Australasian Plant Pathology (2023) 52:293–302
https://doi.org/10.1007/s13313-023-00919-7

ORIGINAL RESEARCH ARTICLE

# First report of DNA barcoding, phylogenetic analysis and transmission study of *Medicago sativa* phytoplasma (16Sr-II-D) and associated insect vectors in Pakistan

**Jam Nazeer Ahmad[1] · M. Z. Sharif[1] · P. Trebicki[2] · S. Tanwir[3]**

Received: 20 October 2022 / Accepted: 19 April 2023 / Published online: 29 May 2023


## Abstract

*Medicago sativa* is a good fodder crop in Pakistan. Its continuous cultivation and sustainable production is important for the better growth and yield of animals. Phytoplasma is an important well spread disease worldwide which has been reporting from more than 150 years ago on different plants including Lucerne or Alfalfa *(Medicago sativa).* The study was conducted to identify phytoplasma and potential insect vectors responsible for its transmission. The sampling of infected plants and sucking insects was done from Multan, RY Khan and Faisalabad, Punjab during 2017–2020. The 50 symptomatic samples were observed for the detection of phytoplasma initially through staining and electron microscope. It was further confirmed by Nested Polymerase Chain Reaction (Nested-PCR), Sequencing and Phylogenetic Analysis. The results indicated the presence of phytoplasma bodies in sieve tube cells of the infected plants that were further confirmed by the amplification of 1.8 kb and 1.2 kb fragment of 16 S rRNA gene using the primer pairs P1/P7 and RI6F2n/R2 respectively. Restriction fragment length polymorphism (RFLP) analysis also showed a similar pattern of bands formation associating with 16 S rRNA of 16SrII-D subgroup linked with sesame phyllody (16SrIID) group. The obtained DNA sequences of Pakistani isolates were submitted on NCBI (MT614018.1 and MT614019.1). The phylogenetic analysis using Clustal W and MEGA6 software showed that submitted sequences have > 99% nucleotide identity with phytoplasma strain "*Ca.* P. *australasia*" of 16SrII-D subgroup. The potential insect vectors, *Orosius orientalis, Orosius argentatus, and Laudelphax striatellus* captured from infected plants were detected positive as well as transmission study confirmed their vector status for alfalfa phyllody diseases transmission. To our information, this is first detection of phytoplasma infestation and its insect vectors associated with *Medicago sativa* in Pakistan. The 16Sr-II D group of phytoplasma is spreading widely in many crops so, control is essential to stop it into other economically important crops.

**Keywords** Alfalfa or Lucerne · *Medicago sativa* · Insect vectors · 16SrII-D rRNA phytoplasma · DNA sequencing · Nested PCR

## Introduction

In Pakistan, *Medicago sativa* L, commonly known as Alfalfa or Lucerne is cultivated for the purpose of fodder, pasture as well as seed production. The highly yielding and diseases resistant varieties are very important for its sustainable production. Other than yield limiting factors, phytoplasmas is very devastating bacterial diseases causing severe loss worldwide in different crops. The sap sucking insect-vectors are the main reason of such bacterial transmission having link with multiple symptoms (Gopurenko et al. 2016).

The most worldwide dominant disease in Lucerne is witches' broom (Khan et al. 2002). Sugar beet and alfalfa witches'-broom caused by 16SrII related phytoplasmas are economically significant diseases in Iran (Salehi et al. 2005) and Saudi Arabia (AL-Saleh et al. 2014).

Phytoplasma has been reported to infect vegetation in various countries like Stolbur phytoplasma in Italy (Marzachi et al. 2000) and Serbia (Starovic et al. 2012), little leaf phytoplasma in India (Suryanarayana et al. 1996) while AY phytoplasma

✉ Jam Nazeer Ahmad
jam.ahmad@uaf.edu.pk

[1] Dr. Jam Laboratory, Department of Entomology, University of Agriculture, Faisalabad, Pakistan

[2] Agriculture Victoria Research, Bundoora, Australia

[3] Plant Stress Physiology and Molecular Biology Lab, Department of Botany, University of Agriculture, Faisalabad, Pakistan

from USA (Peters et al. 1999). In addition phytoplasma is documented serving as a reservoir for causing yellows phytoplasma infection in canola (Wang and Hiruki 2001). The recognition of phytoplasmas has been achieved depending upon several factors, of those more important are host choice, symptomatology and vector explicitly, but such kinds of methodologies are not suitable especially for the purpose of confirming genomic connection among various phytoplasmal strains. Staining through light microscopy has been defined as a simple and quick method for diagnosis of phytoplasma diseases (Deeley et al. 1979). For detection of diversified strains of phytoplasma, DNA-dependent techniques have been employed, of that DNA-DNA hybridization using cloned probes as well as reactions including PCR is engaged (Bertaccini et al. 1990; Bonnet et al. 1990; Lee and Davis 1998; Lee et al. 1990). This study was conducted first time to detect and characterize the group of phytoplasma and associated insect vectors responsible for its transmission in the fields of Lucerne field in Punjab, Pakistan.

## Materials and methods

### Field surveys and sample collection

During survey 2017–2020, visual examination of 100 plants following a W-pattern (method of sample collection or observation) was done and infected alfalfa plants exhibiting the phytoplasma symptoms (Fig. 1) were collected from Faisalabad, Multan and R Y Khan Regions of Punjab, Pakistan. The collection of different insects from Lucerne (Alfalfa) fields was done with the help of aerial net. Leafhoppers such as *Orosius orientalis, Orosius argentatus, Laudelphax striatellus, Bemesia tabaci, Empoasca spp* and others were collected (Table 1) and brought to Dr. Jam Laboratory to process for molecular detection and identification of phytoplasma. These potential insect vectors were identified under microscope morphologically by Dr. am Nazeer Ahmad. Different insect vectors causing phytoplasma diseases in Lucerne crops globally are shown in Table 2.

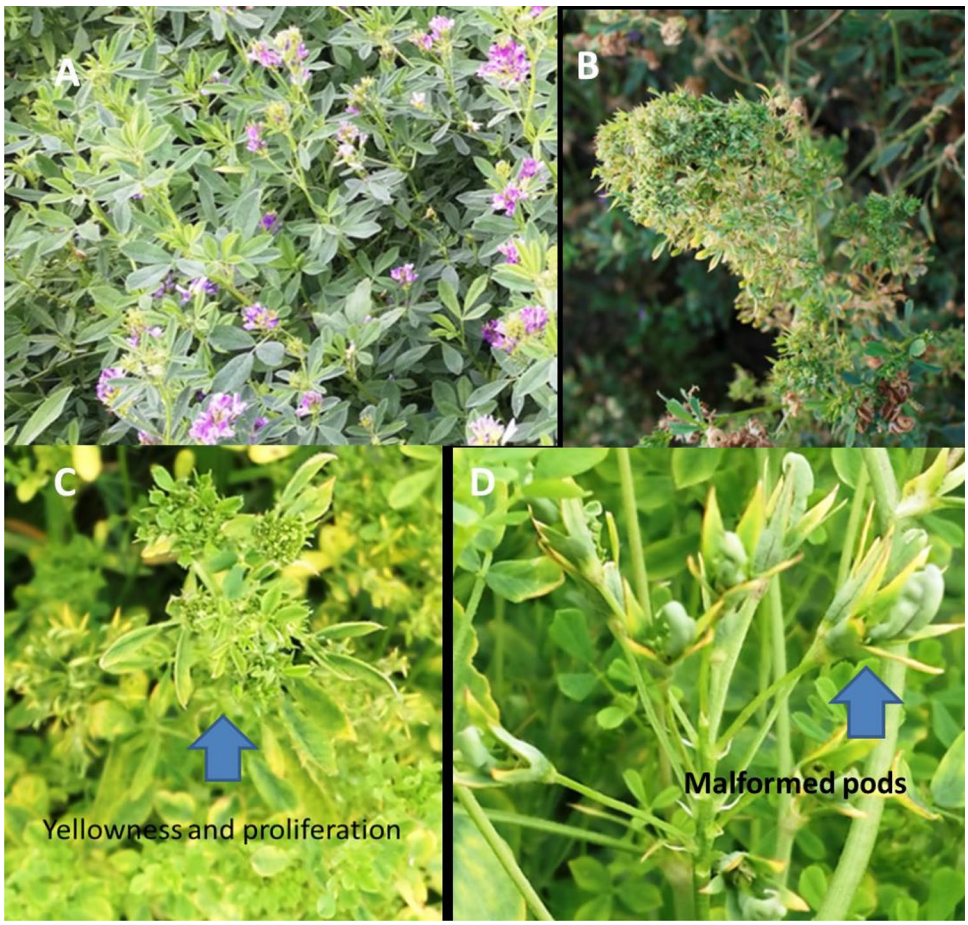


**Fig. 1** **A**, **B**, **C**, **D** Healthy and Phytoplasma infected *Medicago sativa* (Alfalfa or Lucerne) plants in fields at Faisalabad of Punjab, Pakistan during 03-02-18. **A** Healthy plant, **B** which's broom, **C** yellowness and proliferation of leaves, **D** malformed pods formation

**Table 1** Number of different insects captured from *Medicago sativa* (Alfalfa) field crops of Punjab Province during 2017–2020

| S. No. | Insect species | (no.) PCR +/Total Faisalabad | (no.) PCR +/Total Multan | (no.) PCR +/ Total RY Khan |
|---|---|---|---|---|
| 1 | *Orosius argentatus* | 12/20 | 15/30 | 8/30 |
| 2 | *Orosius orientalis* | 13/25 | 10/25 | 14/25 |
| 3 | *Exitianus Spp.* | 0/20 | 3/20 | 0/30 |
| 4 | *Empoasca spp.* | 11/20 | 13/40 | 8/40 |
| 5 | *Bemesia tabaci* | 0/25 | 0/40 | 0/30 |
| 6 | *Amrasca devastans* | 5/20 | 10/20 | 12/20 |
| 7 | *Laudelphax spp.* | 10/20 | 11/20 | 9/30 |
| 8 | *Austroagallia spp.* | 6/30 | 3/20 | 5/30 |

## PCR detection and transmission tests

Insect vectors, *Orosius orientalis, Orosius argentatus, L. striatellus, and Bemesia tabaci* were directly captured from infected fields and surroundings and employed for Nested PCR. For transmission test, *O. orientalis, Orosius argentatus, L. striatellus and Bemesia tabaci* were maintained on healthy periwinkle plants for one week to ten days. Then a batch of fifteen to twenty five insects per plant feeding on infected plants was transferred to 10 caged healthy Lucerne seedlings (4-week-old) for an inoculation access period of 7 days. Insects were then killed after the inoculation feeding period with Confidor (0.8 ml/l H2O) and stored at -20 °C temperature. In addition, a similar set of *Medicago sativa* (Lucerne or Alfalfa) plants was inoculated using the same insects fed on healthy Lucerne plants. Later, tested plants were observed on a weekly basis for appearance of symptoms linked to phytoplasma infection.

## Detection tests: light microscopy

Before molecular identification, for quick detection, hand sections of phytoplasma infected samples of *Madicago sativa* were stained with the assistance of toluidine blue for the period of 15 min at room temperature. Then dipped in distilled water awaiting the water turn clear. When air dried, incubation of sample was carried out in 99.5% ethanol for 0.5 to 15 min by means of 0.5-min intervals to eliminate the dye from the pathogen cells but not from the plant material (Shinkai and Kobayashi 2007). Finally, the examination of section stained by Toluidine was undertaken by using light microscope at a magnification of 40X.

**Electron microscopy** The few infected samples from the batch of collected plants used for light microscopy were also used for electron microscopic detection. For this purpose, healthy and infected samples of the stem of *Medicago sativa* enriched

**Table 2** List of phytoplasma associated insect vectors reported for *Medicago sativa* crop from different countries

| S. No | Insect vectors | Vectored diseases | Country | References |
|---|---|---|---|---|
| 1 | *Orosius argentatus* (Evans) | Australian Lucerne yellows (ALuY) | Australia | Pilkington et al. (2004) |
| 2 | *Austroagallia torrida* (Evans) | Tomato big bud (TBB) | Australia | Pilkington et al. (2004) |
| 3 | *Orosius orientalis* (Mats.) | - | Australia | Gopurenko et al. (2016) |
| 4 | *Austroagallia avicula* | Oman Alfalfa Witches broom (AlfWB) | Oman | Khan et al. (2003) |
| 5 | *Empoasca decipiens* | Papaya yellow crinkle | Oman | Khan et al. (2003) |
| 6 | *Aceratagallia* sp.<br>*Neokolla hieroglyphica*<br>*Macrosteles fascifrons* | Alfalfa Witches broom (AlfWB) | Canada | Khadhair et al. (1997) |
| 7 | *Orosius argentatus* (Mats.)<br>*O. orientalis*<br>*Laudelphax striatellus* | Pakistani Alfalpha phyllody phytoplasma (PAPP) | Pakistan | This study |

in water agar were processed overnight in 5% of glutaraldehyde with 0.2 M PIPES buffer (pH 7.4). Then they were post-fixed in 1% of osmium tetra oxide for the time period of 18 h. at room temperature. The samples were treated with uranyl acetate (5%) for the period of 16–18 h. after washing with distilled H2O. After washing, dehydration was performed using absolute ethanol and entrenched in Spur resin at the temperature of 70 °C for duration of 48 h. The pieces of 120 nm were cut with TRMC MT 7000 ultra-micro- tome and then picked on copper grids. The ultra-thin pieces were stained with uranyl acetate (5%) for 30 min and lead citrate for 10 min respectively. was applied. At the end, observations were made through application of JEOL JEM1010 transmission electron microscope functioning at 80 KV.

### DNA extraction and nested PCR

Extraction of DNA (0.5 g) of symptomatic and non-symptomatic caged plants as well as insect vectors was carried out using CTAB extraction protocol described by (Doyle and Doyle 1990). For every single plant used as test, 2 samples were taken from various portions of plant. Each sample containing 3 leaf midribs was used for DNA extraction. Overall reaction mixture (50 µl) for PCR comprising 1 µl template DNA, Taq polymerase (1.25 units) and buffer comprising MgCl2 (1.4 mM), primers (0.4 µM) and dNTP (0.1 mM). For first round PCR, universal primer pair P1/P7 described by (Deng and Hiruki 1991; Kirkpatrick and Smart 1995) while in case of nested-PCR primers pair RI6F2n/R2 described by (Gundersen and Lee 1996) was used for phytoplasma detection. Conditions applied for PCR cycling were: 1 min of denaturation at 95 °C heat (2 min duration for initial cycle), 1 min annealing process at 55 °C temperature & 1.5 min time for process of extension at temperature of 72 °C for thirty five cycles. Lucerne linked phytoplasmal DNA product extracted from those plants showing phytoplasma associated symptoms and sterile dH2O were applied as positive & negative controls correspondingly. After completion of every nested PCR investigation, PCR product of 2 µL were analyzed with the aid of electrophoresis on agarose gel (1%) and stained with ethidium bromide, then pictured under ultraviolet light by gel documentation system. The collected samples of *Medicago sativa* showing symptoms and surrounding captured insects were tested for Nested-PCR using universal primer pair P1/P7 followed by R16F2n/R16R2 (Fig. 4).

### RFLP analysis

Nested-PCR products of 7uL (1.25 kbp) from 16 S ribosomal DNA) from three isolates of various lucerne fields of Faisalabad, Multan and RY Khan were individually digested by employing *Alu*I, *Hpa*II, (restriction enzymes) regarding manufacturer's guidelines at the temperature of 37 °C overnight. Then, electrophoresis of digestion products was done by means of agarose gels (3%) and pictured or visualized later the staining with illuminating chemical "ethidium bromide" by ultraviolet trans illumination under Gel Documentation System. The resulting patterns of restriction fragments length polymorphism (RFLP) were matched with those already searched and documented for 16 S rRNA of some other phytoplasmas (Marcone et al. 2000).

### Nucleotide sequencing and phylogenetic analysis

Amplification of the product obtained with nested polymerase chain reaction (1.25-bp) of phytoplasma infected plants was achieved through commercial kit and then sequencing was done by Macrogen, Korea. The sequenced DNA fragments of phytoplasma were aligned & examined working with Lasergene v. 7.1 software package (DNASTAR, USA). Phylogenetic analysis of DNA sequence data was performed using a neighbor joining algorithm through MEGA6 (Tamura et al. 2007). The 16 S rRNA gene sequences of several phytoplasma groups applied for comparisons were recovered from website of GenBank database (www.ncbi.nlm.nih.gov) (Fig. 7).

## Results

### Symptomatology of diseases

Characteristic symptoms were detected in Lucerne cultivated in different regions of Pakistan. The naturally occurring symptomatic plants in the fields showed leaf yellowing, stunting, and abnormal flower development. Shoot proliferation and yellowing of leaves, small leaves, phyllody, witches broom and malformed pod malformation like symptoms was observed in more than 70–90 week-old plants (Figs. 1 and 2). It was observed that ratoon crop or alfalfa fields left for seed production purposes was severely infected with phytoplasma diseases as compared to fresh crop. In 2017–2020, such symptoms were prevalent on large scale in fields *Medicago sativa* surveyed in several areas of Faisalabad and Multan. Hence, a widespread survey was carried out to determine the prevalence of this syndrome in Pakistan and to isolate & investigate probable relationship with infection connected to phytoplasmas.

### Microscopy examination

The toluidine stained hand cutting sections of the midrib of *Medicago sativa* leaves was carried out using light microscope (Micros, Austria). Figure 3 showed the navy blue colored scattered zones showing presence of the


**Fig. 2** **A**, **B**, **C** *Medicago sativa* (Lucerne or alfalfa) healthy harvested and phytoplasma infected (seed crops) fields visited at Multan during 30-06-2018 in Punjab, Pakistan. **A** Lucerne field showing phytoplasma infected plants, **B** Lucerne infected seed crops field, **C** healthy field

phytoplasma in infected samples. The phloem of sections taken from samples of healthy plants remained unstained. Further, electron microscopy observation of infected tissues exhibited 200–600 nm in diameter the pleomorphic bodies of phytoplasma that were restricted to the sieve tube elements but healthy samples were lacking such type of bodies (Fig. 4).

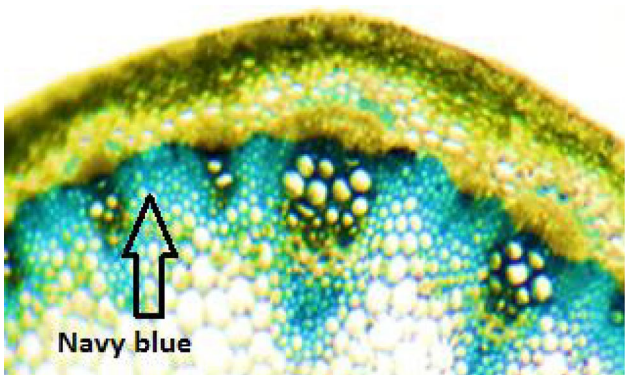


**Fig. 3** Phytoplasma detection through light microscopy by using Toludine staining's in a thin cross section of *Medicago sativa* leaf midrib. Navy blue in cross section shows presence of phytoplasma. Magnification 40X

## Nested PCR detection and RFLP analysis

Regarding PCR detection, 15 out of 18, 13 out of 20, and 10 out of 20 symptomatic *Medicago sativa* samples collected from Faisalabad, Multan and Rahim Yar Khan respectively were positive. The low ratio of PCR detection in field collected samples from Rahim Yar Khan could be due to similar yellowness or small leaves symptoms induced by nutritional deficiency or other abiotic factor. Table 1 showed that *O. argentatus, O. orientalis*, (A) *devastans*, and *L. striatellus* were positive and exhibited bands amplification of 1.2 kb whereas *(B) tabaci* and some other leafhoppers were found to be PCR negative. Digestion of 1st PCR containing 1.2 kb product (using P1/P7 primer pair) & 2nd Nested-Polymerase chain reaction products (using R16F2n/R16R2 primers) (Figs. 5 and 6) from affected *Medicago sativa* plants were undertaken by employing the restriction enzymes (*Hpa*II and *Alu*I). The

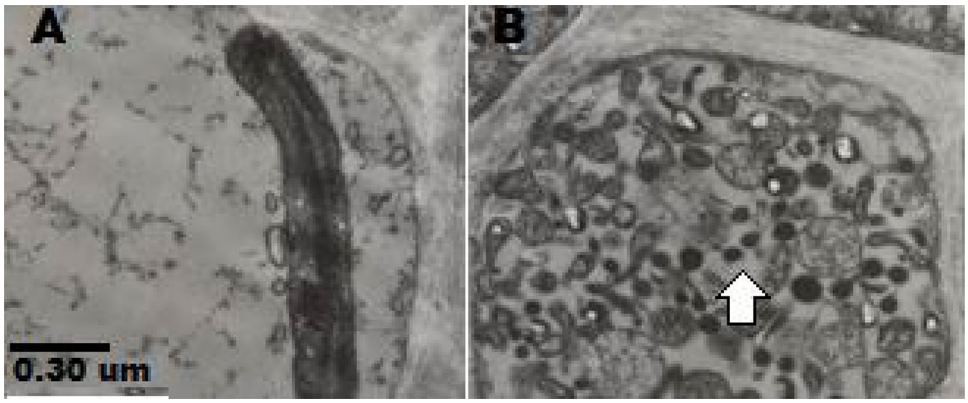


**Fig. 4** **A** healthy cell, **B** phloem cell of phytoplasma affected *Medicago sativa* plant showing phytoplasma bodies (bar = 0.30 µm) in all sieve tube elements observed under electron microscope

resultant pattern of RFLP (Fig. 6) was found consistent with the profile of sesame phyllody of 16SrII-D subgroup phytoplasma used as reference strain.

## Sequences and phylogenetic analysis

Sequencing of nested-PCR products (P1/P7 and RI6F2n/R2) of *Medicago sativa* plants was carried out and then compared the nucleotide identity between one another and with some another groups & subgroups of phytoplasmal 16 S rRNA available in Genbank. The identified isolates or strains of Pakistani phytoplasma associated with *Medicago sativa* (MK611418.1 and MK611419.1) were designated as Alfalfa or (Lucerne) phyllody phytoplasma (Alfph) IGCDB isolate and UAF isolate respectively. The percentage nucleotide identity of both isolates exhibited > 99% sequence identity (Fig. 7) with "*Ca.* P. *australasia*" of 16Sr II-D subgroup. Numerous phytoplasma strains and their groups/subgroups reported along with their accession numbers are presented in Fig. 7.

## Transmission study

Transmission trials were only conducted by *Orosius argentatus*, *O. orientalis*, *Bemicia tabaci and Laudelphax spp.* under green house and insect controlled cages because

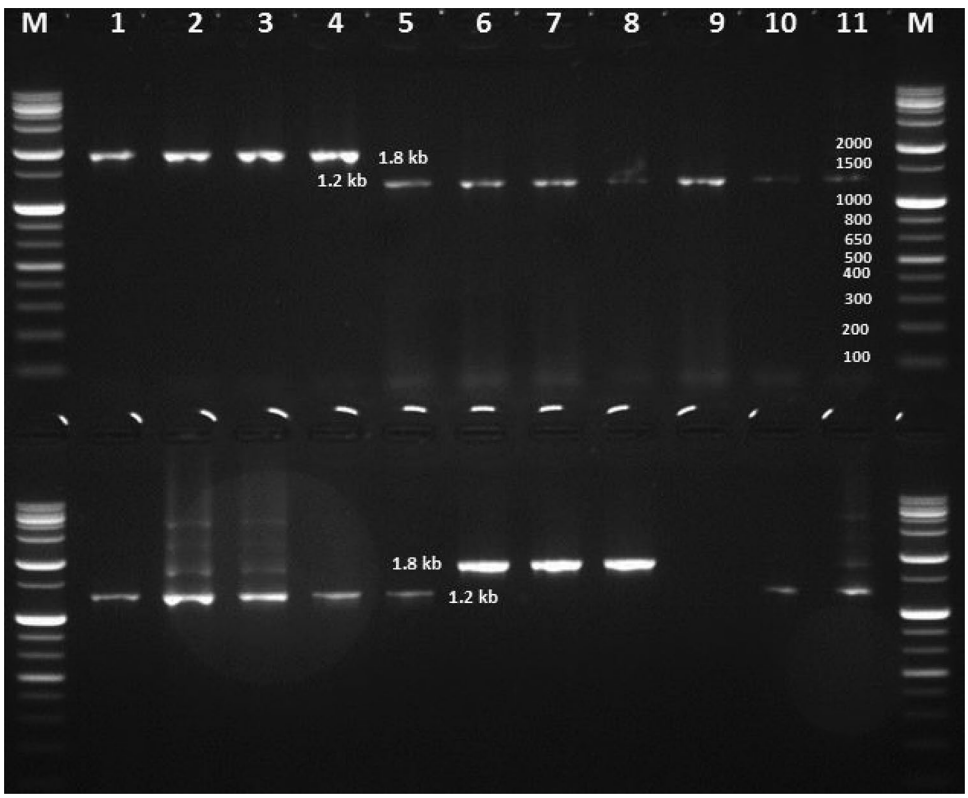


**Fig. 5** Nested-PCR of the 16Sr RNA gene by using universal primers pairs P1/P7 (1.8 kb) followed by R16F2n/R16R2 (1.2 kb) primers. Upper gel: Wells 1–11 infected lucerne plants; Lower gel: wells 1–11 infected insect vectors; 9 is the negative control (water), Well M 1 kb DNA Ladders, Electrophoresis was conducted in 1.5% agarose gel stained with ethidium bromide (1 µg µL-1) in the TAE 1X buffer

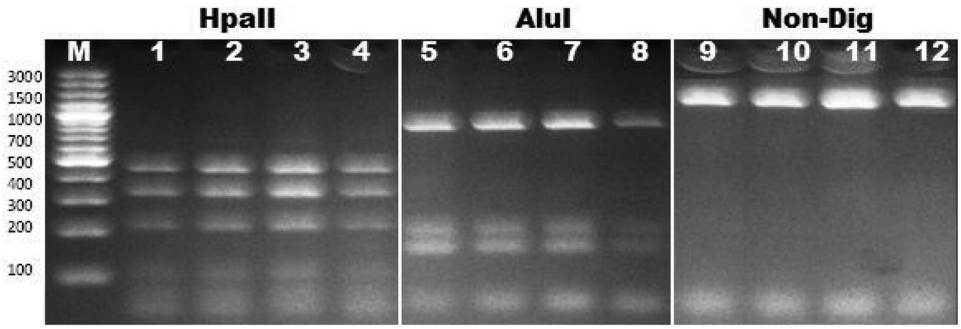


**Fig. 6** Restriction fragment length polymorphism (RFLP) analysis with restriction enzymes (*HpaII* and *Alu*I). M) Molecular weight DNA Ladders (100 bp Invitrogen); wells contain the nested PCR products from the *Medicago sativa* (Alfalfa or Lucerne) samples digested with the HpaII (1–4 wells), none digested (9–12 wells), AluI (5–8 wells). The wells 4, 8 and 12 contained nested PCR products from already identified sesame infected by 16SrII-D (reference strain) phytoplasma. Electrophoresis was conducted in 3% agarose gel stained with ethidium bromide (1 µg µL-1) in the TAE 1X buffer

maximum abundance of these insect vectors were observed during different stages of crop growth as well as their ability to transmit various diseases in different crops. Among them, *O. argentatus*, *O. orientalis, and Laudelphax spp.* were successful, while, *Bemesia tabaci* remained unsuccessful to transmit phytoplasma in Lucerne plantation. The maximum transmission of phytoplasma was noticed when *Medicago sativa* plants were fed on by 30 *O. argentatus, 25 O. orientalis and 20 Laudelphax* respectively. Out of 15 transmitted *Medicago sativa* plants each, 10 exhibited symptoms for *O. argentatus, 12 for O. orientalis* and 5 for *Laudelphax* spp. after 40–50 days of transmission respectively. The transmission for others potential leafhoppers is under trial.

## Discussion

Based on main syndrome symptoms and reaction with toluidine stain, navy blue colored is observed in frequently scattered areas of the phloem zone consisted with similar study conducted by various researchers (Salehi and Izadpanah 1992; Ahmad et al. 2017). In current study, *Orosius species and Laudelphax* were found to be fully capable of transmitting this pathogen to healthy plants. Previous studies showed that 'Ca. Phytoplasma aurantifolia' was detected in leaf hopper "*O. orientalis* (Matsumura)" collected from the Lucerne (*Medicago sativa*) field (Gurr and Gurr 2007). On the other hand, Gopurenko et al. (2016) stated that the presence of phytoplama was not marked among the phloem sucking insect vectors (*A. torrida*, *O. argentatus* and *O. orientalis*) sampled in locality of the phytoplasma infected Lucerne. Moreover, *E. indicus* collected from Bermuda grass in India was reported as phytoplasma positive and a potential vector of phytoplasma group 16SrXIV (Kumar et al. 2015) while PCR positive samples of *E. indicus* collected from *Parthenium hysterophorus* were also detected (Yadav et al. 2015). Bressan and Purcell 2005 confirmed *Deltocephalus flavicosta* as vector of phytoplasma subgroup 16SrIII-A in Canada.

Overall plants fed on by *Orosius species and Laudelphax* exhibited symptoms of phytoplasma infection and the symptoms observed were shoot proliferation, slight leaves yellowing, stem tillering and floral abnormalities (phyllody). The *Medicago sativa* plants fed on by whitefly *(Bemecia tabaci)* did not showed any significant symptoms of phytoplasma infection. There are some other insects which were reported as potential vectors of phytoplasma transmission in *Medicago sativa* crop of different countries as *Austroagallia avicula* was reported as vector of alfalfa witches broom (AlfWB) in Oman, while *Aceratagallia* sp., *Neokolla hieroglyphica* and *Macrosteles fascifrons* were reported as vector of Alfalfa (*Medicago sativa)* Witches broom (AlfWB) in Canada (Khan et al. 2003; Khadhair et al. 1997). Further, some others insect vectors have been mentioned in Table 2. More than 300 diseases associated with phytoplasma in various species of plant belonging to field crops, vegetables, weeds and trees as well as in their insect vectors have been documented (Parrella et al. 2008). While, the authors have also reported the phytoplasma occurrence in symptomatic *Brassica*, fenugreek, sunflower various vegetables and weeds from Pakistan (Ahmad et al. 2015a, b, c, 2017; Sharif et al. 2019; Malik et al. 2020; Aslam et al. 2021). Previously, it was also found that the stolbur phytoplasma affect the methylation status and genes expression regulation in infected tomato plants causing abnormal growth and development (Ahmad et al. 2013, 2014). The sequence analysis of 16S ribosomal RNA has been employed to study the phylgenetic study of

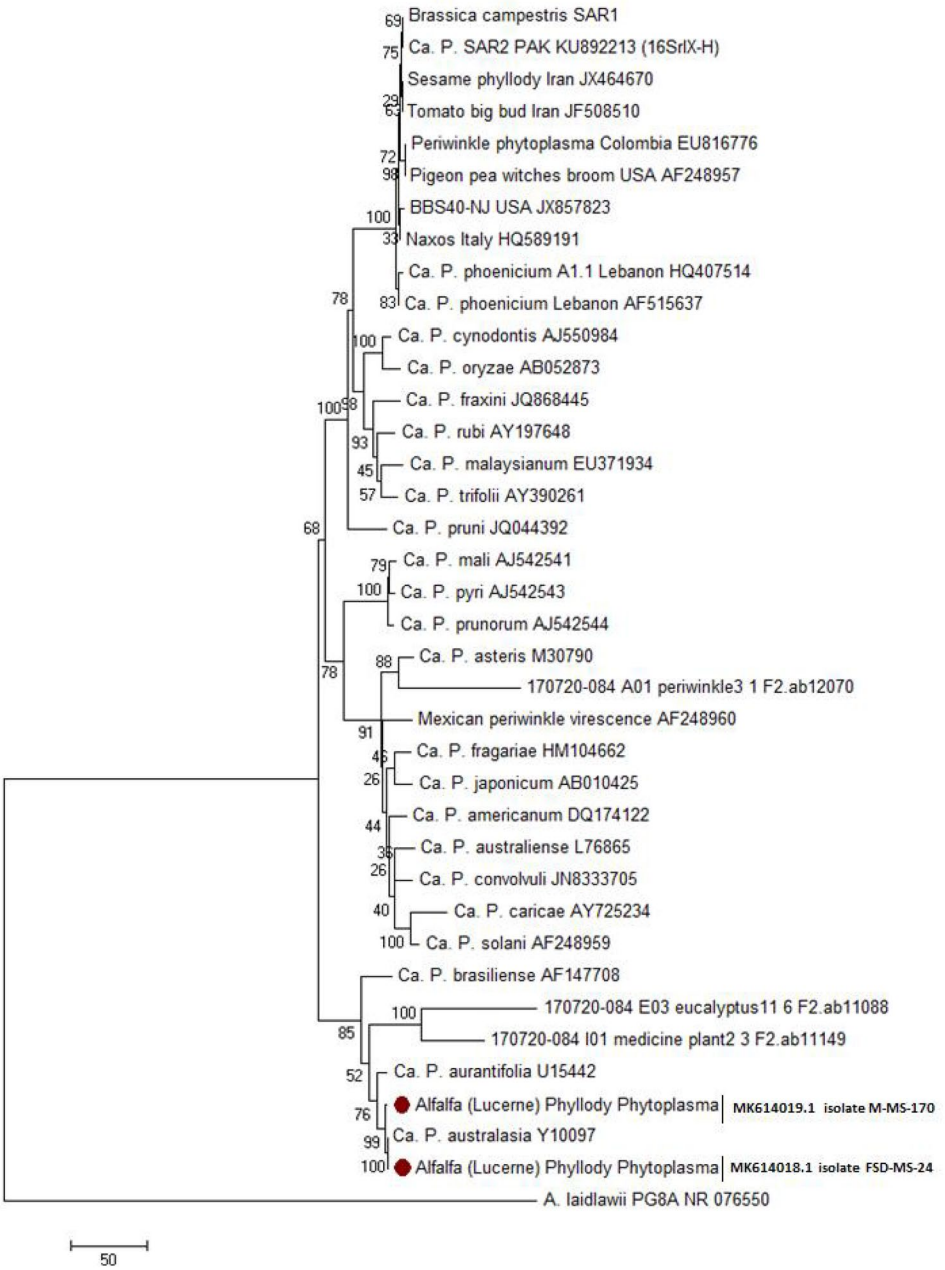


**Fig. 7** Construction of a phylogenetic tree through multiple alignments of nucleotide sequences of genes (16 S rRNA) for isolates of *Medicago sativa* (Alfalfa) from Pakistan (MK611418.1 and MK611419.1) phyllody phytoplasma and other countries achieved from the GenBank database using MEGA6 software by using a methodology designated as "neighbour joining method"

phytopathogens (Seemuller et al. 1994). In current study, the pattern achieved from RFLP of *Medicago sativa* phytoplasma was found to be the same as in sesame phyllody (reference strains), illustrative of 16SrII-D subgroup. This 16SrII-D subgroup was also reported as causative agent of sesame phyllody in India, Pakistan and Turkey (Pamei and Makandar 2016; Ikten et al. 2014; Aslam et al. 2021). The phylogenetic analysis exhibited close association (>99% sequence identity) of current identified Lucerne (*Medicago sativa*) isolates with "Ca. P. *australasia*" strain. It is also reported that '*Candidatus* species' of phytoplasma including '*Ca*. P. *aurantifolia*' and'*Ca*. P. *brasiliense*' had close association with Pakistani phytoplasma isolates (Zreik et al. 1995; Montano et al. 2001). Such groups and subgroups were also reported to infect papaya, Pale Purple Coneflower (Pearce et al. 2011), and tomato plants (White et al. 1998) in Australia but the strains have not been differentiated so far on the basis of genetics. Phytoplasmas from various groups have been documented to be connected with disease in cucurbits (Montano et al. 2000, 2006, 2007). This examination provides evidence that *O. orientalis, O. argentatus and laudelphax* are potential vectors for the Lucerne associated phytoplasma. This is the first report of the vector status of this leafhopper in Lucerne crop of

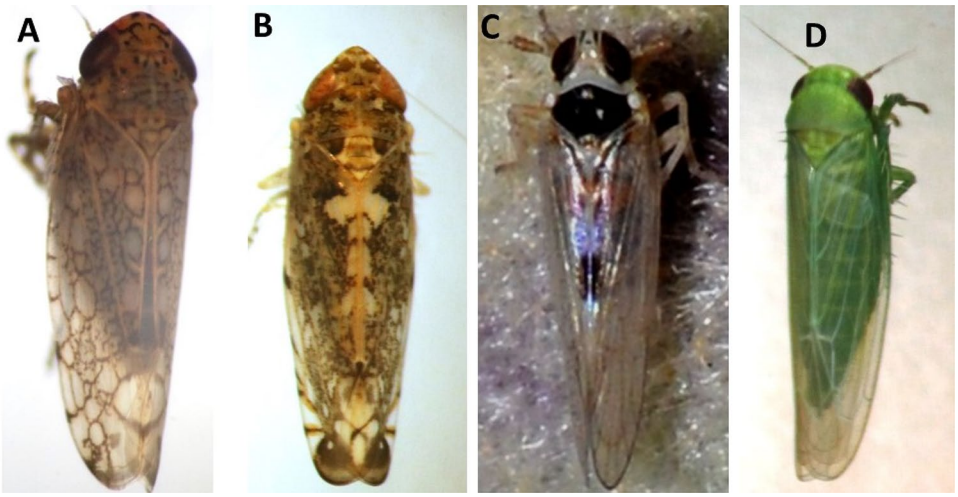


**Fig. 8** *Medicago sativa* phytoplasma-containing leafhoppers detected by nested PCR and 16-23 S rRNA genes sequencing **A** *Orosius orientalis;* **B** *Orosius argentatus;* **C** *Laodelphax striatellus;* **D** *Empoasca spp*

Pakistan. The current studies also confirmed that the Pakistani phytoplasma isolates triggering Lucerne plant infections are members of subgroup 16SrII-D clade connected with phytoplasma 16S rRNA gene and RFLP classification with accession numbers (MK611418.1 and MK611419.1). This study also proposed that Pakistani isolates are being surely transmitted from one crop to another or from wild reservoir to crop by means of these insect vectors. Furthermore, investigations are mandatory to detect the insect vectors responsible for the transmission of phytoplasma in the country and to define its plant and insect host range. Additionally, better genetic discrimination of isolates will be required to find out the geography and dynamics of its epidemics (Fig. 8).

**Acknowledgements** Authors are highly thankful to Higher Education commission of Pakistan (HEC) under grant (No.204535/NRPU/R&D/HEC/14/159 and Framework of Institutional Cooperation Program (FICP-Pak-Norway Grant) to Dr. Jam Nazeer Ahmad and Dr. Samina Jam Nazeer Ahmad) for providing funds for research and to establish Dr. Jam Laboratory (IGCDB) at University of Agriculture Faisalabad, Pakistan.

## Declarations

**Conflict of interest** Authors declare that they have no conflict of interest.

## References

Ahmad JN, Garcion C, Teyssier E, Hernould M, Gallusci P, Pracros P, Renaudin J, Eveillard E (2013) Effects of stolbur phytoplasma infection on DNA methylation processes in tomato plants. Plant Pathol 62:205–216

Ahmad JN, Renaudin J, Eveillard S (2014) Expression of defense genes in stolbur phytoplasma infected tomatoes, and effect of defense stimulators on disease development. Eur J Plant Pathol 139(1):39–51

Ahmad JN, Ahmad SJN, Arif MJ, Irfan M (2015a) First report of oil seed rape (Brassica napus) associated phytoplasma diseases and their insect vector in Pakistan. Phytopathogenic Mollicutes 5:S89–S90

Ahmad SJN, Ahmad JN, Aslam M, Rizwan M, Ijaz M, Shabbir M (2015b) The wide occurrence of Parthenium weed associated disease and its potential insect vectors in the Punjab, Pakistan. International Parthenium News. Tropical and Sub-tropical Weed Research Unit, University of Queensland, Australia, pp 9–10

Ahmad SJN, Ahmad JN, Irfan M, Ahmad M, Aslam M (2015c) Report on phytoplasma new host plants in Pakistan. Phytopathogenic Mollicutes 5:S71–S72

Ahmad JN, Ahmad SJN, Aslam M, Ahmad MA, Contaldo N, Paltrinieri S, Bertaccini A (2017) Molecular and biologic characterization of a phytoplasma associated with *Brassica campestris* phyllody disease in Punjab province, Pakistan. Eur J Plant Pathol 149:117–125

AL-Saleh MA, Amer MA, AL-Shahwan IM, Abdalla OA (2014) Detection and molecular characterization of Alfalfa Witches'-Broom phytoplasma and its Leafhopper Vector in Riyadh region of Saudi Arabia. Int J Agric Biol 16(2):300–306

Aslam M, Tanwir S, Akhtar ZR, Ahmad JN (2021) First report of 16SrII-D phyllody phytoplasma and associated insect vectors infecting multi-flower inbred lines of sunflower (Helianthus annuus L.) in Faisalabad, Pakistan. Pak J Agri Sci 58:985–992

Bertaccini A, Davis RE, Lee IM, Conti M, Dally EL, Douglas SM (1990) Detection of Chrysanthemum yellows mycoplasma-like organisms by dot hybridization and southern blot analysis. Plant Dis 74:40–43

Bonnet F, Saillard C, Kollar A, Seemuller E, Bove JM (1990) Detection and differentiations of mycoplasmalike organism associated with apple proliferation disease using cloned DNA probes. Mol. Plant-Microbe Interact 3:438–443

Deeley J, Stevens WA, Fox RTV (1979) Use of dienes, stain to detect plant diseases induced by mycoplasma like organism. Phytopathol 69:1169–1171

Deng S, Hiruki C (1991) Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J Microb Meth 14(1):53–61

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissues. Focirs 12:13–15

Gopurenko D, Fletcher MJ, Liu J, Gurr GM (2016) Expanding and exploring the diversity of phytoplasmas from lucerne (*Medicago sativa*). Sci Rep 6(1):1–7

Gundersen DE, Lee IM (1996) Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol Medit 35:144–151

Gurr GM, Gurr GM (2007) Australian Lucerne Yellows Disease: testing and extension of disease management strategies. Rural Industries Research and Development Corporation

Ikten C, Catal M, Yol E, Ustun R, Furat S, Toker C, Uzun B (2014) Molecular identification, characterization and transmission of phytoplasmas associated with sesame phyllody in Turkey. Eur J plant pathol 139(1):217–229

Khadhair AH, Hiruki C, Hwang SF (1997) Molecular detection of alfalfa witches'-broom phytoplasma in four leafhopper species associated with infected alfalfa plants. Microbiol Res 152(3):269–275

Khan AJ, Botti S, Al-Subhi AM, Gundersen-Rindal DE, Bertaccini AF (2002) Molecular identification of a new phytoplasma associated with Alfalfa Witches'-Broom in Oman. Phytopathol 92:1038–1047

Khan AJ, Botti S, Al-Subhi AM, Zaidi MA, Altosaar I, Alma A, Bertaccini A (2003) Molecular characterization of the 16S rRNA gene of phytoplasmas detected in two leafhopper species associated with Alfalfa plants infected with Witches' Broom in Oman. Phytopathol Mediterr 42(3):257–267

Kirkpatrick B, Smart C (1995) Phytoplasmas: can phylogeny provide the means to understand pathogenicity? Adv Bot Res 21:188–206

Kumar S, Jadon V, Tiwari AK, Rao G (2015) *Exitianus indicus* (distant): a putative vector for 'Candidatus phytoplasma cynodontis' in India. Phytopathogenic Mollicutes 5(1):51–52

Lee IM, Davis RE (1998) Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms by using cloned DNA and RNA probes. Mol. Plant-Microbe Interact 1:303–310

Lee IM, Davis RE, Dewitt N (1990) Non-radioactive screening method for isolation of disease specific probes to diagnose plant diseases caused by mycoplasma-like organisms. Appl Environ Biol 56:1471–1475

Malik ST, Ahmad* JN, Sharif MZ, Trebicki P, Tahir M, Bertaccini A (2020) Molecular detection and characterization of phytoplasmas in *Trigonella foenum-grecum* and identification of potential insect vectors in Punjab, Pakistan. Pak J Bot 52(5):1605–1613. https://doi.org/10.30848/PJB2020-5(16)

Marcone C, Lee IM, Davis RE, Ragozzino A, Seemuller E (2000) Classification of aster yellows-group phytoplasmas based on combined analysis of rRNA and *tuf* gene sequence. Int J Syst Evol MicroBiol 50:1703–1713

Marzachi C, Veratti F, Aquilio MD, Vischi A, Conti M, Boccardo G (2000) Molecular hybridization and PCR amplification of non-ribosomal DNA to detect and differentiate stolbur phytoplasma isolates from Italy. J Plant Pathol 82:201–212

Montano HG, Davis RE, Dally EL, Pimentel JP, Brioso PST (2000) Identification and phylogenetic analysis of a new phytoplasma from diseased chayote in Brazil. Plant Dis 84:429–436

Montano HG, Davis RE, Dally EL, Hogenhout S, Pimentel JP, Brioso PS (2001) *Candidatus* Phytoplasma brasiliense', a new phytoplasma taxon associated with hibiscus witches' broom disease. Int J Syst Evol Microbiol 51:1109–1118

Montano HG, Brioso PST, Pimentel JB, Figueiredo DV, Cunha JO (2006) *Cucurbita moschata*, new phytoplasma host in Brazil. J Plant Pathol 88:226–226

Montano HG, Paulo STB, Roberta CP, Joao PB (2007) *Sicana odorifera* (Cucurbitaceae) a new phytoplasma host. Bull Insectol 60:287–288

Pamei I, Makandar R (2016) Association of 16SrII-D Phytoplasma with Phyllody Disease in Sesame (*Sesamum indicum*) in Telangana from Southern India. Plant Dis 100(8):1777

Parrella G, Paltrinieri S, Botti S, Bertaccini A (2008) Molecular identification of phytoplasmas from virescent Ranunculus plants and from leafhoppers in Southern Italian crops. J Plant Physiol 90:537–543

Pearce T, Scott J, Pethybridge SJ (2011) First Report of a 16SrII-D subgroup phytoplasma associated with Pale Purple Coneflower Witchs"-Broom disease in Australia. Plant Dis 100(7):1494–1494

Peters RD, Lee ME, Grau CR, Driscoll SJ, Winberg RM, Kutzweil NC (1999) First report of aster yellows phytoplasma in alfalfa. Plant Dis 83:488

Pilkington LJ, Gurr GM, Fletcher MJ, Nikandrow A, Elliott E (2004) Vector status of three leafhopper species for Australian lucerne yellows phytoplasma. Aust J Entomol 43(4):366–373

Salehi M, Izadpanah K (1992) Etiology and transmission of sesame phyllody in Iran. J Phytopathol 135:37–47

Salehi M, Heydarnejad J, Izadpanah K (2005) Molecular characterization and grouping of 35 phytoplasmas from central and southern provinces of Iran. Iran J Plant Pathol 41:62–64

Seemuller E, Schneider B, Maurer R, Ahrens U, Daire X, Kison H, Lorenz KH, Firrao G, Avinent L, Sears BB, Stackebrandt E (1994) Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. Int J Syst Bacteriol 44:440–446

Sharif MZ, Ahmad SJN, Tahir M, Ziaf K, Zhang SH, Ahmad JN (2019) Molecular identification and characterisation of phytoplasma associated with carrot, cabbage and onion crop and their putative insect vectors in Punjab, Pakistan. Pakistan J Agric Sci 56(2):407–414. https://doi.org/10.21162/PAKJAS/19.7152

Shinkai T, Kobayashi Y (2007) Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence in situ hybridization and real-time PCR. Appl Environ Microbiol 73(5):1646–1652

Starovic M, Kuzmanovic S, Veljko G, Josic D (2012) Detection and identification of two phytoplasmas (16SrIII-B and 16SrXII-A) from alfalfa (Medicago sativa) in Serbia. J Phytopathol 160(11–12):758–760

Suryanarayana V, Singh S, Muniyappa V, Reddy H (1996) Little leaf of *Medicago sativa* L.-A new phytoplasma disease in India. Int J Trop Plant Dis 14(2):167–171

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24(8):1596–1599

Wang K, Hiruki C (2001) Use of Heteroduplex mobility assay for identification and differentiation of Phytoplasmas in the Aster Yellows Group and the Clover Proliferation Group. Phytopathol 91:546–552

White DT, Blackall LL, Scott PT, Walsh KB (1998) Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in '*Candidatus* Phytoplasma australiense' and a new taxon', *Candidatus* Phytoplasma australasia'. Int J Syst Bacteriol 48(3):941–951

Yadav A, Thorat V, Bhale U, Shouche Y (2015) Association of 16SrII-C and 16SrII-D subgroup phytoplasma strains with witches' broom disease of Parthenium hysterophorus and insect vector *Orosius albicinctus* in India. Aus Plant Dis Notes 10(1):31. https://doi.org/10.1007/s13314-015-0181-2

Zreik L, Carle P, Bove JM, Garnier M (1995) Characterization of the mycoplasmalike organism associated with witches-broom disease of lime and proposition of a *Candidatus* taxon for the organism, *Candidatus* Phytoplasma aurantifolia. Int J Syst Bacteriol 45(3):449–453