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Identification and impact of phytoplasmas associated with greenhouse cucumber phyllody in Iran

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Abstract: Cucumber phyllody symptoms were observed in greenhouse cucumber plants during 2014-2018 in all surveyed areas of central and west of Iran where the highest disease incidence was up to 82% in Taft (Yazd province). Symptoms exhibited by diseased plants were virescence, phyllody and sterility of the flowers. For verification of phytoplasma presence and identity, total DNAs were extracted from 44 symptomatic and six asymptomatic plants that were subjected to PCR amplifying 16S rRNA genes of phytoplasmas. PCR amplicons of the expected size were obtained only from the symptomatic plants. RFLP analysis of R16F2n/R2 amplicons showed patterns identical to those of the clover proliferation (16SrVI) and "stolbur" (16SrXII) phytoplasma groups. Consensus sequences corresponding to phytoplasma strains from the two localities Taft and Shahrekord showed 99% identity with phytoplasmas enclosed in groups 16SrVI and 16SrXII, respectively. Phylogenetic analysis confirmed that these phytoplasmas cluster with '*Candidatus* Phytoplasma trifolii' and '*Ca*. P. solani', respectively. Virtual RFLP provided profiles identical to the patterns of 16SrXII-A and 16SrVI-A phytoplasma subgroups. These phytoplasma subgroups were previously reported in different plant species growing near to the greenhouse cucumber areas in Iran, and play a possible role in the epidemiology of disease for its dissemination.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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1. Introduction

Among the cucurbitaceous plants grown in greenhouses, *Cucumis sativus* with 7,427 ha is considered the most economical important crop in Iran where about the 77% of the area under greenhouse cultivation is greenhouse cucumber (Iranian Ministry of Agriculture, 2019). These plants need less water than the species cultivated in the fields, and due to the water constraint, this production is expanding. The presence of phyllody disease was reported in cucumber up to 80% in Jiroft and Kahnooj

(Kerman province, Iran) (Azadvar *et al.,* 2004). In some areas, due to high disease incidence and severity, infected plants did not bear fruits and farmers remove cultivated cucumbers and re-sown them. In 2004-2006 surveys in greenhouses the presence of cucumber phyllody was observed in Yazd, Varamin, and Larestan with about 35%, 80% and 3% of disease incidence respectively, and the phytoplasma presence was confirmed (Esmaeilzadeh-Hosseini *et al*., 2006). Phytoplasmas are destructive bacteria infecting more than one thousand plant species worldwide. They are transmitted mainly by leafhoppers and symptoms include yellowing, discoloration, dwarfing, witches' broom, virescence and phyllody (Bertaccini *et al.,* 2014). Their identification relies on molecular classification based on the amplification and/or RFLP analyses of their 16S rRNA gene (Lee *et al*., 1998; IRPCM, 2004). The aim of the present work was to identify the phytoplasmas associated with greenhouse cucumber phyllody in central and west parts of Iran in order to devise the appropriate disease management to reduce its economic impact.

2. Materials and Methods

During 2014 to 2018, greenhouse cucumber growing areas of central and west of Iran were surveyed for evaluation of phytoplasma disease presence. Symptomatic and symptomless cucumber plants were collected in greenhouses located in Akramia, Chah Shahrdar and Taft (Yazd province) and Shahrekord region (Chaharmahal and Bakhtiari province) and subjected to molecular studies for phytoplasma detection and identification. Sampling was carried out randomly in five 1,000 m^2 greenhouses and the disease incidence was calculated by counting the number of symptomatic plants exhibiting phyllody out of the total number of greenhouse cucumber plants in each greenhouse multiplied by 100.

Nucleic acid extraction was carried out as described by Zhang *et al.* (1998) using 0.2 g of fresh midrib tissue from 44 symptomatic greenhouse cucumber plants and from 6 asymptomatic seed grown greenhouse cucumber plants. The DNA from pot marigold phyllody phytoplasma (16SrII-D subgroup) (Esmaeilzadeh-Hosseini *et al.,* 2018) was used as positive control. A total of 100 ng of nucleic acid was used for the PCR to amplify the 16S rRNA gene of the phytoplasmas with primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.,* 1995) followed by nested PCR using R16mF2/R16mR2 and R16F2n/R16R2 (Gundersen and Lee, 1996) primers in a total volume of 50 µl. One µL of the products from direct amplification was diluted in 29 µL of sterile deionized water for the nested amplifications. The PCR reaction was performed in 50 µL mixtures containing 0.4 µM of each primer, 0.2 mM of each dNTP, 1.25 U Taq DNA polymerase and 1X Taq polymerase buffer (CinnaGen, Iran). PCR protocols were done as reported by Salehi *et al*. (2015). Following PCR, 2 µL of each reaction mixture was electrophoresed in a 1% (w/v) agarose gel containing 0.3 µg/mL ethidium bromide in 0.5 X TBE buffer (22.5 mM Tris-borate, 1 mM EDTA, pH 8.0). The amplicons obtained with R16F2n/R16R2 primers were analyzed by single restriction endonuclease digestion with *Alu*I, *Hae*III, *Taq*I, *Hpa*I, *Hpa*II, *Mse*I, *Rsa*I, *Kpn*I and *Hha*I (Thermo Scientific). The digestion products were analyzed through an 8% polyacrylamide gel electrophoresis and the visualization of DNA bands was carried out with a UV transilluminator after staining by ethidium bromide.

Direct sequencing in both directions of twelve samples (three per each greenhouse area) was carried out using R16mF2/R16mR2 amplicons and the same primers. The resulting sequences were trimmed to the R16F2n/R2 fragment (about 1,240 bp) and submitted to GenBank. A database search of homologous sequences was performed by BLAST analyses at the NCBI (www.ncbi.nlm.nih.gov). The R16F2n/R2 sequence of the 16S rRNA gene of greenhouse cucumber phyllody phytoplasma strains SGCP (Shahrekord greenhouse cucumber phyllody), TGCP (Taft greenhouse cucumber phyllody), Fars (GenBank accession number JN574839) and Tehran (GenBank accession number MH004460) and of 16S rRNA gene of selected '*Candidatus* Phytoplasma' species or phytoplasma strains enclosed in the subgroups of groups 16SrVI and 16SrXII were aligned (Table 1). A phylogenetic tree was constructed with the phytoplasma sequences obtained and others retrieved from the GenBank using the neighbor-joining method with MEGA software version 7 (Kumar *et al.,* 2016). *Acholeplasma laidlawii* was used as an out-group to root the tree and bootstrapping was performed 1,000 times to estimate the stability and support for the branches. The ribosomal subgroup affiliation of the detected phytoplasmas was confirmed by virtual RFLP analysis with the *i*Phyclassifier (Zhao *et al.,* 2009).

Table 1 - Phytoplasma sequences used for comparison with the reported greenhouse cucumber strains from Iran

Disease or phytoplasma	GenBank accession number	Country	16S ribosomal subgroup
'*Ca*. P. trifolii'	AY390261	Canada	16SrVI-A
Strawberry multiplier disease	AF190224	Canada	16SrVI-B
Illinois elm yellows	AF409069	USA	16SrVI-C
Periwinkle little leaf	AF228053	Bangladesh	16SrVI-D
Centaurea solstitialis virescence	AY270156	Italy	16SrVI-E
Catharanthus phyllody	EF186819	Sudan	16SrVI-F
Portulaca little leaf	EF651786	India	16SrVI-H
'Ca. P. sudamericanum'	GU292081	Brazil	16SrVI-I
'Ca. P. solani'	AF248959	Serbia	16SrXII-A
'Ca. P. australiense'	L76865	Australia	16SrXII-B
Strawberry lethal yellows	AJ243045	Australia	16SrXII-C
'Ca. P. japonicum'	AB010425	Japan	16SrXII-D
'Ca. P. fragariae'	DQ086423	Lithuania	16SrXII-E
"Bois noir" strain BN-Op30	EU836652	Italy	16SrXII-F
"Bois noir" strain BN-Fc3	EU836647	Italy	16SrXII-G
'Ca. P. convolvuli'	JN833705	Italy	16SrXII-H

3. Results and Discussion

Greenhouse cucumber diseased plants showed flower virescence, phyllody and sterility (Fig. 1), the disease was named greenhouse cucumber phyllody (GCP).

The symptoms were observed in all the greenhouses of the four surveyed areas. The disease rate was up to 11.2%, 33.5%, 82% and 8.7% in Akramia, Chah Shahrdar, Taft (Yazd province) and Shahrekord region (Chaharmahal and Bakhtiari province), respectively.

PCR amplicons of about 1.8, 1.4 and 1.25 kb were obtained from all the symptomatic greenhouse cucumber samples but not from the symptomless ones. Restriction fragment length polymorphism (RFLP) analysis of R16F2n/R16R2 amplicons using *Alu*I, *Hae*III, *Taq*I, *Hpa*I, *Hpa*II, *Mse*I, *Rsa*I, *Kpn*I and *Hha*I restriction enzymes showed two RFLP pattern identical to those reported for the 16SrVI and 16SrXII phytoplasma groups, respectively (Fig. 2).

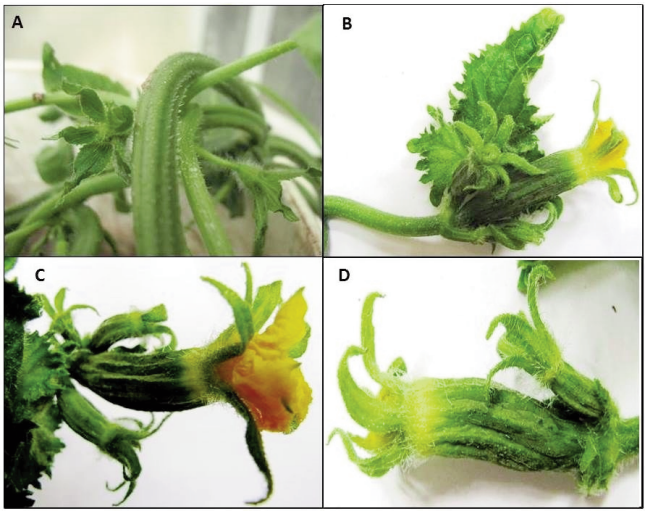

Fig. 1 - Flower virescence, phyllody and sterility in a greenhouse cucumber plant from Yazd (A) and Chaharmahal and Bakhtiari (B, C, D) provinces.

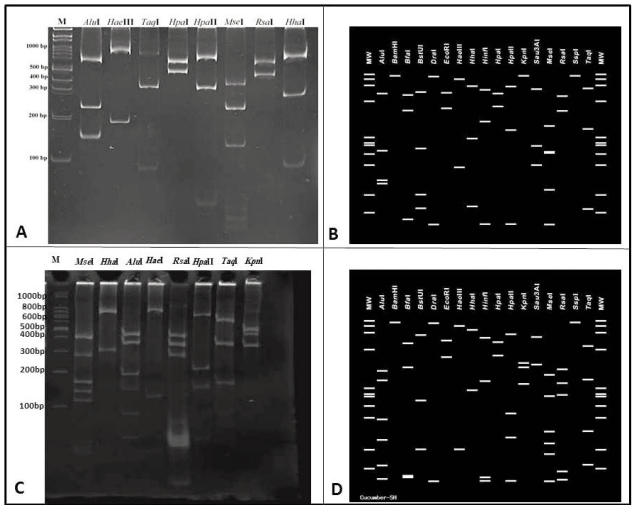

Fig. 2 - Real and virtual RFLP patterns (Zhao *et al*., 2009) respectively of 1.2 kb amplicons from TGCP (A and B) and SGCP (C and D) phytoplasma strains 16S ribosomal gene sequence. Lane M, 100 bp DNA ladder (Biobasic, Canada). DNA products were digested with the enzymes listed at the top of the figures.

The DNA fragments obtained from twelve samples after direct sequencing were aligned and the consensus sequences corresponding to a representa-

tive of GCP phytoplasmas in Taft (TGCP) and Shahrekord (SGCP) were deposited in GenBank under the accession numbers MF438041 and MK402983, respectively. The BLAST search of these sequences showed that TGCP (1,251 bp) and SGCP (1,243 bp) phytoplasmas had 99.60% and 99.28% identity with phytoplasmas enclosed in subgroups 16SrVI-A ('*Ca*. P. trifolii', GenBank accession number AY390261) and 16SrXII-A ('*Ca*. P. solani', GenBank accession number AF248959), respectively. The phylogenetic analysis confirmed that TGCP phytoplasmas cluster with phytoplasmas classified in the 16SrVI group and were therefore confirmed as closely related to '*Ca.* P. trifolii', while the SGCP phytoplasmas cluster with those enclosed in the 16SrXII group and were therefore related to '*Ca*. P. solani' (Fig. 3). The R16F2n/R2 amplified regions from TGCP and SGCP phytoplasmas digested *in silico* with 17 restriction enzymes

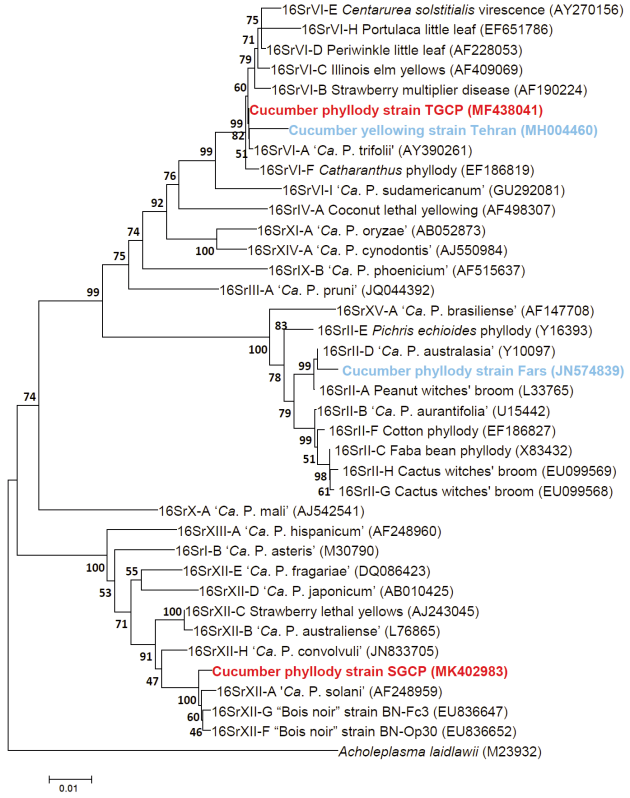

Fig. 3 - Phylogenetic tree constructed by the Neighbor-Joining method of the R16F2n/R16R2 sequence of 16S rRNA gene of 38 phytoplasmas including the cucumber strains SGCP, TGCP, Tehran and Fars, and phytoplasmas enclosed in subgroups of the 16SrVI, 16SrII and 16SXII groups. The greenhouse cucumber phyllody phytoplasmas are in color and bolded (in red those sequenced in this work). Numbers at the nodes are bootstrap values based on 1,000 repetitions. 'Ca. P.': 'Candidatus Phytoplasma'. GenBank accession numbers for sequences are given in parentheses while the phytoplasma ribosomal grouping is before the strain name.

using online *i*PhyClassifier program exhibited virtual RFLP profiles identical to the reference pattern of 16SrVI-A and 16SrXII-A, respectively (Fig. 2).

Phytoplasmas belonging to diverse ribosomal groups have been detected in *Cucurbitaceae* species worldwide showing different arrays of symptoms. In particular, 16SrI in *Cucurbita pepo* L. in Italy (Minucci *et al.,* 1995) and in *Sechium edule* (Jacq.) Sw. in Costa Rica (Villalobos *et al.,* 2002), 16SrII in *C. sativus* and *C. pepo* in Australia, Egypt and Iran (Davis *et al.,* 1997; Omar and Foissac, 2012; Salehi *et al.,* 2015), 16SrIII in *Luffa cylindrica* L. (Rox.) and *Sicana odorifera* (Vellozo) Naud in Brazil (Montano *et al.,* 2000, 2007a, 2007b) and 16SrVIII in *L. cylindrica* in Taiwan (Davis *et al.,* 2017).

Phyllody is an important phytoplasma disease of cucurbitaceous plants in Iran (Salehi *et al.,* 2015) and it was associated with the presence of a peanut witches' broom phytoplasma (16SrII) in greenhouse cucumber plants showing phyllody (Dehghan *et al.,* 2014) and of a clover proliferation phytoplasma (16SrVI) in Tehran (Ghayeb Zamharir and Azimi, 2019). Molecular assays confirmed the phytoplasma presence in the symptomatic greenhouse cucumber analyzed in this work and allow their identification as '*Ca*. P. trifolii' (16SrVI-A) and '*Ca*. P. solani' (16SrXII-A)-related strains (Esmaeilzadeh Hosseini *et al*., 2019).

In the present work the identification of phytoplasmas in subgroups 16SrVI-A and 16SrXII-A allows epidemiological considerations. The 16SrVI-A-related phytoplasma strain was identified in the central areas of Iran, in the Yazd province where the most important plant species harboring 16SrVI phytoplasmas are tomato and eggplant (Salehi *et al.,* unpublished) and alfalfa (Esmaeilzadeh Hosseini *et al.,* 2015a, 2015b; Purmohammadi *et al.,* 2017). Greenhouse cucumber phyllody associated with the presence of 16SrXII-A ("stolbur") phytoplasmas was present in Chaharmahal and Bakhtiari province where this phytoplasma was detected also in grapevine showing diverse symptoms (Mirchenari *et al.,* 2015) and alfalfa showing witches' broom (Esmaeilzadeh Hosseini *et al.,* 2016a, 2016b). Phytoplasma diseases associated with "stolbur" were present in plant host species adjacent to greenhouses cucumber areas but their role in the epidemiology of the disease needs to be proved.

Due to the problems of water constraint, the cucumber greenhouse cultivation in Iran has been widely increased. In the majority of cucumber production greenhouses, aeration valves are usually not

Fig. 4 - Aeration valves are usually not covered with netting by greenhouse owners and this allows the entry of insect vectors.

covered with netting by greenhouse owners (Fig. 4) which allows also the possible entry of insect vectors. Furthermore, the major cucumber greenhouses are located close to the agricultural fields and rangelands where during the recent droughts, the insect vectors are attracted and possibly transmitted the phytoplasmas from sources outside the greenhouse.

It is therefore probable that infection in these plants play a role in the epidemiology for the dissemination of this bacterium in the greenhouses also considering that the plants are not completely isolated from the environment. The presence of consistent populations of *Orosius albicinctus* and *Circulifer haematoceps* both recognized vectors of the cucumber phyllody disease was detected in plants grown adjacent to these greenhouses (Salehi *et al*., 2015). Their feeding activity during the year, especially in the Yazd province, leads to the widespread dissemination of phytoplasmas; therefore, preventing the entry of insect vectors into the greenhouses is the most recommended management to reduce the disease incidence.

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