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SHORT COMMUNICATION

Molecular detection of *Candidatus* Phytoplasma spp. causing witches' broom disease of acid lime (*Citrus aurantifolia*) in India

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Abstract Phytoplasma infected acid lime plants in India develop characteristic symptoms like small chlorotic leaves, multiple sprouting and shortened internodes. Leaves drop prematurely and infected branches have distorted twigs resembling witches' broom appearance which eventually show die-back symptoms. During its first report in 1999, witches' broom disease identification was made on the basis of symptomatology and electron microscopy. However, molecular techniques have proved to be more accurate and reliable for phytoplasma detection than the conventional methods. During survey in the year 2010 six samples were collected from infected acid lime plants showing typical field symptoms from Vidarbha region of Maharastra. Initially, phytoplasma bodies were observed in phloem tissues of all six symptomatic samples under JEM 100S transmission electron microscope and all these six samples were subsequently screened using different set of phytoplasma specific universal primers by nested PCR, a widely recommended molecular technique for phytoplasma detection. In the present study P1/P7 "universal" phytoplasma-primer set was used for first round of PCR and amplified products were processed separately for nested PCR with three different nested primer pairs viz. R16F2n/R16R2, R16mF2/R16mR1 and fU5/rU3. The presence of phytoplasma was confirmed in all six suspected samples and one representative ~1.2 kb size amplicon was sequenced and deposited in GenBank as *Candidatus* Phytoplasma species AL-M (JQ808143). This is the first report of PCR based molecular detection of phytoplasma-induced witches' broom disease of acid lime (WBDL) in India. Further molecular evaluation to determine the identity to the species level is in progress.

Keywords *Candidatus* Phytoplasma · PCR · Acid lime · Witches' broom · India

Abbreviations

WBDL	Witches' Broom Disease of Acid Lime
UAE	United Arab Emirates
RFLP	Restriction fragment length polymorphism
EM	Electron microscopy
PVPP	Polyvinylpolypyrrolidone

In India citrus is the third most important fruit crop after banana and mango, with an estimated production of 7.46 million tonnes of fruits and area coverage of ~0.9 million hectares (Kumar et al. 2011). Among different commercial citrus cultivars, acid lime (*Citrus aurantifolia* (L) Swingle) constitutes nearly 20 % of total citrus production. The plants in the field are often infected by many virus and virus-like pathogens and are considered a major factor for causing citrus decline. What were previously described as virus diseases of citrus are actually an array of pathogens which include a phytoplasma, spiroplasma, viroids and at least five different groups of viruses. These pathogens cause systemic diseases and persist in the vegetative parts of the plant as long as infected trees remain alive. Hence losses are not confined to the season in which infection occurs but incur sustained loss through-out the life of the plant. Many of these diseases are universally distributed while some are

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recently discovered diseases and endemic to a region or country. These pathogens are known to be transmitted by infected budwoods, insect vectors or by mechanical means under field experimental conditions.

Occurrence of phytoplasma, a graft transmissible phytopathogenic mollicute, to cause Witches' Broom Disease of Acid lime (WBDL) first noticed from Oman during 1975 currently infests over 98 % of lime cultivation and is highly devastating in Oman and adjoining regions (Bove 1986; Bove et al. 2000). Similar disease was later reported from United Arab Emirates (UAE) (Garnier et al. 1991), India (Ghosh et al. 1999a, b), Iran (Bove et al. 2000), Saudi Arabia (Alhudaib et al. 2009) and Pakistan (Mannan et al. 2010). The Phytoplasma associated with WBDL disease in Oman and UAE is known as *Candidatus* Phytoplasma aurantifolia belongs to 16SrIIb (Peanut WB group) based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences (Zreik et al. 1995; Lee et al. 2000; Bertaccini and Duduk 2009). Our recent survey of citrus orchards conducted during 2010 indicated phytoplasma infection in acid lime that conspicuously develops witches' broom symptoms in infected plants in different citrus growing belts of India. With the climate change taking place globally and potentially creating favourable environment for the plant pathogens and their insect vectors, changes in the cropping systems, movement of infected planting material with increasing international travel and trade and introduction of host susceptibility genes through the exchange of germplasms, WBDL may cause serious threat to the citrus industry in future if not managed immediately. Till date, destruction of severely infected twigs or tree is the only management strategy for WBDL. Hence a sensitive and reliable diagnostic tools for quick and early detection of phytoplasma are essential for accurate disease identification even before the symptom appearance in infected plant.

Phytoplasmas are cell wall-less, non helical prokaryotes having very small genome that inhabit plant phloem. They are poorly understood plant pathogens because they have not been cultured in vitro and hence designated with *'Candidatus'* status (Lee et al. 2000; Bertaccini and Duduk 2009). The concentration of phytoplasma in woody plants like citrus remains very low and is unevenly distributed within the infected plant (Lee et al. 2000). Until recently identification of phytoplasma induced witches' broom disease in acid lime in India was primarily based on symptomatology, host range, vector specificity and limited EM studies (Ghosh et al. 1999a, b) and so far no attempts have been made to identify the pathogen using molecular diagnostic tools. However, molecular techniques for phytoplasma detection have proved to be more accurate and reliable than the conventional methods (Lee et al. 1993, 1998; Santos-Cervantes et al. 2008; Manimekalai et al. 2011). Present paper deals with PCR (Polymerase Chain Reaction) based rapid and reliable molecular diagnosis of phytoplasma – induced witches' broom disease of citrus by amplifying highly conserved 16 S ribosomal DNA and is the first report of such study from India.

During the survey conducted of citrus orchards in Vidarbha region of Maharashtra in the year 2010, characteristic witches' broom symptoms were found in four acid lime plants (Fig. 1a) in one orchard having a total sixty plants. Initial disease symptom included appearance of small chlorotic leaves, multiple sprouting and shortened internodes. Leaves drop prematurely and infected branches have distorted twigs characteristic of witches' broom symptoms. In advanced stages, infected branches show die-back symptoms. In an another orchard having a total seventy plants, two acid lime plants with shoot proliferation in one branch of each infected tree symptoms somewhat similar to Witches' broom were observed. Only symptomatic branches from these plants were collected for further study. Initially,

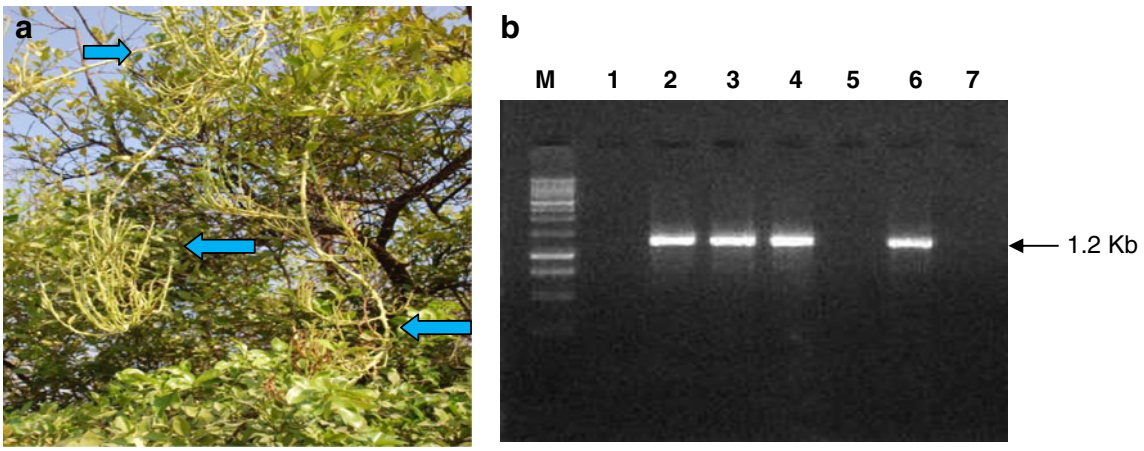

Fig. 1 Symptoms of Witches' broom in acid lime and its diagnosis. **a** Characteristic shoot proliferation in infected acid lime plants. **b** PCR amplified products with R16F2n/R16R2 primer set. Lane M: 1Kb ladder, Lane 1 to 6: Symptomatic acid lime samples. Lane 7: Healthy plant sample as negative control

Table 1 Plant samples used for PCR based molecular detection of Phytoplasma-induced WBDL

Sample no.	Sample details	Symptoms
1	Acid lime P1 orch-1	Typical witches' broom symptoms
2	Acid lime P2 orch-1	Typical witches' broom symptoms
3	Acid lime P3 orch-1	Typical witches' broom symptoms
4	Acid lime P4 orch-1	Typical witches' broom symptoms
5	Acid lime P5 orch-2	Shoot proliferation symptoms
6	Acid lime P6 orch-2	Shoot proliferation symptoms
7	Acid lime P7 (NRCC orchard)	Healthy plant as negative control

ultrathin sections of leaf midrib of these symptomatic and one healthy acid lime plant (as a control) were fixed on copper grids, stained with uranyl acetate and lead acetate, and examined in a JEM 100S transmission electron microscope (Ghosh et al. 1999b). Numerous bodies having characteristic morphology of phytoplasma were observed in phloem sieve tubes of all six acid lime symptomatic samples but not with healthy samples. Few representative branches were graft transmitted into healthy acid lime seedlings which subsequently developed similar symptomatic appearance at about 12–14 months.

All six phytoplasma infected samples and one non-symptomatic sample as negative control were used for the molecular detection and characterization of the pathogen (Table 1). DNA was extracted from ~300 mg leaf midribs and young bark portion of symptomatic branches using a modified phytoplasma enrichment protocol with addition of 5 % polyvinylpolypyrrolidine (PVPP, MW of 40,000) during tissue grinding with liquid nitrogen (Ahrens and Seemuller 1992; Manimekalai et al. 2010). The presence of phenolics and polysaccharides inhibit DNA amplification

Table 3 Amplification of Phytoplasma specific PCR products by nested primers

Lane No.	Sample	Nested PCR results with different Primers		
		R16F2n/ R16R2	R16mF2/ R16mR1	fU5/ rU3
1	Acid lime P1 orch-1	–	+	–
2	Acid lime P2 orch-1	+	+	–
3	Acid lime P3 orch-1	+	+	–
4	Acid lime P4 orch-1	+	+	+
5	Acid lime P5 orch-2	–	+	+
6	Acid lime P6 orch-2	+	+	+
7	Acid lime P7(NRCC orchard)	–	–	–

['–' no amplification, '+' positive amplification]

by PCR and was overcome by the addition of PVPP. Generally the concentration of phytoplasmal DNA from total plant DNA is low and often the pathogen is unevenly distributed in the infected plant. It also varies from plant to plant, seasonal variation and distribution in different organs (Davis and Lee 1993; Firrao et al. 2007). Because of these limitations one step positive phytoplasma specific DNA amplification by PCR is not considered as an adequate proof of phytoplasma infection and hence nested PCR is always recommended for the phytoplasma detection. In the present study P1/P7 "universal" phytoplasma primer set was used for first round of PCR and amplified products were processed separately for nested PCR with three different nested primer sets viz. R16F2n/R16R2, R16mF2/R16mR1 and fU5/rU3 (Table 2).

The reaction mixture of 25 μl contained 100 ng of total DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.4 mM of plus and minus primers, 1.5 U of GoTaq Hot Start DNA polymerase (Promega). PCR amplification was carried out in My Cycler thermal cycler (Bio-Rad, USA) with one cycle of 4 min at 95 °C followed by 35 cycles of 1 min at 94 °C, 2 min at 55 °C, 3 min at 72 °C, and final

Table 2 Sequence of universal primers P1/P7 and nested primer pairs (nested primers) used for Phytoplasma detection in acid lime

Sr. No.	Primer	Nucleotide sequences (5′-3′)	Amplicon size	References
1.	P1 P7	AAGAGTTTGATCCTGGCTCAGGATT CGTCCTTCATCGGCTCTT	1.8 kb	(Lee et al. 1998; Seemueller et al. 1998; Santos-Cervantes et al. 2008)
2.[a]	R16mF2 R16mR1	CATGCAAGTCGAACGA CTTAACCCCAATCATCGAC	1.45 kb	(Lee et al. 1998; Santos-Cervantes et al. 2008; Mannan et al. 2010)
3.[a]	R16F2n R16R2	GAAACGACTGCTAAGACTGG TGACGGGCGGTGTGTACAAACCCCG	1.2 kb	(Lee et al. 1993, 1998; Santos-Cervantes et al. 2008; Mannan et al. 2010)
4.[a]	fU5 rU3	CGGCAATGGAGGAAACT TTCAGCTACTCTTTGTAACA	0.88 kb	Chen et al. 2009

[a] nested primers

extension at 72 °C for 10 min. In the present study we screened by PCR six phytoplasma suspected samples using primer pairs P1/P7 and the amplified products were used as template for nested PCR using three different sets of primer; R16F2n/R16R2, R16mF2/R16mR1 and fU5/rU3. For all three primer sets total master mixture of 25 μl contained 1 μl of P1/P7 amplified product, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP each, 0.4 mM of each primer, 1.3 U of GoTaq Hot Start DNA polymerase (Promega) and nuclease free water. In case of R16mF2/R16mR1 and R16F2n/R16R2 primer sets, the amplification conditions were; one cycle of 3 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1.5 min at 56 °C (50 °C for R16mF2/R16mR1), 2 min at 72 °C, and final extension at 72 °C for 10 min. For fU5/rU3 primer set reaction was carried out as one cycle of 5 min at 95 °C followed by 35 cycles of 45 s at 95 °C, 45 s at 53 °C, 1 min at 72 °C, and final extension at 72 °C for 10 min. All these PCR products were analyzed by electrophoresis in a 1.5 % agarose gel and visualized by staining with ethidium bromide and UV GelDoc system (Bio-Rad, USA).

Amplification of a prominent ~1.2 kb band characteristic of Phytoplasma were observed with R16F2n/R16R2 nested primers in four samples (Fig. 1b; Lane 2,3,4 and 6 and Table 3) which suggested that these samples were infected with phytoplasma. Again amplification of a 1.45 kb band was observed with R16mF2/R16mR1 nested primer set with all six samples. With fU5/rU3 primer pair an amplification product of fragment of 0.88 kb characteristic of phytoplasma was observed with samples 4, 5 and 6 (Table 3). No amplification was observed with any of the phytoplasma primer pairs in healthy acid lime sample (Fig. 1b; lane 7). Out of all the amplicons, a representative R16F2n/R16R2 1.2 kb amplicon from sample 2 (Fig. 1b; lane 2) was purified from gel using the GenElute Gel Extraction Kit (Sigma) as per manufacturer's protocol and sequencing was done at automated DNA sequencing facility of Chromous Biotech Private limited, Bangalore, India using the ABI BigDye v 3.1 terminator cycle sequencing protocols (Applied Bio-systems, Foster City, CA). The fluorescent-labeled extension products were analyzed by the dye terminator method on DNA sequencer Model ABI 3500xL (Applied Bio-systems). The sequence has been deposited in GenBank as *Candidatus* Phytoplasma species AL-M (Accession no. JQ808143). Similarity search using Blastn revealed that sequence showed the maximum identity with Phytoplasma sp. RYL-GD (EU093079) with 98 % of query coverage followed by Peach yellow leaf roll phytoplasma (Y16394) and *Candidatus* Phytoplasma pyri isolate P-16SrXC (JN563609) with 82 % identity (Seemueller et al. 1998).

The study presents PCR based molecular detection of phytoplasma – induced witches' broom disease of citrus in India. All phytoplasma universal nested PCR primer sets used in the study viz. R16F2n/R16R2, R16mF2/R16mR1 and fU5/rU3 represents the primer sets used previously with detection of Phytoplasma in citrus, coconut and tomato and presently were successfully used for the detection of phytoplasma in acid lime in India. However minor differences were observed with amplification as shown in Table 3. For example, in sample number 2 amplification was observed with R16F2n/R16R2 and R16mF2/R16mR1, while amplification was not observed with fU5/rU3. Similarly, in sample number 1 amplification was observed with only R16mF2/R16mR1 but not with primer sets R16F2n/R16R2 and fU5/rU3. Amplified products were observed with samples 4 and 6 with all three primer sets. Intensity and quality of the bands observed with fU5/rU3 primer set was better than other primer sets. This discrepancy probably reflects minor variations in the sequence in the template which potentially would indicate diversity in the pathogen population. Consistent and reproducible amplifications observed with R16F2n/R16R2 and fU5/rU3 primer sets suggest that these primer sets can be routinely used for the diagnosis of citrus phytoplasma in India. BLAST analysis of JQ808143 sequence confirmed the *Candidatus* Phytoplasma spp. as a causal organism for the witches' broom disease of acid lime in India. Thus the present study reports rapid and sensitive molecular diagnosis by PCR of causal *Candidatus* Phytoplasma infecting acid lime in India. Further molecular evaluation to identify the nature phytoplasma to the species and group is in progress.

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