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To detect the genetic variability of phytoplasma affecting some weed species in Uttar Pradesh, India

Shoeb Ahmad[1], Akil A Khan[2]
[1-2] Department of Botany, GF College, Shahjahanpur, Uttar Pradesh, India

Abstract
Phytoplasmas cause diseases in various weeds that can act as alternative natural hosts, facilitating the spread of phytoplasmas to other economically important plants and, therefore, increasing economic losses. Suspicious symptoms of phytoplasma of typical leaf chlorosis, witch's broom and small leaf were recorded in sixteen species of weeds in and around agricultural fields in different parts of Uttar Pradesh, India in 2017 to2019. to determine the association of phytoplasma through nested PCR assays using pairs of universal primers specific for phytoplasma, P1 / P7 and R16F2n / R16R2. Of sixteen species of symptomatic weeds collected, ten species of weeds, *viz*. Digitaria ciliaris, Phalaris minor, Eleusine indica, Cynodon dactylon, Parthenium hysterophorus, Dichanthium annulatum, Cannabis sativa, Amaranthus spinosus, Digitaria sanguinalis and Oplismenus burmanni have tested positive for phytoplasma. BLAST and phylogenetic analysis of 1.2 kb products of 16Sr DNA phytoplasma sequences from ten species of positive weeds confirmed the association of three different phytoplasmic groups, i.e. 16SrI, 16SrII and 16SrXIV. Association relationships of phytoplasma with
D. ciliaris (16SrXIV group), E. indica (16SrXIV group) and P. minor (16SrI group) were the newest records of phytoplasma hosts in the world. However, the two weeds, *viz*. P. hysterophorus and O. burmanni have been reported as hosts for the new phytoplasma group (16SrII) in India. This article discussed detailed and updated information on occurrence, symptomatology, molecular characterization, transmission, taxonomy, genetic diversity and phytoplasma management approaches of weeds. Knowledge of the diversity of phytoplasmas will be expanded through recent studies and the availability of molecular tools for the identification of pathogens.

Keywords: genetic variability, phytoplasma, weed species, India

1. Introduction

Phytoplasmas are prokaryotic plant moliclutes that are bacterial pathogens limited by phloem that cause many serious diseases of woody and herbaceous plants all over the world (Bertaccini *et al*., 2014).

In addition to many economic crop species, several weeds are important phytoplasma reserves and play an important role as alternative / natural collateral hosts (Tran-Nguyen *et al*., 2000 [39]; Blanche *et al*., 2003 [6]; Joomum *et al*., 2007; Pasquini *et al*., 2007 [23]; Harrison and Oropeza, 2008 [12]; Mall *et al*., 2010). Over 30 weed species have been reported to host phytoplasmas belonging to four different groups (16SrI, 16SrII, 16SrVI and 16SrXIV) across India and most of them belonged to 16SrI and 16SrXIV phytoplasma groups (Raj *et al*., 2008 a, b, c; Baiswar *et al*., 2010 [3]; Rao *et al*., 2007, 2010, 2011; Mall *et al*., 2011; Tiwari *et al*., 2012; Babu *et al*., 2015) [2].

Early detection of these phytoplasmas associated with weed diseases is very important to verify the possibility of further spread of phytoplasma diseases to other crops. Therefore, this study attempted a detailed study of several pest species in and around agricultural fields during the autumn and spring seasons from August 2017 to April 2019 to report any new pest species as a host of important phytoplasmas that infect crops.

Sixteen species of weeds (Table 1) belonging to the Asteraceae, Poaceae, Cannabinaceae and Amaranthaceae families were collected which show suspected symptoms of phytoplasma

2. Materials and Methods

Total DNA was extracted from synthetic herb samples using the CTAB method (Ahrens and Seemüller, 1992). A nested PCR approach was used for the detection of phytoplasmas from synthetic herb samples collected with P1 / P6 primer pairs (Deng and Hiruki, 1991) [8], followed by nested R16F2n / R16R2 primers (Gundersen and Lee, 1996) [11]. The details of the PCR tests were followed as described by Rao *et al*. (2014).

The nested PCR products (1.2 kb amplicon) were purified using the Pure Link gel extraction kit (Invitrogen, Germany) and sequenced directly in both directions.

The sequences obtained from nested PCR products were assembled using the DNA Baser online tool (www.dnabaser.com).

The consensus sequence was sent to GenBank and used in BLASTn research. A database search for homologous sequences was performed using BLAST analysis at NCBI (http: // ncbi.nlm.nih.gov/BLAST) to compare and construct phylogenesis. The 16D rDNA gene sequences were aligned with the representatives of the phytoplasma groups / subgroups available from GenBank using Clustal (Thompson *et al*., 1994) [37].

The 16Sr DNA sequence generated by the present study and the 19 deformation sequences of the reference phytoplasma recovered from GenBank (Table 2) were used to construct the phylogeny using the MEGA version 6.0 software (Tamura *et al*., 2007) [36] using the neighborhood method with default parameters using 1000 repeats for bootstrap.

3. Detection of Genetic Variability of Phytoplasmas

The first round of PCR amplification did not produce the expected 1.5 kb product of the 16D rDNA region of any of the symptomatic herb samples with the pair of primers P1 / P6 (data not shown). However, an amplification of ~ 1.2kb was obtained in PCR assays nested with R16F2n / R16R2 primer of ten species of symptomatic weeds such as Digitaria ciliaris, Phalaris minor, Eleusine indica, Cynodon dactylon, Parthenium hysterophorus, Dichanthium annulatum, Cannabis sativa, Amaranthus spinosus (L.), Digitaria sanguinalis and Oplismenus burmanni together with positive control (Phytoplasma of herbaceous sugarcane shoots, SCGS, Rao *et al.*, 2014, Fig. 1, Table 1).

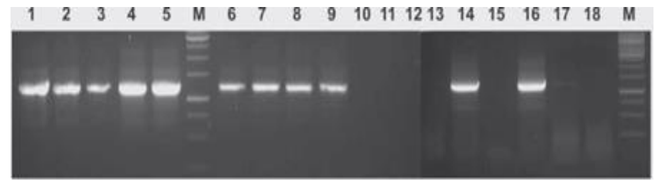

Fig 1: Agarose gel with ~ 1.2 kb of bands amplified by PCR nested with R16F2n/R16R2 primer from symptomatic weed samples (lanes: 1 (Cynodon dactylon), 2 (Phalaris), 3 (D. ciliaris), 4 (Eleusine), 5 (Parthenium), 6 (Oplismenus), 7 (Dicanthium), 8 (Cannabis), 9 (Digitaria sanguinalis) and 14 (Amaranthus) are positive PCR products for symptomatic weeds and Lane 16 (SCGS positive control).

Table 1

Weed species collected	Name of City (state)	Symptoms	Nested PCR result	Identif ied Phytop lasma group
Cynodon dactylon (L.) Pers.	Shahjahanpu r (UP)	White leaf	+	16SrX IV
Phalaris minor Retz.	Hardoi (UP)	Grassy appearance, white leaf	+	16SrI
Digitaria ciliaris (Retz.)	Kanpur (UP)	chlorotic leaf	+	16SrX IV
Eleusine indica (L.) Gaertn	Bharaic h (UP)	chlorotic leaf	+	16SrX IV
Parthenium hysterophorus L.	Luckno w (UP)	Witches‟-broom	+	16SrII
Oplismenus burmanni (Retz.) P. Beauv.	Pilibhit (UP)	White leaf	+	16SrII
Dichanthium annulatum (Forssk.)	Bareilly (UP)	White leaf	+	16SrX IV
Cannabis sativa L.	Rampur (UP)	witches‟ broom and yellowing	+	16SrI
Digitaria sanguinalis (L.)	Morada bad (UP)	White leaf	+	16SrX IV
Amaranthus spinosus (L.)	Aligarh (UP)	yellowing	-	16SrX IV

Presence or absence of phytoplasmas indicated by + and –, UP = Uttar Pradesh respectively, Photograph of the Sample, naturally infected diseased and healthy which used in Survey like 10 isolate from different locations.

Fig 2: Healthy and Infected Cynodon dactylon (L.) Pers.

Fig 3: Healthy and Infected Phalaris minor Retz

Fig 4: Healthy and Infected Digitaria ciliaris (Retz.)

Fig 5: Healthy and Infected Eleusine indica (L.) Gaertn

Fig 6: Healthy and Infected Parthenium hysterophorus L.

Fig 7: Healthy and Infected Oplismenus burmanni (Retz.) P. Beauv

Fig 8: Healthy and Infected Dichanthium annulatum (Forssk.)

Fig 9: Healthy and Infected Cannabis sativa L.

Fig 10: Healthy and Infected Digitaria sanguinalis (L.)

Fig 11: Healthy and Infected Amaranthus spinosus (L.)

Neither direct ("one turn") nor nested PCR analyzes DNA amplified from model DNA isolated from any of the non-symptomatic weeds in the study (data not shown). Of the ten phytoplasma positive weed species, the four weeds, namely D. annulatum, C. sativa, D. stramonium and D. sanguinalis previously reported, were not sequenced and the available 16Sr sequence presented above in GenBank was used for the comparison of sequence analyzes between phytoplasma isolates tested on these weed species. Nested ~ 1.2 kb PCR amplicons from the rest of six weed samples (C. dactylon, O. burmanni, D. ciliaris,P. minor, P. hysterophorus and E. indica) were directly sequenced and sent to GenBank with access numbers. KF760445, KF760446, KJ661543, KJ622368, KJ676961 and KJ661544 respectively. BLAST analysis of nested ~ 1.2 kb ribosomal PCR 16S DNA sequences from C. dactylon, E. indica and D. ciliaris shared a 99% sequence identity with phytoplasma strains of" "Candidatus Phytoplasma cynodontis" (group 16SrXIV), or BGWL (AF248961); Brachiaria herb and white leaf (AB052872). Therefore, these three weed isolates were identified as members of the 16SrXIV group of phytoplasma. The 16Sr sequences of P. hysterophorus and O. burmannii phytoplasma isolates shared a 99% sequence identity with 'Candidatus Phytoplasma aurantifolia' (KF811205), Bushehr sesame (KC429655) and Tylophora indicate a small leaf (KF773149).

While the isolate of phytoplasma P. minor shared the identity of 99% with the phytoplasma isolates of "Candidatus Phytoplasma asteris", that is the yellow of the sugar cane leaves (KJ491100).

The phylogenetic analysis of the 1.2 kb product of the 16S rRNA gene of the six weed species confirmed the results of the BLASTn analysis according to which the herbs D. ciliaris, C dactylon and E. indica shared a close affinity with the group strains 16SrXIV of phytoplasmas, while P. minor shared a close affinity with the 16SrI groups of phytoplasmas and the isolates of P. hysterophorus and O. burmannii belonged to the 16SrII group of phytoplasmas (Fig. 2).

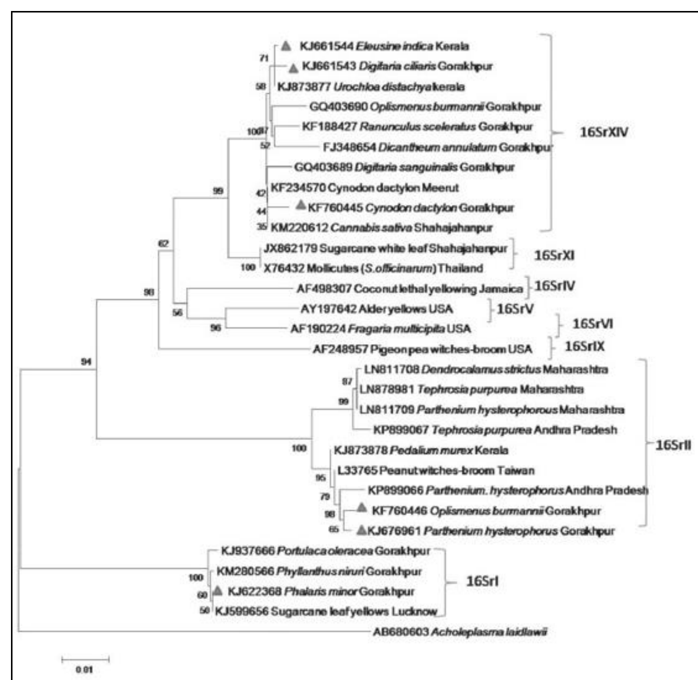

Fig 2: Phylogenetic tree built by the MEGA 6 version using the neighboring binding method that shows the relationships between the different phytoplasmas identified in the weeds. Acholeplasma laidlawii was used as an external group

In the present study, three weed species, e.g. D. ciliaris, E. indica and P. minor, were reported as new phytoplasma hosts in India belonging to the 16SrI and 16SrXIV phytoplasma groups respectively. While P. hysterophorus and O. burmannii were registered as guests of the new phytoplasma group (16SrII) in India. Previously, P. hysterophorous had been reported to host "Ca. The group of P. asteris and
O. burmannii have been reported as guests of" Ca. P. cynodontis group from India (Rao *et al*., 2007, 2010). However, D. annulatum and D. sanguinalis belonged to the 16SrXIV group (Rao *et al*. 2009), while Cannabis sativa belonged to 16SrI (Mall *et al*., 2011) and Datura stramonium belonged to the 16SrVI group of phytoplasma (Singh *et al*. 2012). previously reported as phytoplasmas from India have also been reported positive in our study. In addition to the ten species of phytoplasma positive weeds, the other six species of symptomatic herbs, namely O. corniculatum, O. corymbosa, P. indica, A. aspera, P. fraternus and G. globosa, which clearly showed a phytoplasma, white leaf symptoms, yellowing, witch's broom and grass sprouts were suspected and floral deformation could not produce the desired amplification in PCR tests nested with universal phytoplasma primers that indicated a phytoplasma titer insufficient and / or absence of phytoplasmas in these symptomatic weed test samples (Table 1) To date, over 53 pest species have been reported as phytoplasma hosts worldwide (Tran-Nguyen *et al*., 2000 [6]; Blanche *et al*., 2003 [6]; Mall *et al*., 2011; Duduk and Bertaccini, 2011 [9]; Bekele, 2011; Sunpapao, 2014). Nucleotide sequence comparison studies showed that weeds infected with weeds belonged mainly to five main groups, *viz*. 16SrI, 16SrII, 16SrVI, 16SrXI and 16SrXIV. Among these, the 16SrI, 16SrII and 16SrXIV phytoplasmas have a greater presence in nature all over the world (Mall *et al*., 2011; Bertaccini and Duduk, 2009) [5]. In India, phytoplasmas of the 16SrXIV group ("Candidatus Phytoplasma cynodontis") are reported as the main group associated with weed species followed by the group of yellow aster phytoplasmas (16SrI) (Mall *et al*., 2004, 2010).

4. Genetic Diversity Weed Species in India

The phytoplasmas of weeds show a wide geographical distribution (Table 2). So far, more than several species of pests have been reported to have phytoplasma infections worldwide. Nucleotide sequence studies have shown that phytoplasmas that infect weeds belong mainly to the main groups.

Table 2: 16Sr phytoplasma DNA sequences used for phylogenetic relationship studies of the weed phytoplasma strains identified in the present study.

Weed species	Phytoplasma group identified	Accession no.
Eleusine indica	16SrXIV	KJ661544
Digitaria ciliaris	16SrXIV	KJ661543
Cynodon dactylon	16SrXIV	KF760445
Oplismenus burmanni	16SrII	KF760446
Parthenium hysterophorus	16SrII	KJ676961
Phalaris minor	16SrI	KJ622368
Cannabis sativa L	16SrI	GSE56964
Amaranthus spinosus (L.)	16SrXIV	GQ403690
Dicanthium annulatum	16SrXIV	FJ348654
Digitaria sanguinalis	16SrXIV	GQ403689

5. Management

Currently, there are no more effective methods for treating phytoplasma diseases. The impact of these diseases depends on several factors, such as the virulence of the strains within a given taxon and their tendency to change, the presence and dynamics of the vectors, the concentration of phytoplasma in the host plants and the vectors of insects and environmental conditions and agronomic practices. Consequently, it is not possible to adopt a single control strategy. The most important factors to consider before intervening are: the severity of the disease, whether to plant infected or not plants, routing strategies, availability of vector insects, alternative reservoirs of sensitive plants and the economic impact of the disease (Osler and Carraro, 2004).
The management of these diseases, the use of disease-free plant propagation material is very important, since phytoplasmas spread mainly through the grafted parts of infected plants. A study was also conducted on the effectiveness of plant bodies directed against phytoplasmas. Tetracyclines are bacteriostatic for phytoplasmas and inhibit their growth (Singh *et al*., 1978). However, without continued use of the antibiotic, the symptoms of the disease will reappear. Therefore, tetracycline is not a valid control agent in agriculture, but is used to protect ornamental cocci. It has been a long time since heat therapy has helped eliminate pathogens such as viruses and possibly phytoplasma from plants and mainly from fruit trees (Kunkel, 1936; Kassanis, 1954; Nyland, 1962). For the elimination of phytoplasma, Laimer and Balla (2003) reported a combination of heat therapy and culture of meristems without treatment with tetracycline. These strategies can be applied in the case of infesting infoplasma. Weed phytoplasmas can be best managed by eliminating perennial and biennial weeds, stripping and destroying symptomatic plants, avoiding the planting of sensitive crops together with the crops that host phytoplasmas, the control of buzzer crops in the crop and herbs close to the start of the season (Welliver *et al*., 1999).

6. Conclusion

In the present study, we reported new hosts of phytoplasma weeds that could play a role as a reservoir for phytoplasma. The potential reservoir of phytoplasmas in Indian weed species could be even greater, for which more regular studies would be needed in different areas. It is also important to note that 16SrI group phytoplasmas have been reported in a large number of important agricultural plant species in India (Rao *et al*., 2010; Kumar *et al*., 2014). Therefore, the ratio of P. minor as an additional host for the 16SrI group of phytoplasma can play a key role in the transmission of the 16SrI group phytoplasma from weeds to important crop species and vice versa through vector species of leafhopper.

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