آفات و بیماری‌های گیاهی
جلد 93، شماره 2، اسفند 1404
Applied Entomology and Phytopathology
Vol. 93, No. 2, March 2026
DOI: 10.22092/JAEP.2026.372164.11572

مقاله پژوهشی

همه‌گیرشناسی فیتوپلاسماهای مرتبط با جاروکِ نخود در ایران

مریم غایب زمهریر ✉، علی دهقانی، سارا سیاهپوش

به‌ترتیب دانشیار بخش تحقیقات بیماریهای گیاهان، موسسه تحقیقات گیاهپزشکی کشور، سازمان تحقیقات، آموزش و ترویج کشاورزی، تهران، ایران،

استادیار و محقق بخش تحقیقات گیاهپزشکی، مرکز تحقیقات کشاورزی و منابع طبیعی استان لرستان، سازمان تحقیقات، آموزش و ترویج کشاورزی، خرم آباد، لرستان، ایران

چکیده

بیماری جاروک نخود با نشانه های زردی، افزایش شاخه‌دهی، باریک شدن برگ‌ها و کوتاه شدن میان‌گره‌ها اولین بار در سال 1399در مزارع نخود استان لرستان، ایران مشاهده شد. نمونه‌هایی از گیاهان نخود دارای علائم و بدون علائم جمع آوری و از نظر حضور فیتوپلاسما به روش زیستی و مولکولی بررسی شدند. نتایج تجزیه فیلوژنتیکی و الگوهای RFLP مجازی نشان داد که فیتوپلاسمای عامل بیماری جاروک نخود در گروه 16SrIX قرار دارد. مایه زنی گیاهان نخود سالم با پیوندک آلوده و گیاه سس، در شرایط گلخانه‌ای عاری از حشره، منجر به بروز علائم بیماری شد. از میان گیاهان علفی جمع‌آوری‌شده از اطراف مزارع آلوده، علف هرز گونه *Galium aparine* به عنوان میزبان فیتوپلاسمای گروه 16SrIX-B و 16SrIX-C شناسایی شد. در بررسی مولکولی حشرات مکنده (پسیل، زنجرک و شته) جمع آوری شده از مزارع آلوده، هیچ یک به عنوان ناقل کاندید شناسایی نشدند. نتایج واکنش زنجیره ای پلیمراز آشیانه‌ای نشان داد که ۳۸٪ از نشاءهای حاصل از بذور جمع آوری شده از گیاهان آلوده، ۲۰ روز پس از جوانه‌زنی دارای فیتوپلاسما بودند، در حالی‌که نشاءهای حاصل از گیاهان بدون علائم، سالم بودند.بذرهای آلوده دو نوع علامت متفاوت ایجاد کردند که بررسی‌های زیستی و مولکولی آن‌ها نشان‌دهنده وجود گروههای متفاوت فیتوپلاسما شامل گروه 16SrIX-B و 16SrIX-C بود. آلودگی همزمان گیاهان نخود به دو سویه سبب تشدید علائم و بروز واکنش‌های نکروتیک شد. این نتایج بیانگر نقش برهم‌کنش سویه‌های فیتوپلاسما در شدت بیماری جاروک نخود است.

واژه‌های **کلیدی**: ردیابی مولکولی، شناسایی فیتوپلاسما، انتقال بذری

✉ **E-mail:** zamharir2005@yahoo.com

Epidemiology of phytoplasmas associated with chickpea witches' broom in Iran

MARYAM GHAYEB ZAMHARIR✉, ALI DEHGHANI, SARA SIAHPOUSH

Associate Professor, Plant Diseases Research Department, Iranian Research Institute of Plant protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran,

Assistant Professor and Resercher of Plant protection Department, Lorestan Agricultural and Natural Resource Research Center, Agricultural Research, Education and Extension Organization (AREEO), Khoramabad, Iran,

Abstract

Chickpea witches' broom disease, characterized by symptoms including yellowing, excessive branching, leaf narrowing, and shortened internodes, was first observed in 2020 in chickpea fields of Lorestan Province, Iran. Samples from symptomatic and asymptomatic chickpea plants were collected and examined for the presence of phytoplasma using biological and molecular approaches. Phylogenetic analysis and virtual RFLP patterns revealed that the phytoplasma associated with chickpea witches' broom disease belongs to the 16SrIX group. Inoculation of healthy chickpea plants with infected scions and dodder (Cuscuta spp.) under insect-free greenhouse conditions resulted in the development of disease symptoms. Among the weed species collected from around infected fields, Galium aparine was identified as a host of phytoplasma groups 16SrIX-B and 16SrIX-C. Molecular analysis of sap-sucking insects (psyllids, leafhoppers, and aphids) collected from infected fields did not identify any candidate insect vector. Nested PCR assays showed that 38% of seedlings grown from seeds collected from infected plants were phytoplasma-positive 20 days after germination, whereas seedlings derived from asymptomatic plants were free of infection. Infected seeds produced two distinct symptom types, and biological and molecular analyses indicated the presence of different phytoplasma groups, namely 16SrIX-B and 16SrIX-C. Simultaneous infection of chickpea plants with both strains intensified symptom severity and induced necrotic reactions. These findings suggest that interactions between phytoplasma strains play an important role in determining the severity of chickpea witches' broom disease.

Keywords: Molecular detection, Phytoplasma identification, Seed transmission.

Introduction

With the growing human population, food security, particularly the availability of protein-rich crops, has become a major concern. Chickpea (*Cicer arietinum* L.) is one of the most important legumes cultivated in Iran and worldwide. Legumes, including chickpeas, are among the earliest domesticated plants, with a cultivation history dating back to the Neolithic period (Gaur et al. 2009). Globally, chickpea is grown on approximately 13.54 million hectares, producing an average of 1.13 million tons annually (FAOSTAT, 2024). In Iran, chickpea cultivation covers 550–650 thousand hectares, with an average annual production of 300–350 thousand tons (INCP, 2026).

Chickpea productivity is adversely affected by various biotic and abiotic stresses. One emerging disease of concern is chickpea witches' broom (ChpWB), first observed in summer 2017 in Noorabad, Lorestan province, Iran (34.0720°N, 47.9738°E). Infected plants exhibited symptoms such as little leaf, dwarfism, and witches' broom, and preliminary analysis revealed the presence of a 16SrIX phytoplasma (Ghayeb Zamharir and Dehghani, 2019).

Phytoplasmas are phloem-limited bacterial pathogens transmitted by phloem-feeding insects, including leafhoppers and psyllids (Weintraub *et al*., 2019; Wang *et al*., 2024). These pathogens are associated with various diseases in legumes. For instance, proliferation disease has been reported in cowpea (*Vigna unguiculata*) in India and China (Raj *et al*., 2006; Yan *et al*., 2010), while phyllody disease has been observed in chickpea in Myanmar, where *Candidatus* Phytoplasma aurantifolia was identified (Balol *et al.*, 2021). Chickpea plants infected with 16SrII-D phytoplasma in Oman displayed yellowing, phyllody, and small leaves (Al-Subhi *et al*., 2020). Witches' broom disease has also been reported in peas (*Pisum sativum*) from Poland (Zwoliñska *et al*., 2012), India (Raj *et al.*, 2006; Rao *et al*., 2018), China (Che et al., 2010), Australia (Yang *et al*., 2013), Mexico (Luna-Esquivel *et al.*, 2004), Puerto Rico (Baquero, 1980), and Florida, USA (McCoy et al., 1983).

The present study aimed to investigate the biology of ChpWB phytoplasma in Iran by identifying phytoplasma strains, assessing transmission, characterizing host plant symptomatology, and determining the alternative host.

Materials and Methods

Plant Samples.

collected between 2023 and 2024 from both symptomatic and asymptomatic chickpea (*Cicer arietinum* L.) plants exhibiting witches' broom (ChpWB) dwarfism and proliferation, characterized by grassy growth, reduced leaf size, and overall stunted development (Fig. 1) symptoms. In addition, herbaceous plants surrounding ChpWB-affected fields, including German chamomile (*Matricaria chamomilla* L.), cleavers (*Galium aparine* L.), licorice (*Glycyrrhiza glabra* L.), and chicory (*Cichorium intybus* L.), were sampled to investigate potential alternative hosts.

Insect Collection

Concurrent with plant sampling in May and June 2024, more than eight insect populations were collected from ChpWB-affected fields and adjacent herbaceous plants using an insect net and sticky traps. The collected insects were preserved in 100% ethanol for subsequent identification and molecular analyses.

شکل ۱. علائم کوتولگی، جاروک و نخی شدن برگ‌ها در گیاهان نخود (A) در مقایسه با گیاه سالم (B)

Figure 1. Symptoms of dwarfism, witches broom, and narrowing of leaves in chickpea plants (A) compared with healthy chickpea plants (B)

DNA extraction and phytoplasma detection

The total genomic DNA was extracted from 0.5 g of leaf midrib tissue (Doyle and Doyle 1987). The extracted DNA were electrophoresed in 1% agarose gel, quantified using a Nano-drop spectrophotometer and adjusted to the concentration of 100 ng per µl. DNA was extracted from insects according to Maixner et al. (1995). Insects tissue was ground in extraction buffer (100 mM TrisHCl at pH 8.0, 2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM EDTA, and 0.2% 2-mercaptoethanol) at a ratio of 1:5 (wt/vol, tissue/buffer). The slurry was incubated for 20 min at 60°C and centrifuged for 10 min at 3,000 × g. The supernatant was collected and extracted with an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol), followed by centrifugation and precipitation with 1 volume of isopropanol. Following 30-min incubation at –20°C, the DNA was pelleted at 12,000 × g for 30 min. The pellet was washed twice with 70% ethanol and resuspended in 50 µl of 10 mM Tris and 1 mM EDTA at pH 8.0 (TE).

To detect phytoplasmas by the amplification of a 16S rDNA fragment, two universal phytoplasma primer sets were used in nested PCR reaction. The outside primer set was P1/Tint (Deng and Hiruki, 1991; Smart et al. 1996) followed by R16F2n/R16R2, or R16mF1/mR1 (GTCTTTACTGACGCTGAGGC) (Gundersen and Lee, 1996) or 16R758f/16R1232r (CTTCAGCTACCCTTTGTAAC) (=M1/M2) (Gibb et al. 1995). Phytoplasma-positive controls consisted of DNA extracted from phytoplasma reference strains maintained in almond, while reactions lacking DNA template were included as negative controls. First round PCR reactions were carried out in a total volume of 20 µL using Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark) DNA was amplified with 35 cycles consisting of denaturation at 94°C for 60 s (5 min for cycle 1), annealing at 53°C for 2 min, and primer extension at 72°C for 3 min (5 min for final extebtion). For nested PCR, the thermal conditions were the same as first round except the annealing was at 55ºC for 2 min. The amplifications were carried out in a programmable thermocycler (Bio-Rad, USA); the PCR products were electrophoresed in 1.2% agarose gels in a TAE buffer and visualized with a UV transilluminator following ethidium bromide staining. The molecular weight of the PCR products was estimated by comparison with the 1 kb DNA ladder (Fermentas, Vilnius, Lithuania) .

Sequencing and phylogenetic analysis

R16F2n/R2 (1,248 bp) and M1/M2 (550 bp) amplified fragments (Table 1) from the phytoplasmas detected in symptomatic chickpea chickpea (leaf and seedling) and a Cleavers weed, were directly sequenced in both directions. The 10 sequences obtained were assembled, aligned and compared with nucleotide sequences in the GenBank database, using BLAST (version BLASTN 2.2.18) (National Center for Biotechnology Information, Bethesda, MD, USA). Sequence alignments were performed using ClustalX (Thompson et al., 1997). Phylogenetic analyses were done with maximum parsimony (MP) analysis and close-neighbour-interchange algorithm, using 16S rDNA sequence from this study and from 40 '*Candidatus* Phytoplasma' strains, using *Acholeplasma laidlawii* as the outgroup. The analysis was replicated 100 times. A bootstrap analysis was performed to estimate the

stability and support for the inferred clades (Tamura *et al*., 2004).

جدول 1. خصوصیات جدایه های فایتوپلامایی تعیین توالی شده در این تحقیق

Table 1. Phytoplasma strains sequenced properties

Strain	R16F2n/R2 primed PCR product	M1M2 primed PCR product	Host	Tissu	Location	Geographichal altitude
C51	-	+	chickpea	Plant leaf	Gandabeh	33.18313°N, 47.94694°E
C52	+	+	chickpea	Plant leaf	Darvish	37.67971°N 47.94694°E
C53	+	+	chickpea	Plant leaf	Darvish	37.67971°N 47.94694°E
C96	-	+	chickpea	Plant leaf	Haiatlgeib	,33.18313°N 47.94694°E
C99	-	+	Cleavers (*Galium aparine*)	Plant leaf	Haiatlgeib	33.18313°N 47.94694°E
C56	+	-	chickpea	Plant leaf	Zali	34.0720° N, 47.9738° E
Cs5	-	+	Chickpea seed	seedling	Haiatlgeib	,33.18313°N 47.94694°E
Cs7	-	+	Chickpea seed	seedling	Haiatlgeib	33.18313°N47.94694°E
Cs9	-	+	Chickpea seed	seedling	Haiatlgeib	33.18313°N 47.94694°E

+: PCR product shows expected size band in elechtrophoresis

-: PCR product does not show expected size band in elechtrophoresis

Restriction fragment length polymorphism (RFLP) analyses
Virtual RFLP patterns were obtained for the partial sequences of 16S rDNA from selected sequenced R16F2n/R16R2 fragments using the iPhyClassifier (Zhao et al., 2009) with 17 distinct restriction enzymes (AluI, BamHI, BfaI, BstUI, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI, MseI, RsaI, TspI and TaqI) (Wei et al., 2007). The virtual RFLP patterns generated from chickpea-derived strains were compared with each other and with representatives of 16SrIX subgroups using a Perl program developed by Wei et al. (2008). For actual RFLP analysis 3 µl (about 300 ng of DNA) of R16F2n/R2 or M1/M2 PCR products from 17 amplified samples was separately digested with fast digest enzymes Tru1I, TaqI, and Tsp509I at 65ºC, as recommended by the manufacturers (Fermentas, Vilnius, Lithuania). Restricted fragments were separated through 6.7% polyacrylamide gel electrophoresis in TBE buffer. Patterns were then observed with UV transilluminator following ethidium bromide staining.

Insect vector identification
The collected insects were identified by Dr. Shahab Manzari and Dr. Fariba Mozaffarian (Department of entomology, the Iranian Research Institute of Plant Protection). Among the 8 insect populations tracked from the infected chickpea fields and the surrounding weeds, there were no species belonging to the psyllidis, grasshoppers, and leafhoppers groups and in all of them the dominant identified genus was aphid. Since they

all resulted negative to phytoplasma presence no attempt was made to determine their species.

Graft and dodder transmission

Chickpea seeds collected from asymptomatic field plants were planted in the greenhouse. Symptomatic chickpea plants collected from infected fields were grafted into a one-month-old seedlings with 5 repetiitions using side wedge grafted grfting, tested negative for phytoplasma presence by nested PCR. Plants were checked and monitored for 30-45 days. The presence of phytoplasmas in symptomatic tissues above the grafting region was confirmed by nested PCR 45 days after grafting .

The seeds of the dodder (*Cuscuta campestris*) were treated with 60% sulfuric acid for 10 minutes. As soon as the seed wall dissolved in the acid and its color changed to black, the seeds were washed with distilled water and placed on wet filter paper in a Petri dish. After germination, the resulting seedlings were placed on healthy potato or tomato plants used as a source of dodder in transmission experiments. After the dodder was established on grafted chickpea plants with different symptoms (including little leafing and threadlike growth of leaf), shoots grown from dodder on these plants were placed on healthy chickpea plants. After one month, the contact between the healthy and infected chickpea plants was cut off and the plants were free from dodder and kept under insect proof greenhouse conditions to monitor the appearance of symptoms and confirmed with nested PCR analysis.

Seed transmission

To verify the seed germination and the percentage of possible seed transmission, 90 seeds were prepared from symptomatic plants with incomplete pods and 90 seeds from symptomless plants. The chickpea seeds were placed in a Petri dish on filter paper moistened with sterile distilled water in the dark for 24 to 48 hours. Then, the germinated seeds were transferred to a pot with sterile soil. The percentage of germinated seeds was calculated for both groups of seeds from symptomless and symptomatic plants. After one month of plant growth in the greenhouse, samples were taken from leaves of symptomatic and asymptomatic plants. DNA was extracted from the leaves and phytoplasma detection was evaluated as described above. The plants were kept in the insect proof greenhouse until flowering and podding stages.

The seeds collected from infected plants in the field showed two different types of symptoms after growing in insect proof greenhouse conditions. The phytoplasmas associated with each symptom type was identified using molecular methods. These plants with different symptoms were then used to inoculate healthy plants using dodder and grafting. Mixed infection that dimostrated with sequencing and actual RFLP analysis with both phytoplasma strains was also performed using dodder and grafting.

Results

A total of l43 plant samples were collected from chickpea fields and surrounding herbaceous plants in Lorestan Province, Iran. Of these, l23 chickpea samples, including both symptomatic and asymptomatic plants, were analyzed for phytoplasma presence (Table 2). PCR amplification targeting the l6S rRNA gene produced the expected 1.25 kb fragment in 86 out of 90 symptomatic chickpea samples, whereas only 2 out of 33 asymptomatic samples tested positive.

In addition, 20 samples of potential alternative host plants exhibiting symptoms such as yellowing or small leaves were collected from and around ChpWB-affected fields. These included German chamomile (*Matricaria chamomilla*), cleavers (*Galium aparine*), licorice (*G. glabra*), and chicory (*C. intybus*). Molecular analyses revealed that *G. aparine* tested positive for phytoplasma infection, while the other species were negative.

Sequence analyses of the amplified fragments from chickpea samples (GenBank accessions: PX952847, PX952848, PX952849, PX952850, PX952852, PX952854) (Table l) revealed 99.2% to 99.8% similarity with previously reported sequences and 99.4% to 99.8% similarity with '*Ca.* Phytoplasma phoenicium' strain ChicBSl92 (GenBank accession KY986921) and periwinkle virescence phytoplasma clone pv2 (GenBank accession KR706446.1). These results confirm that the phytoplasma associated with ChpWB in chickpea plants in Lorestan belongs to the *Ca.* Phytoplasma phoenicium (l6srRNA IX-B and IX-C).

The results of sequence analysis of Ml/M2 nested PCR productes indicated that the nucleotide sequences from cleavers (*G. aparine*) that were collected from chickpea symptomatic fields, had 99.8%–100% sequence identity to previously reported sequences from chickpea plants infected with phytoplasmas (GenBank accessions in Table l).

The virtual and real RFLP pattern derived from the l6S rDNA R16F2n/R2 fragments is most similar to the reference pattern of the l6SrIX-B and l6SrIX-C subgroup, with a similarity coefficient of 0.98; the phytoplasma under study is a therefore a molecular variant that could be enclosed in this subgroup IX-B and IX-C (Fig. 2 and 3).

The results of nested PCR analysis showed that among the 8 insect populations, no phytoplasma presence was detected.

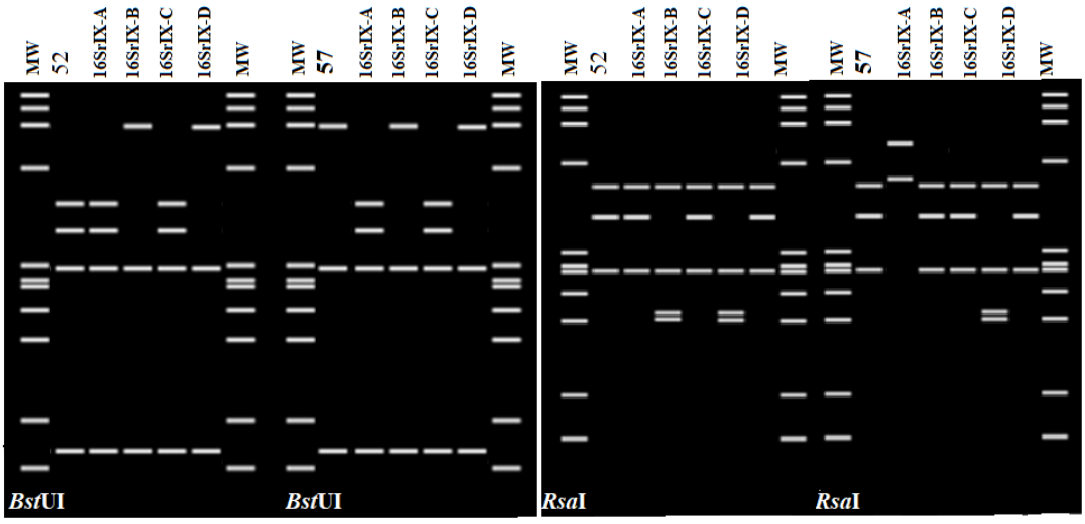
جدول 2. نتایج شناسایی فیتوپلاسما در نمونه‌های جمع‌آوری‌شده از مزارع نخود دارای علائم کوتولگی و افزایش شاخه‌دهی

Table 2. Results of the phytoplasma identification in samples collected from chickpea fields with symptoms of witches broon and stuntimg.

Symptoms and host species	No. of tested samples	No. of positive samples	'Ca. Phytoplasma'	Subgroup	GenBank Accession numbers
Symptomatic chickpea (*Cicer arietinum*)	90	86	'*Ca.* P. phoenicium' (KF923877)	16SrIX-B	MG748600, MG748601 PX952852
			periwinkle virescence phytoplasma clone pv2 (KR706446)	16SrIX-C	PX952847 PX952848 PX952850
Asymptomatic Iranian chickpea (*Cicer arietinum*)	33	2	'*Ca.* P. phoenicium' (KF923877)	16SrIX-B	PX952854
Chicory *(Cichorium intybus)*	6	0			
Cleavers *(Galium aparine)*	8	7	*Ca.* p. phoenicium (AF455040)	16SrIX-B	PX952849
licorice *(Glycyrrhiza glabra)*	3	0			
German chamomile *(Matricaria chamomilla)*	3	0			

The above identification was confirmed by the sequencing of selected phytoplasma strains from chickpea and cleavers (*G. aparine*) (Table 2).

The obtained aligned sequences of the chickpea, and cleavers phytoplasma cluster with 40 phytoplasmas were classified into 16SrIX-B and 16SrIX-C subgroups (Fig. 4). Inside the group 16SrIX branch, the chickpea and cleavers phytoplasma strains, that were

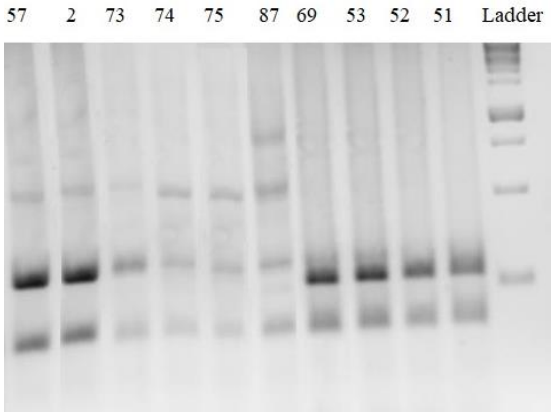


شکل ۲. نتایج RFLP مجازی با استفاده از نرم‌افزار iPhyClassifier مربوط به جدایه‌های 52 و 57

Figure 2. Virtual RFLP results using iPhyClassifier software for isolates 52 and 57.

identified in this study, clustered in the same subclade with previously characterized strains of '*Ca.* P. pheonicium' and periwinkle virescence phytoplasma. The results showed that only the cleavers (*G. aparine*) might serve as alternative host for ChpWB phytoplasma. Two-month-old grafted chickpea plants grown in the greenhouse developed typical ChpWB symptoms, including narrow leaves and proliferation, 1–1.5 months post-grafting (Figure 5). Nested PCR analysis confirmed that the grafted plants were infected with a phytoplasma closely related to previously reported sequences, including Candidatus Phytoplasma phoenicium strain ChicBS192 (GenBank accession KY986921) and periwinkle virescence phytoplasma clone pv2 (GenBank accession KR706446).

Results of dodder transmission from chickpea infected plants to healthy plants resulted in symptoms appearing 20 days after the dodder was established on healthy plants (Fig. 5).

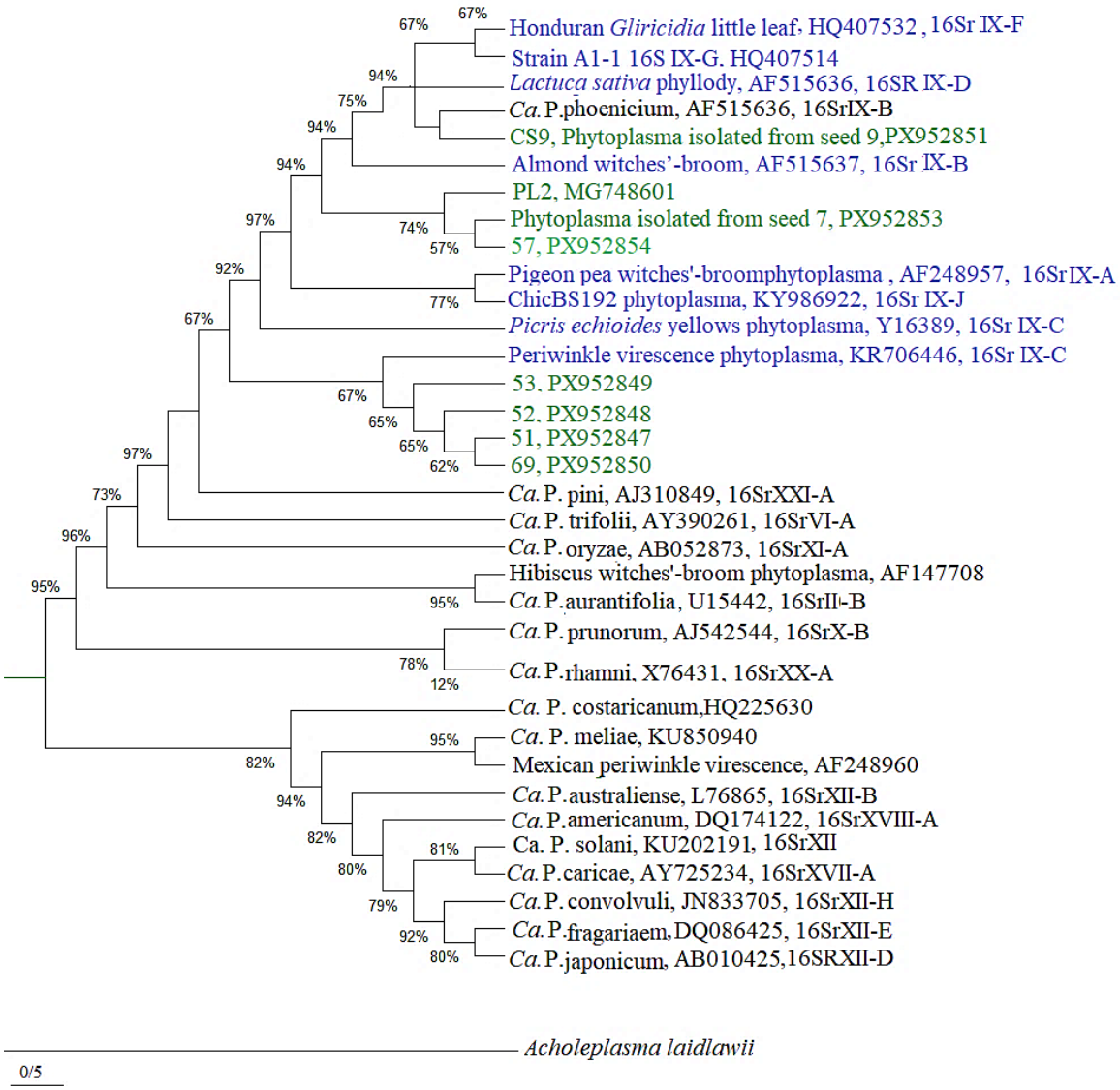


شکل 3. نتایج RFLP حقیقی با استفاده از آنزیم *Tsp* 5091 مربوط به نمونه های 57، 2، 73، 74، 75 (زیر گروه 16SrIX-B) و 69، 53، 52، 51 (زیر گروه 16SrIX-C)، در مقایسه با دی ان ای سایز مارکر (100 bp, Fermentase).

Figure 3. Actual RFLP results obtained using the *Tsp*509I enzyme for samples 57, 2, 73, 74, and 75 (subgroup 16SrIX-B), and samples 69, 53, 52, and 51 (subgroup 16SrIX-C), compared with the DNA size marker (100 bp, Fermentas).

were sampled. The 38% of plants grown from seeds collected from symptomatic plants resulted positive to phytoplasma presence 40 days after the plants growth from seeds. These plants showed two types of symptoms: growth retardation, dwarfism, and leaf thinning, and more severe growth retardation, yellowing, and necrosis of the leaves along with leaf thinning (Figure 7). At the flowering and podding stages, plants grown from these seeds in the greenhouse also showed symptoms of the disease, and in addition to small leaves and reduced growth, pods did not form in them compared to healthy control plants., and necrosis of the leaves along with leaf thinning (Figure 8).

The results of the study showed that the germination percentage of seeds from symptomless plants in laboratory conditions was 99% and the germination percentage of seeds from symptomatic plants was 78% (it is important to note that this 78% is the result of cultivating 40% of seeds with a healthy appearance collected from infected plants in the field (Fig. 6)). Comparison of means using the t-test method showed that these germination percentages are significantly different. After 20 to 30 days of seedling plants growth in the insect proof greenhouse, leaves from seedlings from infected and healthy plants



شکل 4. درخت فیلوژنتیکی سویه‌های فیتوپلاسمای همراه با جاروک نخود ایرانی با استفاده از نرم‌افزار MEGA12 و به روش Neighbor-Joining دادههای روی شاخه‌ها ضرایب بوت‌استرپ با ۱۰۰ تکرار هستند. توالی نمونه های استاندارد زیرگروه های اصلی گروه با رنگ آبی و توالیهای مربوط به این مطالعه با رنگ سبز مشخص شده است. توالیهای مربوط به سایر گروه های فایتوپلاسمایی با رنگ سیاه در نمودار مشخص است. در هر شاخه گونه ;کاندید نماینده گروه، شماره پذیرش در بانک ژن و گروه و زیر گروه مربوطه نشان داده شده است.

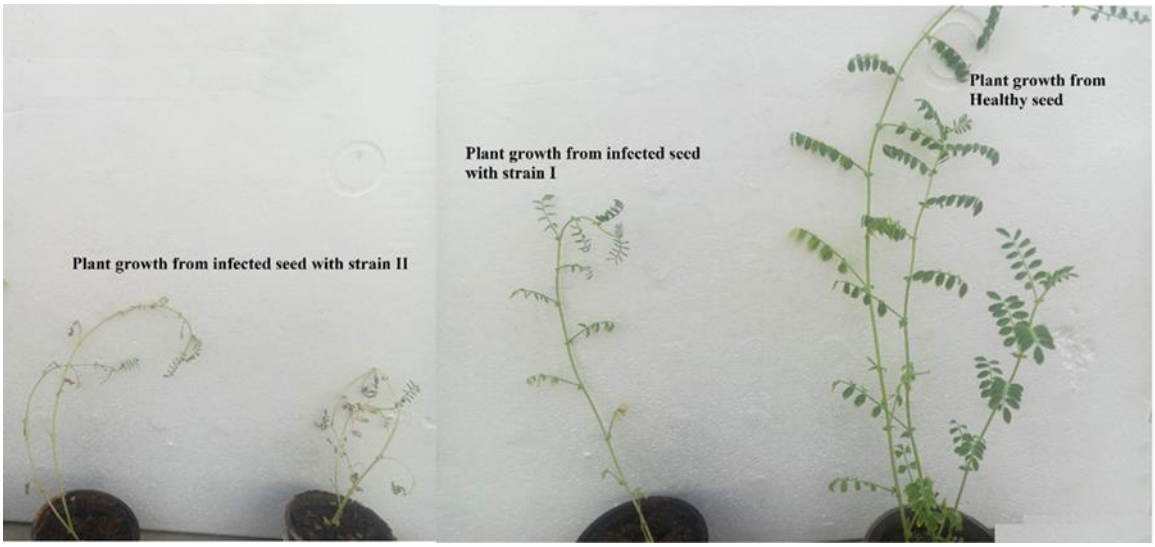
Figure 4. Phylogenetic tree of chickpea phytoplasma strains using Mega12 software by neighbor-joining method . Numbers on branches are bootstrap coefficients with 100 replicates. The sequences of standard reference strains representing the main subgroups of the group are indicated in blue, while the sequences obtained in this study are shown in green. Sequences belonging to other phytoplasma groups are shown in black in the diagram. In each branch, the representative candidate species, GenBank accession number, and the corresponding group and subgroup are indicated.

شکل 5. علایم حاصل از انتقال فایتوپلاسمای همراه با بیماری جاروک نخود توسط سِس از یک گیاه آلوده شده با پیوندک آلوده (سمت چپ) در مقایسه با یک گیاه سالم (سمت راست)

Figure 5.
.Symptoms of chickpea witches' broom disease caused by phytoplasma transmission via dodder (Cuscuta spp.) from a plant infected through infected grafting (left) compared with a healthy plant (right).

شکل 6. بذرهایی با ظاهر سالم (چپ)در مقایسه بابذرهای چروکیده حاصل از گیاهان نخود ایرانی آلوده به جاروک نخود (راست)

Figure 6. Seeds with healthy appearance (left) compared with shriveled seeds produced in witches' broom infected chickpea plants (Right).



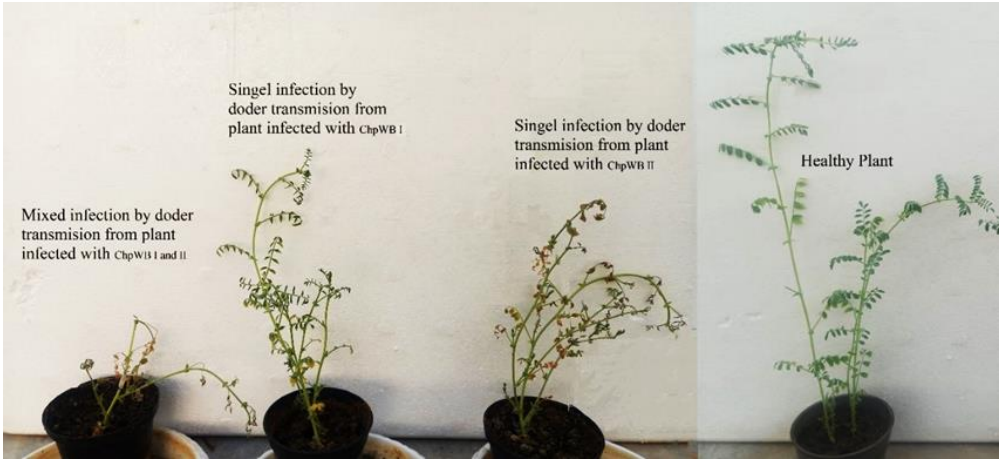
شکل 7. گیاهان رشدیافته از بذرهای جمع‌آوری‌شده از گیاهان دارای علائم در مقایسه با گیاهان حاصل از بذرهای گیاهان سالم، و بروز علائم کوچک‌برگی (ChpWBI (IX-B) or starin I) و رشد نخی‌شکل (ChpWBII (IX-C) or Strain II) در این گیاهان تحت شرایط گلخانه‌ای عاری از حشره.

Figure 7. Plants grown from seeds collected from symptomatic plants compared to plants grown from seeds from healthy plants and the appearance of symptoms of little leafing (ChpWBI (IX-B) or starin I) and threadlike growth in (ChpWBII (IX-C) or Strain II) these plants under insect proof greenhouse condition

Molecular analysis of the plants grown from infected seeds confirmed that two different strains related to 16SrIX subgroups were detected ìn these chickpea plants that names Strain I (ChpWBI (IX-B)) and Strain II (ChpWBII (IX-C)).

days after the transmissions (Fig. 8). Mixed infection of dodder transmission from infected plant with ChpWB I and II induce decline of inoculated plants (Fig 8).

Grafting and dodder transmission from these chickpeas infected samples to healthy plants resulted in symptoms appearing 20



شکل 8. انتقال آزمایشی تک‌سویه‌ای و آلودگی مخلوط سویه‌های فیتوپلاسما (ChpWB I و ChpWB II) از گیاهان آلوده به گیاهان سالم

Figure 8. Experimental single and mixed phytoplasma strains transmission of ChpWBI (IX-B) and ChpWBII (IX-C) from infected plant to healthy ones

Discussion

Preliminary studies showed that chickpeas collected from Noorabad, Lorestan Province in Iran, with symptoms of stunting, little leaves and witches broom accompanied by phytoplasma were in 16SrIX group (Ghayeb Zamharir and Dehghani, 2019). ChpWB disease is one of the new emerging diseases of chickpea with unknown etiology. The results presented here showed that ChpWB in Iran is a phytoplasma disease where some symptoms are similar to those reported befor (Yang *et al*., 2013; Luna-Esquivel *et al.*, 2004; Baquero, 1980).

In this study, the results of sequencing, RFLP and phylogenetic analysis of 16S ribosomal RNA gene of phytoplasma strains from chickpea samples with symptoms of witches' broom disease showed that a phytoplasma strain related to '*Ca.* P. pheonicium' was associated with the symptomatic samples. This result was confirmed by molecular analyses after grafting and dodder transmission. Among the collected weeds, chamomile (*M. chamomilla*), cleaver (*G. aparine*), licorice (*G. glabra*) and chicory (*C. intybus*), only cleaver (*G. aparine*) can serve as source for the phytoplasmas since the strains from this plant were molecularly similar to the phytoplasmas detected in chickpea witches' broom and proliferation disease. This species therefore could potentially act as a secondary or overwintering host of this pathogen. There is no report of cleaver phytoplasma infection in Iranian orchards and fields and this is the first report of this phytoplasma in this weed. The presence of mixed infection is suggested based on evidence from both RFLP analysis and sequence data, and that the use of high-throughput sequencing approaches may provide more accurate confirmation of this issue in future studies.

Biological analysis of *Ca*. P. phoenicium associated with ChpWB can be transmitted through seeds, although at a lower rate than expected. Seeds from symptomless plants exhibited a high germination rate (99%), while seeds from symptomatic plants germinated at a significantly lower rate (78%), suggesting that phytoplasma infection negatively affects seed viability. This reduction in germination may be linked to the physiological stress induced by the pathogen in developing seeds, consistent with previous reports of seed-associated phytoplasmas reducing germination and seedling vigor (Lee *et al*., 2000; Zhao *et al*., 2016).

Nested PCR analysis confirmed that 38% of seedlings derived from symptomatic seeds were positive for phytoplasma 40 days after germination, demonstrating vertical transmission from infected parent plants. The seedlings exhibited two distinct symptom types: mild symptoms, including growth retardation, dwarfism, and leaf thinning, and severe symptoms, including pronounced growth retardation, yellowing, necrosis, and leaf thinning. These observations suggest that the severity of seedling symptoms may depend on the phytoplasma titer, strain variation, or interactions with host plant physiology.

Importantly, seedlings that continued growth to the flowering and podding stages-maintained disease symptoms, including small leaves, reduced growth, leaf necrosis, and failure to form pods. This indicates that seed-transmitted phytoplasma infection can have long-term effects on plant development and reproductive success, potentially contributing to yield losses in affected fields. These findings align with previous reports in other legume crops, where seed transmission of phytoplasmas led to early growth impairment and reduced reproductive output (Al-Subhi *et al*., 2020; Yan *et al.*, 2010).

Overall, the results highlight the role of seeds as a potential source of inoculum for ChpWB phytoplasma, emphasizing the importance of using pathogen-free seeds and monitoring seedling health in integrated disease management programs. Further studies on phytoplasma distribution within seeds and the influence of environmental factors on vertical transmission could provide deeper insights into disease epidemiology and control strategies.

The results of the mixed infection experiments demonstrated that the simultaneous presence of two distinct ChpWB phytoplasma strains in chickpea plants significantly increased disease severity compared to single-strain infections. Co-infected seedlings exhibited not only reduced growth, narrow leaves, and increased internode spacing, but also severe symptoms such as leaf necrosis and hypersensitive reactions (Figures 7–8).

Under natural conditions, plants can be simultaneously infected by phytoplasmas from different 16Sr groups (Yu). Mixed infections in plants caused by phytoplasmas has been reported befor in some wooden and herbaceous plants (Marchi et al., 2015; Trivellone et al., 2026; Ghayebzamharir et al., 2022). Mixed infections in plants caused by phytoplasmas and diverse pathogens lead to challenges and complexities in diagnosing and controlling the associated plant diseases ().

These findings indicate the existence of synergistic interactions between the strains ChpWBI (IX-B) and ChpWBII (IX-C), whereby their combined presence amplifies tissue damage and disrupts normal physiological processes more than either strain alone. Such synergistic effects may result from several factors including (I) increased pathogen load: The two strains may collectively establish higher active phytoplasma populations in stems and leaves, intensifying stress on the host (Bertaccini *et al.*, 2014). (II) Differential tissue colonization: Each strain may preferentially infect different tissues, and together they accelerate the spread and severity of the disease (Seemüller & Schneider, 2007). (III) Molecular interactions between strains: The presence of one strain may induce expression of genes in the other, enhancing

the production of virulence factors that damage the host (Bertaccini *et al*., 2014).

This synergistic effect has important epidemiological implications for ChpWB phytoplasmas strains, as the coexistence of multiple strains in a region can increase disease severity and complicate management strategies. These results also highlight that studies focusing solely on single-strain infections may underestimate the true impact of the disease in the field (Kumar *et al*., 2011).

Therefore, effective management of ChpWB should consider the diversity of circulating strains, the potential for mixed infections, and implement strategies such as the use of pathogen-free seeds, monitoring of alternative host plants, and surveillance of multiple strains to reduce disease severity.

In conclusion, ChpWB associated with the presence of a phytoplasma strain belonging to the 16SrIX-B and 16SrIX-C group in Iran. The *G. aparine* could were the alternative host of ChpWB phytoplasma. Identification and characterization of the ChpWB agent is useful for developing strategies to control this phytoplasma-induced disease in chickpea in Iran.

References

BALOL, G., C. CHANNAKESHAVA and M.S. PATIL, (2021). Molecular characterization of Candidatus Phytoplasma aurantifolia isolate infecting chickpea (Cicer arietinum) in Dharwad, Karnataka. Legume Research. 44(7): 854-858. doi.org/ 10.18805/LR4156

BERTACCINI, A., B. DUDUK, S. PALTRINIERI, N. CONTALDO, (2014). Phytoplasmas and their interactions with hosts. Frontiers in Microbiology, 5, 1–12. Doi.og/ 10.1016/j.tplants.2005.09.008

CHE, H.Y., D.Q. LUO, R.Y. FU and Y.F. We, (2010). Molecular detection and identification of a phytoplasma associated with pigeon pea witches' broom disease in Hainan Province, China. Acta Phytopathologica Sinica, 40: 113-121.

DENG, S., C. HIRUKI, (1991). Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. Journal of Microbiological Methods, 14, 53-61. doi.org/10.1016/0167-7012(91)90007-D

DOYLE, J.J., J.L. DOYLE, (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemichal Bulletin, 19,11-15.

FAO (Food and Agriculture Organization). (2024). FAOSTAT Database faostat.fao.org/.

GAUR, P.M., TRIPATHI, S., GOWDA, C.L.L., PANDE, S., SHAMA, H.C., SHARMA, K.K., KASHIWAGI, J., VADEZ, V,. KRISHNAMURTHY, L. and VARSHENY, R.K. 2009. International efforts in chickpea improvement. In: Legumes for Ecological Sustainability: Emerging Challenges and Opportunities (Ali, M., Gupta, S., Basu, P.S., Niamuddin, G., eds.). Indian Society of Pulses Research and Development, Kanpur, India. pp. 359–377.

GHAYEB ZAMHARIR, M., S. SHAMELI, A. BERTACCINI, (2022). Epidemiology of soybean bud proliferation and seed pod abortion disease in Iran. Australasian Plant Pathology, doi.org/10.1007/s13313-022-00866-9.

GIBB, K.S., A.C. PADOVAN, B.D. MOGEN, (1995). Studies on sweet potato little-leaf phytoplasmas detected in sweet potato and other species growing in Northern Australia. Phytopathology, 85, 169–174. Doi.org/ 10.1094/Phyto-85-169

GUNDERSEN, D.E., I.M. LEE, (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea, 35, 144-151. DOI: 10.2307/42685262

HIRUKI, C. (1999). Paulownia witches' broom disease important in Asia. ISHA Acta Horticulture, 495, 63–68. Doi.org/ 10.17660/ActaHortic.1999.496.6

HOGENHOUT, S. A., K. OSHIMA, D. AMMAR, S. KAKIZAWA, H. N. KINGDOM, S. NAMBA, (2008), Phytoplasmas: Bacteria that manipulate plants and insects. Molecular Plant Pathology, 9(4), 403. https://doi.org/10.1111/j.1364-3703.2008.00472.x

INCP (2026), www.incp.ir

KUNAR, S., K. REDDY, RAJ, (2011). Witches' broom disease of legumes: epidemiology and management. Legume Research, 34(3), 173–182.

LEE, I. M., M. MARTINI, K. D. BOTTNER, Y. ZHAO, D. E. GUNGERSEN, (2000). Detection of phytoplasmas in plant and insect hosts using polymerase chain reaction. Phytopathology, 90(11), 1097–1107. Doi.org/10.1094/PHYTO.2001.91.8.741

LUNA-ESQUIVEL G, R.I. ROJAS-MARTINEZ, E. ZAVALETA-MEJIA and MARTINEZ-DE LA VEGA, (2004). First report of pigeon pea witches' broom phytoplasma in Altamirano, Guerrero, Mexico. Revista Mexicana de Fitopatología, 22: 125-128.

MAIXNER, M., U. AHRENS, and E. SEEMULLER, (1995). Detection of German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and vector by a specific PCR procedure. European Journal of Plant Pathology, 101:241-250. Doi.org/10.1007/BF01874780

MARCHI, G, T. CINELHI, D. RIZZO, L. STEFANI, E. GOTI, M. DELLA BARTOLA, A. LUVOST, A. PANATTONI, A. MARTERAZZI, (2015). "Occurrence of different phytoplasma infections in wild herbaceous dicots growing in vineyards affected by bois noir in Tuscany (Italy)", Phytopathol. Mediterr., 54 (3): 504–515. Doi.org/ 10.14601/Phytopathol_Mediterr-16239

MCCOY R.E., J.H. TSAI, R.C. NORRIS and G.H. GWIN, (1983). Pigeon pea witches' broom in Florida. Plant Disease, 67: 443-445. Doi.org/ 10.1094/PD-67-443.

RAJ, S. K., M. S. KHAN, S. K. SNEHI, S. SRIVASTAVA, H. B. SINGH, (2006). Candidatus Phytoplasma asteris isolate associated with a little leaf disease of pigeon pea in India. Plant Pathology, 55, 823–830. https://doi.org/10.1111/J.1365-3059.2006.01483.X

RAO G.P., M. KUMAR, S. TOMAR, S.K. SINGH and I.M. JOHNSON, 2018. Detection and identification of four 16Sr subgroups of phytoplasmas associated with different legume crops in India. European Journal of

Plant Pathology, 150(2): 507-513. Doi.org/10.1007/s10658-017-1278-6

SEEMULLER, E., & B. SCHNEIDER, (2007). Phytoplasmas: diversity, detection, and interactions with plants and insects. Journal of Plant Pathology, 89(2), 105–118. doi.org/10.3390/insects17020223

TAMURA, K., M. NEI, S. KUMAR, (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101: 11030-11035. Doi.org/10.1073/pnas.0404206101

THMPSON, J.D., T.J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN, D.G. HIGGINS, (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 25, 4876–4882. doi.org/10.1093/nar/25.24.4876

TRIVELLONE, V., W. NOOR SYEDA, M. GHAYEB ZAMHARIR, C. H. DIETRIICH, (2026). Diversity of Phytoplasmas Infecting Plants and Insects in Iran Reveals Two Novel Ribosomal Subgroups. Insects, 17 (223): 1-16. Doi.org/103390/Insectes17020223.

WANG, R., B. BAI, D. LI, J. WANG, W. HUANG, Y. WU, L. ZHAO, (2024). Phytoplasma: A plant pathogen that cannot be ignored in agricultural production—Research progress and outlook. Molecular Plant Pathology, 25(2), e13437. doi.org/10.1111/mpp.13437

WEI, W., R.E. DAVIS, M. LEE, Y. ZHAO, (2007), Computer-simulated RFLP analysis of 16SrRNA genes: identification of ten new phytoplasma groups. International Journal of Systematic and Evolutionary Microbiology, 57,1855–1867. Doi.org/ 10.1099/ijs.0.65000-0

WEI, W., I.M. LEE, R.E. Davis, X. Suo, Y. Zhao, (2008). Automated RFLP pattern comparison and similarity coefficient calculation for rapid delineation of new and distinct phytoplasma 16Sr subgroup lineages. International Journal of Systematic and Evolutionary Microbiology, 58, 2368-2377. Doi.org/ 10.1099/ijs.0.65868-0

WEINTRAUB, P., T. VALERIA, K., KERSTIN, (2019), The Biology and Ecology of Leafhopper Transmission of Phytoplasmas: Transmission and Management of Phytoplasma - Associated Diseases. 10.1007/978-981-13-2832-9_2. Doi.org/10.1007/978-981-13-2832-9_2

ZWOLINSKA A, K. KRAWCZYK, H. POSPIEZNY, (2012) Molecular Characterization of Stolbur Phytoplasma Associated with Pea Plants in Poland. J Phytopathol 160 (7-8): 317–323. doi.org/10.1111/j.1439-0434.2012.01903.X.

ZHAO, Y., R.E. DAVIS, (2016). Criteria for phytoplasma 16Sr group/subgroup delineation and the need of a platform for proper registration of new groups and subgroups. International Journal of Systematic and Evolutionary Microbiology, 66, 2121-2123. doi.org/ 10.1099/ijsem.0.000999

ZHAI, Y., W .WEI, I.M. LEE, J. SHAO, X. SUO, R.E. DAVIS, (2009). Construction of an interactive online phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). International Journal of Systematic and Evolutionary Microbiology, 59, 2582–2593. doi.org/10.1099/ijs.0.010249-0

ZHANG, J., X. XANG, W. WEI, (2020). Mixed infections of phytoplasmas: molecular interactions and symptom expression. Plant Disease, 104(6), 1545–1555.

YAN, J., S. RAJ, G. P. RAO, (2010). Effects of phytoplasma infection on growth and reproductive performance in legume crops. Crop Protection, 29(10), 1128–1135. doi.org/0.3389/fmicb.2019.01349.

YANG, S.Y., N. HABILI, A. AODA, I. DUNDAS, J.G. PAULL and J.W. RANDLES, (2013). Three group 16SrII phytoplasma variants detected in co-located pigeon pea, lucerne and tree medic in South Australia. Australasian Plant Disease Notes, 8: 125–129. Yu, S_S. (2022). Understanding Mixed Infections of Phytoplasmas with Diverse Plant Pathogens. In" Phytoplasmas: Genomes, Plant Hosts and Vectors." 265-280. Doi.org/ 10.1079/9781800627031.0017