

NESTED PCR ASSAY FOR DETECTION OF RICE ORANGE LEAF PHYTOPLASMA IN THE ZIGZAG LEAFHOPPER VECTOR, *RECILIA DORSALIS* (MOTSCHULSKY): A FIRST REPORT

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INTRODUCTION

Rice Orange Leaf was widely distributed in South and South-east Asia (Hibino *et al.*, 1987), including Thailand, Malaysia, Indonesia, China, Sri Lanka and the Philippines. ROL is transmitted by the leafhopper *Recilia dorsalis* Motchulsky, which also transmits rice dwarf virus and rice tungro virus. These diseases differentiate from ROL on symptoms and geographic distribution. In the Philippines, the ROL phytoplasma is transmitted by *R. dorsalis* in a persistent manner, with an incubation period of 15-33 days (Hibino *et al.*, 1987). 'Candidatus Phytoplasma asteris' group phytoplasmas have been reported in many crops in India, Rice Orange Leaf phytoplasma is the first report of 16SrI aster yellows group phytoplasma infection of rice (*Oryza sativa* L.) in India (Valarmathi *et al.*, 2013).

Phytoplasmas are generally present in phloem sieve tubes and in the salivary glands of insect vectors (McCoy, 1983). Most phytoplasmas are transmitted from plant to plant by leafhoppers in a persistent manner (Sinha and Chiykowski, 1967; Purcell, 1982). Phytoplasmas also present in the alimentary canal, haemolymph, salivary glands, and intracellularly in various body organs of their insect vectors (Purcell, 1982). Numerous attempts to culture phytoplasmas on artificial nutrient media or cell-free media have been unsuccessful so far (Lee and Davis, 1986). In India, the leafhopper *Proutista moesta* (Westwood) was shown to transmit Sugarcane grassy shoot (SCGS) phytoplasma and recently *Deltocephalus vulgaris* Dash and Viraktamah has been identified as a potential vector for SCGS (Srivastava *et al.*, 2006). Nested PCR analyses showed that 58.3% of plants exposed to *Recilia banda* Kramer (Hemiptera: Cicadellidae) were positive for phytoplasma and developed characteristic stunt disease symptoms while 60% of *R. banda* insect samples were similarly phytoplasma positive. Persistence of stunt pathogen in *R. banda* and positive transmission of stunt pathogen to healthy plants incriminate *R. banda* as a vector of Napier stunt phytoplasma in Kenya (Obura *et al.*, 2009). In the present study, nested PCR assay has been carried out to detect the rice orange leaf phytoplasma in the Zigzag leafhopper.

MATERIALS AND METHODS

Extraction of DNA

The insects were crushed using a homogenizer in 1.5 mL tube containing 500 µL of 2 % CTAB buffer and one µL β-mercaptoethanol and incubated for 30 min at 65°C followed by cooling to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added. This mixture was centrifuged at 9677 g for 15

ABSTRACT

Phytoplasmas are known to be transmitted by plant hoppers, leafhoppers and psyllids in a persistent, propagative manner. In our present study, Nested PCR assay using primer pairs (p1/p7 and R16F2n/R16R2) amplified at ~ 1.2 kb of 16S rRNA gene of phytoplasma from the vector collected from infected plant. It was found that the nested PCR assay had proven to be a reliable molecular tool for detection of the Rice Orange Leaf (ROL) phytoplasma in leafhopper vector. These results suggested that rice orange leaf phytoplasma was detected in Zigzag leaf hopper and reported for the first time in India.

KEY WORDS

Rice Orange leaf phytoplasma
'Candidatus Phytoplasma asteris'
Zigzag leafhopper

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min, then the aqueous phase was transferred into a clean tube and 0.6 volume of isopropanol was added, then incubated for 30 min at -20°C followed by centrifugation at $9677g$ for 15 min. The clear supernatant was discarded carefully leaving the pellet. The pellet was rinsed with 70 % ethanol for 10 min and air dried and then the tube was kept face down on a paper tissue. Finally it was suspended with $35\ \mu\text{L}$ sterile deionized water and stored at 4°C (Doyle and Doyle, 1990).

Nested PCR assay

Nested-PCR assays with the universal primer pair P1/P7 followed by the universal primer pair R16F2/R2, designed to amplify a portion of the 16S rRNA gene (Lee *et al.*, 1993; Gundersen and Lee, 1996) were employed. One microlitre of DNA was used for first round amplification with primer pair P1/P7 and $0.5\ \mu\text{L}$ of first round product was used as template in nested-PCR without dilution with phytoplasma specific primers R16F2n/R16R2. A total of 35 thermal cycles were carried out which included denaturation for 1 min (2 min for first cycle) at 94°C , annealing for 2 min at 50°C and extension for 3 min (10 min in final cycle) at 72°C . The nested-PCR was repeated thrice using the same samples. The DNA fragments were gel purified using a gel extraction kit (Qiagen, New Delhi, India) and cloned in plasmid (pGEMT[®] vector- Promega). The Plasmid DNA was directly sequenced in both orientations at SciGenom Labs Pvt Ltd, Kerala, India.

RESULTS AND DISCUSSION

The different hoppers in rice ecosystem namely Green leafhopper (GLH), Zigzag leafhopper (ZLH), Brown planthopper (BPH) and White backed planthopper (WBPH) from the phytoplasma infected rice plants in the farm fields of TNAU were collected. The total DNA isolated from the above hoppers was subjected to nested PCR assay. Among the different hoppers, Zigzag leafhopper (ZLH) showed positive in the nested PCR assay using primer pairs (p1/p7 and R16F2n/R16R2) (**Table 1**). No phytoplasma specific amplification was observed in the templates of the other three hoppers implying

that the pathogen was transmitted by the Zigzag leafhopper (ZLH).

Adult hoppers have characteristic zigzag white and brown pattern on the front wings. The microscopic observations of dorsal and ventral view of Zigzag leafhopper (*Recilia dorsalis* (Motschulsky)) were exhibited in Fig. 1. The Zigzag leafhopper (ZLH) collected from the phytoplasma infected rice fields were subjected to nested PCR assay using primer pairs (p1/p7 and R16F2n/R16R2). The DNA fragment amplified at $\sim 1.2\ \text{kb}$ of 16S rRNA gene of phytoplasma from the vector of infected plant. No amplification was observed from the vector collected from healthy rice plants (Fig. 2). The amplified product of 16S rRNA gene of rice phytoplasma from the Zigzag leafhopper by using primer pairs, p1/p7 and R16F2n/R16R2 were cloned into pGEM-T[®] vector and transformed into *Escherichia coli* strain DH5 α . The PCR products obtained from the vector were cloned and sequenced on both the orientations. Sequence analysis of the 16S rRNA gene sequences of phytoplasmas was analysed from GenBank using the tool NCBI BLAST. The analysis revealed that the 1178-bp nucleotide sequences shared 98% nucleotide sequence identity with 16S rRNA gene sequences of 16SrI (*Candidatus* phytoplasma asteris) phytoplasmas namely rice orange leaf phytoplasma from Coimbatore (Gen Bank acc. JX290547), from Thailand (GenBank acc. JQ965687).

A nested PCR approach is often needed for detection of phytoplasmas when they occur at low levels or are distributed unevenly in their plant or insect hosts (Goodwin *et al.*, 1994 and Andersen *et al.*, 1998). Srivastava *et al.* (2006) found that the nested PCR using p1/p7 and p4/p7 has proven to be a reliable molecular tool for detection of the Sugarcane grassy shoot phytoplasma in leafhopper vector, *Deltocephalus vulgaris*. Polymerase chain reaction (PCR), based on the highly conserved 16S gene, primed by P1/P6-R16F2n/R16R2 nested primer sets was used to diagnose phytoplasma in insects (*Recilia band*) which transmits Napier stunt phytoplasma in western Kenya. In India, the leafhopper *Deltocephalus vulgaris* and *Exitianus indicus* were identified as a potential vectors for



Dorsal view



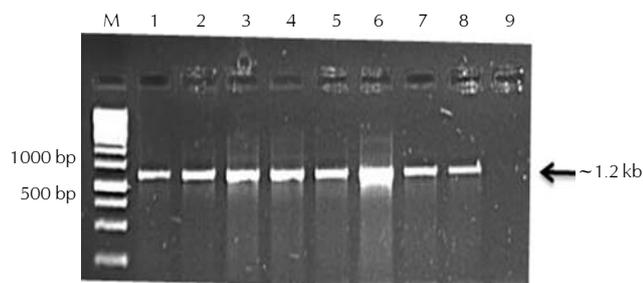
Ventral view

Figure 1: Microscopic view of Zigzag leafhopper (*Recilia dorsalis* (Motschulsky))

Table 1: Detection of Rice Orange Leaf (ROL) phytoplasma from the different hoppers in rice ecosystem

S. No	Samples	Green leafhopper (GLH), <i>Nephotettix virescens</i>	Zigzag leafhopper (ZLH), <i>Recilia dorsalis</i>	Brown Planthopper(BPH) <i>Nilaparvata lugens</i>	White backed planthopper (WBPH), <i>Sogatella furcifera</i>
1.	Coimbatore- ADT 43	X	√	X	X
2.	Coimbatore- CO 39	X	√	X	X
3.	Coimbatore-White Ponni	X	√	X	X
4.	Erode- BPT 5204	X	√	X	X

x Absent; √ Present

**Figure 2: Detection of 16S rRNA gene of phytoplasma in Zigzag leafhopper by Nested PCR using primers, p1/p7 and R16F2n/R16R2**

Sugarcane grassy stunt virus (SCGS) (Srivastava *et al.*, 2006, Rao *et al.*, 2014).

In our study, no phytoplasma-specific amplification was observed in the templates of the other three hoppers namely Green leafhopper (GLH), Brown plant hopper (BPH) and White backed plant hopper (WBPH) implying that the pathogen was transmitted by the Zigzag leafhopper (ZLH). Nested PCR assay using primer pairs (p1/p7 and R16F2n/R16R2) amplified at ~ 1.2 kb of 16S rRNA gene of phytoplasma from the vector collected from infected plant. These results suggested that the Rice Orange Leaf (ROL) phytoplasma was detected in Zigzag leafhopper through nested PCR assay for the first time in India

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