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MOLECULAR DETECTION AND CHARACTERISATION OF PHYTOPLASMA IN TRIGONELLA FOENUM-GRAECUM AND IDENTIFICATION OF POTENTIAL INSECT VECTORS IN PUNJAB, PAKISTAN

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Abstract

Discerning with crop health issues, this study was conducted to detect and identify phytoplasmas and their potential insect vectors in symptomatic Fenugreek (*Trigonella foenum-graecum*) plants collected from Punjab, Pakistan. The detection of phytoplasma in collected leafhopper species and Fenugreek plants was confirmed by nested PCR amplification of 16SrDNA by employing primer pairs (P1/P7 & R16F2n/R16R2). Our results indicated that all the symptomatic fenugreek plant and specimens of leafhoppers, *Orosius albicinctus, Empoasca spp.,* and *Balclutha incisa* resulted positive in PCR. Sequencing of amplified DNA products and phylogenetic analysis of our Fenugreek phyllody phytoplasma (Accession number MH398586) showed that the phytoplasma strains detected has maximum identity (100%) with '*Candidatus* Phytoplasma australasiae' subgroup 16Sr-II-D (Gen Bank number Y10097. This is the first detection and identification of phytoplasma presence in fenugreek seed plants with putative potential insect vectors in Faisalabad, Punjab, Pakistan.

Key words: *Trigonella foenum-graecum*; 16SrII-D phytoplasma; Phylogeny; Potential insect vectors.

Introduction

Fenugreek (*Trigonella foenum-graecum*) is a self-pollinating vegetable crop, which is native to Indian subcontinent as well as the Eastern Mediterranean region. This crop ranges to central Asia and North Africa, and in recent time, has been grown in UK, Central Europe and North America. It has also been utilized for conventional medical treatments (Ng *et al*., 2007; Vyas *et al*., 2008). Various pathogens can affect fenugreek crop, including; bacteria, fungi, viruses and nematodes causing symptoms like damping off (*Pythium aphanidermatum*), root rot (*Rhizoctonia solani*), wilt (*Fusarium oxysporum*), collar rot (*Sclerotium rolfsii*), leaf spot (*Cercospora traversiana*), leaf blight (*Corynespora cassicola*), downy mildew (*Peronospora trigonella*), powdery mildew (*Erysiphe polygoni*), rust (*Uromyces anthylidis*), turnip mosaic and fenugreek mosaic wilt ensuing yield reduction (Khare *et al*., 2014). Total fenugreek crop losses in Australia were associated with Cucumber mosaic virus (CMV), Pea seed-borne mosaic virus (PSbMV) and *Turnip yellows virus* (TuYV) (Aftab *et al*., 2018). In addition, insects and mites also attack fenugreek crops and cause severe yield losses. Such insects include stem fly (*Ophiomyia* spp), cowpea aphid (*Aphis craccivora* Koch), serpentine leaf miner (*Liriomyza trifolii* Burgess), thrips (*Scirtothrips dorsalis* Hood), lucerne weevil (*Hypera postica* Gyllenhal) and spotted pod borer (*Maruca testulalis* Geyer), whilst mites include *Tetranychus cucurbitae* Rahman and Sapra (Arachnida: Tetranychidae) (NIPHM, 2014).

Huge crop losses have been caused by stolbur phytoplasma contamination in vegetable plantations. These same crops have simultaneously harbored multiple viruses, bacterial and fungal symptoms, causing plant destruction. Additionally, nested-PCR studies utilizing a set of primer pairs (P1/P7 and R16F2n/R2) also determined phytoplasma incidence in symptomatic vegetable plants (tomato, onion, brassica and *Parthenium hysterophorus*) in Pakistan (Fialova *et al*., 2009; Ember *et al*., 2011; Ahmad *et al*., 2015b, Ahmad *et al*., 2017).

Phytoplasmas are obligate parasite, affecting a number of different plant species globally. They are also phloem limited and have no cell wall (Lee *et al*., 2000; IRPCM, 2004). Phytoplasmas cause multiple infections in various crop species comprising vegetables, cereals, fruits as well as trees (Lee *et al*., 2000). Characteristic phytoplasma symptoms were noticed in several medicinal plants including *Trigonella foenum greacum* in Serbia, which were further verified via electron microscopy (TEM) as well as molecular techniques like nested PCR, RFLP analyses and sequencing (Pavlovic *et al*., 2014). Different phytoplasma groups and subgroups have been detected from plants of the leguminosae family; among these are chickpeas, mung beans and soybeans, which are linked with 16SrII-D and 16SrXXXI respectively (Akhtar *et al*., 2009a; Akhtar *et al*., 2010; Lee *et al*., 2011). A variety of symptoms following phytoplasma infection include leaf yellowing, small leaf size, virescence, developmental aberrations (proliferation, dwarfism), and more commonly flower abnormalities. Whereas symptoms for diseases like stolbur and big bud comprise of deformed flowers, reduced fruit size and reduction of yield in tomato plants (McCoy *et al*., 1989; Del Serrone *et al*., 2001; Anfoka *et al*., 2003; Ahmad *et al*., 2013). Phytoplasma infection alter phytohormone based gene expression in tomato *(Solanum lycopersicum*) therefore application of phytohormones can mitigate not only phytoplasma stress (Ahmad *et al.,* 2014) but also drought stress in pea (*Pisum sativum* L.) alongwith combined application of rhizobacteria (Bashir *et al.,* 2020).

The main mode of phytoplasma transmission is via sap-sucking insects, including *Psyllidae*, *Cicadellidae*, and *Cixidae*. Phytoplasma can be also transmitted by grafting and vegetative/asexual propagation (tubers, cuttings, rhizomes & bulbs) (Lee & Davis, 1992). Different dodder species (*Cuscuta campestres*, *epilinum* and *trifolli*), which are the plant parasites affecting various plants species, are also responsible for the transmission of phytoplasmas (Salehi *et al*., 2014).

Tissue staining and light microscopy has been identified as a simple and quick method for detection of phytoplasma infection (Deeley *et al*., 1979). Fluorescent microscopy tends to be more preferred (Hibben *et al*., 1986; Franova *et al*., 2007). Previous tactics were primarily depended upon observations of symptoms associated with various pathogenic strains followed by the detection of phytoplasma occurrence in phloem tissues linked segments when stained with Dienes' stain (Musetti, 2013).

Members of the phytoplasma subgroup "16SrII-D" in taxonomy, previously known under currently cancelled species designation '*Ca. P. australasiae*' (White *et al*., 1998; Firrao *et al*., 2004), are linked with crop impairment of economic importance. Such infections include PpYC (Papaya yellow crinkle), TBB in Australia, PpM and Pale Purple Coneflower Witches"-Broom (White *et al*., 1997; Schneider *et al*., 1999; Pearce *et al*., 2011).

There were multiple objectives to conduct this productive study. Of all those, the main objective of current investigation were associated to get confirmation, identification and characterization of phytoplasma in fenugreek crops by using molecular analysis. While other aims included were symptomatology and recognition of phytoplasma transmitting insect vectors.

Materials and Methods

Field surveys and sample collection: During 2017, plant samples from fenugreek plants cultivated for seeds were collected from Faisalabad, Multan and Rahim Yar Khan Districts within the province Punjab, Pakistan. W-pattern was used as a sampling procedure during visual inspection of fenugreek crop plants. Visual inspections of 1000 plants were also carried out to assess percentage of plants exhibiting phytoplasma symptoms (Fig. 1). Thirty plant samples of fenugreek from each above-mentioned region were randomly collected as test samples and then evaluated for percentage infection of phytoplasma (Table 2). Potential insect vectors sucking on symptomatic plants were collected using sweep net. Insects and plants exhibiting symptoms were collected, enclosed in zip-lock bags and then brought to the IGCDB Laboratory, PARS Campus, University of Agriculture Faisalabad. Samples were frozen at -40°C until DNA extraction. Molecular examinations of all the plants and insects samples were performed.

Fig. 1. Healthy fenugreek plant with normal flower (C,D), Phytoplasma infected Fenugreek plants exhibiting leaf yellowing, phyllody, flower virescence, proliferation and tillering of shoots (A,B and E).

DNA extraction: Extraction of DNA was carried out from field-collected insect and plant samples, which were crushed with the help of mortar and pestle following CTAB extraction protocol as documented by Doyle and Doyle (1990).

PCR assays for phytoplasma in test plants and insects: Each reaction mixture (50 mL) for PCR comprised of 1 µL of DNA, Taq polymerase (1.25 units), Taq buffer comprising 1.4 mM MgCl2, primers (0.4 µM) and dNTP (0.1 mM). For the first round PCR universal primer pair P1/P7 (Deng & Hiruki, 1991; Kirkpatrick *et al*., 1995) while in case of nested PCR primers pair RI6F2n/R2 (Gundersen & Lee, 1996) were used for phytoplasma detection. Conditions applied for PCR cycling were: 1 min denaturation at 95°C (2 min duration for first cycle), 1 min annealing at 55°C temperature and 1.5 min time for the process of extension at the temperature of 72°C for 35 cycles (9.5 min on final cycle). Fenugreek phytoplasma DNA product, collected from those plants showing phytoplasma associated symptoms and sterile dH2O (SDW) were used as positive and negative controls respectively. After the completion of each nested PCR investigation, PCR product of 2 µL were analyzed with the aid of electrophoresis on agarose gel (1%), stained with ethidium bromide and then visualized under UV light using Gel documentation system.

Light microscopy of plant samples: Toludine and Dienes' stain was used for detection of phytoplasma infection in fenugreek plants. Dienes' stain enclosed 10 gram Maltose, 2.5 gram Methylene blue, 1.25 gram Azure II and 0.25 gram sodium carbonate dissolved in water (100ml). The filtration of stain was directed by means of Whatman No.1 filter paper. The midrib of plant leaves were used to prepare free-hand sections. These sections were transferred onto ethanol (70%), stained by using the Dienes' stain for 10 min, and then washed with distilled water. Finally, phytoplasma presence was determined using the light microscope at 40X magnification (Hibben *et al*., 1986).

Toludine staining's (0.5% toluidine blue [wt/vol] was also used for the detection of phytoplasma infection in fenugreek plants. Hand sections of infected plant samples were stained with the assistance of toluidine blue for 15 min at room temperature and then dipped in distilled water until the water turned clear. Following air-drying, incubation of samples were carried out in 99.5% ethanol for 30 second to 15 min intervals to remove the dye from the pathogen cells, but not from the plant material (Shinkai & Kobayashi, 2007). Finally, the examination of sections was undertaken using light microscope at magnification of 40X.

RFLP analysis of plants: Nested-PCR products of eight microliters (1.25kbp from 16S ribosomal-DNA) from three isolates of various fenugreek fields in Punjab were individually digested by employing *Hpa*II, *Alu*I (restriction enzymes) regarding manufacturer's guidelines at 37°C temperature overnight. Electrophoresis of digestion products was then done using agarose gels (2%) and pictured or visualized after staining with ethidium bromide (1µg µL-1) in the TAE 1X buffer 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 16S ribosomal-DNA of other phytoplasmas (Lee *et al*., 1998; Marcone *et al*., 2000).

Sequencing and phylogenetic analysis: Amplification of nested polymerase chain reaction product (1.25-bp) of test plants was done through commercial kit and then sequenced using AbiPrism 3100 Genetic Analyzer apparatus (Applied Biosystems, USA). Data obtained through sequencing of plant samples was aligned & examined with Lasergene v. 7.1 software package (DNASTAR, USA) and homology phylogenetic studies were performed with MEGA6 software using "neighbour joining method" (Tamura *et al*., 2007). The phytoplasma strains used for the construction of phylogenetic tree are shown (Table 1 and Fig. 5).

Table 1. Phytoplasma groups with their accession numbers used for construction of phylogenic analysis tree.

Sr. No.	Phytoplasma group	Accession numbers
1.	Sesame phyllody Iran 16Sr II-D	JX464670
2.	*Ca.* P. *australasia*	Y10097
3.	*Ca.* P. *aurantifolia*	U15442
4.	*Ca.* P. *brasiliense*	AF147708
5.	*Ca.* P. *solani*	AF248969
6.	*Ca.* P. *caricae*	AY725234
7.	*Ca.* P. *convulvoli*	JN8333705
8.	*Ca.* P. *australiense*	L76865
9.	*Ca.* P. *americanum*	DQ174122
10.	*Ca.* P. *japonicum*	AB010425
11.	*Ca.* P. *fragariae*	HM104662
12.	Mexican periwinkle virescence	AF248960
13.	*Ca.* P. *asteris*	M30790
14.	*Ca.* P. *parunorum*	AJ542544
15.	*Ca.* P. *pyri*	AJ542544
16.	*Ca.* P. *mali*	AJ542541
17.	*Ca.* P. *pruni*	JQ044392
18.	*Ca.* P. *trifolii*	AY390261
19.	*Ca.* P. *malaysianum*	EU371934
20.	*Ca.* P. *rubi*	AY197648
21.	*Ca.* P. *fraxini*	JQ868445
22.	*Ca.* P. *oryzae*	AB052873
23.	*Ca.* P. *cynodontis*	AJ550984
24.	*Ca.* P. *phoenicium* A1.1 Lebanon	HQ407514
25.	Naxos Italy	HQ589191
26.	BBS40-NJ-USA	JX857823
27.	Pigeon Pea Witches Broom USA	AF248957
28.	Periwinkle Phytoplasma Colombia	EU816776
29.	Tomato Bigbud phtoplasma Iran	JF508510
30.	Sesame phyllody Iran	JX464670
31.	*Ca.* P. SAR 2PAK (16Sr IX-H)	KU892213
32.	*Brassica compestris* SARI	-
33.	*A. laidlawii* PG8A	NR076550

Table 2. Symptomatic plants observed in fenugreek seed field during field surveillance and their PCR detection from samples collected from different districts of Punjab

Sr No	Region	PCR (+)/total	(%) PCR (+)	Symptom/ total	(%) infection
1.	Faisalabad	5/30	16.66	51/1000	5.1
2.	Rahim Yar Khan	2/30	6.66	43/1000	4.3
3.	Multan	5/30	16.66	46/1000	4.6

Results

Symptomatology and insect population: Distinctive symptoms of phytoplasma were identified on fenugreek plants grown in certain regions of the Punjab, Pakistan. Phyllody (floral abnormality), leaf yellowing, shoot proliferation and stem tillering were observed (Fig. 1). Around 4-5% of field plants from three different districts were identified to be infested by phytoplasma through visual symptoms assessment (Table 2). In addition, number of insect species collected from fenugreek field included *Orosius albicinctus, Empoasca spp., Circulifer haematoceps, Balclutha incisa, Bemesia tabaci*, thrips and some unidentified small leafhoppers. Overall numbers of these insects collected from different regions are given in Table 3.

Light microscopy of Dienes' stained tissues: Staining techniques (Diene's stain) upon microscopic examination resulted coloring (deep navy blue) of cross sections of symptomatic plant samples (Fig. 3A). However, such results confirm the presence of pathogenic bodies within tissues of plant samples. On the other hand, sections taken from samples of healthy plants remained unstained indicating the non-existence of bodies of pathogenic infection (Fig. 3B).

Testing phytoplasma infection in plant and insect samples: A 1.25 kb specific phytoplasma PCR product was amplified in the samples of symptomatic fenugreek plants collected from different regions, while healthy plant samples showed no phytoplasma presence (Fig. 2). The overall infection in plant samples detected by nested PCR ranged from 6 to16% (Table 2). Nested-PCR results for insects including; *Bemisia tabaci,* thrips and another unidentified small leafhoppers did not show phytoplasma infection, but in following leafhopper species; *O. albicinctus*, *Empoasca spp., C. haematoceps* and *B. incisa* phytoplasma was detected. However, analysis revealed that the average number of 1.38 insects amongst overall insects (recognized hoppers species and others) was detected as phytoplasma infective species (Table 3).

RFLP analysis of fenugreek plants: Digestion of the product obtained from nested PCR of affected plant samples using *Hpa*II, *Alu*I (restriction enzymes) profile of RFLP analysis (Fig. 4). The profile resembled 16SrII-D subgroup of sesamum phyllody phytoplasma, which was used in reference strain.

Phylogenetic analysis: Phylogenetic investigation for percentage homology (Fig. 5) was determined between the 16SrDNA sequences. The percentage homology of our *Trigonella foenum-graecum* IGCDB isolate exhibited 99-100% association with *Ca. P. australasia* strain of 16Sr II-D subgroup (acc.no Y10097).

Table 3. Insect specimens collected from different fields and their nested-PCR results for phytoplasma presence.

Sr No	Region	Insect Species	Total insect	PCR tested	PCR (+)/total	(%) infection
1.	Faisalabad	*Orosius albicinctus*	20	15	4/15	26.66
		Empoasca spp.	15	15	3/15	20
		Balclutha incisa	19	15	2/15	13
		Unidentified small leafhoppers	37	15	0/15	0
		Bemesia tabaci	150	15	0/15	0
		Thrips	142	15	0/15	0
2.	Rahim Yar Khan	*Empoasca spp.*	22	15	3/15	20
		Orosius albicinctus	20	15	3/13	20
		Unidentified small leafhoppers	41	15	0/15	0
		Bemesia tabaci	234	15	0/15	0
		Thrips	141	15	0/15	0
3.	Multan	*Orosius albicinctus*	19	15	5/15	33.33
		Empoasca spp.	20	15	1/15	6.66
		Unidentified small leafhoppers	33	15	0/15	0
		Bemesia tabaci	120	15	0/15	0
		Thrips	124	15	0/15	0
Total			**1157**	**240**	**21**	

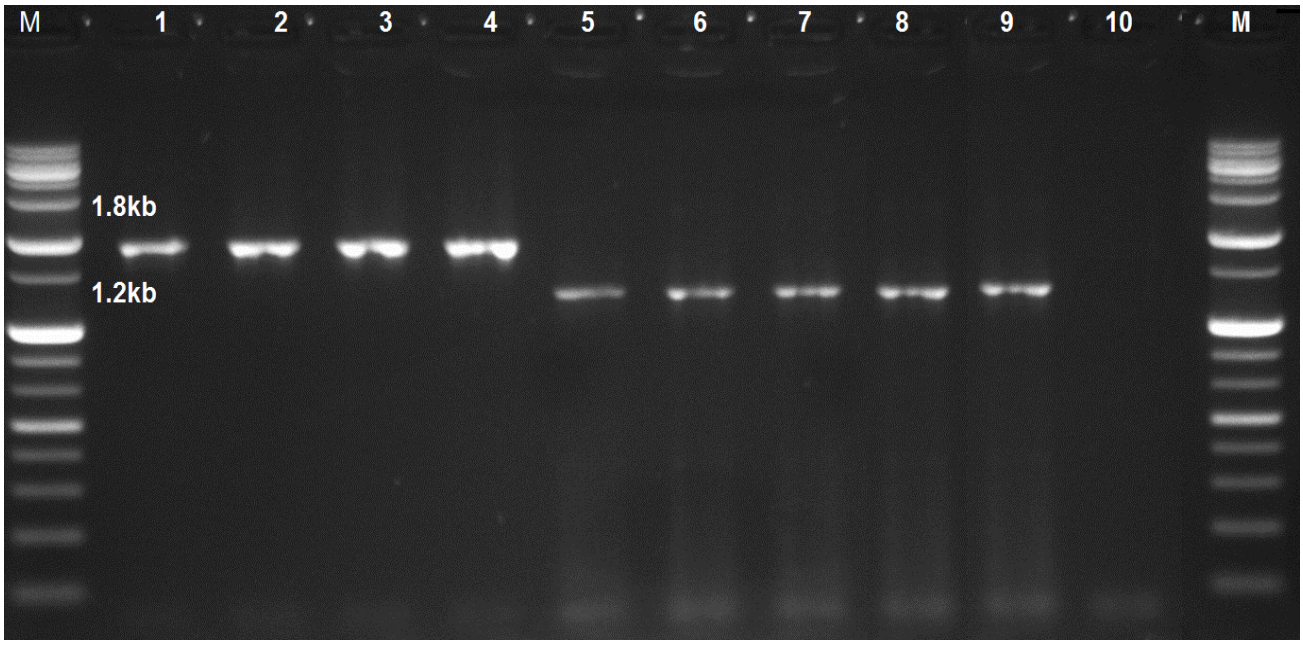

Fig. 2. Nested PCR detection of Fenugreek associated phytoplasma by using universal primer primers P1/P7 and RI6F2n/R2. Lane 1-4 (P1/P7 based PCR amplicons (1.8 kb) of infected Fenugreek samples); Lane 5-8 (RI6F2n/R2 based PCR amplicons (1.2kb) infected samples); Lane 9- (+ control); Lane 10- (Healthy sample); Lane M- I kb (+) DNA ladder Marker (GeneMark).

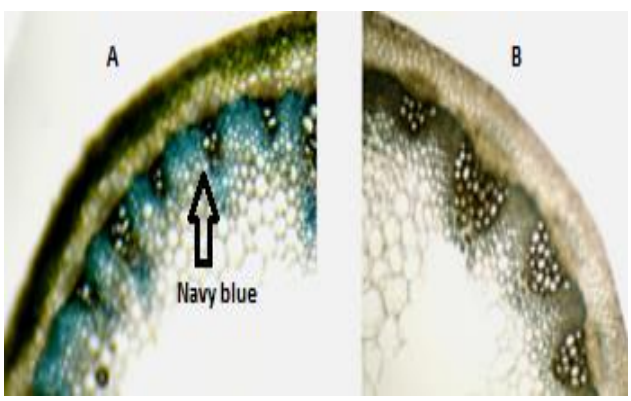

Fig. 3. Phytoplasma detection through light microscopy by using staining in a thin cross section of fenugreek leaf midrib. Navy blue (A) in phytoplasma infected cross section shows presence of phytoplasma as compared to healthy one (B). Magnification at 40X.

Discussion

Phytoplasma diseases in vegetable crops are a crucial agricultural problem, causing severe yield and quality losses. In our investigation, we confirmed phytoplasma infection in indicative plant samples as well as insect specimens by nested PCR amplification. The symptoms we observed in phytoplasma-infected fenugreek plants were similar to the symptoms of various phytoplasma infected plants mentioned in previously publications and exhibited no unique symptomatology. Here we also employed Dienes staining of symptomatic and phytoplasma infected plants that revealed frequently scattered regions in phloem zone analogous to zones detected for other phytoplasmas (Salehi & Izadpanah, 1992).

In this investigation, the phytoplasma was detected in insect species including *O. albicinctus*, *Empoasca Spp.,* and *B. incisa* (Table 3). These insects have also been documented for transmission of 16Sr- II group of phytoplasma in various plants. For example, Salehi *et al.*, (2016) showed *O. albicinctus* as a potential vector of 16Sr-II group, linked with disease of carrot witches' broom (CarWB). Seljak (2013) described *H. hamatus* as a new alien leafhopper species and highlighted this insect as vector of phytoplasma in the case of ornamental plants in Europe. The presence of *Balclutha incisa* and *Circulifer haematoceps* was reported from Pakistan and Israel (Khatri and Rustamani, 2011; Weintraub *et al*., 2004). Similarly, phytoplasma incidence in Pakistan may be due to wide spread of such insect vectors. Phyllody diseases linked with subgroup "16SrII-D" and some particular symptoms in chickpea, sesame (Akhtar *et al*., 2008a, 2009b; Ahmad *et al*., 2015a), and in *Parthenium* hysterophorus, *Raphanus sativus* and *Solanum lycopersicum* were also reported in Pakistan (Ahmad *et al*., 2015a, 2015b, 2015c) respectively.

Results for PCR positive insect species are not conclusive as phytoplasma occurrences in the bodies of insects do not necessarily mean that the insect is able to transmit the pathogen (Vega *et al*., 1993). Therefore, to confirm their vector status we have to go through transmission trials. Moreover, for efficient control of this syndrome we will need good understanding of vectors as well as a reservoir of phytoplasmal pathogens. More investigations on insect ecology and biology can also be utilized to spot potential diseases management strategies. On another hand, the ranges of up to 16% of phytoplasma infected plant samples and 4-5% of surveyed field crops sown for seeds showed phytoplasma infection in this study (Table 2) put greater emphasis on the importance of management decisions.

Current study confirmed *Ca. P. australasia* of subgroup "16SrII-D" as the causative agent of fenugreek plant infection. Phytoplasmas under the 16SrII group are gaining greater importance economically (Salehi *et al*., 2008) as the member of this group is causing massive infestation globally. The PpYC phytoplasma previously designated as '*Ca*. P. australasiae' (White *et al*., 1997; White *et al*., 1998) had also reported for having a close connection to subgroup 16SrII-D. In addition, the phytoplasma strain sesame phyllody (acc. no. KP297862) of 16SrII-D subgroup was also documented to cause phyllody disease in sesame crops in India (Pamei and Makandar, 2016) and Turkey (Ikten et al., 2014). Phylogenetic analysis positioned the phytoplasma strain "*Ca*. P. *aurantifolia* (acc.no FJ410489)" in 16SrII-D subgroup causing mung bean phyllody in Pakistan (Akhtar *et al*., 2010). Such groups were also documented 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 associated with disease in cucurbits (Montano *et al*., 2000; Montano *et al*., 2006; Montano *et al*., 2007). Six diverse phytoplasma taxonomic groups infest *Solanum lycopersicum* plants globally (Arocha *et al*., 2007; Blancard, 2009). To the best of our knowledge, this report is documenting the first record of phytoplasma subgroup "16SrII-D" in fenugreek plantation in Pakistan.

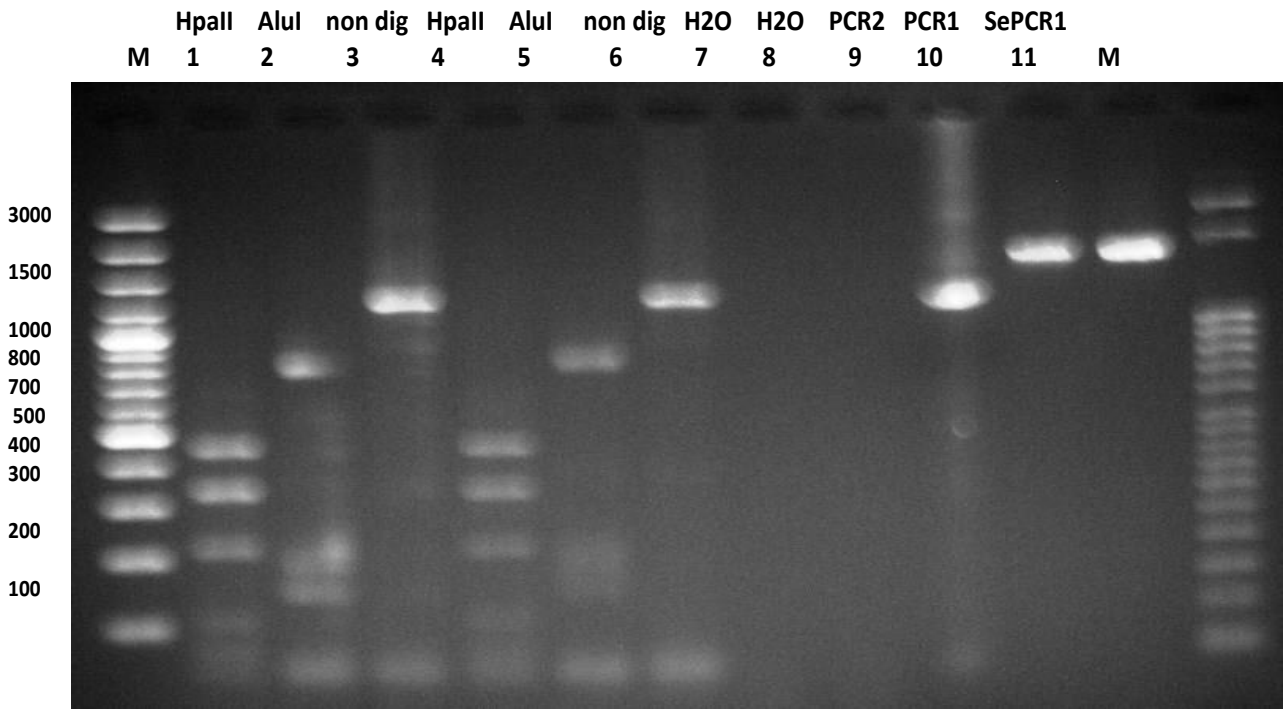

Fig. 4. RFLPs using *Hpa* II and *Alu*I restriction enzymes; M, The molecular weight DNA Ladders (100 bp Invitrogen left) and DNA ladder 50 bp right (Novagen); Other wells contain the RFLP and nested PCR products from the fenugreek samples digested with the *Hpa*II (1, 4 wells), non-digested (3-6 wells) of PCR2 Product, *Alu*I (2 and 5 wells). The wells 7 and 8 contain Healthy samples; well 9 contain nested PCR2 fenugreek product and 10 contain PCR/P1/P7 amplified fragment of fenugreek (infected), well 11 contain PCR products from identified sesamum infected by 16SrII-D (reference strain) phytoplasma. Electrophoresis was conducted in 2 % agarose gel dyed with ethidium bromide (1 µg µL-1) in the TAE 1X buffer.

Conclusions

In this manuscript, we presented phytoplasma identification and its infection in fenugreek seed plants and insects. It is surprising because phytoplasmas infection has never been stated to cause disease in fenugreek plantations in Pakistan. But, the lack of transmission trials of this particular pathogen through insect species has hindered investigation to confirm their status as potential vectors. However, the presence of pathogenic infection in the above-mentioned insects and fenugreek plants highlight the future research areas to explore the vector potential of insect species and pathogen–host interactions. Moreover, massive infestation of 16Sr-II group (and IID subgroup) out of all other phytoplasma groups have been reported in Pakistan and globally. So, it provides an area for researchers to explore the causes of spread of this particular group in the natural environment. Due to high economic importance, this study also stresses the need to take curative measures to hinder the spread of this destructive pathogen in fenugreek crops in Pakistan.

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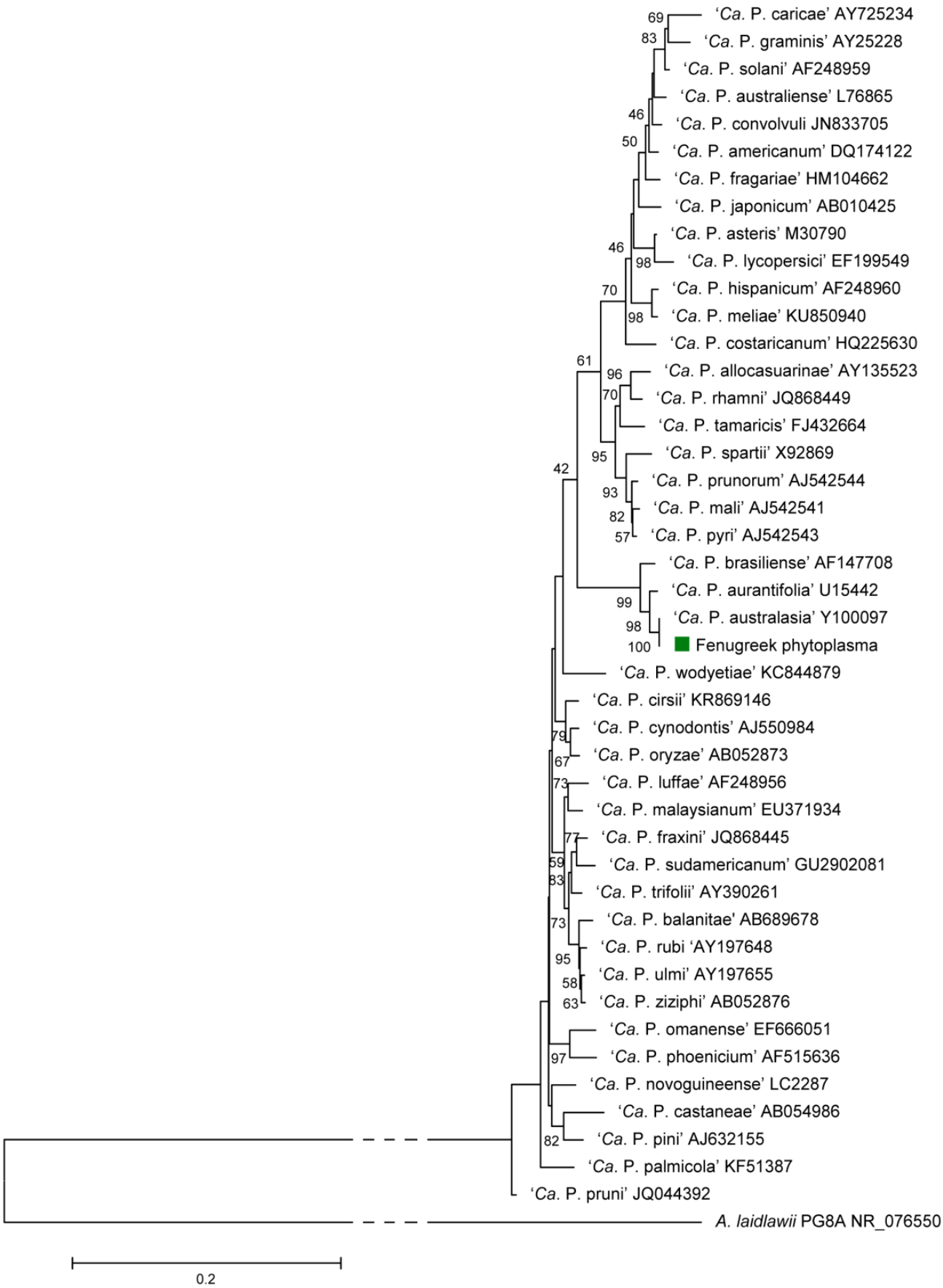

Fig. 5. Phylogenetic tree through multiple alignment of nucleotide sequences of genes (16S rRNA) for fenugreek phylody phytoplasma (MH398586) and GenBank available '*Candidatus* species' using MEGA6 software with the Neighbor-Joining method (Saitu & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). *Acholeplasma laidlawii* is used as out-group.

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