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A brief review on molecular detection and characterization of phytoplasma associated with pulses

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Abstract
In India phytoplasma associated with plants including crops, fruits, trees, ornamental, sugarcane, grasses and weeds was increasing at alarming rate. Recent evidences showed that phytoplasma associated with pulse crops in India. Phytoplasma cause diseases in pulse crops are chances to causing severe losses. In earlier days very few phytoplasma diseases were identified in India based on bright field, fluorescence, electron microscope observations, tetracycline treatment and to a lesser extent by serological assays. Among these, microscopic methods do not attain pathogen identification, and all of them are not always sufficiently sensitive to detect phytoplasma infections in low titer hosts. Today detection of phytoplasma based on molecular methods including PCR assays are efficiently carried out in India and based on this technique phytoplasma infection on pulse crops was reported in India. The "*Candidatus* Phytoplasma asteris", "*Candidatus* Phytoplasma aurantifolia" belong to 16SrI and 16SrII group of phytoplasmas are the major groups associated with pulse crops reported to be infected with phytoplasma throughout India. In this paper, we have discussed on phytoplasma disease on pulse crops in India.

Keywords: phytoplasma disease, pulse crops and reviews

Introduction
Pulses are consumed as dal, which is a cheap source of plant protein and also have medicinal properties. By products of pulses like leaves, pod coats and bran are fed to animals in the form of dry fodder. Some pulse crops like chickpea, urdbean and mungbean are fed to animals as green fodder. Mungbean plants are also used as green manure which improves soil health and adds nutrients into the soil (Status paper on pulses).

In the world, pulses are cultivated by 171 countries. At triennium ending 2010-11, the total area under pulse cultivation was 723 lakh ha, with production of 644.08 lakh t with a productivity of 890 kg/ha. The highest area and production was contributed by India (32.24%) and the highest productivity was of France (4219 kg/ha) followed (Status paper on pulses).

The first report on association between wall less prokaryotes and plants was reported in 1967. Doi and his colleagues (1967), discovered wall-less prokaryotes resembling mycoplasmas in the phloem of plants with mulberry dwarf**,** potato witches' broom, aster yellows and paulownia witches' broom by electron microscopy.

Last few years pulse crops are infected by phytoplasma diseases in India. Lakshmanan *et al.* (1988) [13] first reported phytoplasma associated with green gram phyllody in India. Characteristic symptoms of phytoplasma infected green gram plants shows stunted growth, production of little leaves in initial stage, at flowering stage floral parts are transformed into green leaf like structure followed by abundant vegetative growth.

Two phytoplasma species cause major phytoplasma diseases of pulses in India, they are the '*Candidatus* Phytoplasma aurantifolia' and '*Candidatus* Phytoplasma asteris' and these species infect the major leguminous plants such as black gram, greengram, cowpea, chickpea and pigeonpea. (Reddy *et al*., 2014; Ragimekula *et al*., 2014; Kumar *et al*., 2012; Pallavi *et al*., 2012. and Raj *et al*., 2006) [21, 18, 12, 17, 19].

The available literature on molecular characterization of phytoplasma associated with pulses (Table 1) and development of PCR based diagnostic methods for detection of phytoplasma infecting pulses has been reviewed and presented here.

Ghanekar *et al.* (1988) [8] reported association of MLO's with chickpea phyllody at Coimbatore, Tamilnadu. Infected plants showed that pale green colour with smaller leaflets and bushy due to axillary shoot proliferation, abnormal green structure develops in place of flowers. Phytoplasma infected chickpea plants examined under electron microscopy.

Lakshmanan *et al*. (1988) [13] reported phytoplasma associated with green gram phyllody first time in India from Coimbatore. The disease was characterized by stunted growth and production of small leaves in initial stages, later at flowering stage floral parts are transformed into green leaf like structures. They conducted the experiment on transmission of phytoplasma from infected to healthy plants by sap inoculation and side wedge grafting. The disease was successfully transmitted on 25 days old green gram plants through side wedge grafting, but it was unsuccessful through sap inoculation.

Deng and Hiruki. (1991) [6] designed five primer pairs based on 16S rRNA sequences of eukaryotes. These PCR primer pairs had high sequence homology to the conserved 16S rRNA genes of various culturable and nonculturable Mollicutes. Full length 16S rRNA genes and partial-length 16S rRNA genes of variable regions were successfully amplified when DNA preparations from culturable Mollicutes such as *Mycoplasma flocculare* and three Spiroplasma strains and nonculturable Mollicutes associated with various plant diseases were used as PCR templates. Amplifications were not detected from healthy plants. The results suggest the possibility that 16S rRNA genes of culturable and nonculturable Mollicutes can be amplified for detection and for a phylogenetic study using crude Mollicutes DNA preparations under appropriately controlled thermocycling conditions.

Gundersen *et al*. (1996) [9] developed a new universal primer R16mF2 /R1, a modified universal primer pair R16mF2/R2 and used for amplification of 16S rDNA from phytoplasma by nested PCR, allowed identification of primary phytoplasma associated with each tissue sample.

Rayachaudhuri *et al*. (1997) [20] observed typical phytoplasma like bodies in tubes of phloem of diseased brinjal plants. Size of the bodies varies from 245 to 737 mm. Mycoplasmal etiology of the disease was further conformed when spraying of achromycin and ledermycin to infected plants starting six hours after graft inoculation, the established disease was completely prevented.

Castro *et al*. (2004) [4] observed phytoplasma disease symptoms such as shoe stringed leaves, phyllody and flower abortion with faba bean fields of Spain. The etiology of this disease was investigated using graft transmission to periwinkle plants. Cloning of PCR amplicon, 16S DNA sequencing and phylogenetic analysis indicated that this phytoplasma belonging to 16SrIII group.

Das and Mitra. (2004) [5] studied detection of phytoplasma in tissues of little leaf disease affected brinjal plant using DAPI stain and to compare its efficiency with Dienes stain. Twigs from little leaf disease affected brinjal, collected from fields of IARI Research Farm, New Delhi, were side grafted on to healthy brinjal seedlings in insect proof green house. All the grafted plants developed symptoms within 21-25 days after inoculation. These plants were screened for the presence of phytoplasma. In the comparative study of both the detection methods, it was observed that DAPI could detect little leaf infection within two weeks after graft inoculation whereas, Dienes method failed.

Saqib *et al.* (2005) [25] observed phytoplasma disease symptoms such as leaf stunting, little leaf and proliferating branches with chickpea in Australia. The PCR product from symptomatic leaves was sequenced and confirmed that presence of a phytoplasma with high similarity to the 16SrII group i.e., '*Candidatus* Phytoplasma aurantifolia'.

Raj *et al*. (2006) [19] reported little leaf disease of pigeon pea in experimental field at NBRI, Lucknow, India. Phytoplasma infected pigeonpea plants shows production of little leaves, shortening of internodes and petioles giving a bunchy appearance and whole plant stunting. Nested PCR with phytoplasma 16S rDNA primers P1/P6 followed by R16F2n/R16R2 and gave product size of 1.5 Kb and 1.2 Kb respectively. Cloning and sequencing of 1.2 Kb size amplicon, confirm that '*Candidatus* Phytoplasma asteris' is the causal agent of little leaf disease of pigeon pea.

Saady *et al.* (2006) [22] reported phytoplasma disease associated with Chickpea plant at Nizwa Region, Oman. Restriction fragment length polymorphism (RFLP) analysis of nested PCR products showed that chickpea phyllody causing phytoplasma belongs to group 16SrII peanut witches' broom group.

Saqib *et al*. (2006) [24] reported phytoplasma disease symptoms on snakebean and tomato at horticultural region in Broome, Western Australia. They amplified the phytoplasma 16S rRNA gene by nested PCR, and sequence analysis of 16S rDNA indicated that causal agent as '*Candidatus* Phytoplasma australiense'.

Alvarez *et al.* (2007) [3] conducted the experiment on transmission of phytoplasma via vegetative propagation from infected to healthy cassava plants in insect proof greenhouse at 20 to 25°C and 50 to 90% relative humidity (RH). Graft transmission using leaf midribs, petioles, and shoots from each of five infected and healthy cassava plants via cleft, chip budding. Graft unions were carefully covered with parafilm and a plastic bag for 1 to 3 weeks. Plants were incubated in the greenhouse and observed for symptom development for 5 months. The presence and identity of phytoplasmas was assessed in all the plants before and after transmission experiments using nested PCR assays.

Hodgetts *et al*. (2007) [11] developed terminal restriction fragment analysis (T-RFLP) technique for simple and rapid detection and diagnosis of phytoplasmas in plants. The technique was tested for 37 isolates from 10 of the 16Sr groups. To confirm the presence of phytoplasma DNA and to know the 16Sr group, amplification by nested PCR was done for 37 samples using primers P1 and P7, followed by R16F2 and R16R2. PCR products were digested with *Alu*I, *Hae*III and analysed by agarose gel electrophoresis. The digest patterns confirmed the group classifications for all the isolates.

Akhtar *et al*. (2008) [2] reported association of phytoplasma with chickpea plants in Pakistan. An infected plant shows proliferation of branches with smaller leaflets, giving a bushy appearance to the plants. The flowers developed abnormal green leaf like structures instead of normal flowers. Tissue samples from infected and healthy plants were examined under transmission electron microscope, typical pleomorphic bodies were observed from infected samples but such bodies were absent from healthy samples. The DNA was extracted from infected plants and amplified using the universal phytoplasma PCR primers P1/P7 followed by R16F2n/R16R2. Partial sequencing confirmed that the phytoplasma had the greatest homology to 16SrII phytoplasma.

Akhtar *et al*. (2010) [1] reported phyllody disease caused by

Phytoplasma on mung bean in Pakistan. Total DNA was extracted from infected plants and used as a template for nested PCR amplification using 16S rDNA phytoplasma primers P1/P7 followed by R16F2n/R16R2, resulted 1800bp and 1250bp amplicons respectively. Direct sequencing of the 1.25Kb PCR amplicon showed highest sequence identity with 16SrII group and phylogenetic analysis placed the phytoplasmas in sub group 16SrII-D.

Viswanathan. (2010) [29] detected phytoplasmas causing grassy shoot disease in sugarcane by immunofluorescence technique. The results clearly established that the fluorescent antibody technique could be a useful technique in the detection and localization of phytoplasmas causing GSD in infected sugarcane tissues.

Makarova *et al.* (2011) [14] conducted experiment on use of quantitative real time PCR for a genome wide study of aster yellow witches broom phytoplasma gene expression in plant and insect hosts. To study whether a relative gene expression quantification method could be applied to phytoplasmas in a high throughput manner, an AYWB pathosystem was used. AYWB phytoplasma has a unique life cycle that includes both plant and insect hosts. They hypothesised that the adaptation to such different habitats would be reflected in gene expression in the respective hosts, which in turn could be detected by the delta delta (dd) Ct method.

Oberhansli *et al*. (2011) [16] development of a duplex TaqMan real-time PCR for detection of phytoplasmas and 18S rRNA host genes in fruit trees and other plants. The method was successfully employed for the screening of phytoplasmas in samples of fruit tree, raspberry and grapevine.

Shahriyari *et al*. (2011) [26] generated of a specific monoclonal recombinant antibody against causal agent of witches' broom disease of lime *'Candidatus* Phytoplasma aurantifolia' by using phage display technology.

Kumar *et al.* (2012) [12] reported bud proliferation disease on cowpea in New Delhi, India. Nested PCR amplification with phytoplasma specific primer pairs P1/P7 and R16F2n/R2, resulted amplicon product size of 1.8Kb and 1.25Kb respectively. The 1.25Kb amplicon product was cloned and sequence analysis with other phytoplasma species in database shows that 16S rDNA sequence of the cowpea phytoplasma share 99% identity with phytoplasmas of 16SrI group members i.e., '*Candidatus* Phytoplasma asteris'.

Nguyen *et al*. (2012) [15] reported sweet potato little leaf strain V4 phytoplasma associated with snake bean in the Northern Territory, Australia. Phytoplasma disease symptoms such as little leaf, big bud, phyllody, deformation and proliferation of stems, leaves and flowers were observed on 12 week old snake beans plants. DNA was extracted from infected snake bean and *Phyllanthus amarus* plants and amplified with P1/P7 primers. The 1,800 bp PCR product was sequenced and bioinformatics analyses were conducted using the Geneious Pro software. Genetic relation between the snake bean and *Phyllanthus amarus* phytoplasmas were determined by restriction fragment length polymorphism using the restriction enzymes, *Alu I* and *Rsa I.* The phylogenetic dendrogram of the 16S rRNA gene sequences from 25 phytoplasmas showed that phytoplasma from snake bean grouped with the sweet potato little leaf phytoplasma i.e., 16Sr II group.

Pallavi *et al*. (2012) [17] reported association of phytoplasma with phyllody disease of chickpea in India. Infected chickpea plants shows that phytoplasma disease symptoms such as pale green leaves, bushy appearance due to excessive stunting of shoots, reduced internodal length and excessive axillary proliferation. DNA was isolated from infected and healthy chickpea leaves and this DNA used as a template in nested PCR amplification with phytoplasmas 16S rDNA primers P1/P7 followed R16F2n/R16R2 and observed product size of 1.8Kb and 1.2Kb respectively. The 1.8Kb amplicon was cloned into the plasmid vector pTZ57R/T and sequenced. BLAST analysis shows that more than 98% similarity to the members of the 16S rII group i.e., '*Candidatus* Phytoplasma aurtentifolia.

Singh *et al*. (2013) [27] conducted experiment on association of phytoplasma with faba bean at ICAR Research farm, at Patna in Bihar. Early symptoms of faba bean phyllody consisted of light yellowing of leaves, gradual greening of petals, mild yellowing, vein clearing and slightly inward folding of leaves in the apical region of plant. Afterwards successive yellowing and shedding of older leaves and development of phyllody was noticed. They were concluded that ambient temperature found to be contributing positively on disease development.

Hamed *et al.* (2014) [10] reported phytoplasma associated with faba bean witches broom disease in Egypt. Total DNA was isolated from infected faba bean plants and amplified by nested PCR with phytoplasma specific primers P1/P7 and R16F2n/R16R2 and direct PCR amplification with witches' broom specific primers SR1/SR2, resulted product size of 1200bp and 325bp respectively. The transmission experiments were carried out to transmit the phytoplasma from the symptomatic phyllody faba bean plants to periwinkle and healthy faba bean by dodder plant. The phytoplasma that causes phyllody disease was successfully transmitted from infected to healthy plants. The causative agent was successfully transmitted to healthy plants, producing disease Symptoms within 25-35 days.

Ragimekula *et al*. (2014) [18] reported mungbean phyllody in Andhra Pradesh, India. Infected plant shows stunting, proliferation of branches with small leaves and phyllody. Nested PCR amplification with P1/P7 and R16F2n/R16R2 primers, resulted amplicon product size of 1.8Kb and 1.2Kb respectively. The 1.2Kb amplicon was cloned and sequenced. BLAST analysis revealed that 100% sequence identity with 16SrII group '*Candidatus* Phytoplasma aurantifolia.

Reddy *et al*. (2014) [21] reported '*Candidatus* Phytoplasma aurantifolia' associated with blackgram phyllody in India. Phytoplasma disease symptoms such as yellowing, stunting, witches' broom, little leaves and reduced size of flowers were observed with infected blackgram plants. DNA was isolated from infected plants and nested PCR amplification with P1/P7 followed by R16F2n/m23sr, resulted product size of 1.8Kb and 1.6Kb respectively. A representative 1.6Kb PCR fragment was cloned into a pTZ57R/T vector and sequenced. Analysis of 16S rDNA sequence of the *Vigna mungo* phytoplasma with other isolates revealed the highest sequence identity with phytoplasmas of the 16SrII group.

Saleh *et al*. (2014) [23] worked on molecular characterization of the 16Sr II group of phytoplasma associated with faba bean in Saudi Arabia. Faba bean symptomatic leaves were collected and DNA was isolated from these samples. The DNA was amplified by nested PCR with P1/P7 primer followed by R16F2n/R16R2 primer pair and obtained product size of 1.8Kb and 1.25Kb respectively. Phylogenetic analysis of the 16S rRNA gene of the obtained nucleotide sequence indicated that the faba bean phytoplasmas isolates were more closely related to peanut witches' broom group i.e., '*Candidatus* Phytoplasma aurantifolia.

Table 1: List of phytoplasma species associated with important crop plants, their groups and accession numbers.

S. No	Crop name	Reference	Phytoplasma species	Group name	Accession number
1	Green gram	Lakshmanan *et al*. (1988) [13]			
2	Chickpea	Ghanekar *et al.* (1988) [8]			
3	Faba bean	Castro *et al*. (2004) [4]		16SrIII group	
4	Chickpea	Saqib *et al.* (2005) [25]	*Candidatus* Phytoplasma aurantifolia	16SrII group	
5	Pigeon pea	Raj *et al*. (2006) [19]	*Candidatus* Phytoplasma asteris	16SrI group	DQ343287
6	Chickpea	Saady *et al.* (2006) [22]		16SrII group	
7	Snakebean and tomato	Saqib *et al*. (2006) [24]	*Candidatus* Phytoplasma australiense	16SrII group	
8	Chickpea	Akhtar *et al*. (2008) [2]		16SrII group	
9	Mung bean	Akhtar *et al*. (2010) [1]	*Candidatus* Phytoplasma aurantifolia	16SrII-D group	FJ410489
10	Cowpea	Kumar *et al.*(2012) [12]	*Candidatus* Phytoplasma asteris	16SrI group	HM449952
11	Snake bean and tomato	Nguyen *et al*. (2012) [15]	Candidatus Phytoplasma aurantifolia	16SrII group	DQ375777 DQ375778
12	Chickpea	Pallavi *et al*. (2012) [17]	Candidatus Phytoplasma aurantifolia	16SrII group	
13	Faba bean	Singh *et al*. (2013) [27]			
14	Faba bean	Hamed *et al.* (2014) [10]			
15	Green gram	Ragimekula *et al*. (2014) [18]	*Candidatus* Phytoplasma aurantifolia	16SrII group	KF811205
16	Black gram	Reddy *et al*. (2014) [21]	*Candidatus* Phytoplasma aurantifolia	16SrII group	KJ540943
17	Faba bean	Saleh *et al.* (2014) [23]	*Candidatus* Phytoplasma aurantifolia	16SrII group	JQ861532 JQ861533

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