MOLECULAR DETECTION AND CHARACTERIZATION OF SANDAL SPIKE PHYTOPLASMA

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DEPARTMENT OF PLANT PATHOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE – 560 065

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Thesis submitted to the
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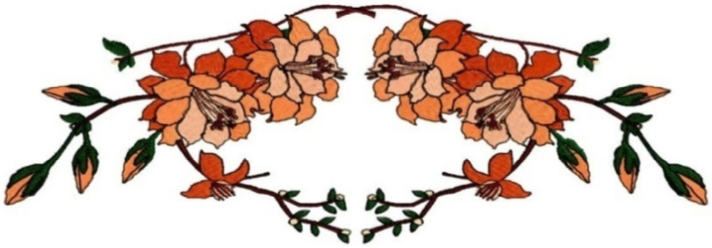

AFFECTIONATELY DEDICATED

to

My

beloved parents

Sri Revanasiddappa

Smt. Vinoda

Uncles

Mr. Jagadeesh, T.,
Mr. Shivamurthy

Chairman

Dr. K.T. Rangaswamy

DEPARTMENT OF PLANT PATHOLOGY
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CERTIFICATE

This is to certify that the thesis entitled **“Biological relationship and molecular characterization of Sandal spike phytoplasma.**” submitted by **Mr. MURALI, R., I.D. No. PAL-243** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT PATHOLOGY** to the **University of Agricultural Sciences, Bangalore**, is a record of research work done by him during the period of his study in this University, under my guidance and supervision and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

Bangalore
4th July, 2012

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4th July, 2012 ***(MURALI, R.)***

Molecular detection and characterization of Sandal spike phytoplasma

Murali, R.

M.Sc(Agri) THESIS ABSTRACT

Sandal is an economically important tree species which has been grown by farmers in some states in India. Spike disease caused by phytoplasma is a major problem in Sandal cultivation particularly in the states of Karnataka, Kerala and Tamil Nadu. Polymerase chain reaction (PCR) and Nested polymerase chain reaction (N-PCR) techniques for the detection of Sandal spike and *Stachytarpheta* phyllody phytoplasmas were handled by modifying PCR conditions. PCR and N-PCR products of approximately 1800bp and 1250bp corresponding to 16S rDNA of phytoplasmas was amplified using P1/P7 universal phytoplasma primers and R16F2n/R16R2 nested PCR primers respectively. The annealing temperature of 55^{o}C for one minute was found to be suitable for amplifying Sandal spike and *Stachytarpheta* phyllody phytoplasmal 16S rDNA fragments. Survey of Sandal plantations in southern Karnataka for assessment of Sandal spike incidence revealed the occurrence of spike disease ranging from 0-18 per cent. The disease was lowest in Shimoga district and highest in Tumkur district. The remaining districts were free from Sandal spike disease. The *Stachytarpheta* a common weed found in the Sandal ecosystem was also infected with phytoplasma and the typical characteristic symptoms were recorded. The cross infectivity transmission between Sandal spike and *Stachytarpheta* phyllody phytoplasmas through dodder revealed that the *Stachytarpheta* is not a collateral host for spike disease as it was not transmitted. RFLP (Restriction fragment length polymorphism) profiles of 1.8kb PCR product of Sandal spike and *Stachytarpheta* phyllody phytoplasmas digested with *AluI* and *HinfI* restriction endonuclease enzymes showed differences in 16S rDNA genome. Distinct RFLP pattern obtained with respect to Sandal spike and *Stachytarpheta* phyllody phytoplasmal 16Sr DNA fragments revealed that the Sandal and *Stachytarpheta* phytoplasmas are different.

July, 2012
Department of Plant pathology
UAS, GKVK, Bangalore

Signature of the Chairman
(Dr. K. T. Rangaswamy)

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Introduction

I. INTRODUCTION

Sandal (*Santalum album* L.) is an economically important, semi root parasite, perennial, evergreen tree species belongs to the family Santalaceae. It is known for its scented wood and fragrance oil. Although the tree grows in several countries, the best heart wood formation takes place only in India. In India it occupies 8513 square kilometres area in the states of Karnataka, Kerala, Madhya Pradesh, Maharashtra, Andra Pradesh and Tamil Nadu. (Srinivasan *et al.,* 1992). The average annual production of Sandal wood has been drastically come down from 4000 tonnes to 1044 tonnes in 1965 to 1995 (Srinivasan *et al.,* 1992).

Sandal is affected by few diseases, of which the spike disease is most destructive one (Selisker and Wilson, 1981). The disease was first reported in Coorg in 1898 is now found in the southern parts of Tamil Nadu. In natural forests radial spread of the disease is commonly encountered. Trees of all ages and sizes are attacked by the disease and diseased tree die within few years after the onset of spike disease. The disease is noticed in all major Sandal-growing states of India *viz.*, Karnataka, Tamil Nadu and Kerala. Conservative estimates indicate that the annual loss due to the disease to be around Rs.$5x10^6$ (10,00000) (Iyengar, 1969).

Phytoplasmas are single celled, wall-less prokaryotes, resembling mycoplasmas in morphology. Obligate parasites, cannot be grown in *in-vitro* cell free culture. They are transmitted by phloem feeding insects like leafhoppers, plant hoppers and psyllids. These are sensitive to tetracyclines but resistant to penicillin. Trival name “Phytoplasma” adopted in 10th congress of International Organization of Mycoplasmology.

Phytoplasmas formerly MLO's have been found to be associated with disease in several hundred plant species. Doi *et al.* (1967) first discovered the presence of cell wall less prokaryotes with in sieve cells of plant exhibiting yellow symptoms. These unique plant pathogenic prokaryotes have been termed as MLOs and these organisms belonging to class Mollicutes. The members of the class Mollicutes were characterized by lack of cell wall, small genome size (680 to 1600 kb) low Guanine plus-Cystine content (G+C), inability to culture.

Most of the studies carried out in the past work confined to understand the nature of pathogen, insect vector, the role of other hosts and biochemical and physiological aspects. Very little attention has been given to understand the pattern of disease spread, the precise role of other host, identification of the natural vector and developing effective management strategies.

The inability to culture MLOs has made it difficult to characterize and determine their taxonomic status by traditional methods. The current circumstances necessitate emphasis on molecular characteristics and phylogeny rather than phenotypic characteristics in determining the taxonomic status of MLOs.

The recent introduction of Polymerase Chain Reaction (PCR) assays has greatly advanced the capacity to detect and identify phytoplasmas. PCR assay using primer pairs designed on the basis of clone phytoplasmal DNA fragments or 16S rDNA gene sequences. The phytoplasma group-specific primer pair allows sensitive detection and simultaneous classification of specific Phytoplasmas strains of the plants (Lee *et al.,* 1993).

The high price of Sandal wood declared by the government of Karnataka and recent government policy to allow the private cultivation of Sandal by valid license from the forestry department has encouraged the farmers to go for planting of Sandal on even very fertile soils. A number of Sandal plantations have also come up in the states of Gujarat, Tamil nadu, and Karnataka. However recently in one of the farmer's Sandal plantations of Karnataka the severe incidence of spike disease has been observed. There are many reports in the literature which implicates a number of forest plant species/weeds associated with Sandal ecosystem in the forest are reported to act as natural sources of infection. Some of the important species includes *Zizyphus oenoplea, Vinca rosea, Dodonea viscose, Eucalyptus grandis, Dendrocalamus strictus.* Recently a new weed with characteristic symptoms of phytoplasma also been noticed in spike prevailing farmer's Sandal plantations. However, no systematic studies have been made in the past using molecular work to understand the exact role of those plant species/weeds in the epidemics of spike disease associated with Sandal ecosystem as the reservoir hosts for the spike disease.

In all of these reports the role of the hosts has been speculated on association of phytoplasmal symptoms with the plant species associated no systematic studies done to establish their role either through cross transmission studies on molecular approach. Further there is no report on *Stachytarpheta* acting as host for the disease in the literature. In view of the high incidence of phytoplasmal infection on *Stachytarpheta* associated with the Sandal spike prevailing plantations, the present study was taken up to find out the interrelationship with each other by RFLP analysis of the 16S rDNA of the phytoplasmas.

Objectives of Investigation:

1. Standardization of PCR technique for detection of phytoplasmas associated with Sandal spike and *Stachytarpheta* phyllody.
2. PCR based survey of the Sandal plantations to assess the incidence of Sandal spike disease in southern Karnataka.
3. Cross infectivity studies between Sandal spike and *Stachytarpheta* phytoplasma isolates.
4. Characterization of Sandal spike and *Stachytarpheta* phyllody phytoplasmas by RFLP analysis of 16S ribosomal gene.

Review of Literature

II. REVIEW OF LITERATURE

The spike disease of Sandal is prevalent in the states of Karnataka and Tamilnadu. It was first reported in 1898 by Mc Carthy from forest of Coorg in Kodagu district of Karnataka. It was considered to be first as a nutritional deficiency (Butler, 1903). Later (Barber, 1903) concluded that the disease was due to death of root ends of the Sandal tree. It was believed that the disease caused by virus which in some cases may be spread by insects (Coleman, 1917). (Hole, 1917) stated that the disease was provoked by an unbalanced circulation of sap, which might be caused by different factors such as fire, death or damage Sandal host plants, partial suppression of Sandal by lantana or other plants or exposure of Sandal trees growing under shade. However he does not believed that spike disease was really infectious. (Hodgoon, 1980) believed that spike disease was seed borne. (Lushington, 1970) strongly opposite the theory of "unbalanced circulation of sap". (Fischar, 1980) stated that the disease was caused by the bacterial agency known as "ultra-microscopic organisms". (Coleman, 1917) confirmed that the disease was transmissible by grafting. Until 1968, the disease was believed to be caused by virus, but recent studies by different workers revealed that the disease was caused by mycoplasma like organisms (Dijkstra and Lee, 1969; Hull *et al.,* 1969; Anupam Varma *et al.,* 1969).

2.1 DISRTIBUTION

Muthanna (1995) stated that in Karnataka the disease was limited to only 80 square miles in 1899; it had spread to 2137, 5715 and 7100 square miles in 1926, 1934 and 1950 respectively. The per cent incidence of the disease ranged from 1 to 55 in Karnataka (Muniyappa *et al.,* 1980). This disease was not observed in northern districts of Karnataka.

Incidence of the disease in trees of various girth class groups of Sandal was critically studied by Mitchell and Rangaswamy (1933). They observed maximum incidence gradually reduced in successive higher girth classes. Iyenger (1961) recorded the maximum disease incidence on Sandal trees of 15-30cm girth class.

2.2 SYMPTOMATOLOGY

2.2.1 External symptoms

The characteristic symptoms of the disease as described by Butler (1903), Coleman (1917) and Hearsey (1917) were formation of long thin leaves, shortening of internodes, and yellowing of leaves. Spiked leaves may be normal or copper coloured. Ultimately there was defoliation and death of the tree in advanced stage. Phylloid or virescent flowers were occurred after infection. Rama Rao (1917) differentiated three types of symptoms i. Nanky form, ii. Spike with normal or copper coloured leaves and iii. Weeping type.

The disease was characterised by witche's-broom symptoms, consisting of small, narrow leaves which turn pale-green or yellow on branches. Affected leaves become over crowded due to internodes shortening and standout stiffly from branches, acquiring a spike-like appearance leaves and branches dry out and affected plants eventually die-off within a couple of years from the first appearance of symptoms. (Khan *et al.,* 2004)

Venkat Rao and Gopal Iyengar (1934) described two types of symptoms: i. Penduous type ii. Rosette type. In pendulous type, the shoots become nalky showing continuous apical growth and attained length of 30-90 cm, the leaves being confined to one-third of the length from the growing tip. The shoots grew disappropriately along the length.

The spiked shoots were less than one-third the length of pendulous type and grew stiff and erect bearing clusters of shoots.

Plants infected by phytoplasmas exhibit an array of symptoms that suggest profound disturbances in the normal balance of plant hormones or growth regulators. Symptoms include virescence (the development of green flowers and the loss of normal flower pigments), phyllody (the development of floral parts into leafy structures), sterility of flowers, proliferation of auxiliary or auxillary shoots resulting in a witches'-broom appearance, abnormal elongations of internodes resulting in slender shoots, generalized stunting (small flowers and leaves and shortened internodes), discolorations of leaves or shoots, leaf curling or cupping, bunchy appearance of growth at the ends of the stems, and generalized decline (stunting, die back of twigs, and unseasonal yellowing or reddening of the leaves) (Lee *et al.*, 2000).

2.2.2 Internal symptoms

Butler (1903) and Narasimhan (1964) noticed cellular hypertrophy and accumulation of starch in parenchyma pith and medullary rays. Proliferating intercellular inclusion bodies were observed in leaf cells of diseased plants. The inclusion bodies were mostly found in association with nuclei. Coccoid to spherical inclusion bodies were also observed in phloem and xylem cells of infected tissues (Ananthapadmanabha *et al.,* 1973).

The phloem in the diseased plants especially old ones proliferated due to increased production of secondary phloem and development of necrotic areas mainly consisting of collapsed sieve tubes (Dijkstra and Vanderwant, 1970).

2.3 CAUSAL ORGANISM

Barber (1906) considered it to be a root disease. Hole (1917) attributed it to be physiological disorder resulting from prolonged unbalanced circulation of sap caused by adverse factors such as forest fires.

Dijkstra (1968) reported that, Round or slightly oval inclusion bodies were found in low numbers in epidermal cells of spiked (expressing spike disease symptoms) leaves. He could not be demonstrated in epidermal tissue of apparently healthy leaves. The intracellular inclusions stained with phloxine and rose bengal.

Hiruki and Dijkstra (1973) reported that, detection of phloem cells containing mycoplasma-like bodies associated with the Sandal spike disease was made easier by observing sections from stems of infected *Vinca rosea* plants by means of fluorescence microscopy. The aniline blue-stained cells that contained mycoplasma-like bodies fluorescenced yellow in ultraviolet light (366 nm). The presence of mycoplasma-like bodies was confirmed by examination of ultrathin sections obtained from the same stems in the electron microscope. Based on the infectious nature of the disease, graft transmissibility, accumulation of starch in diseased tissues and absence of any bacterium, fungus, Butler (1903) considered this disease to be of viral nature. However, investigations carried out during late sixties revealed the association of mycoplasma-like organisms (MLO’s) with the disease.

Sunil Thomas and Balasundaran (1998) detected yellow-green fluorescence in phloem of diseased Sandal infected with spike disease using DAPI stain.

Anupama Varma *et al.* (1969) Observed mycoplasma-like bodies in the tissues infected with Sandal spike and little leaf of brinjal, spherical to ovoid structures were found in the phloem cells in both the cases ranging from 40-460mn in case of Sandal spike and 40-400nm in case of little leaf of Brinjal.

Hull *et al.* (1969) for the first time observed numerous pleomorphic bodies measuring 60-750nm in ultra thin sections of infected stem and leaves. Such bodies were not observed in sections of healthy plants. Spherical or ovoid structures 40-460nm in diameter was observed in the phloem cells of the Sandal spike infected tissues (Anupam Varma *et al.,* 1969).

Dijkstra and Lee (1969) observer irregular or round bodies measuring 300-600 nm with strands of presumed DNA in the less electron dense in sieve elements of infected tissue. In the centre and at the periphery, round bodies measuring 150-200 nm in diameter.

Electron microscopy of healthy looking leaves from tetracycline treated spiked treated spiked Sandal trees revealed no mycoplasma in their sieve elements (Hull *et al.,* 1970) indicating the mycoplasmal etiology of the disease. In sieve elements of ultra thin sections of periwinkle (*Carthamus roseus*) infected by Sandal spike agent, Hiruki and Dijkstra (1973) observed three types pleomorphic bodies *viz.,* spherical (160-280 nm), large bodies (400-800 nm) and small bodies (50-160 nm), unit membrane (8-10nm thick), ribosomes of bacteria type (12-13 nm diameter) and the nucleoid (DNA strands) devoid of membrane. Ghosh *et al.* (1985) also confirmed that association of mycoplasma-like bodies with the disease.

Ghosh *et al.* (1988) studied the differences in morphology of spike MLO in various tissues collected from the same spike infected plant. Highly pleomorphic (only a few spherical) bodies were observed in young bark. In the old bark, a large number of both spherical and pleomorphic

MLO's were seen. In sieve elements of petiole and main vein of the leaf, MLO's were found highly uniform and spherical in shape.

2.4 SURVEY

Subba Rao and Muniyappa (1980) carried out a systematic survey to know the disease incidence of Sandal spike in Karnataka. They found maximum disease incidence in Kodagu (32.32%) followed by Hassan (27.88%) and minimum was in Kolar (1.6%).

Rangaswamy (1995) carried out a systematic survey to know the prevalence and disease incidence of Sandal spike disease in different forest divisions of Karnataka and Tamilnadu.

In India, some of important phytoplasma diseases in horticultural crops reported are Sandal spike (Barber, 1903; Anupam Varma *et al.,* 1969), little leaf of brinjal (Thomas and Krishnaswami, 1939; Anupam Varma *et al.,* 1969), Zizyphus witches' broom (Hull *et al.,* 1970), Potato purple top (Giri and Nagaich,1971), coconut root wilt (Solomon *et al*., 1983), yellows and little leaf of *Hibiscus rosa-sinensis* (Chaturvedi *et al*., 2009) and *Zinnia elegans* (Agrahari *et al*., 2010).

Samuitiene *et al.* (2007) surveyed plant collections in botanical gardens and floriculture farms in Lithuania for phytoplasmal diseases. Thirty seven ornamental species belonging to eighteen plant families exhibiting disease symptoms including general yellowing and stunting, proliferation of shoots, phyllody, virescence and reduced size of flowers, and reddening of leaves were collected in commercial floriculture farms and botanical gardens.

Phytoplasma caused diseases have been identified in Israel in numerous species of various botanical families, primarily from crop plants. Northern Israel has the most diversity of phytoplasma groups followed by the northern Negev desert and Arava valley, with the centre of the country having the least. There were proportionally more infected flowers than orchards (including vineyards) or than vegetable crops. Of the 15 known 16S rDNA phytoplasma groups, seven are present in Israel (Weintraub *et al.,* 2006).

Sertkaya *et al.* (2007) carried out a survey during 2002-2004 in Turkey to screen for phytoplasmas in sesame and periwinkle plants with phyllody symptoms, in pepper with stolbur symptoms, in eggplant with little and yellow leaves and in tomato with big bud symptoms. *Empoasca* sp. and *Orosius orientalis* (Matsumura) [= *albicinctus* (Distant)] leafhoppers were collected from infected sesame fields in 2003. Phytoplasmas detected in sesame, periwinkle, pepper, eggplant and *O. orientalis* showed 16S rDNA RFLP patterns that were most closely related to strains belonging to clover proliferation group (16SrVI-A). Phytoplasma associated with tomato big bud in Turkey belongs to the stolbur phytoplasma group (16SrXII-A).

Gungoosingh-bunwaree *et al.* (2007) reported the incidence of phytoplasma in field grown tomatoes which rarely exceeded 10 per cent, but under hydroponics up to 100 per cent incidence has been recorded.

Ghosh *et al.* (1999) first observed witches' broom disease of lime (WBDL) in Nagpur district in eastern Maharashtra in 1995 .Subsequent surveys in 1995–1998 revealed the presence of this disease up to 5 per cent in Maharashtra State and in other major acid lime growing states of Andhra Pradesh, Tamil Nadu and Karnataka.

Orenstein *et al.* (2001) sampled four of the most prevalent grapevine cultivars in northern, central and southern regions of the Golan Heights, Israel. There were significant differences in the levels of phytoplasma infection between different sub regions of the Golan

Heights; highest levels were found in the south followed by the centre, and the lowest levels in the north. Stolbur was found to be the predominant phytoplasma (70 per cent), although aster yellow (11 per cent), western X (5 per cent) and mixtures of two phytoplasmas (13 per cent of all infections, 90 per cent of which involved Stolbur and aster yellow) were also found.

Since March 2005, 30 to 50 per cent of *Portulaca grandiflora* plants in the ornamental gardens as well as in pots at the Central Institute of Medicinal and Aromatic Plants, Lucknow, India have displayed symptoms resembling phytoplasma infection (Samad *et al.,* 2008). Disease symptoms start as a typical bud proliferation, downward curling, and diminishing size of leaves, followed by overall stunted growth and yellowing of the whole plant from April to June. Some plants also showed rosettes symptoms and proliferation of auxillary shoots resulting in a witches'-broom appearance.

Arocha *et al.* (2008) recorded the presence 16SrII phytoplasma isolate in lettuce,carrot, french bean, and *Amaranthus* sp. at GBPUA&T, Uttarakhand, Pantnagar, India based on nested PCR that target the phytoplasma 16S rRNA, RFLP pattern and sequencing results.

During field visits to Barapani, Meghalaya (Northeast India) in June-July, 2008, typical witches’ broom symptoms were observed in almost 46 per cent of *Crotalaria tetragona* plants (Baiswar *et al.,* 2009).

Phytoplasma diseases have been identified at Israel in numerous species of various botanical families, primarily from crop plants. Northern Israel has the most diversity of phytoplasma groups followed by the northern Negev desert and Arava valley, with the centre of the country having the least. There are proportionally more infected flowers than orchards (including vineyards) or than vegetable crops. Of the 15 known 16S rDNA phytoplasma groups, seven are present in Israel (Weintraub *et al.,* 2007).

2.5 Dodder transmission

Dijkstra and Lee (1972) reported transmission of Sandal spike to *Carthamus roseus* and back using *Cuscuta subinclusa.* Symptoms such as little leaf, phyllody and yellowing of leaves were observed 4-8 weeks after connecting healthy plants through strands of dodder established on the diseased twig.

Kaminska and Korbin (1999) reported that the periwinkle plants that were bridged by *Cuscuta odorata* from the diseased lilies and *Alstroemerias* grafting resulted in stunted growth, dull yellowing and malformation of the leaves in 4-6 weeks.

Marcone *et al.* (1999) reported dodder transmission of pear decline, European stone fruit yellows, rubus stunt, *Picris echioides* yellows and cotton phyllody phytoplasmas to periwinkle. They reported that transmission depended on both type of phytoplasma and *cuscuta* vector. The rubus stunt and cotton phyllody phytoplasmas were transmitted at high efficiency by *C. europia* and *C. campestris* respectively whereas the other phytoplasmas were transmitted only at a low rate.

Ghosh *et al*. (1999) used dodder (*Cuscuta reflexa* Roxb.) to transmit the causal agent of witches' broom disease (WBD) from citrus to periwinkle (*Catharanthus roseus* (L.) G. Don) plants and the disease was successfully transmitted from infected acid lime to periwinkle plants and vice-versa by dodder.

2.6 PCR detection of phytoplasma

PCR is the most employed molecular method to confirm/identify the phytoplasmal involvement with plant diseases. The universal primers designed based on the conserved sequences 16S rDNA have been used to obtain phytoplasmal specific products.

Disease affected tissues of plants were tested for the presence of pathogen by using polymerase chain reaction (PCR) technique (Sunil Thomas and Balasundaran, 1999). A set of Oligonucleotide universal primers (P1 and P7) specific to the conserved region of 16S-rDNA gene were used to amplify 558 bp sequence of the phytoplasma associated with Sandal spike phytoplasma and other phytoplasmas. DNA fragments were obtained from the PCR products after 20 cycles of amplification, and then subjected to restriction fragment length polymorphism analysis (RFLP) with *Alu*-I restriction endonuclease. The technique confirmed that Sandal spike phytoplasma belongs to group I of the eleven major phytoplasma groups. This technique has also been applied for identification of phytoplasmas infecting plant species.

Total DNA extracted separately from leaves of either healthy or diseased Sandal was used as template in a nested PCR assay employing phytoplasma 16S rDNA universal primer pairs P1/P7, followed by R16mF2/R16mR1, as previously described (Khan *et al.,* 2004). A nested PCR product of about 1.2 kb was amplified from a diseased plant but not from symptomless plants.

Khan *et al.* (2004) developed a non-specific PCR assay for the detection of phytoplasma in Sandal the associated phytoplasma remains unidentified.

PCR-based assays developed in the late 1980s and early 1990s further facilitated the detection and classification of phytoplasmas by providing a much more sensitive means for phytoplasma detection (Lee *et al.,* 2010).

The PCR products obtained from symptomatic leaves of five separate diseased plants were digested with restriction endonucleases *Alu*I, *Hinf*I and *RsaI* (Khan *et al*., 2006). The resulting Restriction Fragment Length Polymorphism (RFLP) profile for each enzyme was identical, indicating that all PCR positive plants contained a similar phytoplasma. The PCR product was purified, cloned and sequenced. Pair wise comparison of the16S rDNA Sequence by BLAST analysis revealed 99% similar to *'Candidatus* phytoplasma asteris'-related strains.

Based on sequence analysis of DNA products from polymerase chain reaction (PCR)-amplified 16S ribosomal DNA and 16S–23S spacer region using universal phytoplasma primers were studied (Hee-Young *et al*., 2003). The phytoplasmas associated with plant diseases were grouped in to several groups (I-XI). Potato witches' broom disease (PWB) was identified as a member of 16S-group VIII. It was more closely related to elm AH phytoplasma (99.7% similarity), under clover proliferation (CP) subgroup.

Rao *et al.* (2003) used nested polymerase chain reaction was conducted to identify the phytoplasma associated with grassy shoot disease (GSD) of sugarcane in the Gorakhpur district of India. A 1.25 kbp DNA fragment encoding the phytoplasma 16S rDNA gene was consistently amplified by nested PCR. The results showed that samples of diseased plants from two sugarcane varieties gave the same restriction fragment length polymorphism (RFLP) patterns with two restriction enzymes (*HaeIII* and *RsaI*) similar to that of other known sugarcane phytoplasmas. This is the first report on molecular characterization of phytoplasma associated with GSD of sugarcane in India.

Khan *et al.* (2004) developed sensitive nested-PCR (polymerase chain reaction) for the detection of phytoplasma in diseased Sandal trees.

Two sets of universal primer pairs based on 16S rDNA sequences were employed in direct/nested- PCR assays. In direct-PCR, templates consisting of *Santalum album* spike (SAS) phytoplasma total crude DNA extracts in dilutions up to 1: 90,000 were primed. The direct-PCR products thus obtained were subsequently used in nested-PCR. The nested-PCR was sensitive enough to re-amplify the direct-PCR product (obtained from 90,000 times diluted crude DNA extract), resulting in a DNA fragment of 1.2 kb. The efficacy of nested-PCR showed that it could re-amplify the direct-PCR product in dilutions of 1: 60,000. The specificity of nested-PCR fragments was confirmed by Southern hybridization. The availability of a sensitive nested-PCR-based system should facilitate detection of the phytoplasma in potential vector insects and identification of other host plants of *Santalum album* spike (SAS).

Phytoplasmal DNA was amplified by the PCR using the universal primer pairs P1 and P7 (Deng & Hiruki, 1991; Smart *et al.,* 1996) derived from highly conserved ribosomal sequence primers within the 16S and 23S RNA gene sequences and intragenic spacer regions, were used in direct PCR. The P4 and P7 primers (Smart *et al.,* 1996) were used for nested PCR assays that successfully detected the SCGS phytoplasma and obtained an amplified product of 1600 bp and 500bp respectively (Srivastava *et al.,* 2006).

Rao *et al.* (2008) used phytoplasma specific primers P1/P7 for detection of SCGS phytoplasma by PCR and another universal primers P4/P7, amplified a fragment of about 1800 bp and 530 bp.

Rojas-Martinez *et al*. (2003) examined for the presence of phytoplasma in marigold phyllody by nested PCR using the universal primer pair R16mF2/R16mR1 followed by the primer pair R16F2n/ R16R2 and obtained a product of 1.2 kb.

PCR technique with phytoplasmal universal primers P1/P7 and R16F2n/R16R2 were used to detect the chickpea phyllody phytoplasma. Amplified product of approximately 1250 bp was obtained (Akthar *et al*., 2009; Al Saady *et al*., 2006).

The causal agent of phytoplasmal disease in ornamental plant, sneezeweed was detected by using polymerase chain reactions (PCR) using phytoplasma universal primer pairs P1/P7 (1.8 kb) and R16F2n/R16R2 (1.2 kb) and yielded a product of 1.2 kbp (Navalinskiene *et al*., 2006).

Khan *et al*. (2007) tested phytoplasma associated with witches' broom and virescence diseases of sesame by nested PCR using primer pairs R16F2n/R16R2n and obtained a products of 1.2kb fragment from phytoplasma 16S rDNA gene.

Schneider *et al.* (1995) determined a phytoplasma associated with Toria phyllody by direct and nested PCR using universal phytoplasma specific P1/P7 primer and R16F2n/R2 primer pairs yields the (Gundersen & Lee, 1996) amplified product of approximately 1.8 kb and 1.25 kb respectively.

Khalid Akhtar *et al*. (2008) determined the 16Sr group of the phytoplasma associated with Mung bean phyllody by nested PCR using 16S rDNA phytoplasma primers R16F2n/R16R2 and obtained a product of 1250bp.

Amplification of phytoplasmal 16S rDNA of *Catharanthus roseus* (L.) was done with the universal phytoplasma primer pairs P1/P6 and R16F2n/R16R2. Amplified product of approximately 1.5kb and 1.2kb were obtained (Chaturvedi *et al.,* 2009).

Phyllody diseased plants were examined for phytoplasma infection by means of PCR using universal primers P1 and P7. PCR products of about 1800bp were obtained after amplification of DNA isolated from fresh and dried *Parthenium* specimens showing phyllody symptoms (Taye Tassema *et al*., 2003).

PCR assay was employed by using P1/P7 universal phytoplasma primers and secA primers designed for identification and classification of periwinkle proliferation phytoplasma, amplified 1.8kb fragment that encompasses the entire 16S rRNA gene, the 16S - 23S intergenic spacer region and the beginning of the 23S rRNA gene as well as the 840bp from part of *secA* gene, respectively (Nejat *et al*., 2009).

Phytoplasma disease in strawberry was assessed for phytoplasma infection by nested PCR primed by phytoplasma universal primer pairs R16mF2/R1 and F2n/R2 (2) or P1/P7 (3) and F2n/R2. Phytoplasma-characteristic of 1.2kbp DNA sequences were amplified from all diseased plants (Jomantiene *et al*., 1999).

Sreenivasan (2001) purified the Sandal spike phytoplasma for the production of polyclonal antibody. Phytoplasma from diseased plants was purified using the techniques – differential centrifugation method, celite pad filtration technique and percoll density gradient centrifugation was used to raise polyclonal antibodies for the subsequent detection of the pathogen by immunological techniques.

The Polymerase Chain Reaction (PCR) incorporating phytoplasma-specific ribosomal RNA primer pair P1 and P7 consistently amplified a product of expected size (1·8 kb) when template DNA for PCR was extracted from leaf and shoot samples of *Gliricidia sepium* exhibiting symptoms of little-leaf disease (LLD). Phytoplasma detection in trees was enhanced by re- amplification of the P1/P7-primed PCR products with nested primers PPf1 and Tint. Nested reactions enabled additional positive detections in foliage of several *G. sepium* trees showing only mild or no apparent LLD symptoms (Kenyon *et al*., 1998).

Raj *et al*. (2006) determined phytoplasma associated with pigeonpea phyllody by direct PCR using universal phytoplasma specific P1/P6 primer (Deng and Hiruki, 1991) resulted in the production 1·5 kb and the PCR products were used as templates in a nested PCR using primers R16F2n/R16R2 (Gundersen and Lee, 1996) giving a product of around 1·2 kb.

Azadvar *et al*. (2009) determined phytoplasma associated with Toria phyllody by direct and nested PCR using universal phytoplasma specific P1/P7 primer (Schneider *et al.*, 1995) and R16F2n/R2 primer pairs (Gundersen and Lee, 1996). Amplified products of approximately 1.8 and 1.25 kb were obtained.

Phytoplasmal DNA was amplified by the PCR using the universal primer pairs derived from highly conserved ribosomal sequences. Sesame infected with witches'-broom disease was assayed by PCR. The results showed the presence of phytoplasma specific to 1.8 kb product with direct PCR and a product of 1.2 kb with nested PCR from infected sesame sample. This was thought to be the first report of a phytoplasma of the 16Sr II group causing witches'-broom disease on sesame in the Sultanate of Oman (Al-Sakeiti *et al*., 2005).

Amplification of phytoplasmal rDNA of *Catharanthus roseus* (L.) was done with the universal phytoplasma primer pairs P1/P6 and R16F2n/R16R2. Amplified products of approximately 1.5 kb and 1.2 kb were obtained (Yamini Chaturvedi *et al.,* 2009).

Akhtar *et al*. (2009) examined for the presence of phytoplasma in sesame phyllody by nested PCR assay using primer pair P1/16S-Sr followed by R16F2n/R16R2n was used to amplify a 1.53 kb and 1.2 kb fragment of the phytoplasma 16S rRNA gene, respectively.

Saquib *et al*. (2006) employed PCR technique using phytoplasma universal primers P1/P7 and R16F2n/R16R2 to confirm the association of phytoplasma with *Vigna unguiculata* (L.) and *Lycopersicon esculentum* L.

2.7 PCR RFLP Analysis of phytoplasma

The partial 16S rDNA sequences amplified from various MLOs were compared by restriction fragment length polymorphism (RFLP) analyses. Based on similarity coefficients derived from RFLP analyses, 40 MLOs were classified into 9 distinct 16S ribosomal RNA (16Sr) groups and 14 subgroups. Type MLO strains designated for each group and subgroup were as follows: 16SrI-A, tomato big bud; 16SrI-B, Maryland aster yellows; 16SrI-C, clover phyllody; 16SrI-D, Paulownia witches' broom; 16SrI-E, blueberry stunt; 16SrII, peanut witches' broom; 16SrIII-A, Canada peach X; 16SrIII-B, clover yellow edge; 16SrIV, palm lethal yellowing; 16SrV, elm yellows; 16SrVI, clover proliferation; 16SrVII, ash yellows; 16SrVIII, Loofah witches's broom; and 16SrIX, pigeon pea witches' broom (Lee *et al*.,1993).

Sunil Thomas and Balasundaran (1999) DNA fragments were obtained from the PCR products after 20 cycles of amplification, and then subjected to restriction fragment length polymorphism analysis (RFLP) with *Alu*I restriction endonuclease. The technique confirmed that Sandal spike phytoplasma belongs to group I of the eleven major phytoplasma groups. This technique has also been applied for identification of phytoplasmas infecting plant species.

Schneider *et al*. (1999) reported that phytoplasmas were found in 33 plant species. RFLP analysis revealed 13 different restriction patterns. The majority of the phytoplasmas showed a restriction pattern identical to that of either of the tomato big bud (TBB) or sweet potato little leaf V4 (SPLL-V4) phytoplasma. Phytoplasmas from 6 plant species showed a restriction pattern similar to that of the pigeonpea little leaf (PLL) phytoplasmas. Seven new restriction fragment patterns have been detected and sequence analysis of 16S/23S spacer region revealed that three of these phytoplasmas are related to the Faba bean phyllody (FBP) group. The spacer region of the graminaceous phytoplasma was most similar to phytoplasmas from the sugarcane white leaf group.

Based on RFLP analyses with 17 restriction enzymes, Lee *et al.* (1993, 1998 and 2000) constructed a comprehensive classification scheme for phytoplasmas. Separation of major groups was based on similarity coefficients of collective RFLP patterns of a 1.2kb PCR amplicon of near full length 16S rDNA gene (Lee *et al.*, 1998). The similarity coefficients of RFLP patterns between two distinct groups were 90 per cent or below.

The highly conserved 16S rDNA gene sequence has been used as the primary molecular tool for classification of phytoplasmas. A total of 19 distinct groups, termed 16S rDNA groups (16Sr groups), based on actual RFLP analysis of PCR-amplified 16S rDNA sequences or 29 groups based on *in silico* RFLP analysis have been identified (Lee *et al.*, 1998, 2000; Wei *et al.*, 2007).

The RFLP profiles of the PCR fragments produced using *Rsa*I, *Sau3A*I, *Msp*I, *Hpa*II or *Kpn*I were compared with those predicted for sugarcane grassy shoot (SCGS) and sugarcane white leaf (SCWL) sequences (GenBank accession numbers DQ459439, AB052874and X76432). The actual and virtual RFLPs were identical. BLAST results of

452bp of the PCR product showed 98% sequence homology to three phytoplasma strains infecting sugarcane (Ariyarathna *et al.,* 2007).

DNA fragments amplified with primer pairs R16F2/R16R2 and P1/Tint from coconut palms (*Cocos nucifera*), marigold (*Tagetes erecta*), potato (*Solanum tuberosum*), periwinkle (*Catharanthus roseus*), jipi palm (*Carludovica palmata*) and cassava (*Manihot esculenta*) were digested with restriction endonucleases *EcoRI* or *AluI*. Marigold and periwinkle showed an identical pattern and was similar to some extent to that of coconut palm (Almeyda-Leon *et al*., 2001).

Onion phyllody phytoplasma was classified in group 16SrI (aster yellows group), subgroup I-M. Previously, subgroups 16SrI-A, 16SrI-B and 16SrXII-A phytoplasmas were identified in *Allium* spp based on RFLP analysis (Valiunas *et al*., 2007).

The 1.2kbp 16S rDNA product of nested PCR, primed by primer pair R16F2n/R16R2 was subjected to single enzyme digestions with 8 restriction endonucleases. RFLP analysis revealed that the *Celosia argentea* plants were infected by a phytoplasma belonging to group 16SrI (aster yellows phytoplasma group), subgroup I-M (Samuitiene *et al.,* 2007).

Restriction fragment length polymorphism (RFLP) analysis of amplified 16S rDNA indicated that diseased oats were infected by phytoplasma belonging to the group 16SrI (aster yellows, AY, group) and subgroups 16SrI-B (Urbanaviciene *et al*., 2006).

Al-Saady *et al*. (2006) reported that, restriction profiles of chickpea were identical with those of Lucerne witches' broom phytoplasma, a known subgroup 16SrII-B strain

The 1.2kbp 16S rDNA product of ornamental plant sneezeweed was subjected to single enzyme digestions with 10 different restriction endonucleases. Restriction fragment length polymorphism (RFLP) analysis revealed that sneezeweed plants were infected by phytoplasma belonging to group 16SrI (Aster yellows phytoplasma group). (Navalinskiene *et al*., 2006).

Firarao *et al*. (2005) identified the phytoplasmal infection in forage fruit, vegetable, oil, medicinal and ornamental plants and classified them as members of 16SrI-B, 16SrI-C, 16SrIII-B, 16SrX-A, 16SrX-B, 16SrX-C and 16SrXII-A ribosomal subgroups in single or mixed infections.

The analysis of putative restriction sites in 16S rRNA gene sequences of safflower phyllody (SP) revealed that it had identical restriction profiles as Brinjal little leaf phytoplasma and that both differed from the '*Candidatus Phytoplasma trifolii*' reference strain (Salehi *et al*., 2009).

RFLP analysis of P1/P7 PCR products digested with *AluI, haeIII, HhaI*, *HinfI, HpaII, MseI, TaqI* and *TasI* identified two BGWL isolates FBGWL and JBGWL as members of the Bermuda grass white leaf group, 16SrX-IV (Salehi *et al.,* 2009).

Hee-Young *et al.* (2003) Symptoms of witches' broom disease caused by phytoplasma, including general stunting and yellowing, were observed in potatoes (*Solanum tuberosum* L.) in a storehouse on Jeju Island, Korea in 1998. Based on sequence analysis of DNA products from polymerase chain reaction (PCR)-amplified 16S ribosomal DNA and 16S–23S spacer region using universal phytoplasma primers, the phytoplasma associated with potato witches' broom disease (PWB) was identified as a member of 16S-group VIII. It was most closely related to elm AH phytoplasma (99.7%) similarity (Accession no. AF268895), which is in the clover proliferation (CP) subgroup. This report is the first from the East Asian continent of a plant pathogenic phytoplasma belonging to the CP subgroup and includes the nucleotide sequence of most of the potato phytoplasma 16S rDNA.

Restriction Fragment Length Polymorphism analysis (RFLP) of amplified rDNA products indicated that phytoplasmas detected in LLD-affected *G. sepium* were very similar although not identical to phytoplasmas associated with pigeon pea witches-broom (PPWB) disease (Kenyon *et al*., 1998).

Digestion of amplified DNA of 1800bp by using universal phytoplasma primers P1 and P7 from *Parthenium* specimens showing phyllody symptoms with *AluI* restriction enzyme revealed restriction patterns for all samples corresponding to patterns typically observed for the Faba bean Phyllody Phytoplasma (FBP) group (Taye Tassema *et al*., 2003).

RFLP profiles obtained with restriction enzyme *Taq*I from ribosomal DNA amplified with primer pair P1/P7 differentiated elm-associated phytoplasmas from strains originally detected in *Apocynum cannabinum, Prunus* spp., *Rubus fruticosus, Vitis vinifera,* and *Ziziphus jujuba.* RFLP profiles obtained similarly with *Bfa*I differentiated strains from *A. cannabinum* and *V. vinifera* from other phytoplasmas of group 16SrV (Griffiths *et al*., 1999).

Based on restriction fragment length analysis of the amplified 1.1 kbp product of *Lilium martagon* phytoplasma was clssified into the aster yellows cluster (16SrI), subgroup I-C, with clover phyllody phytoplasma as a type strain (Poncarova-Vorackova *et al*., 1998).

Material and Methods

III. MATERIAL AND METHODS

The present investigations on Sandal spike disease and other related hosts of phytoplasma were carried out during 2011-12 at the Plant Virology Laboratory, Department of Plant Pathology and in collaboration with Indian Institute of Wood Science and Technology Bangalore, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bangalore. The details regarding the materials used and methodology followed for these studies are presented in this chapter.

3.1 Molecular detection of the phytoplasma associated with Sandal spike disease and Stachyterpeta phyllody

3.2.1 Standardization of PCR techniques for detection of phytoplasmas associated with Sandal spike and *Stachytarpheta* phyllody.

Characterization of phytoplasmas was described on nucleotide sequence and RFLP (Restriction Fragment Length Polymorphism) of the 16S rRNA gene. The relative locations of the primers in the spacer regions of 16-23S rRNA genes are as depicted in Fig. 1 (Khan *et al.,* 2004). This gene is present in all the prokaryotes and its conserved and variable regions make it ideal for phylogenetic classification (Sunil Thomas and Balasundaran., 1999).

DNA extraction from host plant

I. Protocol:

Initially, total genomic DNA from healthy and Sandal spike affected plants were extracted by following CTAB (Cetyl Trimethyl Ammonium Bromide) method as described Sunard *et al*. (1991), which is as detailed below:

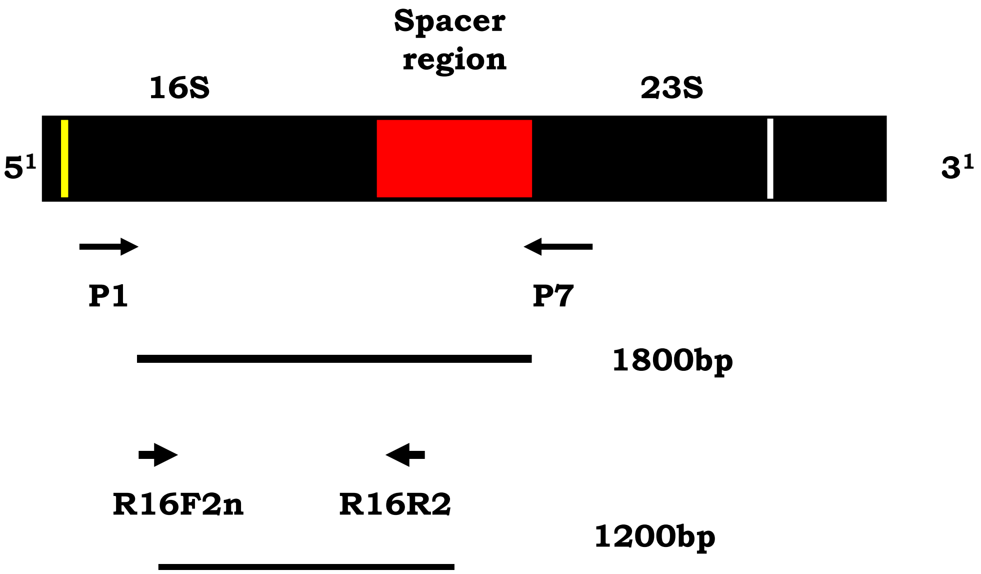

Fig. 1 : Schematic diagram of a phytoplasma rRNA operon showing the 16S and 23S genes and intergenic spacer region. Positions of oligonucleotide primers are shown by arrows.

DNA extraction buffer:

2 per cent (w/v) CTAB

1.4 M NaCl

20 mM EDTA

100 mM Tris- HCl (pH 8.0)

0.2 per cent (v/v) ß -Mercaptaethanol (add after autoclaving)

Procedure:

1. 150-200 mg of spike infected sample, *Stachytarpheta* phyllody and were ground using separate pestle and mortar for each sample.
2. 1.5-2 ml CTAB extraction buffer was added and thoroughly ground.
3. 0.75 ml of ground sap was transferred into 1.5 ml micro centrifuge tube and incubated at 65^{o}C for 30 min.
4. After incubation period, 0.75 ml of Chloroform: Isoamyl alcohol (24:1) mixture was added and mixed to form an emulsion by inverting the tube.
5. The mixture was centrifuged at 13,000 rpm for 10 min.
6. The supernatant was collected and mixed thoroughly with 300 µl of Isopropanol and incubated at -20^{o}C for atleast one hour or overnight.
7. The contents were centrifuged at 13000 rpm for 10 min and supernatant was discarded without losing pellet.
8. The pellet was washed with 500 µl of 70 per cent ethanol and centrifuged at 14000 rpm for 5 min.
9. The ethanol was removed and pellet was dried in a vacuum drier (make) for five min.
10. Finally the dried pellet was resuspended in 1X TE buffer based on the quantity of DNA obtained and stored at -20^{o}C.

PCR Reagents

a. PCR buffer:

200 mM Tris (pH 8.3)	1.0 ml
500 mM KCl	2.5 ml
0.01per cent Gelatin	0.5 mg
H_20 (SDW)	1.0 ml

b. dNTP mixture:

Each 25 l of dATP, dCTP, dGTP and dTTP from a 100 mM stock was mixed. The concentration of each dNTP in this mixture was 25 mM. Further the final concentration of each dNTP was made upto 2.5 mM by diluting it by ten times.

c. Primers:

The primer pair P1 (Deng and Hiruki, 1991) and P7 (Kirkpatrick *et al*., 1992) were used to amplify 1800 base pair (bp) DNA fragment of the phytoplamsa ribosomal DNA.

Primer P1 (Forward): 5’ AAGAGTTTGATCCTGGCTCAGGATT-3’ (25 nt)
Primer P7 (Reverse): 5’ CGTCCTTCATCGGCTCTT-3’ (18 nt)

Primers used for PCR amplification of the 16S rRNA genes of phytoplasmas

	Primer name	**Sequence (5’_-3’_)**	**Reference**
First round PCR primer pair	P1-Forward primer	AAGAGTTTGATCCTGGCTCAGGATT	Deng and Hiruki (1991)
	P7-Reverse primer	CGTCCTTCATCGGCTCTT	Smart *et al*. (1996)
nested primer pair	R16F2-Forward primer	ACGACTGCTGCTAAGACTGG	Lee *et al*. (1993)
	R16R2-Reverse primer	TGACGGGCGGTGTGTACAAACCCCG	

d. Protocol:

1. 0.5 ml Eppendorf tubes were selected, labelled and kept on ice crystals.
2. Samples were taken for PCR along with positive control (sunhemp) and negative control (distilled water).
3. 25 l PCR mixture was prepared by adding the following ingredients into the Eppendorf tube.

Sterile distilled water	13.3 l
10X PCR buffer (supplied with the enzyme)	2.5 l
25 mM $MgCl_2$	1.0 l
2.5mM dNTP mixture	2.0 l
Primer P1 (20 mM)	2.0 l
Primer P7 (20 mM)	2.0 l
Taq Polymerase	0.2 l
DNA	2.0 l
Total	25.0 l

In order to standardize the PCR for the detection of Sandal spike and *Stachytarpheta* isolates, the PCR primers available in the literature were got synthesized and tested for suitability to use them for phytoplasma detection.

The PCR conditions were standardized by using gradient function of Eppendorf gradient thermocycler. Different annealing temperature 53 ± 5 were set and PCR was carried out. The PCR programme were standardized with different annealing time, elongation time and the number cycles by keeping multiple gradient PCR simultaneously.

After preparing PCR mixture, the DNA template was added and tubes were spun briefly and inserted into the wells of a thermal cycler (Eppendorf thermocycler). The PCR was performed in a thermal cycler (Eppendorf) using the following parameters: 95^{o}C for 2 min. initial denaturation followed by 35 cycles of 94^{o}C for 30 sec. denaturation, 55^{o}C for 1 min. primer annealing, 72^{o}C for 2 min. primer extension and finally at 72^{o}C for 10 min final extension. After completion of the reaction, the products were kept at 4^{o}C, prior to gel analysis.

3.2.2 Purification of amplified PCR products using enzene^{R} PCR cleanup kit

PCR amplified products were purified using enzene^{R} PCR cleanup kit (Enzene Biosciences, Bangalore, India) based on the manufacturer's instructions. 50 l of amplified PCR product was mixed with 150 l of binding buffer which facilitates the binding of DNA to the silica membrane of the binding column in the presence of chaotropic salts from the binding buffer. Reaction mixture was loaded into a spin column placed in 2-ml collection tube and centrifuged for 1 min at 10,000 rpm. The flow-through was discarded. The column was washed by adding 750 l of washing buffer into spin column and subsequent centrifugation for 1 min at 10,000 rpm. The flow-through was discarded. The traces of washing buffer retained in the column were removed by centrifugation for an additional 1 min at 10000 rpm. The column was dried for 5-10 min under a fan at room temperature. The column was placed in a fresh 1.5-ml tube. DNA was eluted by adding 30 l of elution buffer on to the center of silica membrane in column, keeping for 5 min and centrifuging for 1 min at 10,000 rpm. To increase the concentration of DNA, eluted DNA was again added to the same column, incubated for 3-5 min and eluted by centrifugation for 2 min at 10,000 rpm. The eluted DNA was stored at -20^{0}C and used for studies.

3.2.3 Elution of DNA from agarose gel using QIAquick gel extraction kit

DNA from agarose gel in TBE buffer was extracted and purified using QIAquick gel extraction kit (Cat. No. 28704; Qiagen, Germany) according to the instructions given by the manufacturer. The brief description of the protocol given as below:

PCR amplified product (about 50-100 l) containing desired fragment or restriction digested PCR product was loaded into 0.8 per cent low melting agarose fresh gel in fresh buffer. The desired DNA fragment was excised from the agarose gel with a clean sharp scalpel while observing through UV illuminator at 260 nm. The gel slice was weighed in a 1.5-ml eppendrof tube. Three volumes of Buffer QG (300 l buffer for each 100 mg gel weight) were added. The sample was then incubated at 50^0C in a water bath for 10 min until the gel slice completely dissolved with occasional mixing the tube 2-3 times during the incubation. One volume of isopropanol was added into the yellow colored mixture which was subsequently mixed properly by inverting the tube several times. The sample was loaded into a QIAquick spin column placed in a 2-ml collection tube and kept for 3-5 min. The spin column with mixture was centrifuged for 1 min at 13,000 rpm. The flow-through was discarded. The spin column was placed back in the same collection tube and centrifuged at 13,000 rpm for 1 min after adding 500 l of Buffer QG into the column. 750 l of Buffer PE (or 70 per cent ethanol) was added into the column and kept for 5 min. The column was centrifuged for 1 min at 13,000 rpm. After discarding flow-through, column was centrifuged for an additional 1 min at 13000 rpm to remove traces of Buffer PE. The column was placed in a fresh 1.5-ml effendrof tube and dried for 5 min to remove any ethanol smell. 30 l of elution buffer (Buffer EB) was added carefully onto the center of the QIAquick membrane and incubated for 5 min at room temperature. The DNA was eluted by centrifuging the column at 13,000 rpm for 1 min. To increase the concentration of DNA, eluted DNA was again added onto the center of the QIAquick membrane of the same column and allowed to stand for another 3-5 min. The column was then centrifuged for 1 min at 13,000 rpm. The eluted DNA was stored at -20^{0}C for further studies.

3.2.4 Analysis of PCR products by agarose gel electrophoresis

Reagents

10X TBE (Tris borate EDTA buffer, 1L): 108 g Tris base, 55 g Boric acid, 0.5 M EDTA (pH 8.0); volume was made up to 1 litre with distilled water and stored at RT.

6X Loading dye: 10 mM Tris HCl (pH 7.6), 0.03 per cent bromophenol blue, 0.03 per cent xylene cyanol, 60 per cent glycerol, 60 mM EDTA

0.1 per cent ethidium bromide stock solution (10 ml): 100 mg ethidium bromide was dissolved in 10 ml distilled water; Stored in dark bottle at 4^{0}C

1kb DNA ladder (Cat. No. SM0311, Fermentas Life Sciences, Germany, 60 µl): 10µl 1 kb ladder (0.5 µg/µl), 10µl 6X DNA loading dye, 40 µl deionized water. Six microlitres of loading mixture is required for an agarose gel lane.

Procedure

The gel casting tray was cleaned by washing and subsequent wiping with 70 per cent ethanol. Required volume of 1 per cent agarose was prepared by melting agarose in 1X TBE buffer (300 mg in 30 ml 1X TBE buffer) in a microwave oven. Once agarose solution was cooled up to 45-50^{0}C, ethidium bromide was added at the rate of 0.5 µg/ml of agarose. Gel casting tray was prepared with the desired number of wells and taping the ends. After comb was placed in the boat, molten agarose was poured in to the boat without forming air bubbles and allowed 30 min for proper solidification (polymerization). Five microlitres of each PCR product was separately mixed with 2 µl of loading dye on a clean polythene strip. After removing the tapes from the ends of the casting tray, tray was placed in the electrophoresis tank filled with 1X TBE buffer maintaining the buffer level 2 mm above the gel slab. The comb was removed carefully. Samples of PCR amplified products were loaded into wells of the gel along with 1 kb DNA ladder (Fermentas Life Sciences, Germany) and run at 60 V for 1-2 hr until the dye front reached the other end of the gel. Gel was removed from the casting tray and visualized under UV and photographed in a gel documentation unit.

3.2.5 Nested PCR

a. Primers: Universal Phytoplasma specific primers designed by Gundersen and Lee, 1996 based on the nucleotide sequences of conserved region of 16-23S rRNA gene are as given below.

Primer R16F2n : 5’-GAAACGACTGCTAAGACTGG-3′ (20nt)
Primer R16R2 : 5′-TGACGGGCGGTGTGTACAAACCCC -3′ (24nt)

b.Procedure:

1. 1µl of direct PCR product resulting from total DNA was diluted with sterile deionised water in various dilutions, i.e. 1:25 and 1:50.

2. 25 l PCR mixture was prepared by adding the following ingredients into the eppendorf tube.

Sterile distilled water	13.3 l
10X PCR buffer (supplied with the enzyme)	2.5 l
25 mM MgCl$_2$	1.0 l
2.5mM dNTP mixture	2.0 l
Primer R16F2n (20 mM)	2.0 l
Primer R16R2 (20 mM)	2.0 l
Taq Polymerase	0.2 l
DNA from diluted PCR product	2.0 l
Total	25.0 l

After preparing PCR mixture, the DNA template was added and tubes were spun briefly and inserted into the wells of a thermal cycler (eppendorf thermocycler). The PCR was performed in a thermal cycler (Eppendorf) using the following parameters: 94^{o}C for 4 min. initial denaturation followed by 30 cycles of 94 ^{o}C for 1 min. denaturation, 56^{o}C for 1 min. primer annealing, 72^{o}C for 2 min. primer extension and finally at 72^{o}C for 10 min primer extension. After completion of the reaction, the products were kept at 4^{o}C, prior to gel analysis. Nested PCR product were subjected agarose electrophoresis analysis.

3.2 PCR based Survey of the Sandal plantations to assess the incidence of Sandal spike disease in southern Karnataka.

A Systematic roving survey was conducted from Sept 2011- June 2012 in different districts of Karnataka state like Bangalore rural, Shimoga, Chitradurga, Chickmangalore. For this purpose 23 Sandal plantations were examined by employing the complete enumeration method to assess the status of Sandal spike disease (Muniyappa, *et al.,* 1980). All the trees/plants were carefully observed for typical symptoms of Sandal spike disease for assessing the disease status. The per cent disease incidence was calculated using the following formula

$$\text{Per cent disease incidence} = \frac{\text{Total number of infected plants}}{\text{Total number of plants observed}} \times 100$$

The suspected plant samples were further tested by PCR for the confirmation of Sandal spike phytoplasma using specific primers.

3.2.1 Complete enumeration (Muniyappa *et al.,* 1980)

This method was employed in the farmer's Sandal plantations where the trees were distributed in the limited area. The total numbers of healthy and diseased Sandal trees were counted in the whole plantation and per cent infected trees were calculated.

Different weed species were also observed during the survey at different locations and data was recorded. The diseased samples were also collected during survey for the characteristic symptoms of phytoplasmas and the incidence was recorded and tested for asssocistion of phytoplasma by PCR protocol.

3.2.2 Occurrence of other phytoplasmal diseases in farmer's Sandal plantations

The incidence of phytoplasmal symptoms on other plants in farmers Sandal plantations was carefully observed on other weed species associated with the Sandal ecosystem. They were later analysed for phytoplasma by PCR.

3.3 Cross infectivity studies between Sandal spike and *Stachytarpheta* phytoplasma isolates

Seeds of *Cuscuta subinclusa* was germinated in earthern pots already planted with periwinkle and *Vadelia* seedlings (Pate 1), after

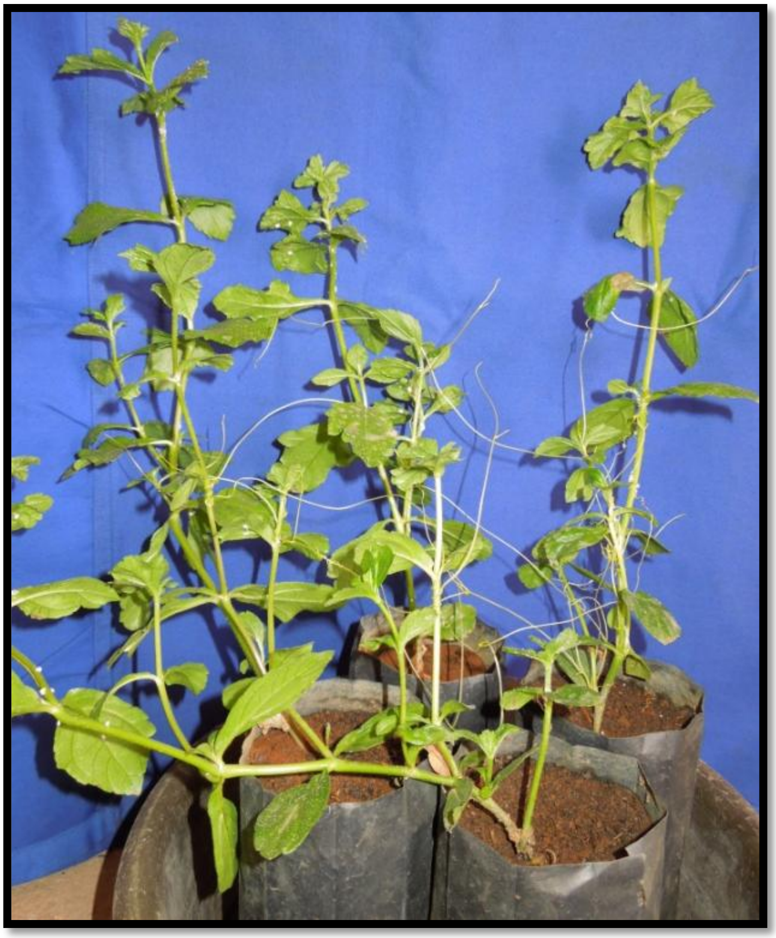

Plate 1 : Dodder ***(Cuscuta subinclusa)*** **maintained on** ***Vadelia chinensis***

establishment, the shoots of the dodder were trained on to diseased Sandal plants. Subsequently the growing tip of the dodder was trained to the test plant i.e *Stachytarpheta indica* and allowed to establish. The inoculated plants were maintained for three months for development of typical symptoms of phytoplasmas.

3.4 Characterisation of Sandal spike and *Stachytarpheta* phyllody phytoplasmas by RFLP analysis of 16S ribosomal gene.

16S rDNA of the two phytoplasmas *viz*., Sandal spike and *Stachytarpheta* phyllody were amplified by PCR using the primer pairs P1/ P7 and products were analysed by restriction endonuclease digestion. 2 to 5 l of each PCR product was digested separately with the two different restriction enzymes *AluI* and *HinfI.* 30 l digestion mixture was prepared by adding sterile distilled water (15 l), reaction buffer (3 l), restriction enzyme (2 l) and PCR product (10 l).

The reaction mixtures were incubated at 37^{0}C for 3-4hr and the digestion products were then separated by gel electrophoresis through a 1.2% Agarose gel and stained in ethidium bromide. DNA bands were then visualized using a gel documentation unit. The RFLP patterns of the phytoplasmas were compared and analyzed as described by Lee *et al*. (1993).

3.5 Analysis of PCR- RFLP products by agarose gel electrophoresis

30µl of PCR product was analyzed in 1.2 per cent agarose gel prepared by melting 1.0g of agarose in 100 ml of 1X TBE in microwave oven (BPL 700T) until a clear transparent solution was obtained. The edges of the gel tray were sealed with a tape and the comb was placed at one end of the tray. The agarose solution was cooled to about 50^{o}C after that, 8-10µl ethidium bromide was added and poured into the gel tray to a thickness of 4-5 mm and allowed the gel to set. After gel solidification the tape was removed and platform was kept in electrophoresis tank. Sufficient electrophoresis buffer (1X TBE) was filled into the tank to cover the gel to a depth of 10 mm and then the comb was removed carefully. Each lane of the gel was dispensed with 10 l PCR product, along with 3 l loading dye. One lane was added with 1 kb molecular weight marker (MBI fermentas). The electrophoresis unit was connected to the power pack (Biometra) and the power supply was turned on until the orange G dye reached the bottom of the gel. The bands were visualized with gel documentation unit.

Experimental Results

IV. EXPERIMENTAL RESULTS

The experiments were carried out on various aspects of Sandal spike disease caused by Phytoplasma with reference to standardization of PCR techniques for the detection of Phytoplasma associated with Sandal spike and *Stachytarpheta* phyllody, PCR based survey of the Sandal plantations to assess the incidence of Sandal spike disease in southern Karnataka, Cross infectivity studies between Sandal spike and *Stachytarpheta* phytoplasma isolates and Characterization of Sandal spike and *Stachytarpheta* phyllody phytoplasmas by RFLP analysis of 16S ribosomal gene. The results obtained on these aspects are presented here under.

4.1 Standardization of PCR technique for detection of Phytoplasmas from Sandal and *Stachytarpheta* infected plants

Polymerase chain reaction (PCR) technique was standardized to detect the phytoplasma from spike infected Sandal and *Stachytarpheta* phyllody infected plant materials. PCR was done through amplification of 16S rDNA fragments of phytoplasmas using phytoplasmal universal primers P1/P7.

The infected leaf samples of Sandal and *Stachytarpheta* weed plant were subjected for PCR analysis by fallowing protocol (Lee *et al*., 1999) of Gradient PCR technique with seven temperature conditions for 30 sec, 1 min and 2 min were carried out to determine the specific annealing temperature. The PCR amplified product was approximately 1800bp fragment corresponding to 16S rDNA of phytoplasma was obtained with the universal phytoplasmal specific primers P1/P7.

Annealing temperature of 55^0C for one minute was found suitable for amplifying Sandal spike and *Stachytarpheta* phyllody phytoplasmas and the rest of the temperatures tested showed no amplification. This modified PCR protocol, amplified the phytoplasma specific 1800bp product from total DNA template extracted from the Sandal spike infected leaf materials, phyllody infected *Stachytarpheta*, and a positive control, Sunhemp phyllody No such products were obtained with DNA template from any of the healthy plant samples (Fig 2).

The following PCR conditions were suitable for amplification of Sandal spike and *Stachytarpheta* phyllody phytoplasmas by using universal phytoplasma specific primers P1/P7 (Fig 3 and 4).

PCR Programme

Initial Denaturation	95^{0} C for 2 min
Denaturation	94^{0}C for 30 min
Annealing	55^{0}C for 1 min
Extension	72^{0}C for 2 min
Final extension	72^{0}C for 10 min

4.1.1 Nested PCR analysis

The nested PCR technique was performed using 1800bp product obtained from PCR amplification using P1 and P7 primers. The PCR products were diluted in the ratio of 1:25, 1:50 and undiluted were subjected to nested PCR analysis using universal primers R16F2n/R16FR2. A PCR product of 1250bp was consistently observed with the annealing temperature of 55^{0}C for two minutes (Fig 5).

4.2 PCR based Survey of the Sandal plantations to assess the incidence of Sandal spike disease in southern Karnataka.

A systemic roving survey was conducted to assess the incidence of Sandal spike disease in farmer's Sandal plantations in southern

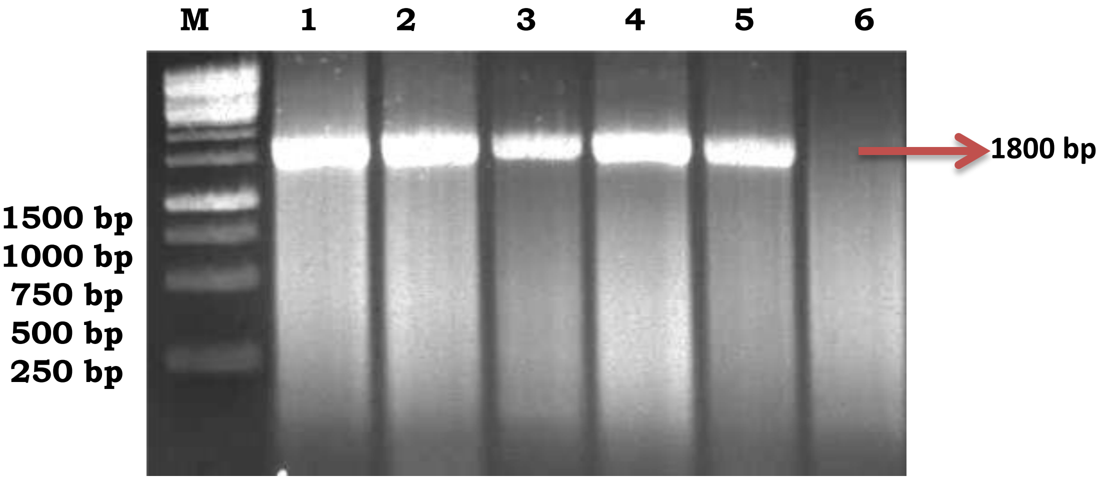

Fig. 2 : Detection of phytoplasma in spike infected and *Stachytarpheta* phyllody plant samples through PCR

M : 1 kb Marker

1 : Sandal spike isolate from B.N.P

2 : Sandal spike isolate from Sira

3 : Stachyterpheta phyllody isolate from Sira

4 : Stachyterpheta phyllody isolate from GKVK

5 : Possitive control (Sunhamp Phyllody)

6 : Healthy sandal sample

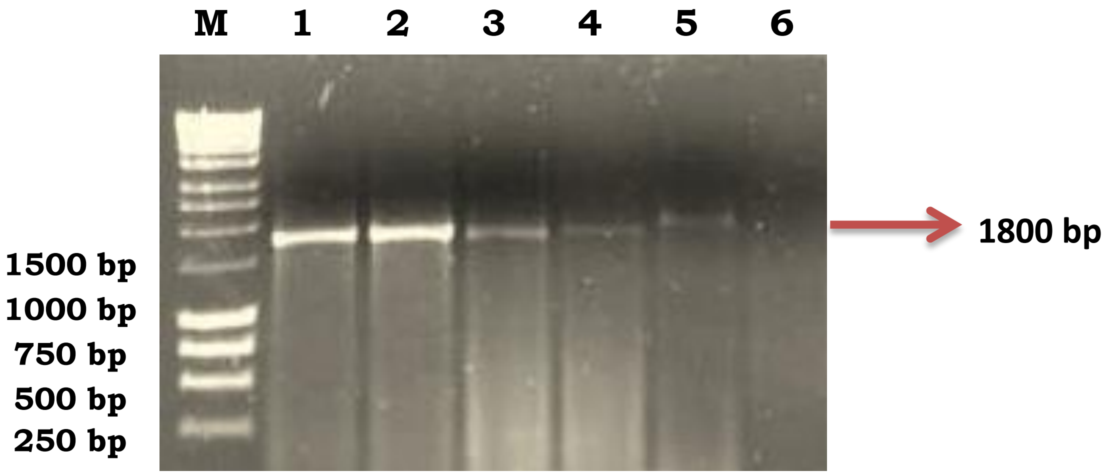

Fig. 3 : **Detection of Sandal spike phytoplasma through Polymerase chain reaction from infected plant samples using P1/P7 primers**

M : 1 kb Marker
1 : Sandal spike disease 1: 25µl
2 : Sandal spike disease 1: 50µl
3 : Sandal spike disease 1: 100µl
4 : Sandal spike disease 1: 200µl
5 : Sandal spike disease 1: 500µl
6 : Sandal spike disease 1: 1000µl

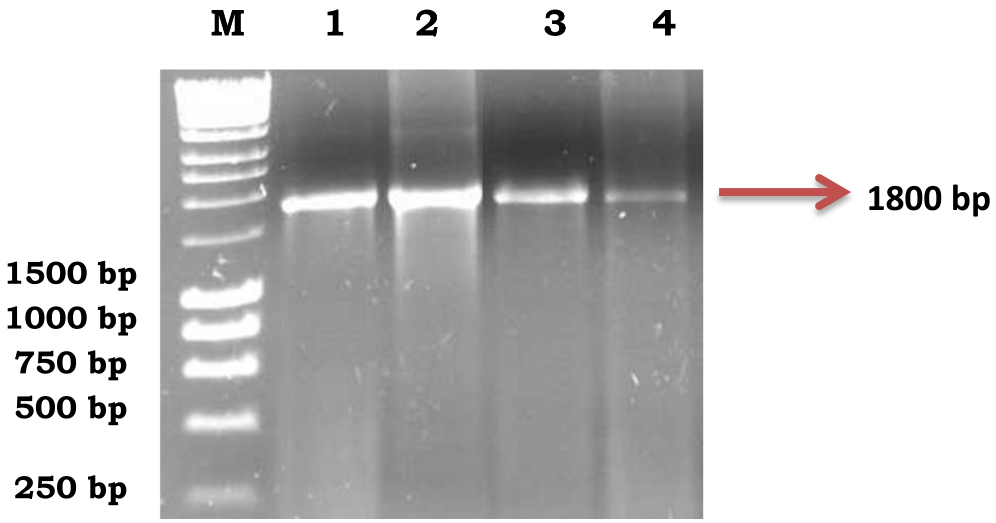

Fig. 4 : PCR detection of phytoplasmal 16S rDNA of *Stachytarpheta* phyllody phytoplasma infected plant samples

M : 1 kb Marker
1 : *Stachytarpheta* phyllody 1: 25µl
2 : *Stachytarpheta* phyllody 1: 50µl
3 : *Stachytarpheta* phyllody 1: 100µl
4 : *Stachytarpheta* phyllody 1: 200µl

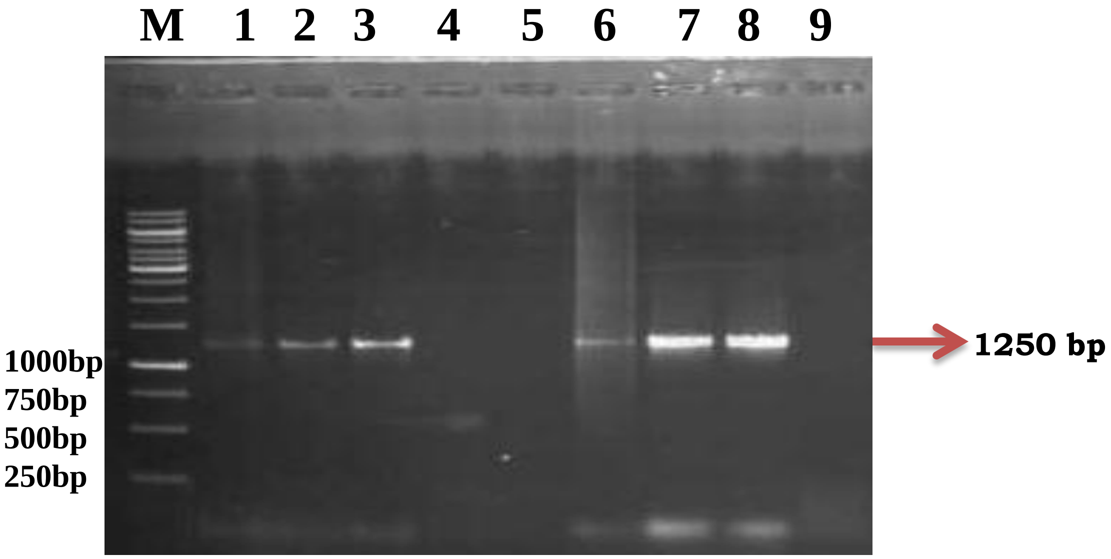

Fig. 5 : Nested-PCR amplification of 16S rDNA of Sandal spike and *Stachytarpheta* phyllody phytoplasma in different dilutions of templates

Lane:

M- 1 Kb Marker

Lane 1- Undiluted PCR product of Sandal spike disease

Lane 2- 1:25 dilution of PCR product of Sandal spike disease

Lane 3- 1:50 dilution of PCR product of Sandal spike disease

Lane 4- Sandal healthy PCR product

Lane 5- Water control

Lane 6- Undiluted PCR product of *Stachyterpheta phyllody*

Lane 7- 1:25 dilution of PCR product of *Stachyterpheta phyllody*

Lane 8- 1:50 dilution of PCR product of *Stachyterpheta phyllody*

Lane 9- *Stachyterpheta* healthy *PCR product*

Karnataka during Sept 2011- June 2012. The surveyed districts include, the farmer's plantations in Tumkur, Chickkballapura, Shimoga, Chitrudurga and Chickmagalore, the results are presented in (Table1)

A total of 24 Sandal plantations were examined. The overall incidence of spike disease varied from 0 to 18 per cent. A maximum incidence of 18 percent was recorded at the one of the farmer's Sandal plantations at Sira, Tumkur district.

In Shimoga district, maximum incidence of Sandal spike disease was recorded at 1.06 per cent Adderi, followed by 1.09 per cent in Thaligere and minimum incidence 0.33 per cent was recorded in Tyagarthi village of Sagara tuluk.

All the five plantations in Chitrudurga, four in Chikkaballapura and five in Chikkamangalore examined for spike incidence, none recorded the spike diseases.

Examination of various weed/plant species associated with the Sandal ecosystem for the incidence of similar type of phytoplasmal infection revealed occurrence of characteristic symptoms of phytoplasmal infection, only on *Stachytarpheta* was found infected. The incidence of the disease was in the range of 2 to 5 per cent (Table 1). Interestingly, the incidence was recorded only in the plantations where the spike disease was noticed.

The symptoms on *Stachytarpheta* (Plate 2 and 3), a common weed species found in the Sandal plantations, were characteristic stunting, reduced leaf size, proliferation of auxillary shoots, shortening of internodes and conversion of floral parts into leaf like structure typically appears like which's broom.

Table 1 : Incidence of sandal spike disease in farmer's Sandal plantations of southern Karnataka

District	Location of plantation	Area under plantation (Acre)	Cropping system	Age of the crop	No. of plants infected / Total No. of plants	% Incidence	Weed species found with characteristic symptoms of phytoplasma and the inicidence	
							Stachytarpheta	
							Type of symptoms	Incidence (%)
Tumkur	Sira	2	ICW Mango	4	79/439	18.00	Phyllody	5
Shimoga (Sagara taluk)	Tyagarthi	5	Sole crop	2	10/2960	0.33	Phyllody	3
	Adderi	1	ICW Arecanut	0.4	4/250	1.60	Phyllody	5
	Thaligere	2	ICW Arecanut	0.6	6/550	1.09	-	0
	Rippenpet,	2	ICW Arecanut	0.5	0/445	0	-	0
	Nedaravalli	1	Sole crop	1	0/220	0	-	0
	Jamburmane	2	ICW mango	0.6	4/522	0.76	Phyllody	5
Shimoga (Therthahalli taluk)	Mandagadde	5	ICW Arecanut	4	10/2785	0.35	Phyllody	5
	Singanbidare	1	Sole crop	3	0/230	0	Phyllody	5
	Therthahalli	2	ICW Arecanut	3	0/480	0	-	0
	Kuruvalli	2	ICW Arecanut	0.5	0/406	0	-	0

Chitradurga	B G kere	80	ICW Coconut Tamarind	2-14	0/18900	0	-	0
	Gollahalli	5	ICW Mango	6	0/2154	0	Phyllody	2
	Hanagal	15	ICW Mango	5	0/6250	0	-	0
	Kodihalli	5	ICW Mango, coconut	5	0/2210	0	-	0
Chickballapura	Bevinahalli,	6	ICW Mango, Sapota	4	0/2900	0	-	0
	Thondebavi,	4	ICW Mango, Sapota	1	0/1715	0	-	0
	Muddenahalli	4	ICW Mango, Gauva	2	0/1840	0	-	0
	Gowribidanuru	5	ICW Sapota	2	0/2425	0	-	0
Chickmagalore	Kemmannugundi	6	ICW Teak, Oak	4	0/2545	0	-	0
	Kodi camp	4	ICW Coconut	1	0/1625	0	Phyllody	3
	Tharikere	4	ICW Arecanut	2	0/1648	0	-	0
	Chickmangalore	5	ICW Arecanut	2	0/2215	0	-	0

Expansion : ICW- Intercropped with

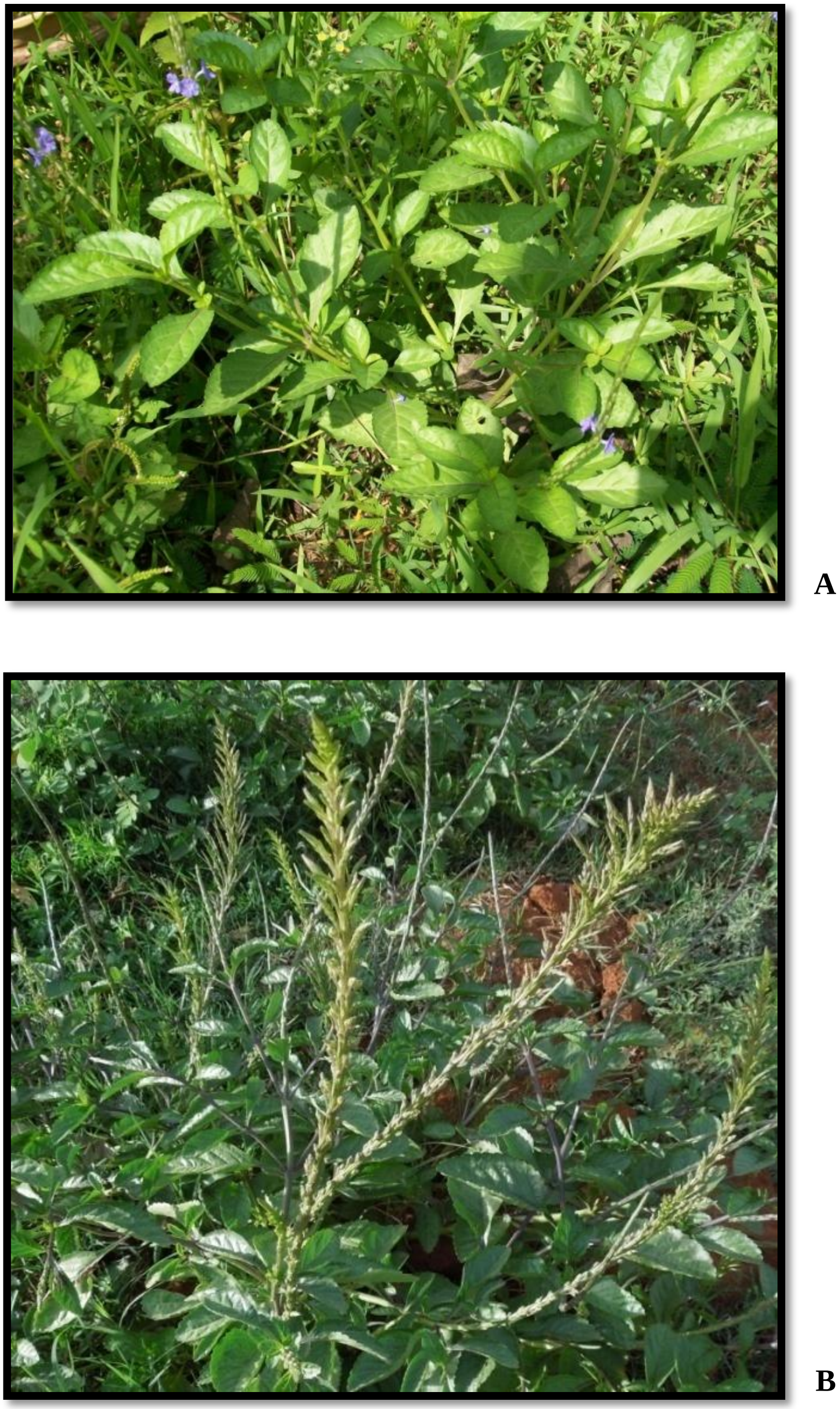

Plate 2 : Healthy *Stachytarpheta indica* (A) and Phyllody infected (B) Weed recorded in Sandal ecosystem

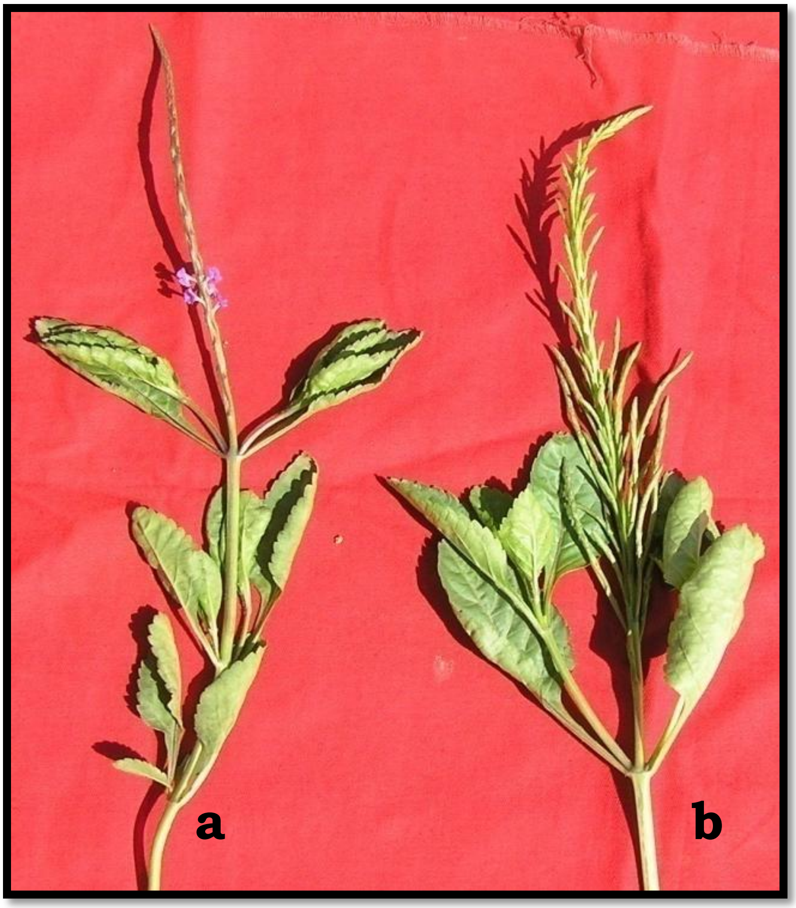

Plate 3 : **Close view of inflorescence of healthy (a) and phyllody Infected (b)** ***Stachytarpheta indica***

Spike infected Sandal (*Santalum album*) plants showed characteristic symptoms consisting of small, narrow leaves which turn pale green, the leaf size were reduced than its normal size and eventually the internodes became shortened. The shortened nature of Sandal plant was appeared like the branches with difficulty to protrude from the shortened internodes acquiring a spike like appearance are (Plate 4, 5 and 6). The leaves and branches of infected plants were dry out and eventually die-off within a short span of its life cycle.

All the infected samples collected during roving survey were subjected to PCR analysis and found positive to spike phytoplasma positive results.

4.3 Cross infectivity studies between Sandal spike and *Stachytarpheta* phytoplasma isolates

Cross transmission studies to determine the role of *Stachytarpheta* as a host of Sandal spike diseases. The healthy seedlings of *Stachytarpheta* were inoculated experimentally by connecting the *Cuscuta* dodder established on infected spike Sandal plants (Plate 7). The results revealed that spike phytoplasma could not be transmitted to any of the inoculated *Stachytarpheta* plants, indicating the inability of phytoplasma to infect and establish in the host. The PCR analysis of all dodder inoculated *Stachytarpheta* plants for the spike phytoplasma also gave negative results (Fig 6).

4.4 Characterization of Sandal spike and *Stachytarpheta* phyllody phytoplasmas by RFLP analysis of 16S ribosomal gene.

PCR-RFLP analysis of 1800 bp product of 16S rDNA products of Sandal spike and *Stachytarpheta* phyllody phytoplasmas were analysed by digesting with restriction endonuclease enzymes, *AluI* and *HinfI*.

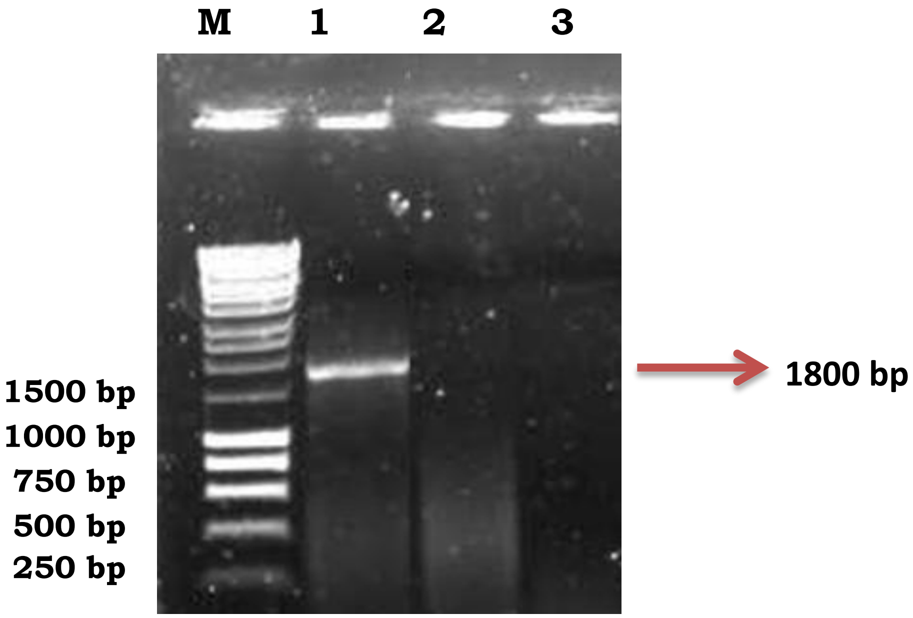

Fig. 6 : **Detection of Sandal spike phytoplasma through PCR from infected plant samples and inoculated *Stachytarpheta* plants**

M- 1 kb marker

1- Sandal spike sample

2 & 3- tested stachytarpheta plantas

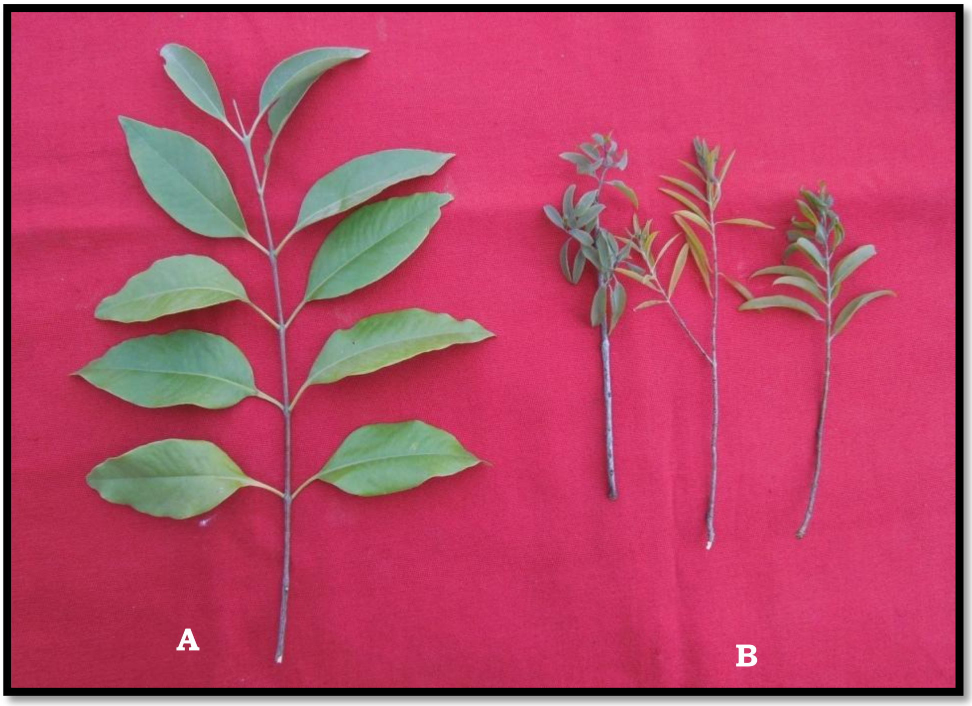

Plate 4 : Leaves from spike infected tree (B) with reduced leaf size in comparison with healthy leaves (A)

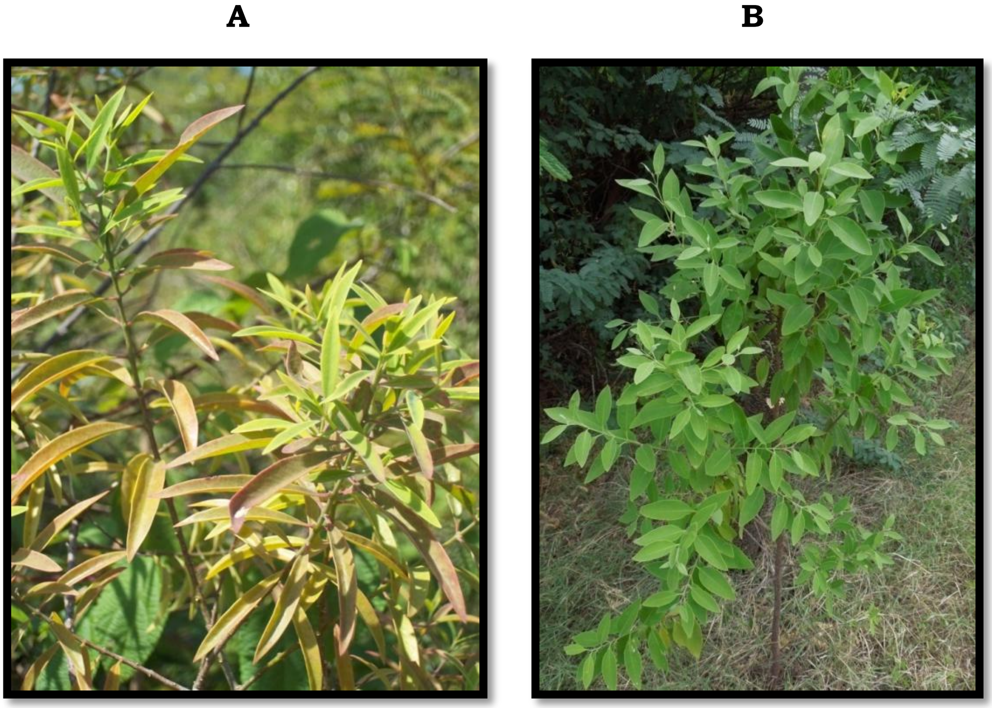

Plate 5 : A: Leaves of spike infected plants with phytoplasma brownish discoloration, reduced size and erectness
B: Healthy Sandal plant

Plate 6 : Sandal spike infected tree with symptoms of complete defoliation and reduced leaf size of new fleshes

Plate 7 : Experimental inoculation of spike phytoplasma to *Stachytarpheta* through dodder

The RFLP profiles of Sandal spike and *Stachytarpheta* phyllody phytoplasmas were quite different from each other (Fig 7). In Sandal phytoplasma the restriction enzyme *AluI* cut the 16S r DNA fragment in to three distinct fragments of 190, 380 and 570 bp length But with the PCR product from *Stachytarpheta* phyllody phytoplasma the restriction enzyme *AluI* generated the 16S r DNA fragment in to two distinct fragments of 570 and 1040 bp length. The combined size of the different fragments was more or less equal to 1800 bp of 16S r DNA fragments.

The restriction enzyme *HinfI* produced three distinct fragments of 500, 600 and 1080 bp length, with *Stachytarpheta* phyllody phytoplasmal 16SrDNA PCR product, only two fragments of 600 and 1080 bp length were observed (Fig 7).

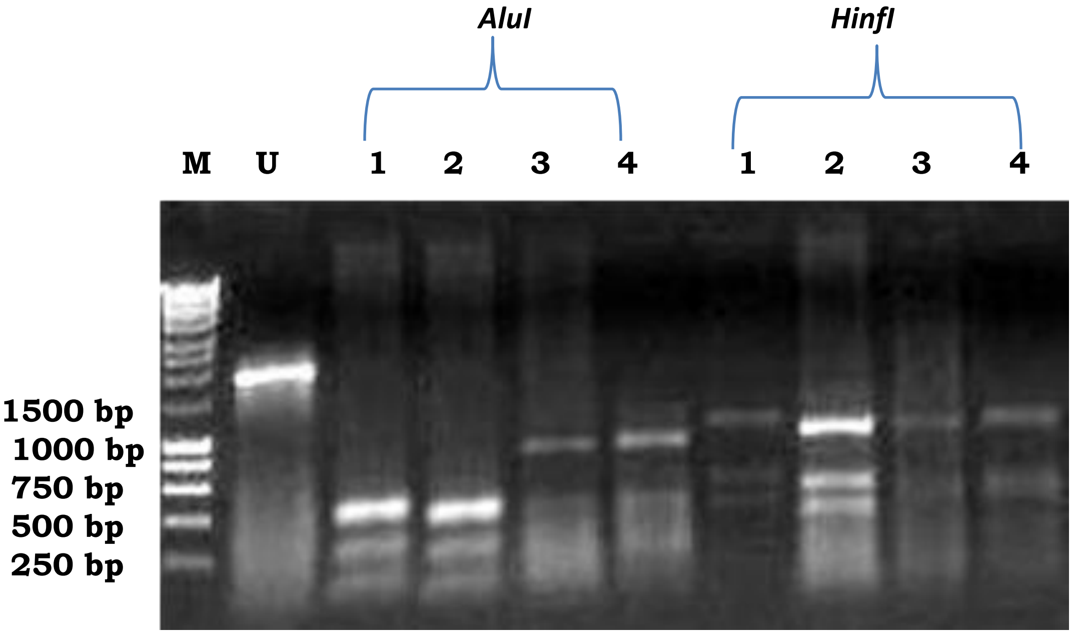

Fig. 7 : Restriction fragment length polymorphism analysis of 16S rDNA amplified PCR product primed by oligonucleotide pair P1/P7

M: 1 kb ladder

U: Undigested PCR product

1: Sandal spike Isolate from B.N.P.

2: Sandal Spike Isolate from Sira

3: *Stachyterpeta* Phyllody Isolate From Sira

4: *Stachyterpeta* Phyllody Isolate From GKVK

Discussion

V. DISCUSSION

Sandal (*Santalum album* L.) is an economically important, semi root parasitic tree, known for its scented wood and fragment oil. Sandal is affected by few diseases, of which the spike disease became most destructive (Selisker and Wilson, 1981). Spike disease in Sandal is caused by phytoplasma and the disease was noticed in all major Sandal-growing states of India *viz.*, Karnataka, Tamil Nadu and Kerala. However, due to liberalization of government policies on cultivation of Sandal, a number of farmer's have started growing on their farm, recent survey of certain farmer's Sandal plantations revealed the occurrence of spike disease.

The present investigations on spike disease in Sandal is focused on the Standardization of PCR technique for detection of phytoplasmas associated with Sandal spike and *Stachytarpheta* phyllody, PCR based survey of the Sandal plantations to assess the incidence of Sandal spike disease in southern Karnataka, cross infectivity studies between Sandal spike and *Stachytarpheta* phytoplasma isolates and Characterisation of sandal spike and *Stachytarpheta* phyllody phytoplasmas by RFLP analysis of 16S ribosomal gene. The results of the investigations are discussed in this chapter.

In the present investigation PCR technique to detect the phytoplasma from infected Sandal and *Stachytarpeta* weed plant was standardized through amplification using phytoplasmal universal primers P1/P7 corresponding to the 16S r DNA. The PCR amplified product of 1800 bp approximately consistently obtained with annealing temperature of 55^{o}C for one minute as compared to Lee *et al.* (1993) reported that annealing temperature of 48^{0}C for one minute results in no amplification of phytoplasma PCR product, the PCR protocol was standardized by altering the PCR conditions especially at annealing temperature as given by (Khan, *et. al*., 2006). Several workers developed PCR technique for detection of phytoplasmas associated with different plant species have been reported (Marcone *et al.,* 1997; Arocha *et al.,* 2005; Rao *et al.,* 2003; Rao *et al.,* 2008) but was unable to amplify.

Further Nested PCR technique was performed with primer pairs R16F2n/R16R2 in order to confirm the association of phytoplasma with Sandal spike and *Stachytarpheta* phyllody through re-amplification of diluted PCR product. Amplification of 1250bp of 16S rDNA fragment was obtained with the diluted and undiluted PCR product amplified using P1 and P7 universal primers. An annealing temperature 55ºC for two minutes supported good amplification of the phytoplasmal 16S rDNA by nested PCR compared to 52ºC for one minute of PCR protocol suggested for other phytoplasmas by various workers (Khan *et al*., 2004; Singh, *et al.,* 1986; Han-MuSeok *et al*., 1997).

Sunil Thomas and Balasundaran (1999) suggested that, conducting nested PCR of direct PCR product assay with primers leads to lack of visibility chances or less visibility due to presence of DNA concentration above the detection limit in ethidium bromide-stained agarose gel and also due to phytoplasma concentration in DNA template or the presence of *Taq* polymerase inhibitors in the PCR mixture.

Nested PCR carried out using primers R16F2n/R16FR2 and also consistently produced 1250 bp PCR product with the anneling temperature of 55^{0}C for two minutes for diluted PCR product and these results were supported by various workers (Singh, *et al.,* 1986; Han-MuSeok *et al*., 1997).

PCR based survey to assess the incidence of spike disease in 24 Sandal plantations from five districts in Karnataka, indicated that occurrence of spike disease in range of 0.00 to 18.00 per cent in one of the plantation at Sira with maximum incidence of 18.00 per cent.

The varied levels of disease incidence could be due to the differences in temperature, relative humidity, collateral associated within the plantations and the population of natural vectors. The disease incidence on Sandal was favored by several factors including climatic conditions. The disease varied according to the plantations of the regions, where the climatic conditions were quite different. However, Muniyappa *et al.* (1980) suggested that, the disease was mostly confined to the areas with rainfall of 800-2000mm, with a temperature of 24-29.5^{0}C and 76-86 per cent relative humidity. He also suggested that, these climatic factors might have playing role for vectors (Leaf and tree hoppers) multiplication and their activity, these said to be natural vectors for Phytoplasma disease.

Cross transmission studies conducted to determine the role of *Stachytarpheta* as collateral host by transmitting the spike phytoplasma through dodder (*Cuscuta subinclusa*) revealed that the weed had no role as collateral host for the Sandal spike disease, as the phytoplasma could not be infected through dodder. The inability of spike phytoplasma to infect and multiply in *Stachytarpheta* weed plant was also confirmed through PCR analysis with the help of universal phytoplasma specific primers P1/P7.

Dijkstra and Lee (1972) reported that the disease could be transmitted from the diseased *Zizupus oenoplia* and *Vinca rosea* to healthy Sandal plants and vice versa through dodder. The presence of phytoplasma bodies in the leaves of tested plants was confirmed through electron microscope and PCR. They also suggested that, the transmission of phytoplsma from *Zizupus oenoplia* or *Vinca rosea* to Sandal and vice versa could be due to the presence of favorable host factors including phytoplasma bodies in the infected leaves. However systematic cross transmission studies are need to determine the precise role of other hosts.

PCR-RFLP analysis conducted to delineate the two phytoplasmal diseases using 1800bp 16S rDNA products of Sandal spike phytoplasma and *Stachytarpheta* phyllody phytoplasma PCR products was digested by two restriction endonuclease enzymes, *AluI* and *HinfI*. The RFLP profiles of two restriction enzymes revealed that the Sandal spike and *Stachytarpeta* phyllody phytoplasmas were quite different to each other.

Sunil Thomas and Balasundaran (1999) reported occurrence of two isolates of phytoplasma from Sandal plantations across Karnataka and Kerala. The RFLP analysis with restriction endonuclease enzymes, *AluI* exhibits 90 per cent similarity among the isolates of Karnataka and Kerala locations confirming that, the phytoplasma infecting Sandal belongs to the Group I of major eleven phytoplasma groups was with 90 per cent similarity.

However, Khan *et al.* (2006) suggested that, Sandal isolate upon digestion with three endo-nuclease enzymes and the RFLP profile for each enzyme was identical, indicating with a new subgroup 16SrI-B phytoplasma associated with Sandal spike through pair wise comparison of 16S rDNA sequence. Pallavi (2009) reported that, the phytoplasma isolate from chickpea and Stachytarpeta phyllody were different from each other with different RFLP profiles generated upon digestion with *Alu I,* endonuclease enzyme.

Based on the cross infectivity and RFLP analysis of 16S rDNA, it is concluded that, the two phytoplasma species are different from each other and *Stachytarpheta* is not a host for spike disease phytoplasma. However, analysis of other phytoplasmal genes and cross transmission studies using vector are necessary to understand their molecular/biological relationship.

Future line of work

1. Extensive survey for the prevalence and incidence of Sandal spike disease in other Sandal plantations in Karnataka and other states.
2. Identification of insect vectors responsible for the natural spread of the disease.
3. Systematic understanding the role of phytoplasmas of other plant species particularly crop/tree/plant in the epidemics of spike disease.

Summary

VI. SUMMARY

Phytoplasmas have been found to be associated with disease in several hundred plant species causes serious menace to field crops and plantations throughout the globe. The spike disease caused by Phytoplasma in Sandal was considered a major problem in Sandal plantations in India, particularly in Kerala, Tamil Nadu and Karnataka.

The present study comprised of standardization of PCR technique for detection of phytoplasmas associated with Sandal spike and *Stachytarpheta* phyllody, PCR based survey of the Sandal plantations to assess the incidence of Sandal spike disease in southern Karnataka, cross infectivity studies between Sandal spike and *Stachytarpheta* phyllody phytoplasma isolates and Characterization of Sandal spike and *Stachytarpheta* phyllody phytoplasmas by RFLP analysis of 16S ribosomal gene. The results of the investigations so obtained are summarized here under.

Polymerase Chain Reaction (PCR) technique for detection of Sandal spike and *Stachytarpheta* phyllody was standardized using gradient PCR, PCR product of approximately 1800bp product of 16Sr DNA was consistently amplified with the universal phytoplasma specific primer pairs of P1/P7 from total DNA extracted from the Sandal spike and *Stachytarpheta* phyllody samples, But not from healthy Sandal leaf samples. The annealing temperature of 55^0C for one minute was found suitable for amplifying Sandal spike and *Stachytarpheta* phytoplasmas.

Nested Polymerase Chain Reaction (Nested PCR) technique performed in order to confirm the association of phytoplasma with the Sandal spike and *Stachytarpheta* phyllody resulted in the amplification of Nested Polymerase Chain Reaction product of 1250bp of 16Sr DNA was obtained using primer pair R16F2n/R16R2 with the 1.8kb PCR product was used as template.

Among 24 Sandal plantations from five districts in southern Karnataka, the incidence of Sandal spike disease ranged from 0 to 18 per cent. The maximum incidence (18 %) was recorded in Sira taluk of Tumkur district, followed by Shimoga 1.65 per cent. The least incidence (0.33 %) was recorded in plantations of Tygarthi village.

Stachytarpheta was found associated with Sandal, of the different weed/plant species found in Sandal eco-system, only on *Stachytarpheta* typical characteristics of phytoplasma was recorded. The spike phytoplasma could not be transmitted to *Stachytarpheta*.

RFLP (Restriction fragment length polymorphism) profiles of 1.8 kb PCR products of Sandal spike and *Stachytarpheta* phyllody phytoplasmas digested with *AluI* and *HinfI* restriction enzymes showed differences in 16S rDNA profiles. Distinct RFLP pattern was observed between Sandal spike phytoplasma and *Stachytarpheta* phyllody phytoplasma. This clearly revealed that Sandal spike phytoplasma was different from *Stachytarpheta* phyllody phytoplasma at molecular level.

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