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Phytoplasma Transmission by Seeds in Alfalfa: A Risk for Agricultural Crops and Environment

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Abstract

Recent research has demonstrated a presence inside the seeds of several plant species of endophytic bacteria that can directly or indirectly interact with germination and seedling growth. Phytoplasmas are plant-pathogenic bacteria that severely impact the agricultural productivity of several crops, including alfalfa, a crucial forage crop in which seed transmission was reported. Therefore, understanding the transmission pathways of phytoplasmas is essential for developing effective control strategies. This study investigates the seed transmission of phytoplasmas in alfalfa using seeds collected in Oman in 2002 and kept in a dry environment in a laboratory for 20 years. The sterilized seeds were germinated and grown in agar medium under sterile conditions and transplanted in soil under greenhouse-controlled insect-proof conditions. Utilizing polymerase chain reaction (PCR) and nested PCR followed by RFLP and sequencing analyses, the alfalfa seedlings were screened for the phytoplasma presence. The detection of phytoplasmas in 16SrIII, 16SrV, 16SrX, and 16SrXII groups was achieved, confirming the preliminary results obtained in the 2002 testing of the same seed batches. This finding indicates that seed transmission could be a critical pathway for the spread of these pathogens in alfalfa, considering their survival in seeds for more than 20 years. Further investigations into the mechanisms of seed transmission and the development of resistant alfalfa varieties are essential to enhance the sustainability and productivity of alfalfa cultivation, thereby supporting the agricultural sector's efforts to meet the growing demand for high-quality forages.

Keywords: molecular analysis; 16Sr RNA gene; PCR; RFLP analysis; plant pathogenic bacteria; epidemiology

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1. Introduction

Phytoplasmas that are associated with several plant diseases are special prokaryotic microorganisms without cell walls. A team of Japanese researchers identified plant-pathogenic phytoplasmas in 1967 [1], initially describing them as bacteria resembling mycoplasmas (mycoplasma-like organisms, MLOs). They have a pleomorphic form for the lack of a cell wall and are 200–800 nm in diameter. Their genome is also relatively tiny, ranging from 680 to 1530 kb. They live in the phloem sieve components of infected plants and in the hemolymph of insects. Phytoplasmas are associated with diseases in over 1000 plant species worldwide, including agricultural, horticultural, ornamental, trees, and weed species [2,3]. A number of insects from the families Cicadellidae, Cixidae, Psyllidae, Delphacidae, and Derbidae are the recognized vectors of phytoplasmas [4]. Phytoplasma detection and

identification are carried out by molecular analysis of 16S rRNA genes followed by restriction fragment-length polymorphism (RFLP) and/or sequence analyses [5] and they are classified in '*Candidatus* Phytoplasma' species by sequence comparison [6]. The availability of PCR primers that amplify a fragment of the 16S rRNA-encoding gene from phytoplasmas has made it possible to identify phytoplasmas infecting both plants and insects. The sequence of the amplified fragment could then be subjected to in silico RFLP analysis and compared to RFLP patterns from reference sequences or directly sequenced and compared with sequences available in databases. Understanding the transmission pathways of phytoplasmas is essential for maintaining plant health and agricultural sustainability. In addition, effective disease control and food biosecurity depend on identifying and understanding the various transmission routes. Research on phytoplasma transmission is essential for producing phytoplasma-free and resistant plans for managing diseases, evaluating risks, and helping with epidemiological investigations. The effects of phytoplasma infections and the maintenance of agricultural productivity, economic stability, and ecological integrity can be obtained by completely understanding their transmission processes. While insect vectors are the primary means of phytoplasma spread, increasing attention has been given to alternative transmission pathways, including seed transmission. In some cases, the pathogen can be carried by seeds harvested from symptomatic plants, potentially leading to infected seedlings after germination. Phytoplasmas have been found to invade floral tissues, fruits, seeds, and even embryos. Notably, seeds from infected plants often remain viable and capable of germination, raising concerns about the unintentional spread of phytoplasmas through seed trade. However, the production of seeds from infected plants is severely compromised by the phytoplasma presence in mother plants due to the malformations, withering, small size, and low weight that are very often associated with their presence. They were observed by electron microscopy in several plant organs, including floral structures in which they usually are packed [7]. The presence of phytoplasmas was found in tomato, maize, and oilseed rape seedlings derived from infected plants [8]. "Stolbur" phytoplasmas were identified in pea seedlings from seeds produced from infected plants [9]. The seed transmission of '*Ca*. P. asteris' was also demonstrated in commercial carrot seeds [10,11]. Phytoplasma transmission by the seeds of soybean plants showing bud proliferation and seed-pod abortion was recently suggested [12]. Phytoplasmas enclosed in 16SrI and 16SrXII groups were identified in both the mother plants and the seedlings of tomatoes collected from fields in southern Italy [13], and the presence of phytoplasma seed transmission in brinjal plants with little leaf disease in India was demonstrated [14,15]. Seeds from symptomatic tomato and chili samples infected with '*Ca*. P. asteris' were also tested in Mauritius after their sowing under insect-proof conditions. After 4 to 6 weeks, a total of 1% tomato and 4.2% chili tested positive for the same phytoplasmas [16].

The presence of phytoplasmas in plants is associated with yellowing, stunting, distortion, witches' broom (proliferation of shoots), and general deterioration. In particular, phytoplasma infections in alfalfa induce morphophysiological disturbances that produce abnormal growth and stunted development [17] (Figure 1).

In different parts of the world, alfalfa witches' broom disease is a severe problem for forage production, and globally the presence of the malformation and stunting in this crop is associated with the presence of different phytoplasmas. To confirm the seed transmission of phytoplasmas in alfalfa as reported in a preliminary findings [18], the aim of this study was to investigate the seed transmission of phytoplasmas in the same seed batches collected from different regions of Oman. The seedlings of the same batches were found infected by these bacteria and maintained in laboratory storage for 20 years. The germination and molecular testing were then performed to verify the presence of phytoplasmas in the seedlings grown after storage.



Figure 1. Symptoms of stunting, malformation and witches' broom in diseased alfalfa in Oman.

2. Materials and Methods

2.1. Seed Collection

Seeds of *Medicago sativa* (alfalfa) were collected from various regions across Oman in 2002. The seeds were collected in 13 regions which include Al Hamara, Al Kami, Bahla, Dhank, Ibri, Manah, Moday Bi, Nizwa, Sur, Saham, Salalah, Sohar, Yanqoul. A total of 28 batch samples were tested from the above-mentioned regions (Figure 2). Part of the seeds were tested at the collection time and found to be infected by diverse phytoplasmas [18].

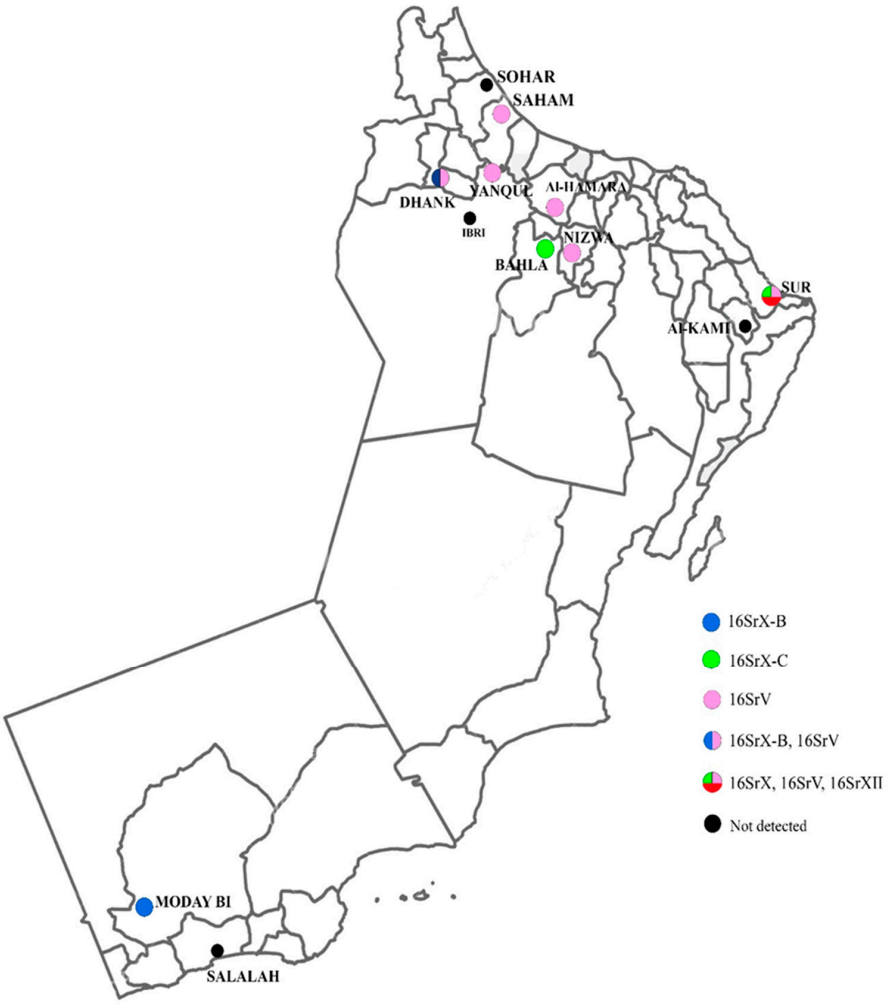

Figure 2. The geographic regions of Oman in which the seed samples were collected: the phytoplasmas detected are reported as ribosomal groups.

2.2. Seed Sterilization, Sowing and Germination

The seed sterilization was performed using hypochlorite 30% for 3 min followed by 70% ethanol for 2 min. After that, the seeds were thoroughly washed three times with sterile distilled water to remove residual chemical agents and prevent potential phytotoxic effects. A total of 268 seeds was sterilized and soaked for about 3–4 h in sterile distilled water and then they were sown in glass tubes (3 seeds per tube) containing sterile agar nutrient medium as described by Murashige and Skoog [19]. The process was carried out under a sterile laminar flow hood. The tubes were closed with a cap and then wrapped with a polythene film to prevent contamination and reduce evaporation and were then kept in controlled conditions at 23 + 2 °C with 16 h artificial light. The seed germination was monitored daily and germination started from the fifth day after sowing (Figure 3).

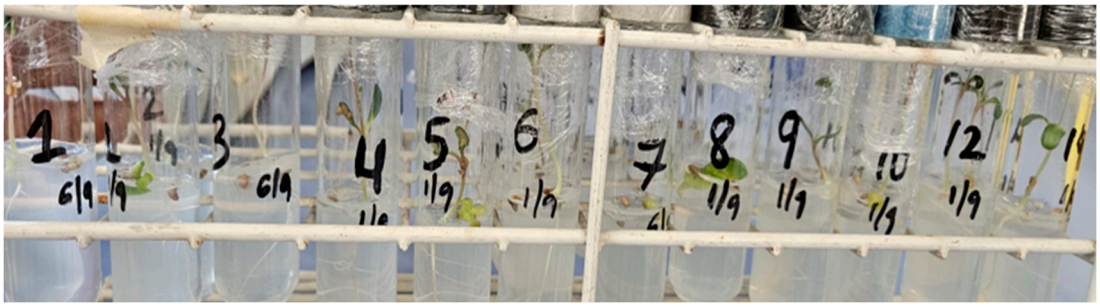

Figure 3. Alfalfa seeds germinating in agar medium in glass tubes (tubes are progressively numbered, below is the day of sowing).

2.3. Transplantation to Pots in Greenhouse

When the seedlings were 3 to 5 cm height, they were transplanted into pots placed in greenhouse under insect-proof condition with controlled temperature 20 + 5 °C and 60–70% relative humidity. To optimize their growth the plantlets were protected with plastic covers to help in the maintenance of humidity and temperature. Over a period of 35 weeks, the seedlings were regularly watered and monitored. The 26 survived seedlings were used for repeated DNA extractions for the evaluation of phytoplasma presence over time. For this purpose, the plants were tested twice at 30 and 150 days after transplanting (DAT).

2.4. DNA Extraction, Phytoplasma Detection and Identification

The DNA extractions were performed for all surviving plants using a cetyltrimethylammonium bromide (CTAB) method [20]. The analyzed tissues were extracted from leaves and midribs, and 1 g of fresh or frozen tissue was employed per plant sample. The tissues were pulverized with liquid nitrogen in a sterile porcelain mortar with sterile pestle into a fine powder and CTAB buffer (CTAB 3%, Tris HCI 100 mM, pH8, EDTA 10 mM, NaCl 1.4 M) plus 0.2% of 2 mercaptoethanol was added under chemical hood. This was followed by incubation for 20 min at 65 °C, the addition of chloroform, and centrifugation for 10 min at 11,000 rpm in a cold centrifuge (4 °C) (Beckman, Allegra, Krefeld, Germany). Then, 1 mL of isopropanol was added to the supernatant, and the mixture was incubated at −20 °C for 10 min and centrifuged for 15 min at 11,000 rpm (4 °C). The pellet was air-dried after washing with 70% ethanol and was maintained at 4 °C or at −20 °C after its resuspension in TE buffer.

The DNA extracted was diluted to 1:30 with sterile distilled H_2O and about 20 to 50 ng were used as a template for PCR with primers P1/P7 and R16F2n/R2 [21–23]. The nested PCRs were carried out on the R16F2n/R2 amplicons diluted with H_2O at 1:30 with primers fU5/rU3 [24] and M1 = $16R_{758f}$/M2 = $16S_{1232r}$ amplifying shorter fragments (about 800 and 550 nt, respectively) form phytoplasmas in all ribosomal subgroups [25]. The negative

controls were employed in each reaction and consisted of replacing the DNA template with sterile distilled H_2O, while the positive controls were not used to avoid carryover contamination. The cycling was performed as described [26]; for the nested PCRs, the denaturation and annealing of 45 s were followed by a 2 min of elongation with the same temperatures as for the PCR cycle. Agarose gel (1%) electrophoresis at 100 V followed by UV examination under transilluminator was used for the visualization of PCR products.

RFLP analyses on M1/M2 and fU5/rU3 amplicons with *Tru1*I and, in some cases, *Tsp509*I (Thermo Fisher Scientific, Vilnius, Lithuania) restriction enzyme provided a preliminary subgroup affiliation that allowed to perform further nested PCR using R16(I)F1/R1, R16(V)F1/R1 and R16(X)F1/R1 primers amplifying phytoplasmas in the 16SrI, 16SrXII, 16SrV, and 16SrX ribosomal subgroups [27,28]. On these latter amplicons further RFLP analyses and/or confirmatory sequencing were performed using enzymes selected according with the detected ribosomal subgroup, i.e., *Tru1*I for R16(I)F1/R1 amplicons, *Taq*I (Thermo Fisher Scientific, Vilnius, Lithuania) for the R16(V)F1/R1 amplicons and *Rsa*I and *Ssp*I (Thermo Fisher Scientific, Vilnius, Lithuania) for the 16Sr(X)F1/R1 amplicons. Around 3–10 µL of each amplicon obtained in nested PCR were used for RFLP analyses carried out with *Tru*I, *Tsp509*I and *Taq*I at 65 °C and *Rsa*I at 37 °C following the manufacturer's instructions. Restriction profiles were visualized in 6.7% polyacrylamide gel run at 140 V and result visualization was performed as described above.

Selected amplicons from the 16S rRNA gene were sequenced with the respective primer in forward and reverse at the Macrogen Company (Milan, Italy) to confirm the identity of phytoplasmas. The sequences obtained were then assembled and manually edited using ChromasPro v1.7.5 (Technelysium, Tewantin, QLD, Australia), and consensus sequences were compared with the nucleotide sequences present at the NCBI GenBank database (National Center for Biotechnology Information) using the BLASTn algorithm.

3. Results

The germination percentages exhibited significant variations among the alfalfa seeds collected from distinct locations in Oman in 2002 and maintained in dry conditions in Petri dishes in laboratory room-temperature environment (Table 1). After 30 days out of the 268 seeds, 47 seeds germinated, which indicates a germination rate of approximately 17.5%; the survival rate after transplantation in the greenhouse was 89.6%. However, two of the batches did not germinate (Manat and Sohar), the lowest germination percentages were recorded for Ibri (1.2%) and Yanqoul (3.8%). The batches with higher germination percentages were Dhank (41.7%) and Saham (40.0%). Among the germinated seeds, 29 seedlings were selected and carefully transplanted into a tray containing the potting mixture.

Table 1. Germination and survival percentages of the alfalfa seedlings.

Sample Location	No. Seeds	No. Seeds Germinated	Germination	Transplanted	No. Plants Survived	Survival Rate
Al Hamara	12	4	33.3%	4	4	100%
Al Kami	13	4	30.8%	3	3	100%
Bahla	18	3	16.7%	3	2	66.7%
Dhank	12	5	41.7%	3	2	66.7%
Ibri	17	2	1.2%	2	2	100%
Manah	17	0	-	-	-	-
Moday bi	15	4	26.7%	3	3	100%
Nizwa	17	2	11.8%	1	1	100%

Table 1. *Cont.*

Sample Location	No. Seeds	No. Seeds Germinated	Germination	Transplanted	No. Plants Survived	Survival Rate
Sur	18	5	27.8%	2	2	100%
Saham	15	6	40.0%	3	3	100%
Salalah	44	9	20.5%	2	2	100%
Sohar	24	0	-	-	-	-
Yanqoul	26	1	3.8%	1	1	100%

Out of the 29 transplanted seedlings, 26 survived and continued to grow without symptoms during the entire period of observation except one plant (alfalfa 4b) which showed stunted growth (Figure 4).



Figure 4. (**Top**): Alfalfa seedlings transferred from small trays to pots in the insect-proof greenhouse. (**Bottom**): On the left, a comparison is shown between an asymptomatic alfalfa potted plant negative to phytoplasma presence (**left**) and the symptomatic plant 4b (**right**) infected by phytoplasmas. On the right, a close up of the alfalfa 4b stunted plant with no major morphological modifications is shown.

When the plants were cut for DNA extraction 30 days after transplanting, they revegetated, showing no phytoplasma-associated symptoms. The stunted plant was used for nucleic acid extraction as a whole, enclosing the roots so it was not possible to retest it at

150 DAT. In the testing performed at 30 days from DAT, a total of 9 samples were positive in nested PCR, and their RFLP profiles on M1/M2 amplicons allowed to identify in distinct samples the presence of phytoplasmas belonging to ribosomal groups 16SrV and 16SrX (Figure 5).

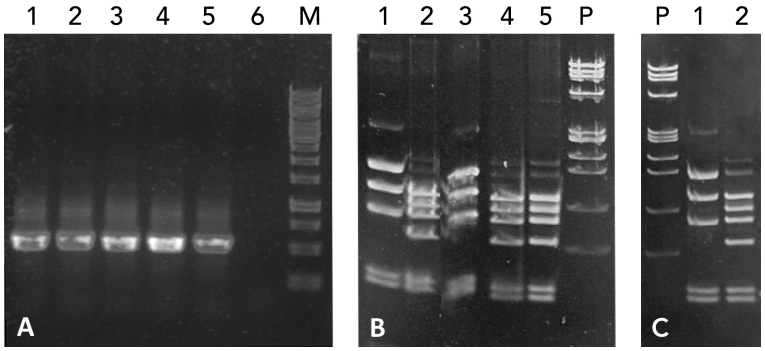

Figure 5. (**A**) Agarose 1% gel displaying the presence of bands amplified with primer pair M1/M2; from 1 to 5, alfalfa samples; 6, H_2O negative control; M, marker 1 kb hyper ladder. (**B**) Polyacrylamide 6.7% gels displaying the positive samples in (**A**) digested with enzyme *Tru1*I. (**C**) Phytoplasma control strains from EPPO-Qbank collection (https://qbank.eppo.int/, 29 March 2025) amplified with primer pair M1/M2 and digested with *Tru1*I: 1, AP15, apple proliferation ('*Candidatus* Phytoplasma mali', 16SrX-A) and 2, EY-C, elm yellows strain C ('*Ca*. P. ulmi' 16SrV-A), (A. Bertaccini, unpublished). P, marker phiX174 DNA/*BsuR*I (*Hae*III) cut.

To cross-verify the identity of the phytoplasmas belonging to groups 16SrV and 16SrX, ribosomal group-specific primers 16Sr(V)F1/R1 and 16Sr(X)F1/R1 were used in nested PCR assays to reamplify the respective R16F2n/R2 amplicons. The obtained amplicons were digested with *Taq*I and *Rsa*I respectively and showed profiles that were identical to those of the reference strains of phytoplasmas in the respective ribosomal groups (Figure 6).

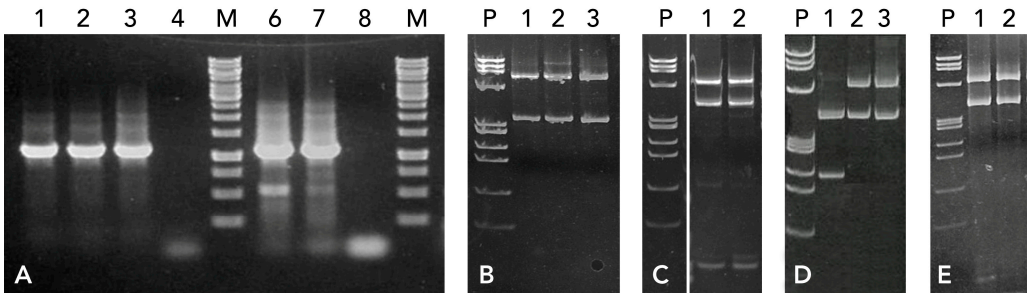

Figure 6. (**A**) Agarose 1% gel displaying the presence of bands amplified with primer pairs R16(V)F1/R1 (left) and R16(X)F1/R1 (right). From 1 to 3 and from 6 to 8, alfalfa samples; 4 and 8, H_2O negative control; M, marker 1 kb hyper ladder. (**B**,**C**) Polyacrylamide 6.7% gels displaying, respectively, (**B**) the 16SrV-positive samples digested with enzyme *Taq*I and (**C**) the 16SrX-positive alfalfa samples digested with the enzyme *Rsa*I. (**D**,**E**) Control strains from EPPO-Qbank collection (https://qbank.eppo.int/, 29 March 2025) (A. Bertaccini, unpublished). (**D**) 1, GESFY-1, German European stone fruit yellows ('*Ca*. P. prunorum', 16SrX-B); 2, AP15, apple proliferation ('*Ca*. P. mali', 16SrX-A); 3, PD, pear decline ('*Ca*. P. pyri', 16SrX-C) amplified with primers R16(X)F1/R1 and digested with *Rsa*I. (**E**) 1, EY-C, elm yellows strain C ('*Ca*. P. ulmi', 16SrV-A); 2, ULW, elm witches' broom ('*Ca*. P. ulmi', 16SrV-A) amplified with primers R16(V)F1/R1 and digested with *Taq*I. P, marker phiX174 DNA/*BsuR*I (*Hae*III) cut.

One each of these 16SrV and 16SrX group phytoplasma amplicons were sequenced and showed 99.80% identity to '*Ca*. P. ulmi' (16SrV-A), and 98.30% identity to '*Ca*. P. pyri' (16SrX-C), respectively, confirming the ribosomal grouping results of the RFLP analysis on

generic and specific amplicons. All the alfalfa samples that were negative in the nested PCR were tested again with M1/M2 primers; however, multiple bands were observed in agarose gel for all samples except for two and their RFLP profiles were unclear. For the two samples with the correct size of the band, the RFLP profiles were referred to phytoplasmas belonging to group 16SrV. A repetition of testing using fU5/rU3 in PCR and M1/M2 in nested PCR was employed on selected samples to cross-verify the phytoplasma presence and identity them. Two samples (alfalfa 18a and 19a) tested positive and their RFLP profiles were confirmed as belonging to ribosomal groups 16SrX and 16SrV, respectively. Two additional samples (alfalfa 10b and 16b) produced the expected length bands, and after the RFLP analysis, phytoplasmas belonging to ribosomal groups 16SrX were identified. As the RFLP profiles were identical, one of the samples was sequenced and the consensus sequence shows 99.58% identity to '*Ca*. P. prunorum' (16SrX-B). Further bands were obtained from a number of samples, but they were either of not expected size or were multiple.

For the amplicon of alfalfa plant 4b (Figure 4) showing stunted growth had an unclear *Tru1*I RFLP profile of M1/M2 amplicon a further digestion with the enzyme *Tsp509*I was performed. After accurate examination and comparison of this latter profile with those of selected reference strains of different phytoplasmas, a mixed phytoplasma profile was distinguished (Figure 7). Despite the difficulty in identifying the specific ribosomal group, phytoplasma profiles referrable to ribosomal groups 16SrII, -IX, and -XII were visible, while a fourth profile was not distinguishable since it is identical to that of phytoplasmas enclosed in diverse ribosomal groups.

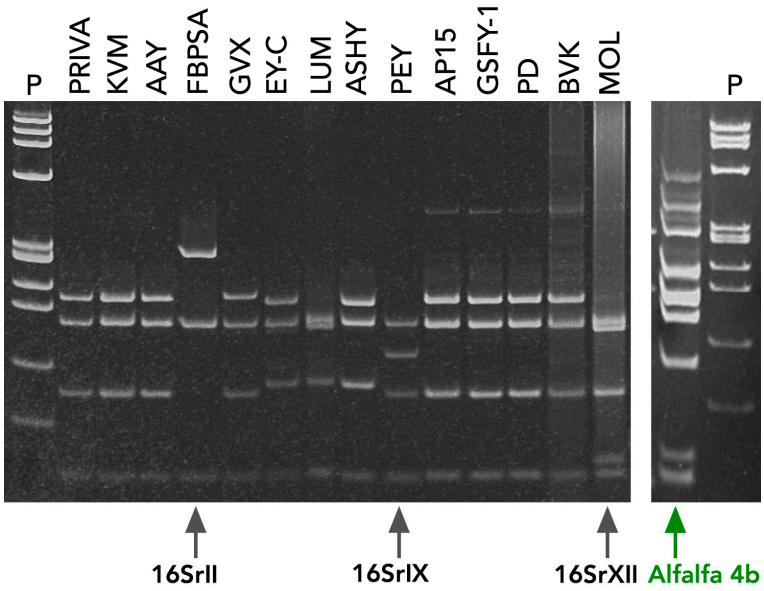

Figure 7. Polyacrylamide 6.7% gels displaying the RFLP profiles (on the left) of phytoplasma control strains belonging to different ribosomal groups/subgroups amplified with M1/M2 primers and digested with *Tsp509*I. The phytoplasma strains from EPPO-Qbank collection (https://qbank.eppo.int/, 29 March 2025) are PRIVA ('*Ca*. P. asteris', 16SrI-A); KVM ('*Ca*. P. tritici', 16SrI-C); AAY ('*Ca*. P. asteris', 16SrI-B); FBPSA ('*Ca*. P. aurantifolia = citri'16SrII-C); GVX ('*Ca*. P. pruni', 16SrIII-A); EY-C ('*Ca*. P. ulmi', 16SrV-A); LUM ('*Ca*. P. trifolii', 16SrVI-C); ASHY ('*Ca*. P. fraxini', 16SrVII-A); PEY, (16SrIX-C); AP15 ('*Ca*. P. mali', 16SrX-A); GSFY-1 ('*Ca*. P. prunorum', 16SrX-B); PD ('*Ca*. P. pyri', 16SrX-C); BVK (16SrXI-C); MOL ('*Ca*. P. solani', 16SrXII-A) from Satta [29]. On the right, sample alfalfa 4b digested with *Tsp509*I is depicted, and the profiles recognized in this alfalfa sample are indicated by the arrow below the reference strains' RFLP picture. The recognized ribosomal groups are 16SrII, -IX, and -XII. P, marker phiX174 DNA/*BsuR*I (*Hae*III) cut.

The second nucleic acid extraction was performed on alfalfa seedlings 150 days after transplanting them from the 24 plantlets that survived. Clear amplification bands were visible in agarose gel for four samples using M1/M2 primers in nested PCR. Other samples showed faint bands, and the remaining samples were negative, showing no bands. Multiple bands were observed in RFLP in some samples which were not linked to recognizable phytoplasma profiles, but profiles of phytoplasmas belonging to the groups 16SrIII and 16SrXII were identified in the seedlings in which the presence of phytoplasmas in group 16SrX was identified at 30 DAT (Figure 8A and Table 2). The testing performed at 150 DAT showed that the majority of the plants with positive results for phytoplasma presence at 30 DAT were negative, while one of the samples from a plant that was negative at 30 DAT was positive to phytoplasmas of the group 16SrXII (Table 2). The detected phytoplasma identities were further confirmed through sequencing analysis in which the 16SrIII sequence of the amplicon showed a 100% match to '*Ca*. P. pruni'. The consensus sequence of the 16SrV RFLP identified amplicon showed a 99.38% match to '*Ca*. P. ulmi'. Since phytoplasmas belonging to groups 16SrXII were detected and confirmed by RFLP and sequencing analysis, to cross-confirm the phytoplasma identity, nested PCR using R16(I)F1/R1 primers detecting phytoplasmas belonging to 16SrI, 16SrII, and 16SrXII was employed on the samples positive for this ribosomal group. Sample (alfalfa 19a) showed an RFLP profile identified as 16SrXII, confirming the phytoplasma ribosomal group affiliation (Figure 8B).

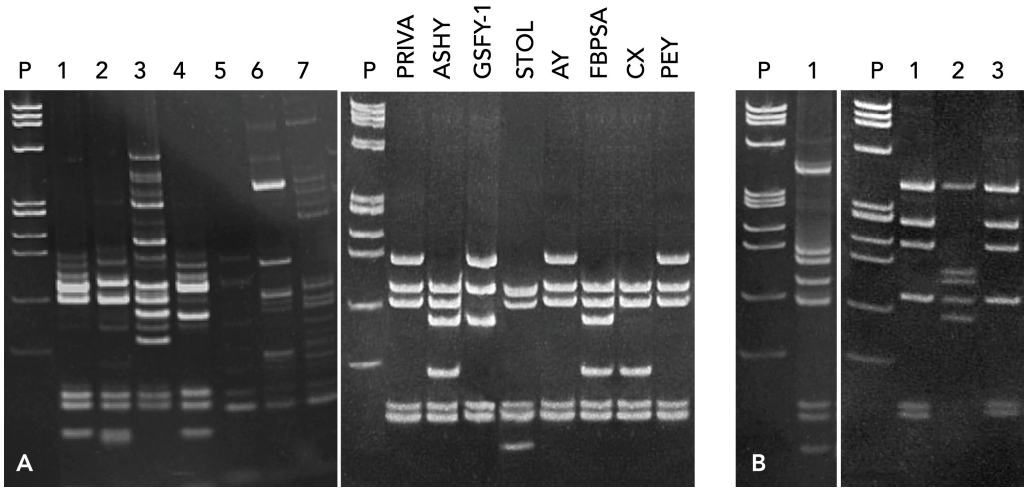

Figure 8. Polyacrylamide gels displaying amplicons in RFLP cut with the restriction enzyme *Tru1*I. In figure (**A**) on the left, amplicons obtained with M1/M2 primers from alfalfa samples: 1: 19a, 2: 10b, 3: 1a, 4: 23a, 5: 20a, 6: 10a, 7: 22a; on the right, the phytoplasma reference strains from EPPO-Qbank collection (https://qbank.eppo.int/ 29 March 2025) amplified with M1/M2 are PRIVA ('*Ca*. P. asteris', 16SrI-A); ASHY ('*Ca*. P. fraxini', 16SrVII-A); 3, GSFY-1 ('*Ca*. P. prunorum', 16SrX-B); STOL ('*Ca*. P. solani', 16SrXII-A); AY ('*Ca*. P. asteris', 16SrI-B); FBPSA ('*Ca*. P. aurantifolia = citri', 16SrII-C); CX ('*Ca*. P. pruni', 16SrIII-A); PEY, (16SrIX-C) from Satta [29]. In figure (**B**) RFLP results of the amplicon obtained using R16(I)F1R1 primers (left) on sample alfalfa 19a and (right) control strains: 1, AY ('*Ca*. P. asteris', 16SrI-B); 2, STOL ('*Ca*. P. solani', 16SrXII-A); and 3, tomato infected with '*Ca*. P. asteris' from Mateeti et al. [13]. P, marker phiX174 DNA/*BsuR*I (*Hae*III) cut.

A nested PCR using M1/M2 was also performed on R16F2n/R2 amplicons to cross-confirm the 16SrV-positive sample. In addition, the 16SrXII identified sample was added to this PCR as positive control. Both samples tested positive with M1/M2 primers. The RFLP profile of the 16SrXII detected sample was again identified as 16SrXII and further confirmed by sequencing results which showed its 99.39% match to the '*Ca*. P. solani' strain from Iranian alfalfa witches' broom-infected plants (GenBank accession number KT763371).

Table 2. Identification of phytoplasmas detected in the alfalfa seedlings at 30 and 150 days after transplant (DAT).

Location	Sample	30 DAT RFLP	Sequencing	150 DAT RFLP	Sequencing
AL Hamara-1	1a	PCR+	-	16SrV+?	99.38% '*Ca*. P. ulmi'
AL Hamara-2	2a	PCR+	-	-	-
AL Kami-1	3a	PCR+	-	-	-
Bahla-I-2	6a	16SrX	98.30% '*Ca*. P. pyri' related	-	-
Bahla-I-2	7a	PCR+	-	-	-
Dhank-2	10a	16SrV	99.80% '*Ca*. P. ulmi'	?	nd
Ibri-2	12a	-	-	-	-
Moday bi-1	15a	-	-	-	-
Moday bi-2	16a	-	-	-	-
Nizwa-2	18a	16SrV	nd	-	-
Sur-1	19a	16SrX	nd	16SrXII	99.39% 'Ca. P. solani'
Sur-2	20a	16SrV	nd	?	nd
Saham-2	22a	16SrV	nd	?	nd
Salalah-1	23a	PCR+	-	?	nd
Salalah-2	24a	-	-	-	-
Yanqoul-II-1	27a	16SrV	nd	-	-
AL Hamara-1	1b	+fb	-	-	-
Al Kami-2	4a	+fb	-	-	-
Al Kami-2	4b	16SrII, -IX, -XII, ?	nd	nd	nd
Bahla-I-2	6b	-	-	-	-
Bahla-II-1	7b	+fb	-	-	-
Dhank-2	10b	16SrX	99.58% '*Ca*. P. prunorum'	16SrIII	100% '*Ca*. P. pruni'
Ibri-2	12b	+fb	-	-	-
Moday bi-2	16b	16SrX	nd	-	-
Saham-1	21a	+fb	-	-	-
Saham-2	22b	+fb	-	-	-

PCR+, multiple bands in PCR; fb, faint band in PCR; nd, not performed; ?, RFLP profile not clear.

4. Discussion

The results indicated the presence of phytoplasmas in seedlings of alfalfa collected in Oman in 2002. The majority of the tested seedlings remained asymptomatic, confirming the previous reports on phytoplasma seed transmission in other herbaceous host species [11,14,15]. In particular, the alfalfa 4b seedling exhibited strong stunting and was simultaneously infected with diverse phytoplasmas, as suggested by the *Tsp509*I restriction profile analyses. At 150 DAT, the phytoplasma presence was no longer detected in the majority of the plants tested in agreement with previous findings for seed transmission in corn, carrot, and tomato [11,14,15,29]. In a few cases, in the same plant, phytoplasmas belonging to a group diverse from the one detected at 30 DAT were identified as reported in similar testing in tomato and eggplant [13,15]. The inconsistency of phytoplasma detection could be explained by assuming the presence of multiple phytoplasmas and/or bacterial endophytes in the seedlings at 30 DAT, which is also suggested by the presence of faint bands in PCR and of unclear profiles in the RFLP analyses. The phytoplasma population could also decrease during the growth of the seedlings following the remodulation of general endophyte populations [30].

The phytoplasma identified as '*Candidatus* Phytoplasma' species [6] was obtained with direct amplicon sequencing, since the amplicon RFLP analyses provide only a preliminary indication of phytoplasma identity (ribosomal group/subgroup) [5].

The alfalfa witches' broom is the most frequent disease associated with phytoplasma presence in this fodder crop worldwide. Globally, it was associated with the presence of different phytoplasmas such as '*Ca*. P. asteris' in the United States of America [31], '*Ca*. P. solani' in Italy and Iran [32], '*Ca*. P australasiae = australasiaticum' in Oman [33], and elm yellows in China [34]. The leafhopper *Orosius albicinctus* is the reported insect vector of this disease in Iran [35], while insect vectors in the other parts of the world were not reported. In Iran, the production of alfalfa has been significantly impacted by this disease, particularly in the province of Yazd in the southern and southeast districts, where alfalfa withes' broom was first reported as a devastating disease resulting in yield losses of up to 100% [35–37]. Later in Fars, Yazd, Kerman, Esfahan, South Khorasan, Khuzestan, Hamedan, and Bushehr province phytoplasmas from both 16SrII-D and -C subgroups were identified as being associated to the disease [35] with 16SrII-D phytoplasmas, resulting in an association with devastating diseases in the tropical provinces [35]. Moreover, 16SrVI-A and 16SrXII-A phytoplasmas were detected in Iran, in the East Azerbaijan and Zanjan provinces [37].

Due to deformities, wilting, small size, and low weight, phytoplasma presence in mother plants substantially affects the amount and quality of seeds produced by infected plants. Early seed germination is a common symptom. De La Rue et al. [38] studied the impact of *Stylosanthes* little leaf disease on the production of *Stylosanthes scabra* seeds. There was a significant decrease in the number of plants showing symptoms at 79 and 110 days after planting. This suggests that infected seed production was linked to the precocity of infection. Additional investigations on coconut palms indicate that the blossoms may have turned necrotic and the fruits cease to be able to ripen if the infection starts in the early plant-development stages. Only fruits that start developing before the infection can fully mature, since the period between pollination and fruit maturity is roughly 12 months, which also coincides with the incubation period of lethal yellowing phytoplasma disease [39]. The percentage of germination varies based on the species and the early onset of the infection. For example, European stone fruit yellows ('*Ca*. P. prunorum')-infected apricot produced seeds that displayed a germination rate 7 times lower (9.4%) and a vitality 4.5 times lower compared to the seeds from healthy control plants [40]. In contrast, higher germination values (72.1%) were reported in coconuts, when compared to healthy ones [41]. Additionally, phytoplasmas in *Citrus aurantifolia* plants derived from seeds of plants infected with witches' broom disease could not be identified after having grown for two years in an insect-proof greenhouse. Phytoplasma DNA was found in the tegument of the seed but not in the embryos, leaves, stems, and roots of these plants. In contrast to the percentages of seeds originating from healthy plants, the germination percentage of the seeds derived from symptomatic plants was higher and individuals derived from infected plants have not shown witches' broom symptoms [42]. However, by germinating and growing a number of seeds from lime fruits collected in an Omani market in vitro, it was possible to detect one symptomatic seedling that was positive to the presence of 16SrII-group phytoplasmas ('*Ca*. P. citri') [43]. Moreover, recent research demonstrated that coconut produced from infected plants produce seedlings infected with '*Ca*. P. palmae' in Mexico (16SrIV-A) [44] and '*Ca*. P. noviguineense' in Papua New Guinea [45].

This research allowed to verify that after more than 20 years in storage, the alfalfa seeds were still able to germinate and test positive for phytoplasma presence at two different growing stages. The germination percentage varied greatly in the seeds collected from various locations in Oman. The overall germination percentage was as low as 17.5%; however, this is mainly related to the fact that parts of the seeds may have lost their viability.

DNA extractions were performed on 26 plants in the greenhouse over the time intervals of 30 and 150 days after transplantation; the best methodology enabling the detection of phytoplasma presence and their identification was a nested PCR assay using R16F2n/R2

and fU5/rU3 and M1/M2 primers. At the time of collection, the seedlings from the same seed batches growing in in vitro conditions allowed the preliminary RFLP identification of phytoplasmas belonging to the 16SrI, 16SrII, 16SrV, 16SrX, and 16SrXII groups [18]. The present research confirmed the presence of 16SrV, 16SrX, and 16SrXII phytoplasmas in the seedlings growing under insect-proof greenhouse conditions (Table 3). This confirms the maintenance of phytoplasmas over time in the seeds, together with their transmissibility through the seeds in vitro [18] and under semi-field conditions (present research). In both cases, one symptomatic and phytoplasma-positive plant was obtained.

Table 3. A comparative summary of ribosomal groups of phytoplasmas detected in the alfalfa seedlings in 2002 and at 30 and 150 days after transplant (DAT) in 2024 in the same seed batches (results identical to those obtained by Bertaccini et al., unpublished, are in bold).

Location	2002 Testing	2024 30 DAT	2024 150 DAT
Al Hamara-1	16SrI, **16SrV**, 16SrX	-	**16SrV**
Al Kami-1	16SrV	-	-
Bahla-I-2	16SrI, 16SrII	16SrX	-
Dhank-2	16SrII, **16SrV**	**16SrV**, 16SrX	-
Ibri-2	16SrI, 16SrII	-	-
Moday bi-2	16SrI, 16SrII, 16SrV, **16SrX**	**16SrX**	-
Nizwa-2	16SrII	16SrV	-
Sur-1	16SrII	16SrX/16SrV	16SrXII
Saham-2	16SrI, 16SrII, **16SrV**, 16SrXII	**16SrV**	-
Salalah-1	16SrII	-	-
Yanqoul-II-1	16SrI, **16SrV**, 16SrX	**16SrV**	-

Although previous studies revealed that most of the plants from seeds obtained from phytoplasma-infected mother plants were able to germinate and survive, the most serious epidemiological concern is the presence in the fields of insect vectors able to transmit these pathogens to the surrounding agricultural crops. Infected seeds are also an important source of spreading phytoplasmas over long distances and to new environments. Therefore, appropriate knowledge and research about phytoplasma seed transmission are important to reduce the spread of these pathogens through seeds and the risk of large economic losses to farmers. Phytoplasma seed transmission was demonstrated in corn, wheat, tomato, carrot, and eggplant, among others, and also in some woody host species such as coconut [46]. Since the presence of these bacteria in seedlings is not very often accompanied by the presence of symptoms (in the present case, as in the first report, only one plant showed symptoms), the seed transmission represents a hidden and dangerous risk for many horticultural crops also considering international seed trade. Despite the low transmission rates (1 to 3%) of these and other bacteria, the widespread presence of insects able to transmit phytoplasmas further facilitates the start of epidemic outbreaks that always lead to severe economic losses.

5. Conclusions

The molecular research results identified the presence of phytoplasmas belonging to different ribosomal groups in the studied alfalfa seedlings. In particular, phytoplasmas

mostly belonging to two ribosomal groups, 16SrV and 16SrX, were detected in the seedlings tested at 30 and 150 DAT. Moreover, all the alfalfa seedlings were cut at 30 DAT except the strongly symptomatic plant (alfalfa 4b), and they grew normally again. In the testing at 150 DAT, phytoplasmas were again detected, indicating that the phytoplasmas were consistently present, viable, and multiplying, and were able to colonize the new foliage. A similar result indicating the viability of seed-transmitted phytoplasmas was achieved by obtaining phytoplasma containing colonies in solid artificial medium after their isolation from corn seedlings [47]. Phytoplasmas detected in early growth stages (30 DAT) differ from those identified in the latter stages (150 DAT), similarly to the results of seed transmission for phytoplasmas identified in eggplants and corn seedlings [14,29]. Further studies are needed to clarify the percentage of phytoplasma transmission by seeds and the impact of their further spreading in the field by insect vector feeding in asymptomatic plants. Moreover, a quarantine should be established worldwide to reduce the possibility of further phytoplasma dissemination.

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