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Detection of phytoplasma infecting greengram by nested PCR

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Abstract
Green gram variety LGG-460 was used to detect the phytoplasma by PCR at different stages of crop growth. Phytoplasma infected three green gram plants were collected for DNA isolation at 30, 45, 60 and 75 DAS from the same field and performed PCR with R16F2n/R16R2 primers. Samples collected at 30 and 45 DAS gave good amplification of expected product size in PCR with R16F2n/R16R2 primers, but weak bands were observed in samples collected from 60 and 75 DAS, thus indicating that the optimum age for the detection of phytoplasma in green gram is 30-45 DAS. The present result shows that the PCR techniques described here allows rapid, sensitive and accurate detection of phytoplasma in plants that are showing typical phytoplasma disease symptoms.

Keywords: Detection technique, greengram, optimum age of the plant and PCR

Introduction
Phytoplasmas are wall-less prokaryotes. They are bounded by a unit membrane and have cytoplasm, ribosomes and nucleic acid. In ultra-thin sections, they appear as a complex of multi branched, beaded, filamentous or polymorphic in shape, bodies ranging from 0.15-1.0 µm in diameter and 0.5-1.8 µm in length (Florence and Cameron, 1978) [1]. Most phytoplasmas are transmitted from plant to plant by sucking insects like leafhoppers and plant hoppers. Phytoplasmas may also be transmitted from infected to healthy plants through the parasitic plant dodder (Cordova *et al.*, 2003) [2]. Phytoplasmas can also be spread via vegetative propagation such as grafting and cutting of infected plants onto healthy plants (Kaminska and korbin, 1999) [3].

Phytoplasmas are generally present in phloem sieve tubes and in the salivary glands of insect vectors. Phytoplasmas also grow in the alimentary canal, haemolymph, salivary glands and intra-cellularly in various body organs of their insect vectors.

Numerous attempts to culture phytoplasmas on artificial nutrient media or cell free media have been unsuccessful so far (Lee and Davis, 1986) [4]. Phytoplasmas are bacterial plant pathogens that cause economically relevant yield losses in different low and high value, annual and perennial crops worldwide, including forest and ornamental trees, grasses, vegetables, flowers and agricultural crops (Bertaccini, 2007) [5]. The severity and nature of disease symptoms depend on the plant and the type of phytoplasma agent.

Before application of molecular techniques, detection of phytoplasmas in diseased plants was difficult. The diagnostic techniques commonly used were therefore observation of symptoms, insect or dodder transmission, electron microscopy. Serological techniques for the detection of phytoplasma began in 1980s with ELISA based methods. In the early 1990s, PCR coupled with RFLP analysis allowed accurate detection and identification of different strains and species of phytoplasmas. The disappearance of symptoms in some cases after tetracycline treatment provided additional evidence that phytoplasma is agents of several plant diseases. The determination of biological properties was laborious and time consuming and often the results are inconclusive. Nested PCR assay increases both sensitivity and specificity and is a valuable technique in the amplification of phytoplasmas from samples in which usually low concentration is present or substantial inhibitors that may interfere with the PCR efficacy are present (Marwitz, 1990; Lee *et al.*, 1993) [7, 6]. In nested PCR universal primers are used for the preliminary amplification and then followed by a second amplification using second group specific primers. Therefore, nested PCR enables the detection of dual or multiple phytoplasmas present in the infected tissues in case of mixed infections (Lee *et al.*, 1993) [6]. The detection and identification of phytoplasma from infected samples were difficult due to their low concentration in infected samples and there is need to find out optimum stage of plant sampling for its diagnosis.

Materials and Methods

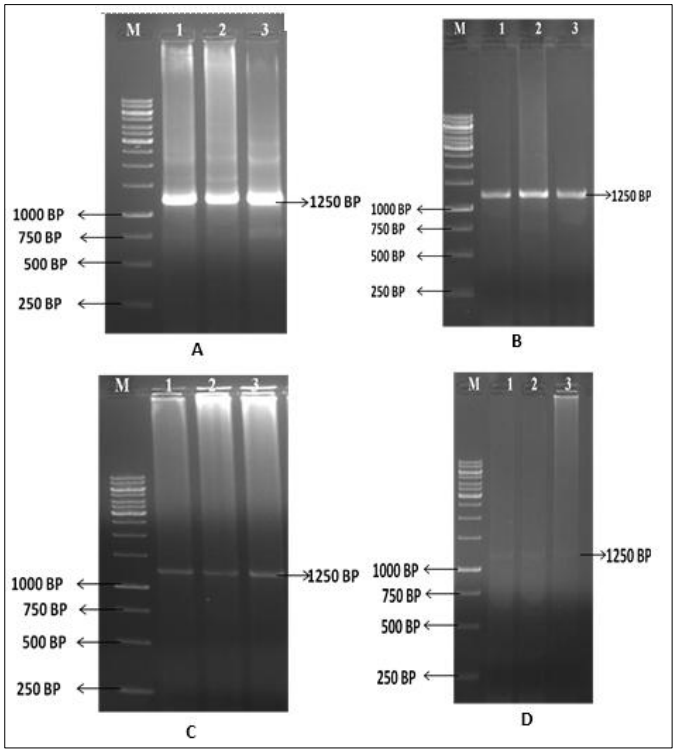
In the present study an attempt was made to detect the phytoplasma by PCR at different stages of crop growth to determine optimum age of the crop. Green gram variety LGG-460 was taken for this study. Phytoplasma infected three greengram plants were tagged in the field and collected one leaf each from lower, middle and top for DNA isolation at 30, 45, 60 and 75 DAS from the same plants. Infected greengram samples were collected first time on 16-01-2015, second time on 31-01-2015, third time on 15-02-2015 and fourth time on 02-03-2015.

The total DNA from phytoplasma infected greengram crop was extracted from leaves using the modified CTAB method (Murray and Thomson, 1980) [8]. Infected plant material (0.5g) was ground in a pre-sterilized pestle and mortar with liquid nitrogen until a fine powder was obtained and transferred to sterile Eppendorf tube. To this added 1ml of pre-heated (65ºC) extraction buffer (1M Tris (pH 8.0), 5 M NaCl, 0.5M EDTA, 2% CTAB, 1% PVP, 0.1 % Mercaptoethanal) and incubated for 1 hour in water bath at 65ºC. Then tubes were centrifuged (Refrigerated Eppendorf centrifuge) at 10,000 rpm for 10 min at room temperature and the supernatant was collected into Eppendorf tubes. To this added equal volumes of phenol-chloroform (1:1) mixed and centrifuged the tubes at 10,000 rpm for 10 min, transferred the supernatant to the fresh Eppendorf tube and added equal volumes of chloroform and Isoamyl alcohol (24:1) mixed well and then centrifuged the tubes at 10,000 rpm for 10 min, collected the supernatant into separate Eppendorf tube and added 0.1 volume of 3M sodium acetate (pH 4.8) and 0.6 volume of ice cold isopropanol then incubated at -20ºC for overnight.

After incubation, the tubes were taken out and centrifuged at 13,000 rpm for 20 min at 4ºC. The supernatant was discarded and the pellet was washed with 70% alcohol and again centrifuged at 13,000 rpm at 4ºC for 10 min, discarded the supernatant, air dried the pellets and dissolved in 50µl of sterile distilled water. The DNA samples were stored at -20ºC for further use.

The total DNA used as a template in PCR with phytoplasma specific primers R16F2n/R16R2 (Gundersen and Lee. 1996) [9]. The PCR were carried out sequentially in a final volume of 25 µl reactions containing 2.5 µl of (10X) PCR buffer, 2.0 µl (25 mM) $MgCl_2$, 0.5 µl (10 mM each) dNTPs, 1.0 µl (10 µM) each primers, 0.2 µl Taq DNA polymerase (5 u/ µl), and 2 µl template DNA (50 ng/ µl). The DNA was amplified by an initial denaturation of 94°C for 4 min followed by 35 cycles of 94°C for 30 seconds denaturation, 56°C for 1 min primer annealing, 72°C for 2 min primer extension and final extension at 72°C for 10 min. The PCR products were analysed by electrophoresis in 1% (w/v) agarose gel. Agarose gel electrophoresis of DNA was performed as described by Sambrook *et al*. (2001). The 1% agarose gel (W/V) was prepared by dissolving 1.0 g of agarose (Sigma, USA) in 100 ml of 1x TBE buffer. The gel was allowed to cool for some time and then 4 µl of ethidium bromide (10 mg / ml) was added and poured into gel casting tray of horizontal electrophoresis unit (Hoefer, USA).

The DNA samples were mixed with loading dye (Fermentas, USA) and the electrophoresis was carried in 1x TBE buffer at 100V (Labemate Power Pack 300, USA) till the dye front reached the lower part of the agarose gel. The migration pattern of the DNA fragments in the gel was recorded using gel documentation system (Alpha Innotech, USA) in an auto exposure mode.



(A) 30 DAS (B) 45 DAS
(C) 60 DAS (D) 75 DAS

Fig 1: Detection of phytoplasma at various growth stages in green gram (cv. LGG460) by PCR with R16F2n/R16R2 primers.

Results and Discussion

In the present study an attempt was made to detect the greengram phyllody phytoplasma by PCR at different stages of crop growth to determine optimum age of the crop. Phytoplasma infected three greengram plants were tagged in the field and collected one leaf each from lower, middle and top for DNA isolation at 30, 45, 60 and 75 DAS from the same plants and performed PCR with R16F2n/R16R2 primers. Samples collected at 30 and 45 DAS gave good amplification of expected product size in PCR with R16F2n/R16R2 primers (Fig. 1), but moderate intensity bands were observed in samples collected at 60 DAS and faint band was observed from samples collected at 75 DAS with same primer indicating that the optimum age for the detection of phytoplasma in greengram is 30-45 DAS.

The application of PCR to the diagnosis of phytoplasma associated diseases has greatly facilitated the detection and identification of a wide array of phytoplasmas in different plant species in India (Singh *et al*., 1978) [10]. Berges *et al*., (2000) [11] conducted an experiment by transmitting apple proliferation phytoplasma onto tobacco by grafting. The phytoplasma concentrations were very low, just reaching $8.9x10^2$ cells per gram of tissue. Rapid increases of phytoplasma numbers were observed and by the end of six weeks, a titre of $3.5x10^6$ cells per gram of tissue was determined. Further growth was slow, and 10 weeks post inoculations, the phytoplasma concentration reached $5.1x10^6$ cells per gram of tissue.

The objective of this study was to develop a PCR method that permits sensitive and accurate detection method for phytoplasma infecting greengram in Andhra Pradesh. The PCR techniques described here allows rapid, sensitive and accurate detection of phytoplasma in plants that are showing typical phytoplasma disease symptoms.

References

1. Florence ER, Cameron HR. Three dimensional structure and morphology of mycoplasrna like bodies associated with albino disease of Prunus avium. Phytopathology. 1978; 68:75-80.
2. Cordova I, Jones P, Harrison NA, Oropeza C. In situ detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease. Molecular Plant Pathology. 2003; 4:99-108.
3. Kaminska M, Korbin M. Graft and dodder transmission of phytoplasma affecting lily to experimental hosts. Acta Physiologiae Plantarum. 1999' 21(1):21-26.
4. Lee IM, Davis RE. Prospects for *in vitro* culture of plant pathogenic mycoplasma like organisms. Annual Review of Phytopathology. 1986; 24:339-354.
5. Bertaccini A. Phytoplasmas diversity, taxonomy, and epidemiology. Frontieres in Bioscience. 2007; 12:673-689.
6. Lee M, Hammond RW, Davis RE, Gundersen DE. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma like organisms. Phytopathology. 1993; 83:834-842.
7. Marwitz R. Diversity of yellows disease agents in plant infections. Zentbl. Bakteriol. Suppl. 1990; 20:431-434.
8. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research. 1980; 8:4321-4326.
9. Gundersen DE, Lee MI. Ultrasensitive detection of phytoplasmas by nested PCR assays using two universal primer pairs. Phytopathologia Mediatory. 1996; 35:144-151.
10. Singh UP, Sakai A, Singh AK. White leaf disease of *Cynodon dactylon* pers., a mycoplasmal disease in India. Experientia. 1978; 34:1447-1448.
11. Berges R, Rott M, Seemuller E. Range of phytoplasma concentrations in various host plants as determined by competitive polymerase chain reaction. Phytopathology. 2000; 90:1145-1152.