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# Molecular Detection of a Phytoplasma Associated with *Stachytarpheta* Witches' Broom in South India

**M S Pallavi, K T Rangaswamy, R Harischandra Naik[$] and B L Divya**

Department of Plant Pathology, GKVK, University of Agricultural Sciences, Bengaluru - 560 065, Karnataka, India.
[$]Department of Agricultural Entomolgy, TNAU, Coimbatore - 641 003, Tamilnadu, India.

## Abstract

Stachytarpheta *(*Stachytarpheta jamaicensis *L.) plants showing typical symptoms of infection by a phytoplasma that causes whitche's broom disease were commonly observed in recent years in parts of south India. The symptoms included excessive production of spikelets in an upright fashion within inflorescence axis, bearing pedicelled phylloid flowers, carpels transformed into a pair of folded upright leafy structures followed by extensive proliferation of side shoots. The causal agent of the witches' broom disease was identified based on symptoms, fluorescent microscopy, amplification of 16S rDNA of the phytoplasma by polymerase chain reaction (PCR) from infected samples. Standard PCR and nested-PCR (N-PCR) protocols were developed for improved efficiency and reliability of the diagnostic protocols. Using the primers P1/P7 and R16F2n/R16R2 primers, 1800 bp and 1200 bp size products were amplified in standard PCR and N-PCR protocols, respectively. The molecular identification of this new disease has been discussed.*

**Key words:** *Stachytarpheta,* witches' broom, phytoplasma, fluorescent microscopy, polymerase chain reaction, nested-PCR

## Introduction

*Stachytarpheta* is an invasive weed believed to be originated in tropical Americas and now occurs widely in India. It is a small perennial shrub frequently grown as an ornamental plant due to its blue coloured flowers and belongs to the family: Verbinaceae. In India, it has become a serious weed both in arable and grazing lands since a decade. It is reported to be the frequent weed (30%) after *Parthenium*(54%) (Suryanarayana , 1993).

Symptomatology has been one of the major criteria for preliminary diagnosis of putative phytoplasmal diseases (Lee and Davis, 1993). Phytoplasmas infect numerous plant species including leguminous and ornamental plants with the symptoms like virescence, proliferation of shoots resulting in Witches' broom, sterility of flowers and compact growth at the end of stems, yellowing, phloem necrosis and dieback of branches of woody plants (McCoy and Thomas, 1981).

The inability to isolate and culture phytoplasma *in vitro* has particularly impeded their identification. This has been a major concern until the introduction of polymerase chain reaction (PCR) assays in which universal primers derived from conserved 16S ribosomal RNA (16S rRNA) gene sequences are used for the identification as well as to classify a broad range of phytoplasmas (Schneider *et al*. 1995; Gundersen and Lee, 1996; Montano *et al*. 2001; Harrison *et al.* 2002; Lee *et al*. 2004; Samuitiene and Navalinskeine 2006). The conserved region of 16S rDNA is used as a standard marker for the phylogenetic classification of phytoplasma groups (Seemuller *et al.*, 1994). PCR has proven to be more sensitive and convenient method for phytoplasma diagnosis than other methods such as serology and nucleic acid hybridization assays. In order to further improve the sensitivity and efficiency of detecting low titre pathogens such as phytoplasma in plant tissue, nested-PCR (N-PCR) assay, in which a preliminary amplification using a universal primer pair followed by the use of a second target-specific primer pair on the products from the first PCR was done (Lee *et al*. 1994; Gundersen and Lee 1996).

No attempts have been done so far in India, to identify the witches broom disease causing pathogen in Stachytarpheta through molecular appraoch. Hence, the search for pathogen causing witches' broom disease to Stachytarpheta at various levels is an essential step for future implementation of weed control in an integrated weed management strategy in India. The objectives of this study were to detect the witches' broom disease causing agent through fluorescent microscopy, and polymerase chain reaction techniques.

## Materials and methods

### Fluorescent microscopic detection

**Plant material.** Leaf samples were collected from naturally infected *Stachytarpheta* plants showing typical symptoms of witches' broom at the Zonal Agricultural Research Station of the University of Agricultural Sciences, Bengaluru, Karnataka, south Indian region (2009-2010). Samples from healthy plants were collected as controls. Phyllody infected samples from *Cicer arietinum* L. were also collected for the study. Another phytoplasma infection causing little leaf disease in periwinkle has been well documented in India (Singh *et al.* 2007; Omar *et al.* 2008) and this was used as positive control in PCR experiments.

**DAPI Staining.** For DAPI fluorescent staining, small pieces of roots, leaf petioles and rachillae of unopened inflorescence of witches' broom affected *Stachytarpheta* plants were fixed in 2.5% glutaraldehyde in 0.2% phosphate buffer for 30 min and were washed in same buffer for the same time span. Free hand thin sections of 20µ thickness of tissues were made with a razor blade and the sections were stained with 0.001% DAPI in 0.01 molar phosphate buffer at $22^0$C for one hour and examined under a fluorescent microscope. The sections were observed under a Zeiss filter set 09 fluorescent microscope using DAFI filter (excitation 450 to 490nm and emission 515nm). The presence or absence of fluorescent spots in the phloem tissues of healthy and diseased samples was observed.

### Extraction of total nucleic acids from infected plants

Total nucleic acids were isolated by following the modified CTAB method (Lodhi *et al.* 1994; Maruthi *et al.* 2002). The protocol followed was: ~0.15g of leaf tissue was frozen in liquid nitrogen and ground in a pre-chilled mortar and pestle. 750µl CTAB extraction buffer [2% CTAB (w/v), 1.4M NaCl, 0.2% 2-mercaptoethanol (v/v), 20mM EDTA, 100mM Tris–HCl, pH 8.0] was added to each sample and thoroughly ground. About 0.75 ml of the sap was transferred into new 1.5 ml micro centrifuge tube and incubated at $60^o$C for 30 min. After incubation, 750 µl of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly to form an emulsion. The mixture was centrifuged at 13,000 rpm for 10 min, and the supernatant was collected and mixed with 300 µl of isopropanol and incubated at -$20^o$C for at least 1 h. The contents were centrifuged at 13,000 rpm for 10 min and supernatant was discarded without losing pellet. The pellet was washed with 500 µl of 70% ethanol and further centrifuged at full speed for 5 min. The ethanol was removed and the pellet was vacuum dried for 5 min. Finally, the dried pellet was re-suspended in 100 µl of 1X TE buffer and stored at -$20^o$C until further analysis by PCR.

### Primers and PCR amplification of 16S rDNA

Phytoplasma-specific primers P1 **(**5'-AAGAGTTTGATCCTGGCTCAGGATT-3') (Deng and Hiruki 1991) and P7 (5'-CGTCCTTCATCGGCTCTT-3') (Smart *et al.* 1996) amplifying ~1800 bp fragment that extends from the 5' end of the 16S rDNA to the 5' end of the 23S rDNA were used for the detection of phytoplasma in a standard PCR. The universal primer pair R16F2n (5'-GAAACGACTGCTAAGACTGG-32 ) and R16R2 (52 -TGACGGGCGGTGTGTACAAACCCC -3) designed to amplify a 1200bp, portion of the 16S rRNA gene was used for N-PCR (Lee and Devis 1993).

The standard PCR and N-PCRs were carried out separately in a final volume of 25 µl reactions containing 2.5 µl 10X PCR buffer, 25 mM $MgCl_2$, 2.5 mM each dNTPs, 20 mM 1.25µl each primers, 0.1µl *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd, Bengaluru, India) and 2 µl template DNA. N-PCR was done using 2 µl of diluted (1:30 or 1:90) standard PCR product. The DNA was amplified by an initial denaturation of $94^o$C for 2 min followed by 35 cycles of $94^o$C for 2 min denaturation, $55^o$C for 2 min primer annealing ($56^0$C for 1 min for N- PCR), $72^0$C for 3 min primer extension, and final extension at $72^o$C for 10 min.

PCR products were separated by electrophoresis in 1% (w/v) agarose gels. A 1 kb molecular weight marker (MBI Fermentas Life Sciences, St. Leon-Rot, Germany; purchased from Genetix, Biotech Asia Pvt. Ltd., Bengaluru, India) was included on each gel. Experimental controls included DNA extracted from healthy plants and double-distilled water. Total DNA extracted from known phytoplasma diseases, chickpea phyllody and periwinkle little leaf were used as positive control (Singh *et al*. 2007; Omar *et al*. 2008).

### Restriction fragment length polymorphism analysis

The N-PCR products of *Stachytarpheta* witches' broom, chickpea phyllody, and periwinkle little leaf phytoplasma 16S rDNA (1200bp) were subjected to RFLP analysis. Five µl aliquots of N-PCR products were digested separately with restriction endonuclease *Alu*I overnight at $37^0$C. The digested products were separated through 1% agarose gel and restriction fragments were visualized with gel documentation unit after staining with 0.5 ml/L ethidium bromide.

## Results and discussion

### Symptoms of the diseased plants

Plants infected with witches' broom disease in *Stachytarpheta* include chlorosis of leaves, severe reduction in leaf size and malformation of floral parts into green leafy like structures. Excessive production of spikelets in an upright fashion within inflorescence axis, bearing pedicelled phylloid flowers, carpels transformed into a pair of folded upright leafy structures followed by extensive proliferation of side shoots (Figure1b). Similar types of symptoms were observed by Sharma and Singh (1988); Suryanarayana (1993).


**Figure 1. Witches' broom disease of *Stachytarpheta* caused by phytoplasma: (a) healthy plant with normal inflorescence; (b) witches' broom affected plants showing excessive production of spikelets in an upright fashion within inflorescence axis**

### Detection of phytoplasma

**Fluorescent microscopy.** Stem sections of infected plants with DAPI showed whitish blue fluorescent spots (Figure 2b) in UV light in the phloem regions indicating the presence of phytoplasmal DNA. Sharma and Singh (1988) reported that phytoplasmas are mycoplasma like organisms bounded by single unit membrane and confined to the phloem region of the plant tissues. No such fluorescent spots were observed in the phloem region of healthy stem tissues (Figure 2a). A number of workers have also employed this technique to establish the phytoplasmal etiology of plant diseases (Suryanarayana, 1993; Akhtar *et al*, 2007).

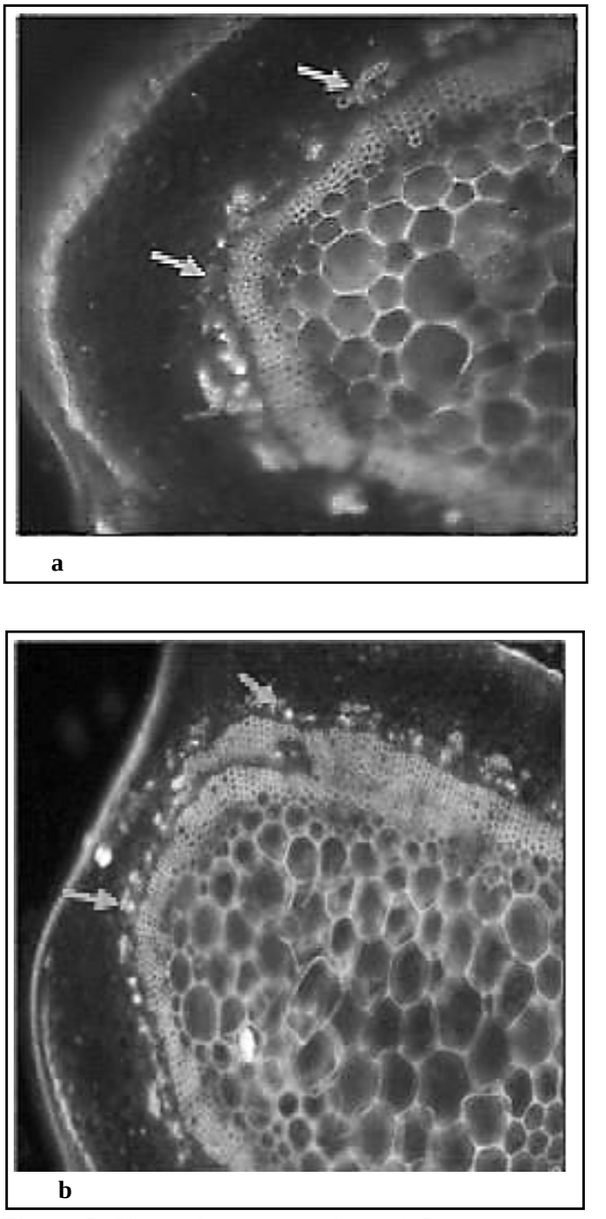


**Figure 2. Ultrathin stem sections of Stachytarpheta showing phytoplasmas of whitish blue fluorescent spots in UV light in the phloem regions. (a) healthy plant (b) witches' broom affected plant.**

### PCR assays

An expected band size of ~1.8 Kb was amplified from 16S rDNA of phytoplasma using P1/P7 primers from *Stachytarpheta* witche' broom, periwinkle little leaf and chickpea phyllody samples except from healthy plants (Figure 2). In N-PCR, expected products of ~1.2 Kb were

amplified using primers R16F2n/R16R2 from diluting the amplicons of P1/P7 to 1:30 (Figure 3). Amplification was not observed when undiluted PCR products of P1/P7 primers were used as template in N-PCR.

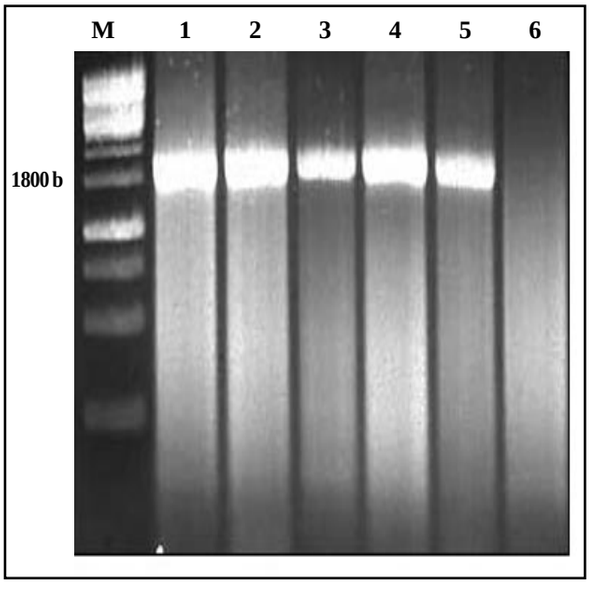


**Figure 3. PCR amplification of phytoplasma DNA from diseased *Stachytarpheta* and reference samples in Chickpea and *Vinca rosea* using the primers P1 and P7. Lanes 1 & 2 *Stachytarpheta* witches' broom, Lanes 3 & 4 chickpea phyllody, Lane 5 periwinkle little leaf, Lane 6 healthy *Stachytarpheta* DNA sample.**

In this study it has been shown that the *Stachytarpheta* witches' broom from south India was infected with a phytoplasma using PCR and RFLP. The standard PCR ( Saqib *et al.* 2006; Akhtar *et al.* 2007) and N-PCR techniques (Al Saady *et al.* 2006) have been used for the detection of chickpea phyllody phytoplasmas from Pakistan and Oman, respectively. However, the use of low stringent conditions (low annealing temperature in particular) in PCR-based diagnostic protocols especially using degenerate primers can often produce misleading results because of the amplification of non-target sequences. In order to overcome these limitations, we adopted a simple diagnostic protocol for the detection of chickpea phyllody phytoplasmas by using the modified CTAB total nucleic acid extraction method (Lodhi *et al*. 1994; Maruthi *et al*. 2002) as well as by optimizing PCR conditions, particularly enhancing the stringency of detection by increasing the annealing temperatures (Lee *et al*. 1994). The specificity and sensitivity of the PCR assays were enhanced by increasing the annealing temperature to 55°C compared to 48°C suggested by others (Lee *et al.* 1993; Khan *et al.* 2004). The N-PCR, performed by preliminary amplification using a universal primer pair followed by the use of a second set of pathogen-specific primers on the products from the first PCR, further increased the sensitivity of detecting low titre pathogens such as phytoplasma in plant tissues (Lee *et al.* 1994; Gundersen and Lee 1996). Using the modified conditions, the PCR results were reproducible in amplifying the 16S rDNA from three diverse phytoplasma diseases of *Stachytarpheta*, chickpea and periwinkle.

## RFLP analysis of phytoplasma 16S rDNA

The digestion of N-PCR products (~1.2 Kb) using the endonuclease *Alu*I revealed the presence of distinct phytoplasmas infecting *Stachytarpheta*, periwinkle and chickpea in south India. Digesting the *Stachytarpheta* witches' broom phytoplasma 16S rDNA generated three products of size 700, 400, 300 and 250 bp (Fig. 4). The infected samples from chickpea (band size 700, 300 and 250 bp) and periwinkle (band sizes 450, 400, 300 and 250 bp) showed bands of different classes.

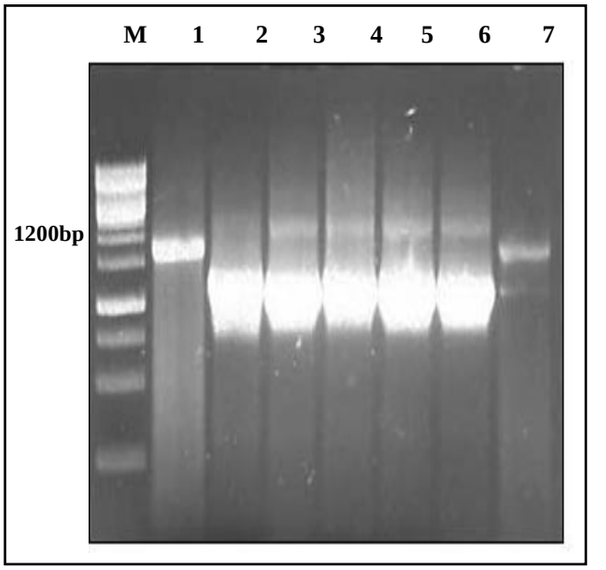


**Figure 4. Amplification of phytoplasma 16S rDNA by N-PCR using the primers R16F2n/R16R2 from infected plants. M 1 Kb marker, lanes 1 & 7 the standard PCR products from infected chickpea and *Stachytarpheta* samples, lanes 2 & 3, and 4 & 5 the N-PCR products obtained from 1:30 dilutions of standard PCR products of infected chickpea and *Stachytarpheta* samples, respectively; lane 6 the N-PCR products obtained from 1:30 dilutions of standard PCR products from periwinkle little leaf.**

The diversity of the phytoplasmas in south India was confirmed by RFLP analysis of the 16S rDNA by using restriction endonuclease *Alu*I. In the similar way Brown and Mclaughlin (2011) adopted RFLP technique for comparative identification of phytoplasma 16S rDNA for the exixtence of LY group phytoplasmas belong to subgroup 16SrIV-E in weed species *Stachytarpheta jamaicensis*. Digestion with AluI showed that the phytoplasma DNA restriction profiles were not similar to those obtained from chickpea phyllody and *Vinca rosea* little leaf phytoplasma strains. This indicates that a phytoplasma associated with the *Stachytarpheta* is different from the two positive controls. Brown and Mclaughlin (2011) tentatively included *Stachytarpheta jamaicensis* in 16SrIV-E group. Hence, continued effort is necessary in order to identify the *Stachytarpheta* witches' broom phytoplasma through feeding test in combination with detection of phytoplasma agent from other suspected phytoplasmal diseases. PCR with phytoplasma group specific primers and/or RFLP analysis of the PCR amplified products with different restriction enzymes coupled with feeding test may be necessary.

Overall, PCR provided a sensitive and rapid means of confirming phytoplasma infection. Such an assay can now be used to determine the host range and distribution of phyllody phytoplasma on economic crops and related weeds, and to determine the relatedness of phytoplasma diseases in India. Therefore, further research works shall attempt to determine the phylogenetic positions of the phytoplasmal diseases of economic crops and other weeds in India by comparison of their RFLP pattern and sequence analysis of the 16S rDNA with those of phytoplasma reference strains. Further survey for the potential vectors in *Stachytarpheta* will allow the determination of species responsible for transmitting diseases from weeds to crop plants and it could provide additional benefit in weed management.

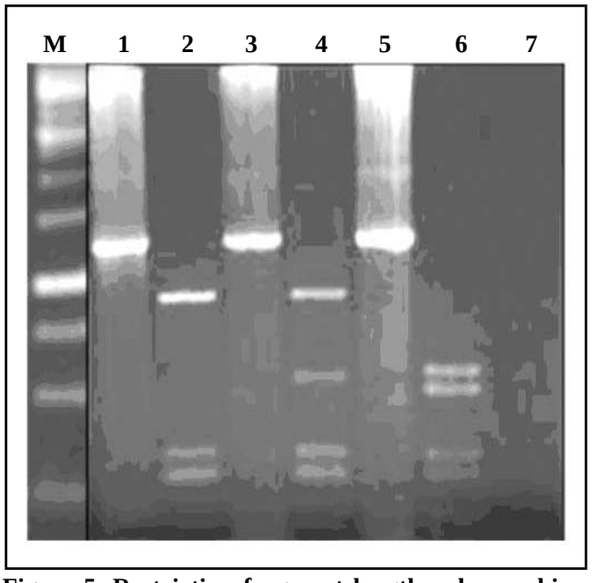


**Figure 5. Restriction fragment length polymorphism analysis of *Stachytarpheta* phytoplasma 16S rDNA N-PCR products using *Alu*I. Lanes 1, 3 & 5 are the N-PCR products from infected chickpea, *Stachytarpheta* and periwinkle, respectively. Lines 2, 4 and 6 are the *Alu*I digests of the respective samples in lanes 1, 3 and 5. Lane 7 water control.**

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Received : 19-03-2011 Accepted : 29-11-2011