Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(5): 2172-2182
Received: 18-07-2020
Accepted: 29-08-2020

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Phytoplasmal diseases in India and its management

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DOI: https://doi.org/10.22271/phyto.2020.v9.i5ad.12669

Abstract
Phytoplasmas are phloem-limited pleomorphic bacteria which lacks the cell wall, mainly transmitted through leafhoppers but also by plant propagation materials and seeds. In India evidences showed that phytoplasma have been found associated with 129 plant species including Cereals, vegetables, fruits, trees, ornamental, sugarcane, grasses and weeds which is increasing at alarming rate. Sesame phyllody, brinjal little leaf, sugarcane grassy shoot, sandal spike, coconut root wilt, areca nut yellow leaf and many diseases in ornamental plants which causes the most severe economic losses in India. In earlier days, very few phytoplasma diseases were identified in India merely based on the bright-field, fluorescence, electron microscope observations, tetracycline treatment and to a lesser extent by serological assays. Recently detection of phytoplasma based on molecular methods including nested PCR assays are efficiently carried out and on that basis several plant species are reported to have phytoplasma infections. Identified phytoplasmas are related to '*Candidatus* Phytoplasma asteris', '*Ca*. P. pruni', '*Ca.* P. ziziphi', '*Ca*. P. trifolii', '*Ca*. P. solani', '*Ca*. P. cynodontis', '*Ca*. P. oryzae', '*Ca*. P. phoenicium', '*Ca.* P. australasia' and '*Ca*. P. pini'. At present the suggested effective management practices in India includes growing resistant varieties, control of insect vectors, weed species as alternative hosts and use of healthy planting materials. It is moreover essential to understand the molecular basis of phytoplasma-vector interaction and other factors involved in disease development in order to reduce the outbreak of phytoplasma. In this chapter, we have discussed overall progress on phytoplasma disease on plant species in India in terms of detection, transmission and management of phytoplasma.

Keywords: Phytoplasma diversity, detection, genetic diversity, transmission, management

Introduction
Phytoplasmas are obligate prokaryotes which present intracellularly that lack cell walls and possess very small genomes (680–1,600 kb). When the first report by Doi *et al*. (1967), phytoplasmas have been identified as pathogens in different plant genera and in some cases it have caused severe epidemics in major crops (Bertaccini *et al*., 2014) [13]. Phytoplasma are fastidious prokaryotes that can survive and multiply only in hypotonic habitats such as plant phloem or insect haemolymph and that's why they are strictly host dependent. They are known to be pathogenic to more than a three hundred plant species (Bertaccini *et al.,* 2014) [13]. Phytoplasmas cause complex syndromes with symptoms such as stunting, proliferating auxiliary shoots, forming sterile deformed flowers, virescence and phyllody in several hundred plant species (Lee *et al*., 2000) [61]. Based on phylogenetic analysis of gene sequences (16S rRNA) phytoplasmas were recently assigned to a provisional genus, '*Candidatus* (Ca.) Phytoplasma' within the class *Mollicutes*. In India, recent evidence showed that phytoplasma associated with plants including cereals, vegetables, fruits, trees, ornamentals, sugarcane, palms, oil crops and weeds are increasing at an alarming rate (Rao, 2017) and till now 129 plant species have been reported to be associated with phytoplasma diseases (Rao *et al*., 2017). In this book chapter, an updated status of progress on research work done on phytoplasmal diseases in India is being presented.

General properties of phytoplasma
Phytoplasmas are prokaryotes with cell wall-less and they are bounded by a "unit" membrane. It consists of cytoplasma, ribosomes, both DNA and RNA. In ultrathin sections, they appear as a complex of multibranched, beaded, filamentous or spheroidal pleomorphic bodies ranging from 175-400 nm in diameter for the spherical and oblong cells and up to 1700 nm long for the filamentous forms (Florence and Cameron, 1978; Waters and Hunt, 1980 and McCoy *et al*., 1989) [28, 150, 83] and they are surrounded by a triple layered single unit membrane as an alternative of cell wall (Lee and Davis, 1992) [59]. Various attempts to culture phytoplasmas on artificial nutrient media or cell-free media have been unsuccessful (Lee and Davis, 1986) [58].

Contaldo *et al*., 2012 [23] demonstrated for the first time that phytoplasmas, can grow independently from their hosts and showed that specific commercial media supported axenic growth of phytoplasmas under defined conditions.

'*Candidatus* phytoplasma': A provisional genus-level taxon and beyond

Modern mollicute systematics has consisted of multiple taxonomy system, developed over the last two decades based on phenotypic, genotypic and phylogenetic criteria for classification of members of the *Mollicutes* (Vandamme *et al*., 1996; Razin *et al*., 1998) [142, 117]. Wide-ranging phylogenetic studies based on 16S rRNA and other housekeeping genes have readily placed phytoplasmas in the class of *Mollicutes* (Namba *et al*., 1993; Gundersen *et al*., 1994; Seemuller *et al*., 1998; Lee *et al*., 2000, 2006; Zhao *et al*., 2005; Martini *et al*., 2007; Hodgetts *et al*., 2008) [92, 35, 124, 61, 156, 80, 44-45]. However, because of the insufficiency of accessible phenotypic criteria, it was inevitable that phytoplasma taxonomy would be heavily based on molecular characteristics and phylogeny.

To resolve the hindrance in phytoplasma taxonomy in 2004 [48], based on agreement among phytoplasmologists and in agreement with the International Committee of Systematic Bacteriology Subcommittee for the Taxonomy of Mollicutes, the International Research Program for Comparative Mycoplasmology, Phytoplasma/ Spiroplasma Working Team – Phytoplasma Taxonomy Group adopted a taxonomic rule that had been established for observing properties of uncultured organisms (Murray and Schleifer, 1994; Murray and Stackebrandt, 1995) [87, 88] and proposed to erect a genus-level provisional taxon '*Candidatus* (*Ca*.) Phytoplasma' to accommodate plant pathogenic, non-helical mollicutes (IRPCM, Phytoplasma / Spiroplasma working Team-phytoplasma Taxonomy, 2004) [48]. To prevent nomenclatural confusion which may arise from description of poorly differentiated taxa, the Working Team established guidelines for naming new taxa within the genus '*Ca*. Phytoplasma': a novel '*Ca*. Phytoplasma' species which describes should refer to a single, unique 16S rRNA gene sequence of greater than 1200 bp and share less than 97.5 % sequence similarity to that of any previously described '*Ca.* Phytoplasma' species unless the phytoplasma under consideration clearly represents an ecologically separated population (IRPCM, Phytoplasma / Spiroplasma working Team-phytoplasma Taxonomy, 2004; Firrao *et al*., 2005) [48].

Diversity of phytoplasmas infecting crops species in India

The history of phytoplasma diseases in India dates more than 100 years old. First report of Root (wilt) disease of coconut in the former state of Travancore (South Kerala) around 1874 and became very much evident during the year 1882 (Butler, 1908; Pillai, 1911; Varghese, 1934) [14, 98, 143]. Later the disease was reported to occur in more than 410 thousand hectares in Kerala (Pillai *et al*., 1973; Solomon *et al*., 2001; Sumi, 2015; Sumi *et al*., 2015) [97, 32, 135-136]. Sandal spike was the first phytoplasma disease to be reported (Varma *et al*., 1969) [144] in India. Thereafter a large number of phytoplasma diseases were described, which includes brinjal little leaf disease (Varma *et al*., 1969) [144], grassy shoot disease of sugarcane (Chona *et al*., 1960) [21], rice yellow dwarf disease (Reddy and Jeyarajan, 1990) [118], Sesamum phyllody (Vasudeva and Sahambi, 1955) [145], Zizyphus witches' broom (Hull *et al*., 1970) [46], Potato purple top (Giri and Nagaich, 1971) [33], white leaf disease of *Cynodon dactylon* (Singh *et al*., 1978) [129], little leaf disease of *Acanthospermum hispidium* (Raju and Muniyappa, 1981) [108] and yellowing disease of *Urtochloa panicoides* (Muniyappa *et al*., 1982) [86]., coconut root wilt (Solomon *et al*., 1983) [31]. These phytoplasama diseases are mainly reported based on bright-field and fluorescence microscopy, electron microscopic observations and tetracycline treatment and to a lesser extent on serological assays. Following the development of molecular tools, phytoplasmas have been characterized as many as 129 plant species in India (Chaturvedi *et al*., 2010; Rao *et al*., 2011a; Rao *et al*., 2017) [17, 111, 55]. Phytoplasma have been associated with many plant species in India. More than ten groups of phytoplasma have been identified and most of them have been reported from North-Eastern parts of the country. Few phytoplasmas have been recorded in Eastern, Western and Central India (Mall *et al*., 2011) [71].

Nucleotide sequence studies of 16S rDNA have shown that the "*Ca.* Phytoplasma asteris‟, "*Ca*. P. aurantifolia‟, "*Ca*. P. ulmi‟, *"Ca.* P. trifolii', "*Ca*. P. phoenicium‟, "*Ca.* P. oryzae‟, "*Ca*. P. cynodontis‟, which representing 16SrI, 16SrII, 16SrV, 16SrVI, 16SrIX, 16SrXI and 16SrXIV groups are the major groups associated with different plant species (Rao *et al*., 2011a, 2017) [111, 55]. The aster yellows phytoplasma group (AY-16SrI) is the most important which affects ornamentals, tree species, vegetables, sugarcane, fruit crops and pulses in India (Raj *et al*., 2011; Reddy, 2012) [104, 119]. Recently association of '*Ca*. P.asteris' with Rice Orange Leaf (ROL) disease in South India in rice was confirmed by Valarmathi *et al*. (2013) [140]. Sugarcane grassy shoot associated with 16SrXI phytoplasmas (Rao *et al*., 2005; 2008) [27, 34] and Nasare *et al*. (2007) [93] reported molecular and symptom analysis reveals the presence of new phytoplasma associated with sugarcane. Sesame phyllody associated 16SrIV (Khan *et al*., 2007) [52] and coconut wilt disease associated 16SrIV and 16SrXI (Sharmila *et al*., 2004; Manimekalai *et al*., 2010) [126, 73] are the most important diseases causing huge economic losses in India. Little work has been done on occurrence and identification of phytoplasma in ornamental plants. Ajaykumar *et al*. (2007) [40] first time recorded "*Ca.* P. asteris" associated with little leaf disease of *Portulaca grandiflora*. Raj *et al*. (2009) [17] reported aster yellows phytoplasma of "*Ca*. P. asteris‟ (16SrI group) associated with malformation and twisting of floral spikes of Gladiolus. Chaturvedi *et al*. (2009a; 2009b, 2009c) [18-19] reported little leaf disease in *Hibiscus rosa-sinensis, Rosa alba* and *Catharanthus roseus* in Gorakhpur. Kumar *et al*. (2012) [53] reported *Mirabilis jalapa* and Chrysanthemum little leaf associated with "*Ca*. P. aurantifolia‟ (16SrII group, Peanut witches' broom). Adkar-Purushothama *et al*. (2009) [1] reported "*Ca*. Phytoplasma asteris‟-related strain associated with a yellows disease of black pepper (*Piper nigrum*). Arocha *et al*. (2009) [4] reported "*Ca*. Phytoplasma aurantifolia‟ (group 16SrII) infection in lettuce, carrot and French bean and acid lime (Ghosh *et al*., 1999) [32] and chickpea (Ghanekar *et al*., 1988) [30]. Raj *et al*. (2008a; 2008c) [101, 107] reported association of "*Ca.* phytoplasma asteris‟ (16SrI) phytoplasma with *Parthenium hysterophorus* showed symptoms of virescence and witches‟-broom and yellow disease of *Achyranthes aspera*.

Singh *et al.* (2009) [10] reported "*Ca.* P. asteris‟ (16SrI) infecting banana (*Musa spp*.). Yellows and little leaf disease in *Zinnia elegans* reported by Agrahari *et al*. (2010) [2]. Azadvar *et al*. (2011) [11] reported association of 16SrI-B subgroup phytoplasma with oil palm stunting disease. Verma *et al*. (2012) [146] reported phytoplasma associated with an axillary shoot proliferation disease in papaya in Maharashtra. The symptoms observed in the papaya fields of Pune, Western

India differed from dieback disease of papaya reported from Gorakhpur, (Rao *et al*., 2011b) [112]. Kumar *et al*., (2012) [53] reported occurrence of phytoplasma disease of periwinkle in northern Karnataka. Tiwari *et al*. (2013) reported association of "*Ca.* P. asteris" (16SrI-B) with the little leaf disease of potato in Uttar Pradesh.

In many parts of Asia and India, sugarcane grassy shoot disease is becoming a major threat to sugarcane cultivation. Mitra *et al*. (2019) [84] reported for the first time the association of '*Ca.* P. asteris' (16SrI-B) with shoot proliferation and witches' broom symptoms of pineapple in the world. Because pineapple has been reported as a new host of 16SrI-B phytoplasma subgroup in India, which has already been reported to have a wide host range infecting various crops (Rao *et al*., 2017) [55]. Gautam *et al.* (2019) [29] reported for the first time the natural presence of 16SrXI group of phytoplasma associated with the present little leaf disease of Vetiver (*Chrysopogon zizanioides*).

Diagnosis of phytoplasma in India

In India, the detection of phytoplasmas were initially based on microscopic methods like transmission electron microscopy (TEM), light microscopy and DAPI (DNA-specific-6-diaminido-2-phenylindole) fluorescence microscopy technique (Deeley *et al*., 1979; Purohit *et al*., 1978; Manimekalai *et al*., 2016) [24, 100, 73]. Serological and molecular diagnostic techniques seem to be emerged in early 2000s (Viswanathan 2001) [148]. PCR-based methods were developed in the early 1990s and found more sensitive than ELISA and RFLP analysis which allows the identification of different phytoplasma strains at easier sense.

During the 21st century, application of PCR for the detection of phytoplasmas in diseased plants and possible insect vectors has significantly facilitated the identification of a wide array of phytoplasmas in different plants species (Muniyappa *et al*., 1982; Padmanabhan, 1982; Raj *et al*., 2007, 2008b, 2009a, 2009b; Rao *et al*., 2007, 2009; Chaturvedi, 2009a, 2009b; Mall, 2009; Gupta *et al*., 2010) [86, 18-19, 55, 27, 34, 70, 18, 19, 133]. A simple differential filtration approach was successfully developed for the enrichment of phytoplasmas DNA. This method compared to conventional methods allowed the enrichment of phytoplasma DNA from the host plant and offered many folds enrichment of phytoplasma particles with a 148-fold increase in sensitivity for their detection (Prabu *et al*., 2008) [99]. Recently a species-specific and 13% more efficient PCR based detection system than the universal 16SrDNA for Sugarcane grassy shoot (SCGS) phytoplasma has been developed using AP-PCR technique (Kawar *et al*., 2010) [51]. Several universal phytoplasma group-specific primers have been developed and used for amplification of 16S rRNA and 23S rRNA, 3F/3R amplified ~1300 bp and IF7/7R2 amplified 490 bp product in direct or nested PCR assays (Manimekalai *et al*., 2010) [76]. Moreover, new sets of multilocus gene primers for gyrA, gyrB and dnaB (dnaBF/dnaBR) were designed and developed for amplification of phytoplasmas belonging to 16SrI, -II, -VI and -XI groups (Madhupriya *et al*., 2015; Madhupriya, 2016) [67, 68]. During the last 5 years more than 40 phytoplasmas are identified in various plant species including crops, fruit trees, ornamentals, sugarcane, grasses and weeds on the basis of sequence analysis comparison of 16S rRNA, sec A gene, tuf gene, gyrA, gyrB, dnaB, groEL and leu C gene (Madhupriya *et al*., 2015; Nabi *et al*., 2015a, 2015b; Yadav *et al*., 2016b; Ghosh *et al*., 2017) [31]. Manimekalai *et al*. (2013) [68, 89, 90, 155, 72] developed a specific and efficient real-time PCR-based detection system for coconut root (wilt) phytoplasma using double-stranded DNA intercalating dye, SYBR Green. Manimekalai *et al*. (2010; 2013) [72, 76] reported '*Ca*. P. oryzae'-related strain (16SrXI group) associated with coconut RWD and Yellow Leaf Disease of Areca palm.

The association of two phytoplasma subgroups (16SrI-B and 16SrII-D) in four ornamental plant species based on pairwise sequence comparison, phylogeny and virtual RFLP analysis of 16S rDNA sequences were confirmed. '*Ca. P. aurantifolia*' subgroup D (16SrII-D) was found associated with chrysanthemum phyllody and leaf yellowing at Delhi and Tamil Nadu, bougainvillea little leaf and yellowing at Delhi and Chinese aster phyllody at Bengaluru, Karnataka. However, jasmine little leaf and yellowing at Bengaluru, Karnataka and chrysanthemum stunting at Pune were found to be associated with '*Ca. P. asteris*' subgroup B-related strains (16SrI-B). The identification of 16SrII-D subgroup phytoplasma infecting bougainvillea and 16SrI-B subgroup infecting jasmine are the new reports to the world (Gopala and Rao, 2018) [34]. Phytoplasmas enclosed in at least sixteen different ribosomal groups infecting vegetable crops have been reported so far across the world (Kumari *et al*., 2019) [56].

***16S RRNA* gene-based system for identification of phytoplasmas**

The differentiation and classification of phytoplasmas were based on several molecular markers. The 16S rRNA gene is the most widely used marker in the phytoplasma research community and proves to be very useful in preliminary classification of phytoplasmas. Several universal or generic oligonucleotide primer pairs based on the 16S rRNA gene, the 16S-23S intergenic spacer region and partial 23S rRNA gene sequences have been designed, which allow amplification of >1200 bp to near full-length 16S rRNA gene sequences of all phytoplasmas associated with various plants and insect vectors (Lee *et al*., 1993b; Namba *et al*., 1993; Schneider *et al*., 1993; Gundersen and Lee, 1996; Smart *et al*., 1996) [65, 92, 122, 62, 36, 130]. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA gene sequences using selected restriction enzymes was employed by Schneider *et al*. (1993) [122] and Lee *et al*. (1993a) [62] for classification of phytoplasmas. Based on RFLP analyses with 17 restriction enzymes, Lee *et al*. (1993a, 1998, 2000) [61, 62, 93] constructed a comprehensive classification scheme for phytoplasmas. Separation of major groups was based on similarity coefficients of collective RFLP patterns of a 1.2 kb PCR amplicon (Lee *et al*., 1998) [93]. The similarity coefficients of RFLP patterns between two distinct groups were 90 % or below. Subgroup delineation within a given group was based on restriction site analysis within this amplicon. A new subgroup was assigned if an unknown phytoplasma strain had one or more restriction sites different from those in all the existing members of the given group. The scheme has been periodically updated (Lee *et al*., 1998, 2000, 2006a [61, 93, 60]; Montano *et al*., 2001; Arocha *et al*., 2005b; Al-Saady *et al*., 2008) [9]. Thus far, it comprises 19 major phytoplasma groups and about 50 subgroups. The grouping is near congruent with the phylogenetic tree constructed by analysis of 16S rRNA sequences. Each group was proposed to represent at least one phytoplasma species (Gundersen *et al*., 1994b) [38]. Recently, the scheme was further updated and upgraded, based on virtual RFLP patterns by Wei *et al*. (2007, 2008) [152, 153] and Zhao *et al*. (2009) [157] through the use of computer-simulated RFLP analysis of vast collections of phytoplasma 16S rRNA gene sequences that

were reported and deposited in GenBank. Currently, the scheme comprises 29 groups and 89 subgroups. Each subgroup is defined by unique collective RFLP patterns. In practice, actual RFLP analysis may be the choice for preliminary characterization of unknown phytoplasmas associated with a given new disease if numerous samples need to be analysed or if there is no sequencing facility available. This updated scheme represents the most comprehensive classification system for phytoplasmas and has provided reliable molecular markers for rapid identification of phytoplasma strains.

Perspective of multiple gene-based systems

Phytoplasmas are insect-transmitted plant pathogens which is capable of multiplication in both vector and plant hosts (Lee *et al*., 2000) [61]. The three-way interactions between phytoplasmas, vectors and plant hosts contribute to the complexity of phytoplasmal bionetwork. These ecologically isolated phytoplasma strains often possess unique biological properties, such as specificity to plant and vector species and symptoms they induce in the affected plants. On the other hand, closely related strains (e.g. 'Ca. Phytoplasma asteris' strains) could cause different diseases and induce different symptoms (Lee *et al*., 2004) [64]. The combination of the 16S rRNA gene with one or more variable gene or DNA fragments, 16S rRNA plus *secY*, 16S rRNA plus rp or 16S rRNA plus *secA*, proved to be sufficient for clearly discriminating two closely related strains (Lee *et al*., 2004, 2006b; Martini *et al*., 2007; Hodgetts *et al*., 2008) [64, 80, 44-45]. The multiple gene based system suitable for classification of the whole spectrum of phytoplasmas will be realized unless there is a near complete sequence database on these potential molecular markers is available.

***Tuf* gene-based system**

The *tuf* gene, encoding the elongation factor, EF-Tu, is another highly conserved gene that has been frequently used for differentiation and classification of phytoplasmas. Schneider *et al*. (1997) [123] designed primer pairs that can be used for amplifications of *tuf* gene sequences from most phytoplasma groups. It was found that the *tuf* gene, like the 16S rRNA gene, represents a potential marker for classification of phytoplasma groups. The nucleotide sequence similarities among the aster yellows (AY), peach X-disease and stolbur (STOL) phytoplasma groups ranged from 87.8 to 97.0 %. Phytoplasma groups and subgroups can be differentiated based on RFLP analyses using several restriction enzymes. The resolving efficacy for separation of distinct lineages among phytoplasmas is slightly lower than that of the 16S rRNA gene (Schneider *et al*., 1997; Marcone *et al*., 2000) [123, 77]. However, in some cases, the *tuf* gene was found to be useful in the differentiation of various ecological strains or strain variants within 16S rRNA subgroups (Langer and Maixner, 2004) [57]. For example, several strain variants were recognized within 16XII-A and 16XII-B, based on analysis of *tuf* gene sequences (Langer and Maixner, 2004; Streten and Gibb, 2005; Andersen *et al*., 2006; Pacifico *et al*., 2007; Riolo *et al*., 2007; Iriti *et al*., 2008) [57, 134, 6, 95, 120, 47]. The main use of *tuf* gene primers to date has therefore been to establish subgroups within the 16Sr groups, particularly within the 16SrI AY group (Marcone *et al*., 2000) [77] and the 16SrXII'*Ca.* Phytoplasma australiense'group (Streten and Gibb, 2005) [134]. In a study on the AY group, the AY-specific primers fTufAy and rTufAy (Schneider *et al*., 1997) [123], which amplify a 940 bp product, were used on 70 phytoplasma isolates in conjunction with the 16S rRNA gene primers (Marcone *et al*., 2000) [77].

***SecY* and *SecA* gene-based system**

The *secY* gene, encoding for a protein translocase subunit, is another molecular marker that is useful for finer differentiation of phytoplasma strains. The *secY* gene sequence variability is similar to that of rp genes. The average *secY* gene sequence similarities between two given 16Sr phytoplasma groups ranges from 57.4 to 76.0 % (Lee *et al*., 2006b) [66]. *SecY* subgroups delineated based on RFLP analyses of *secY* gene sequences from groups 16SrI and 16SrV phytoplasmas generally coincided with those delineated with rp gene sequences (Lee *et al*., 2004, 2006b; Martini *et al*., 2007) [64, 66, 80]. However, due to more informative characters, the resolving power of *secY* is slightly better than rp gene sequences. Complete characterization of the majority of phytoplasma groups and their representative strains is in progress (Lee *et al*., 2004) [64]. The *secY* gene, like the rp gene, could represent a good candidate marker for classification of phytoplasma strains. The *secY* gene has also been used for differentiation of the AY group phytoplasmas (Lee *et al*., 2006b) [66]. In this study, primers were designed based on the published AY and OY sequences to amplify a 1.4 kb near-full-length *secY* gene.

Another protein translocase subunit encoding gene, *secA*, was recently employed for classification of phytoplasmas (Hodgetts *et al*., 2008) [44-45]. A portion of the gene sequence, about 480 bp, was PCR-amplified from various phytoplasma strains representing 12 16Sr groups. The sequence similarity ranged from 69.7 to 84.4 % between two given 16Sr groups. The resolving power of the *secA* gene as a phylogenetic parameter for phytoplasma differentiation is similar to those of rp and *secY* genes.

Restriction fragment length polymorphism (RFLP)

Earlier application of RFLP for differentiation and classification of phytoplasmas was combined with dot or Southern hybridization analysis. Since the introduction of PCR into phytoplasma studies, RFLP analysis of PCR-amplified 16S rDNA has been widely applied for identification and classification of a broad range of phytoplasmas (Lee *et al*., 1993b; Namba *et al*., 1993; Schneider *et al*., 1993; Seemuller *et al*., 1994; Gundersen *et al*., 1994a, b and 1996; Ceranic-Zagorac and Hiruki, 1996) [122, 92, 36, 37, 125, 124, 37, 38, 16]. More than twenty distinct phytoplasma 16S rDNA groups (Seemuller *et al*., 1998) [124] and more than twenty-five subgroups have been identified by using this approach (Lee *et al*., 1998) [93]. However, the closely related phytoplasmas could not be differentiated by analysis of 16S rDNA fragment because of their highly conserved nature (Lee and Davis, 1992., Lee *et al*., 1993b; Griffiths *et al*., 1994) [59, 65,35]. Ribosomal protein genes have a greater potential to reveal variation among closely related strains (Gundersen *et al*., 1996) [36]. Rice Orange leaf (ROL) was identified as belonging to 16SrI-B subgroup by nested PCR assays and *in silico* restriction enzyme analysis by Valarmathi *et al*. 2015 [141].

Heteroduplex mobility assay (HMA)

Heteroduplex Mobility Assay has been employed to detect and to differentiate phytoplasmas (Zhong and Hiruki, 1994) [158] and all results for differentiation of phytoplasmas from HMA were in agreement with those obtained by PCR and in particular RFLP analysis. It has been demonstrated that HMA

provided sensitive differentiation of phytoplasmas when other methods such as RFLP were not readily applicable to differentiate between very closely related 20 phytoplasmas (Ceranic-Zagorac and Hiruki, 1996) [16]. Obviously, HMA combined with PCR will be a very simple, fast, sensitive and reliable method for detection and classification of different strains and groups of phytoplasmas. HMA has been used for differentiation of phytoplasmas in the aster yellows group and clover proliferation group (Wang and Hiruki, 2001) [149]; elm yellows group (Angelini *et al*., 2003) [7] and australian grapevine phytoplasmas (Constable and Symons, 2004) [22]. The investigation on the genetic variability of various isolates of African LYD phytoplasmas associated with Cape St Paul Wilt disease (CSPWD, Ghana), lethal disease (LD, Tanzania) and lethal yellowing (LYM, Mozambique) were done and were also compared to the Caribbean phytoplasma associated with lethal yellowing in cross-linked gels.

Genetic diversity and geographical distribution of phytoplasma

Phytoplasma associated with disease on different plants in India have a wide geographic distribution, but mainly reported so far from north and south parts of the country. So far, these diseases have been identified in 17 different states of India. Ten phytoplasma ribosomal groups were identified mainly from north and south areas while only a limited number of phytoplasma diseases have been recorded in Eastern, Western and Central parts of the country. These diseases affect sugarcane, sesame, ornamentals, oil crops, fruits, palms, vegetables and many weed species. The major number of phytoplasma disease reports, available in ornamental plants followed by weeds, vegetables, spices and medicinal plants, fruit and sugar crops and others. Aster yellows, 16SrI group is the most prevalent and has been associated with 64 plant species followed by 16SrII group (38 plants species), 16SrVI (13 plant species) and 16SrXI (13 plant species) (Rao *et al*., 2017) [55]. Sequencing of 16S rRNA gene allowed to verify the presence of '*Candidatus* Phytoplasma asteris', '*Ca*. P. aurantifolia', '*Ca*. P. ulmi', '*Ca*. P. trifolii', '*Ca*. P. phoenicium', '*Ca*. P. oryzae', '*Ca*. P. cynodontis'-related phytoplasmas as the major 'Ca. Phytoplasma' species present in India. Even though phytoplasma diseases are of common occurrence, only a few of them have been properly studied; therefore, verification of disease incidence, transmission and insect vector identification must be carried out for those not yet studied.

Transmission of phytoplasma by vectors

Host plant range of each phytoplasma is determined mainly by a number of natural insect vector species that are capable to transmit phytoplasma and by the feeding behaviour of these vectors. The overland spread of phytoplasma relies on phloem feeding insects such as leafhoppers (Cicadellidae), planthoppers (Cixiidae) and spittle bugs (Cercopidae). Non-homopteran insects in the genus Halymorpha (Hemiptera: Heteroptera; Tingidae) have also been shown to vector phytoplasmas (Feeley *et al*., 2001) [26]. The phytoplasmas are ingested when insects feed on previously infected plant tissues and probably increase in the midgut of insects and they are then passed to the plant through the insects salivary secretions when feeding on plant tissue (Martinez *et al*., 2000; Maust *et al*., 2003; Weintraub and Beanland, 2006: Alhudaib *et al*., 2009) [78, 82, 154, 4].

Phytoplasmas are known to be transmitted by planthoppers, leafhoppers and psyllids in a persistent, propagative manner (Marzachi *et al*., 2004) [81]. The transmission of phloem-restricted pathogens like phytoplasmas is correlated with the mode of phloem-feeding behaviour. Phloem-, xylem- and parenchyma feeding guilds are not strict categories and especially among vascular-feeder leafhoppers, the distinction between the phloem-feeding and xylem-feeding guilds is blurred (Wayadande, 1994) [151]. Recently, apple aphids were found to be positive in PCR assays for AP phytoplasmas and were suspected to be vectors, but the results of transmission experiments seem to exclude this possibility (Cainelli *et al*., 2007) [15]. A phloem-feeding habit is thus necessary but insufficient for phytoplasma transmission.

'*Ca.* Phytoplasma oryzae' is transmitted by three species of leafhoppers that are found only in Asia (Nakashima *et al*., 1993) [91]: *Nephotettix cincticeps* (Uhler), *N. virescens* (Distant) and *N. nigropictus* (Stal). The phytoplasma can overwinter in leafhoppers and the wild grass *Alopecurus aequalis*, disseminated primarily by the leafhopper. Leafhoppers acquire the phytoplasma by feeding on infected plants for 1–3 h and after a latent period of 20–39 days, the phytoplasma passes from the gut to the salivary gland of the insect. Leafhoppers are then capable of inoculating healthy plants in usually less than 1 h of feeding. The latent period in rice is about 1 month in warm weather and 3 months in cool weather. There is limited evidence for the spread of RYD phytoplasma from rice to other members of the Gramineae, although it is believed to occur through root grafts and occasionally by leafhopper transmission (Jung *et al*., 2003) [50]. The host range of '*Ca.* Phytoplasma oryzae, in nature, may be determined by its vector feeding preferences, which are controlled by biophysical and biochemical mechanisms and ultimately by genetic factors. The plant host specificity may also be due to resistance of a particular plant, since '*Ca.* Phytoplasma oryzae' has not been transmitted to periwinkle or other plants by *Cuscuta* spp. Rice Orange Leaf is transmitted by the leafhopper *Recilia dorsalis* Motchulsky, which also transmits rice dwarf virus and rice tungro virus. These diseases differentiate from ROL on symptoms and geographic distribution. In the Philippines, the ROL phytoplasma is transmitted by *R. dorsalis* in a persistent manner, with an incubation period of 15–33 days (Hibino *et al*., 1987) [43]

In nature, Corn Stunt is transmitted by *Dalbulus maidis* and *Dalbulus elimatus* (Ball) in a persistent and propagative manner (Bedendo *et al*., 2000) [12]. Maize Redness is also associated with the presence of a 16SrXII phytoplasma for which *Reptalus panzeri* (Low) has been identified as the insect vector (Jovic *et al*., 2007) [49]. Only one vector, *Saccharosydne saccharivora* (Westwood), has been proven for SCY group 16SrI in Cuba (Arocha *et al*., 2005a) [8]. In addition, a species of the genus *Cedusa* was found as a putative vector of '*Ca.* Phytoplasma graminis' (Arocha *et al*., 2005b) [9]. *Matsumuratettix hiroglyphicus* (Matsumura) and *Yamatotettix flavovittatus* Matsumura are the known vectors of SCWL (Hanboonsong *et al*., 2002, 2006) [41, 42]. In India, the leafhopper *Proutista moesta* (Westwood) was shown to transmit SCGS and recently *Deltocephalus vulgaris* Dash and Viraktamah has been identified as a potential vector for SCGS (Srivastava *et al*., 2006) [133].

The transmission efficiency of *M. hiroglyphicus* (55 %) is higher than that of *Y. flavovittatus* (45 %) (Hanboonsong *et al*., 2006) [42]. Recent work in Iran has shown that the leafhopper *Exitianus capicola* (Stal), one of the main species

of the Bermuda grass fauna, is both a natural and experimental vector of the BGWL phytoplasma (Salehi *et al*., 2009) [121]. Nested PCR analyses showed that 58.3 % of plants exposed to *Recilia banda* Kramer (Hemiptera: Cicadellidae) were positive for phytoplasma and developed characteristic stunt disease symptoms while 60 % of *R. banda* insect samples were similarly phytoplasma positive (Obura *et al*., 2009) [94].

The phytoplasma infecting rice (Rice Orange Leaf Phyoplasma) was identified as 'Ca. P. asteris' and belongs to 16SrI-B subgroup, it is transmitted by *Recilia dorsalis* in nature (Valarmathi *et al*., 2015) [141]. Kumar *et al*. (2015) [54] suggested that *Exitianus indicus* found to be a putative vector for '*Candidatus* Phytoplasma cynodontis' and may play a role in transmitting 16SrXIV group phytoplasmas in India. Transmission tests and population sampling study further confirmed

that *Maiestas portica* and *Cofana unimaculata* were vectors of the Sugarcane Grassy Shoot (SCGS) phytoplasma from diseased to healthy sugarcane plants. The identification of new vectors of SCGS phytoplasma suggested that these leafhopper species may be responsible for secondary spread of SCGS phytoplasma (Tiwari *et al*., 2016) [139].

Out of five identified leafhopper species from Brinjal Little Leaf infected fields at Uttar Pradesh and Delhi, only *Hishimonas phycitis* was identified as carrier and natural vector of 16SrVI-D subgroup of phytoplasmas by nested PCR assays, sequence comparison, phylogeny, virtual RFLP analysis and transmission assays (Kumar *et al*., 2017) [55]. Among the three major leafhopper species identified, only *Hishimonas phycitis* was identified positive for 16SrI-B and 16SrII-D subgroups of phytoplasmas from chrysanthemum fields at Delhi and jasmine fields at Bengaluru, respectively (Gopala and Rao, 2018) [34].

Management approaches for phytoplasmal diseases

The critical factor for any disease management system is timely and exact identification of plant diseases. At initial stages plant diseases can be successfully managed when preventive measures are applied. Since no effective control measures are available for the phytoplasma associated diseases, effective management practices should be adopted. Conventional management strategies rely mainly on the application of insecticide treatments, rouging of infected plants and production of phytoplasma-free propagation material. However, these strategies are costly and could have undesirable environmental impacts. Several approaches were suggested for the management of phytoplasma diseases and their insect vectors. Unfortunately, not even a single effective control measure has been identified to date. Limited attempt has been made to manage the phytoplasma disease in India. Ajayakumar *et al*. (2007) [3] demonstrated reduction of phytoplasma symptoms by treatment with a tetracycline antibiotic *P. grandiflora* plants at weekly intervals. Earlier, influence of treatment method, duration and concentration of tetracycline on uptake by little leaf affected brinjal plants was demonstrated by Verma and Dubey (1978) [147]. Singh *et al.* (2007) [93] attempted production of phytoplasma-free plants from yellow leaf diseased *C. roseus* by employing *in vitro* chemotherapy with different concentration of oxytetracycline (25-100 mg/ l) for two weeks. An oxytetracycline concentration of 75 mg/l was found optimal for freeing phytoplasma from the infected tissues. About 50% of regenerated plants were phytoplasma-free as confirmed by PCR, and they remained healthy for more than three years (Singh *et al*., 2007) [93]. Elimination of sugarcane grassy shoot disease (SGGS) through apical meristem culture technique for producing clean planting material of sugarcane has been reported (Tiwari *et al.*, 2011) [138]. The results showed that meristems length of 2 and 3 mm were free from the SGGS pathogen. The possibility to contain phytoplasma diseases within an integrated approach include, together with the applied control measures based on clean propagating materials, vector control, and weed management, moreover a stimulation of plant defense can become practically important. A new vector monitoring system has to be developed for identification of phytoplasma vector species, monitor their spread, and to coordinate research into these and other means in which phytoplasmas are spread. Identification of alternative control strategies against these diseases, such as usage of biocontrol organisms or phytoplasma mild strains could also provide effective tools for reducing phytoplasma spread in an environmentally sustainable approach. Breeders should identify the plant varieties showing resistance to the phytoplasma diseases from different regions for the breeding programmes to offer improved hybrid varieties for replanting practices in India.

Conclusion and perspectives

This book chapter presents development and the diagnosis of progress in phytoplasma study and research in India over the past decades. Sesame phyllody, brinjal little leaf, sugarcane grassy shoot, sandal spike, coconut root wilt and arecanut yellow leaf are the major phytoplasma diseases causing serious economic losses to the respective crops throughout the country and hence need immediate attention for the management aspects to reduce considerable losses. A total of 129 phytoplasma diseases are attributed to one of six '*Ca.* Phytoplasma' species. The majority of the reported phytoplasmas have been classified up to subgroup levels on the basis RFLP analysis. Likewise, the largest disease groups are associated with '*Ca*. P. asteris'-related phytoplasmas that should be investigated to determine their phylogenetic diversity. Moreover, some phytoplasmal diseases which were confirmed only by symptoms or transmission electron microscopy observation remain to be identified at molecular levels. Information regarding the vector of the phytoplasma and vector-plant relationships or interactions is necessary for understanding phytoplasma-associated diseases in India needs at most attention. The major thrust area of research in coming years would be complete phytoplasma genome sequencing infecting the majority of crops. Moreover, for understanding host phytoplasma interactions, it is important to identify the function of membrane proteins or secreted proteins and effectors encoded in the phytoplasma genome. The management approaches of widespread phytoplasma disease on major economic crops in India also need attention towards developing resistant genotypes, RNA interference and checking the natural spread of other alternative sources of weed hosts and potential insect vectors are also important information to be achieved. Numerous new phytoplasma strains have been identified in the last decades and a preliminary classification of known and new phytoplasma strains has revealed that phytoplasmas are more diverse than thought. Epidemiological studies should also be carried out to prevent further epidemic spreading of the phytoplasmas in India.

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