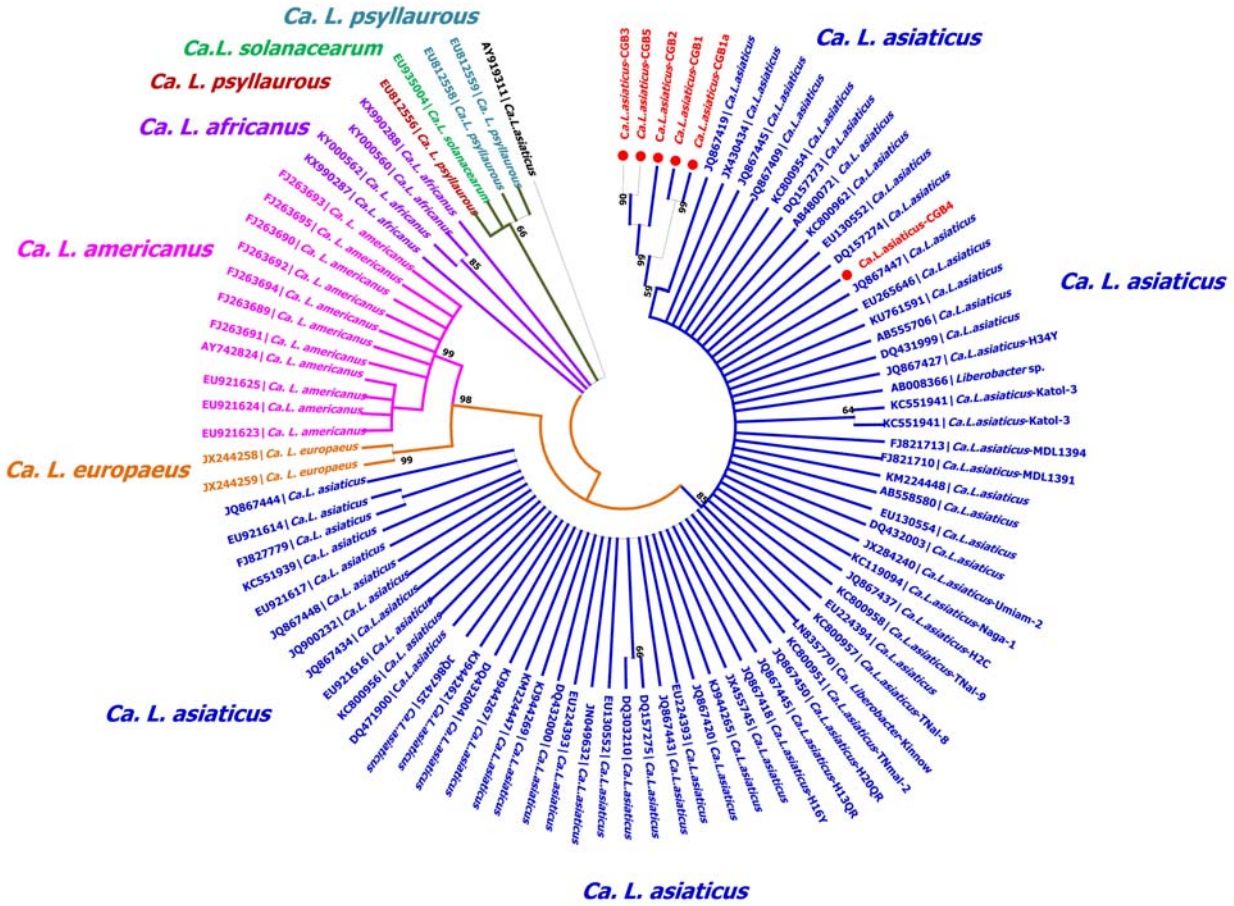

Fig. 6 Phylogenetic tree based on nt sequences of 16S rRNA gene of *Ca. L. asiaticus* with other *Candidatus* species using neighbor-joining algorithm. Horizontal distances are proportional to sequence distances; vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1,000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches.

asiaticus (65 isolates), *Ca. L. americanus* (11 isolates), *Ca. L. africanus* (four isolates), *Ca. L. psyllaurous* (three isolates), *Ca. L. solanacearum* (one isolate) identified previously on wild and cultivated species of *Citrus* and solanaceous crops available in the database was shown in Fig. 6. The analysis showed that only one CGB4 isolate was closed cluster with *Ca. L. asiaticus* isolate infecting different *Citrus* species and other five isolates (CGB1, CGB2, CGB3, CGB4 and CGB5) are form a separate cluster with all other *Ca. L. asiaticus* isolate. This indicates some variability existing in the Coorg mandarin isolates; this can be further addressed through amplification of other region of 16S rRNA and restriction analysis. Further analysis also showed that *Ca. L. asiaticus* isolates from different countries grouped together while "*Ca. L. americanus*", "*Ca. L. africanus*" and "*Ca. L. psyllouraus*" formed a separate and distinct group.

4. Discussion

The genus *Phytophthora* is a cosmopolitan oomycete belongs to the class Oomycetes, under the Phylum Heterokonta [19, 20]. The species of *Phytophthora* have a very wide host range distributed worldwide and cause economically important diseases in vegetables and fruit crops, ornamental plants and forest trees [12, 21]. *Citrus* cultivation globally hampered due to infection of different *Phytophthora* spp., causing heavy yield losses and tree decline. Several *Phytophthora* species have been

Table 4 The sequences of representative *P. palmivora, P. nicotianae* and citrus greening isolates are available in database under following accession number.

Phytophthora isolates	Accession number
P. palmivora-FG7	MF370562
P. palmivora-FG8	MF370563
P. palmivora-FG9	MF370564
P. palmivora-FG10	MF370565
P. palmivora-FG11	MF370566
P. palmivora-FG12	MF370567
P. palmivora-FG30	MF370568
P. palmivora-FG34	MF370569
P. palmivora-FG38	MF370570
P. palmivora-FG41	MF370571
P. nicotianae-FG16	MF370572
P. nicotianae-FG17	MF370573
P. nicotianae-FG18	MF370574
P. nicotianae-FG20	MF370575
Citrus greening isolates	
Ca. L. asiaticus-CGB1	MF370576
Ca. L. asiaticus-CGB1a	MF370577
Ca. L. asiaticus-CGB2	MF370578
Ca. L. asiaticus-CGB3	MF370579
Ca. L. asiaticus-CGB4	MF370580
Ca. L. asiaticus-CGB5	MF370581

reported in different *Citrus* growing regions of the world [12], among which *P. nicotianae*, *P. palmivora* and *P. citrophthora* are the most predominant species infecting *Citrus* [9, 22] in various parts of India, i.e., Assam, Nagpur and Punjab [23-25].

The survey conducted in different location of Kodagu district of Karnataka (India) showed that most of the *Citrus* plants in orchards were severely infected with *Phytophthora*, as well as HLB infection irrespective of age of the plants. The infected plants showed various degrees of disease symptom, such as yellowing, mottling, greening, root rot, trunk slit, wilting and gummosis, respectively. It was observed that budded plants express early susceptibility to both diseases then the plants raised from true seeds [26].

The Coorg mandarin samples (soil and infected feeder root) collected from different places of Kodagu districts revealed the distribution of *P. palmivora* in 60% of samples than *P. nicotianae* (41%). This showed that *P. palmivora* was the most prevailing species infecting Coorg mandarin than *P. nicotianae* in Coorg region. Whereas, in other mandarin growing areas of India, *P. nicotianae* was reported to be more predominate species, which survived at higher temperature and infecting different *Citrus* species rather than *P. palmivora* [9]. Similar results were found from various parts of world that *P. palmivora* and *P. nicotianae* are two pre-dominant species responsible for *Citrus* decline [12, 22, 27-30]. This indicates that existing climatic condition and high rainfalls (average 1,010 mm) in the Coorg region make Coorg mandarin more prone to *P. palmivora* infection.

It is difficult and inconvenient to identify some *Phytophthora* species based on morphological characteristics, as critical morphological features were variable and overlapping under different environmental conditions. Therefore, in the present study, *P. palmivora* and *P. nicotianae* were characterized through the amplification of ITS region that can rapidly distinguish between the two main species of *Phytophthora* involved in causing diseases

in *Citrus* [22]. The phylogenetic analysis based on ITS region revealed that *P. palmivora* and *P. nicotianae* isolates closely clustered with respective *Phytopthora* species infecting different *Citrus* species in India and other different countries [9, 31]. Thus, the study clearly showed that *Phytophthora* isolates collected from 31 places of Kodagu district confirmed the presence of two *Phytophthora* species, namely *P. palmivora* and *P. nicotianae*, respectively.

Another important devastating disease of Coorg mandarin is citrus greening bacteria or HLB disease of Coorg mandarin. The presence of greening disease in different *Citrus* species in India was recorded as early as 1965, and the disease could be transmitted by budding through infected scion and psyllid (*Diaphorina citri*) as natural vector [32]. The association of greening bacterium in different *Citrus* species was reported in India, excluding Coorg region [33]. The sampling survey conducted in the present study focused on major Coorg mandarin growing regions of Karnataka. During these surveys, several commercial Coorg mandarin fields displayed varied kind of disease symptoms, such as sparely foliated, extensive twig die-back, general yellowing, leaf mottling, mild to severe chlorosis, vein yellowing, pale green colour of young leaves. Our preliminary analyses of 523 infected leaf samples of Coorg mandarin by PCR diagnostic tests indicated that the symptomatic plants collected from Coorg region in different commercial fields were infected with citrus greening bacterium. Similarly, technique was used in India to detect the greening bacterium different *Citrus* species grown in different parts of India [6-8]. Greening disease on Coorg mandarin in Coorg region of Karnataka is more prevalent, but there was no information available on definite association and characterization of greening bacterium associated with it. Sequencing of representative six HLB isolates of 16S rRNA gene showed three isolates (CGB1, CGB1a, CGB3) is closely related with *Ca. L. asiaticus* (AB008366) isolate infecting *Citrus* crop in Okinawa, one HLB isolate (CGB2) with *Ca. L. asiaticus* (KJ944269) infecting *Citrus* in China. While two HLB isolates (CGB4, CGB5) are closely related to *Ca. L. asiaticus* (EU265646, FJ821710) and *Ca. L. asiaticus* (EU130552) infecting *Citrus* in Florida, respectively. Therefore, the study clearly showed that Coorg mandarin decline in Coorg region is responsible for the infection of *P. palmivora,* and *P. nicotianae*, as well as HLB bacterium (*Ca. L. asiaticus*), respectively.

5. Conclusion

Coorg mandarin is a famous ecotype of mandarin, grown in southern parts of India, particularly in Western Ghats as component of the multi-tier cropping system in coffee and pepper plantations for more than 150 years. The present study showed that two species *Phytophthora* and different strains of *Ca. L. asiaticus* are causing the rot and greening disease on budded or true seeded Coorg mandarin plants. This may be due to several ground bed and container nurseries, many commercial *Citrus* groves from respective regions were believed to be the hotspots for dissemination of these pathogens into new areas and hence need urgent quarantine attention. Using of disease free planting material along with effective integrated disease management (IDM) strategy is recommended to contain the diseases and to enhance the productivity of Coorg mandarin in Kodagu region.

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