



DETECTION OF PHYTOPLASMA ASSOCIATED WITH CROTALARIA PHYLLODY DISEASE

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Abstract

Crotalaria is a tropical Asian plant grown as a source of green manure, fodder and fiber. Phyllody disease in crotalaria caused by phytoplasma has been gaining importance in the recent years. Symptoms of phyllody infected plants appear pale green and bushy due to excessive stunting, malformation of floral parts into green leaf like structures and production of excessive spikelets in an upright fashion within inflorescence axis. In the present investigation, Fluorescent microscopy and Polymerase chain reaction (PCR) techniques were standardized for detection of crotalaria phyllody phytoplasma. Fluorescent microscopic technique using 4¹-6¹ diamidino-2-phenylindole (DAPI) stain revealed the association of phytoplasma with bright fluorescent spots in the phloem region of infected stem sections, but no such spots were observed in the phloem region of healthy stem sections. Annealing temperature of 55 and 56°C for one minute consistently amplified the phytoplasma specific products of 1800 and 1250 bp using P1/P7 and R16F2n/R16R2 universal primers respectively.

Key Words: Crotalaria phytoplasma, Fluorescent microscopy, CTAB, DNA extraction, polymerase chain reaction.

Introduction

Crotalaria is a genus of herbaceous plants and woody shrubs in the Family Fabaceae (Subfamily Faboideae) commonly known as rattlepods. About 600 or more species of *Crotalaria* are described worldwide, mostly from the tropics. Some species of crotalaria are grown as ornamentals. The common name rattlepod or *rattlebox* is derived from the fact that the seeds become loose in the pod as they mature, and rattle when the pod is shaken. *Crotalaria pallida* (rattlebox) has been used extensively as a green manure and has become naturalized. A disease with characteristic symptoms of phytoplasmal etiology has been observed in a large scale as naturally grown plant in and around cultivated crop plants. The diseased plants were characterized by excessive stunting, malformation of floral parts into green leaf like structures and production of excessive spikelets in an upright fashion within inflorescence axis. Phytoplasmas are cell-wall-less prokaryotic pathogens and cause devastating diseases in a wide range of plant hosts. A study was undertaken to detect the possibility of involvement of a phytoplasma with the disease by using fluorescent microscopy as well as molecular methods.

Materials and Methods

Sample collection: Disease affected crotalaria sample were collected from different districts surveyed in southern Karnataka.

Fluorescent Microscopy

Diamidino-phenylindole staining: For DAPI fluorescent staining, small pieces of stem, leaf petioles and rachillae of unopened inflorescence of phyllody affected and healthy crotalaria were fixed in 2.5% glutaraldehyde in 0.2% phosphate buffer for 30 min. and were washed in same buffer for 30 min. Free hand thin sections of 20µm thickness of tissues were cut using razor blade and the sections were stained with DAPI (0.001%) in 0.01 M phosphate buffer at 22°C for one hour and examined under a fluorescent microscope.

The sections were observed under a Zeiss filterset 09 fluorescent microscope using DAPI filter. The presence or absence of fluorescent spots in the phloem tissues of healthy and diseased samples was observed.

DNA extraction from host plant

CTAB method:

Total DNA from healthy and diseased plants were extracted by following the protocol of Sunard *et.al.*, (1991). 100-200 mg tissues of midrib and young stem were ground with 1.5-2 ml CTAB extraction buffer. About 750 µl of the sample was poured into 1.5 ml Eppendorf tube and the samples were heated at 60°C for 30 minutes in a water bath. The samples were then mixed with an equal volume (750µl) of chloroform: isoamylalcohol (24:1) and centrifuged at 13000 rpm for 10 minutes. The top aqueous phase was transferred into a new 1.5 ml eppendorf tube and DNA was precipitated by adding 0.6 volumes (300 µl) of cold (-20°C) isopropanol and incubated at -20°C for at least 1hr. The samples were centrifuged at 13000 rpm for 10 min and the supernatant was discarded. The pellet was washed in 500 µl of 70 per cent ethanol by

vertexing and then centrifuged for 5 min at 14000 rpm. The ethanol was removed and the pellet was vacuum dried for 5 min and the dried pellet was suspended in 40 µl of 1X TE buffer and stored at –20°C.

DNA amplification:

Phytoplasmal DNA was amplified by the polymerase chain reaction (PCR) using two sets of universal primers (Table 1). PCR that comprised two steps *i.e.*, direct-PCR followed by nested PCR. DNA fragment of 1.8kb expected size (16S rDNA) located in 23S rDNA region of the phytoplasma was amplified using primer pairs P1/P7. The second set of primers *viz.*, R16F2n/ R16R2 were nested within the positions of annealing of primers P1 and P7 along the 16S rDNA of the phytoplasma.

Table 1: Primers used for PCR and Nested PCR

Sl. No.	Name	Primer (Desalted oligo)
1	P1	5 ¹ -AAGAGTTTGATCCTGGCTCAGGATT-3 ¹
2	P7	5 ¹ -CGTCCTTCATCGGCTCTT-3 ¹
3	R16F2n	5-GAAACGACTGCTAAGACTGG-3'
4	R16R2	5'-TGACGGGCGGTGTGTACAAACCCC -3'

In direct-PCR, the template consisted of total DNA extracted from crotalaria phyllody infected and healthy leaves. A total of 25µl PCR mixture contained 10x PCR buffer (Supplied with the enzyme), 25 mM MgCl₂, 2.5 mM dNTP mixture, Primer P1 (20 mM), Primer P7 (20 mM) and Taq DNA polymerase (3U/µl). Amplifications were carried out in DNA thermocycler using the following parameters: 95°C for 2 min. initial denaturation followed by 35 cycles of 94°C for 30 sec. denaturation, 55°C for 1 min. primer annealing, 72°C for 2 min. primer extension and finally at 72°C for 10 min primer extension. An aliquot of 5µl of each direct- PCR product was analyzed by electrophoresis in a 1 per cent agarose gel containing ethidium bromide and visualized on an gel documentation unit.

1µl each of direct-PCR product was reamplified by nested-PCR. The components of nested-PCR mixtures were same as those described for direct-PCR, except the primers. The nested-PCR was primed using the R16F2n/ R16R2. A total of 30 thermal cycles were performed with denaturation of 94°C for 1 min, annealing at 56°C for 1 min and extension at 72 °C for 2 min, which was extended for 10 minutes in the last cycle. An aliquot of 5µl nested-PCR product was analyzed in 1% agarose gel as mentioned in the direct-PCR and photographed on gel documentation system.

To determine the efficacy of nested-PCR, 1µl of direct-PCR product was diluted with sterile deionised water in 1:25, 1:50 and 1:100 dilutions. 1µl of each sample was reamplified in nested-PCR using same as that of normal PCR.

Result

Crotalaria plants infected with phytoplasma were characterized by pale green and bushy appearance due to excessive stunting of shoots, reduced intermodal length (Fig. 1a) and production of excessive spikelets in an upright fashion within inflorescence axis (Fig. 1b). The phloem region of stem sections from crotalaria infected plants when stained with DAPI showed whitish blue fluorescent spots in UV light (300-380nm), but no such spots were detected in the phloem region of healthy sections. Whitish blue fluorescent spots of various shapes and sizes were observed in the phloem region of the infected tissues indicating the presence of phytoplasmas in the phloem tissues (Fig. 2).

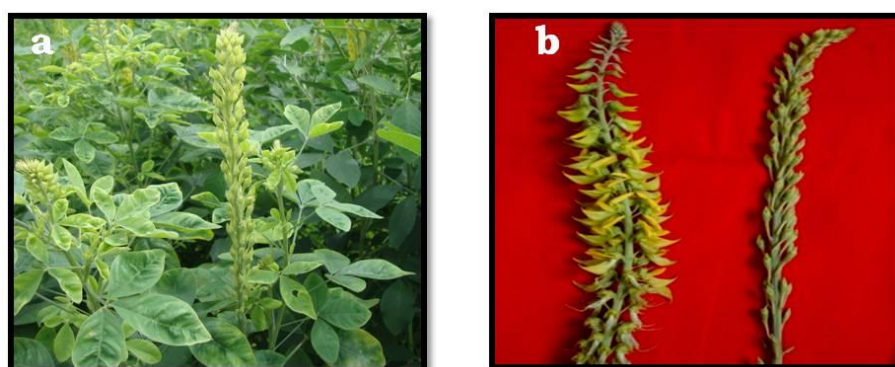


Fig. 1: Phyllody affected crotalaria plant (a). Healthy (left panel) and phytoplasma affected (right panel) crotalaria inflorescence (b).

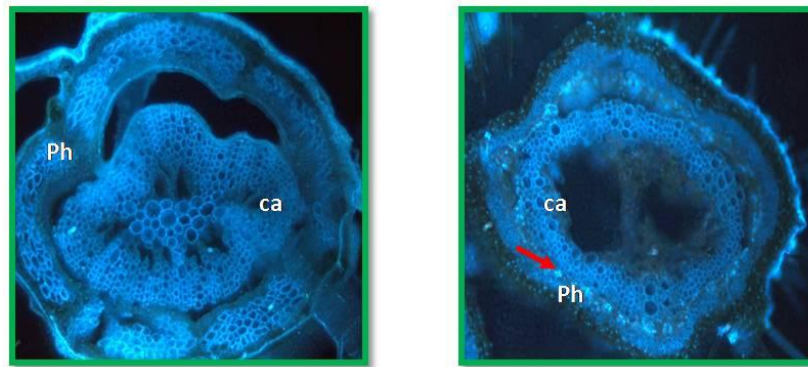


Fig. 2: Fluorescent micrograph of healthy and phytoplasma affected stem sections of crotalaria stained with DAPI.

In case of direct-PCR, use of universal primers P1/P7 yield visible amplified DNA fragment of 1.8kb from infected tissues of crotalaria (Figure 3). Direct-PCR products were diluted and reamplified in the nested-PCR yielded a DNA fragment of 1.25kb.

It also confirmed the presence of phytoplasmal DNA reamplified in nested-PCR using direct PCR products obtained from total DNA diluted up to 1:100 (Figure 4). However, direct-PCR product could not reamplified in nested-PCR without dilution and it did not reveal any positive signal.

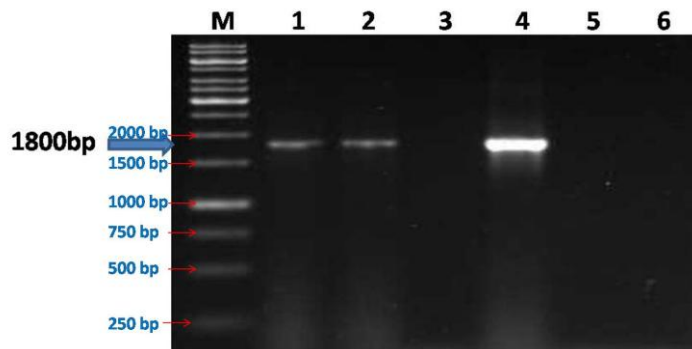


Figure 3: Agarose gel electrophoresis of polymerase chain reaction product of Crotalaria phyllody phytoplasma. M – 1kb Marker, Lane1 and 2–infected crotalaria DNA sample, Lane3 –healthy crotalaria DNA sample, Lane4 – Sesame phyllody infected sample as positive check, Lane5 – healthy Sesame DNA sample, Lane6 – water control.

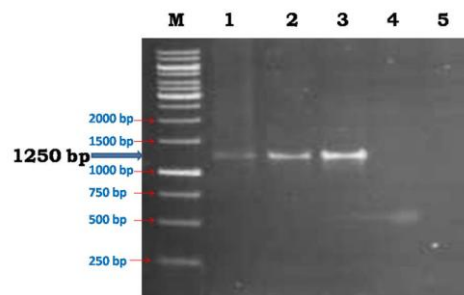


Figure 4: Agarose gel showing nested-PCR of 16S rDNA of Crotalaria phyllody phytoplasma. M- 1Kb Marker, Lane 1, 2 and 3- PCR product of crotalaria phyllody infected sample at 1:25, 1:50 and 1:100 dilution respectively, Lane 4- undiluted PCR product of crotalaria phyllody infected sample, Lane 5- water control

Discussion

The present study has clearly revealed the phyllody disease in crotalaria is caused by a phytoplasma. Weeds are the major reservoirs of phytoplasmas in the nature. They play a very significant role in the survival and perpetuation of not only viruses but also many phytoplasmas. Weed in the open field and cultivated fields known to be a reservoir of phytoplasmas infecting leguminous crops like pigeonpea, crotalaria, soybean and many other legumes (Phatak *et al.*, 1975; Dingra *et al.*, 1983; Shivanathan *et al.*, 1982).

Bright fluorescent spots were found in the phloem region of the phyllody infected tissues indicating the presence of phytoplasmas. Phytoplasmas were confined to the phloem region of the plant tissues Cervantis-Diaz *et al.* (2004) and Rumbos (1986). Whereas, no such fluorescent spots were observed with sections from healthy plants. A number of workers have also employed this technique to establish the phytoplasmal etiology of plant diseases (Suryanarayana, 1993, Rangaswamy, 1995 and Akhtar *et al.*, 2007).

A PCR technique for detection of crotalaria phyllody phytoplasma was standardized by modifying the DNA extraction method of Sunard *et al.* (1991) and modifying the PCR conditions particularly primer annealing temperature, as no phytoplasmal DNA was detected with the standard PCR conditions suggested by Lee *et al.* (1993). The repeated and reproducible phytoplasmal DNA amplification (1800bp) was obtained using phytoplasma specific universal primers P1/P7 only with annealing temperature 55°C for one minute as compared to 48°C for one minute of standard PCR protocol suggested for other phytoplasmas by Lee *et al.* (1993). No such PCR products were amplified from healthy plant samples.

Further Nested PCR technique was performed with primer pairs R16F2n/R16R2 in order to confirm the association of phytoplasma with crotalaria phyllody through reamplification of undiluted and diluted PCR product. Normal as well as nested PCR technique has been employed by various workers for the detection of phytoplasma in the phyllody affected plant (Raj *et al.*, 2006; Schnieder *et al.*, 1999; Lee *et al.*, 1993). Amplification of 1250bp 16S rDNA product was obtained only with the diluted PCR product amplified using P1 and P7 universal primers. No such amplification of 1250bp product was observed with the undiluted PCR product. An annealing temperature 56°C for one minute supported good amplification of the phytoplasmal 16S rDNA by nested PCR compared to 52°C for one minute of PCR protocol suggested for other phytoplasmas by various workers (Khan *et al.*, 2004; Singh, 1986; Han-MuSeok *et al.*, 1997). This clearly indicated the association of phytoplasma with the phyllody disease of crotalaria.

Conclusion

In the present study we have developed a sensitive PCR and N-PCR for the detection of crotalaria phyllody phytoplasma in addition to DAPI staining method. It was demonstrated that phytoplasma DNA could be detected from PCR. Further, direct-PCR product diluted at different concentrations could effectively be reamplified. Thus, the development of this highly sensitive nested-PCR based system will greatly facilitate detection of the phytoplasma. Biological and molecular characterization of the crotalaria phyllody phytoplasma is very much required to understand relationship with other phytoplasma diseases in view of their role in spread and survival of the phytoplasmal diseases.

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