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Research Article

Detection of 16SrXII-A phytoplasma strain associated with *Capsicum annuum* stolbur disease in Iran

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Abstract: Pepper *Capsicum annuum* is one of the important vegetable crops in Iran, especially north of Iran. Various symptoms of stolbur, including limited growth, small and chlorotic leaves, spoon-shaped leaflets, and sterility or fruit alterations, were detected in samples collected from the pepper field in Qazvin province. DNA was extracted from midribs and petioles of pepper leaves using CTAB-based methods. The phytoplasma in all symptomatic pepper plant parts was detected by direct and nested polymerase chain reactions (PCR) using primer pairs P1/P7 and R16F2n/R16R2. The 16S rDNA sequences of phytoplasma isolate were deposited in GenBank (MN877916). Based on phylogenetic studies of the 16S rDNA region, the results of enzymatic digestion of the fragment obtained by amplification with R16F2n/R16R2 primer and virtual RFLP, phytoplasma agent associated with stolbur pepper disease was detected to belong to 16SrXII group and 16XII-A subgroup. According to our knowledge, this is the first report of pepper stolbur disease in Iran.

Keywords: 16SrXII-A, RFLP, Stolbur, Pepper, Phytoplasma

Introduction

Phytoplasmas are small, insect-transmitted, wall-less bacteria associated with devastating plant diseases (Rao *et al*., 2018). To date, 33 groups and more than 118 subgroups of phytoplasmas have been delineated based on RFLP analysis of 16SrDNA sequences, and 44 *Candidatus* Phytoplasma species have been reported (Bertaccini and Duduk, 2009). Many herbaceous and woody plants are subject to phytoplasma infection, which occurs worldwide through insect vectors, human activity, and infected plant material (Bertaccini, 2007; Hogenhout, *et al.*, 2008). Various taxonomic groups and subgroups of phytoplasma affect different plant species. Numerous crops in the Solanaceae family have been infected with phytoplasma (Amaral-Mello *et al*., 2006; Randall *et al.,* 2009; Martini *et al*., 2018). However, pepper is also one of the hosts of phytoplasmas. Significant symptoms on infected peppers which have been reported from different regions of the world include virescence, leaf yellowing, leaf cupping, shortening of internodes, stunting, wilting, fruit deformation, and plant decline (Lee *et al*., 2000; Santos-Cervantes *et al.,* 2008; Zheng-Nan *et al.,* 2013; Martini *et al*., 2018). Due to the diversity of vegetation and various climatic conditions, phytoplasma diseases are increasing in Iran, and significant progress has been made to detect, identify, and

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classify phytoplasmas by using DNA-based methods (Ghandi *et al.*, 2003; Siampour *et al.*, 2019). There are different reports of phytoplasma infection on Solanaceae crops in Iran (Samavi *et al.*, 2012; Jamshidi *et al.*, 2014; Salehi *et al.*, 2014; Sichani *et al.*, 2014; Tohidi *et al.*, 2015; Salehi and Esmailzadeh-Hosseini, 2016). Faghihi *et al.* (2016) detected the 16SrII phytoplasma group from pepper with yellowing, big bud, little leaf, and virescence symptoms for the first time. Despite numerous reports of phytoplasma infection on pepper in Iran, there is little information about the classification of phytoplasma agents in this crop. This study aims to identify pepper phytoplasma diseases in the fields of Qazvin province, Iran.

Materials and Methods

Sample collection and nucleic acid extraction

In July 2019, diseased pepper plants were observed in a field (approx. 800 m2 in area) in Qazvin Province, Iran, with easily distinguishable symptoms of phytoplasma infection, including leaf yellowing and chlorosis curling, deformation, phyllody, and witches' broom. Leaves of healthy and phytoplasma-infected tomato plants with big bud symptoms (Davoodi et al., 2019), respectively, were used as negative and positive controls. Leaf samples from the infected field were collected and stored at 4 °C until the samples were transferred to the laboratory. Total nucleic acids were extracted from 0.1 g ground leaf tissues, including midribs and petioles, using the CTAB method (Doyle and Doyle, 1990). The extracted DNA was stored at -20 °C for further analysis.

Molecular assay of 16S Ribosomal DNA

Detection and characterization of phytoplasma contamination were performed using direct PCR by two primer pairs P1/P7 to amplify the 1800 bp ribosomal operon. It consists of the 16SrRNA gene, the 16S-23S spacer region, and a portion of the 5' region of the 23SrRNA gene. A 1:40 dilution of the direct PCR product amplified by P1/P7 primer pairs was used as a template for nested PCR, using primer pairs R16F2n/R2, which amplifies an internal DNA fragment of 1200bp from the 16SrRNA gene (Lee *et al.*, 1998; Zhao *et al.*, 2009). Detection of phytoplasmas was done using PCR assays. Each 25-µl PCR reaction mix contained 20 ng of template DNA, 2.5 µl of 10 × PCR buffer, 0.8 U of Taq polymerase, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 0.4 mM of each primer. DNA extracted from healthy tomato plants was run as a negative control in each PCR reaction. One microliter of amplicon from direct PCR, diluted 1:40 in sterile distilled water, was used as a template in nested PCR reactions. Thirty-five PCR cycles were performed under the following conditions: 1 min (2 min for the first cycle) for denaturation at 94 °C, 2 min for annealing at 50 °C, and 3 min (10 min for the last cycle) for primer extension at 72 °C. Six microliters of PCR products were separated in 1% agarose gel, stained with ethidium bromide, and photographed under a UV transilluminator.

Restriction fragment length polymorphism and Virtual RFLP

Identification of detected phytoplasmas was made using RFLP analyses with eight restriction endonucleases: *Rsa*I, *Mse*I, *Taq*I, *Alu*I, *Cfo*I, *Hinf*I, *Hae*III, and *Hpa*II (Lee *et al.,* 1998) in restriction fragment length polymorphism (RFLP) analysis. Visualization of RFLP products was performed in a 1% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator. Virtual restriction fragment analysis was performed from the partial sequences of the 16S rDNA gene using the software iPhyclassifier (Zhao *et al.,* 2009) to determine strain subgroup associated with pepper stolbur. Each aligned DNA fragment was digested *in silico* with 17 distinct restriction enzymes (*Rsa*I, *Mse*I, *TaqI*, *AluI, CfoI*, *HinfI*, *HaeIII*, *Hpa*II, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hha*I, *Kpn*I, *RsaI, Sspi,* and *Sau3*AI) that have been used for phytoplasma 16SrRNA gene RFLP analysis.

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DNA sequencing and phylogenetic analysis

After comparing the RFLP patterns, a direct sequence was performed, and the intended isolate was selected to determine its nucleotide sequence (Macrogen Biosystems, South Korea). The sequences were then aligned using the BLAST engine for local alignment (Blast N). Phylogenetic interrelationships among the stolbur strain and other phytoplasma groups were assessed based on 16S rRNA gene sequences. Partial sequences of 16S rDNA from studied phytoplasma and 41 representative phytoplasmas from Gen Bank were aligned using CLUSTAL W software. Then Phylogenetic tree was constructed by the neighbor-joining method with a bootstrap of 1,000 replicates using MEGA6 (Tamura *et al*., 2013). *Acholeplasma laidlawii* was designated as the outgroup to root the tree.

Results

Detection and Molecular assay of phytoplasmas from pepper

Phytoplasma isolates were detected from plants showing small and chlorotic, spoon-shaped leaflets and sterility of fruit by nested PCR with universal primer pairs R16F2n/R16R2. Products of 1250bp were amplified from extracted DNA of infected pepper samples and the positive control. The results of nested PCR indicated that the plants might be infected by a phytoplasma (Fig. 1). No amplification was observed when DNA from asymptomatic plants was used as the template.

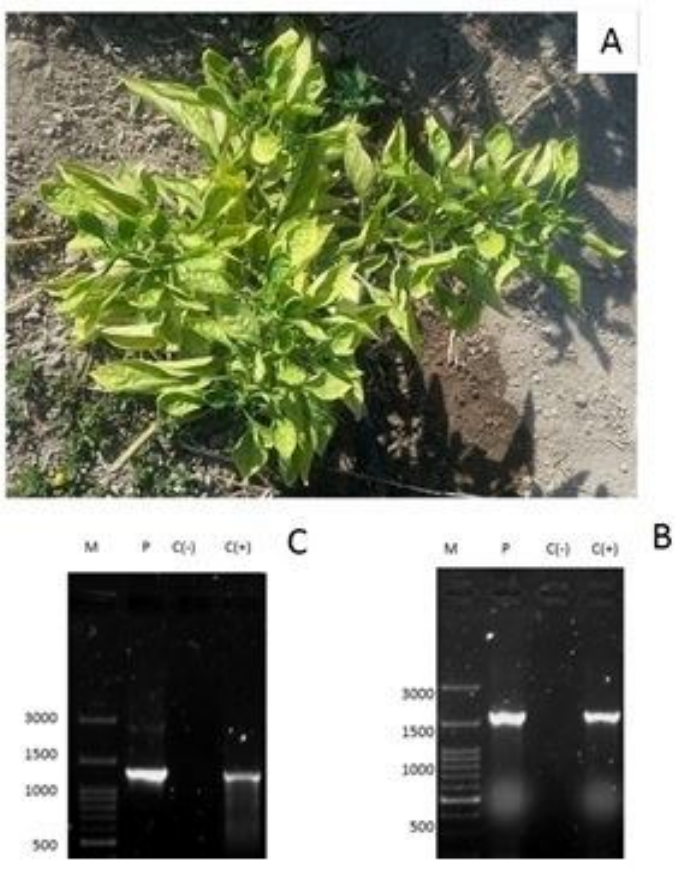

Figure 1 A. The appearance of small and chlorotic leaves, spoon-shaped leaflets in naturally phytoplasma-infected pepper. B. Electrophoresis pattern of 1800bp of rRNA operon amplified by direct PCR using primer pairs P1/P7, C. Nested-PCR primed by primer pairs R16F2n/R16R2n. Lane M: DNA ladder (100 bp). C (-): Healthy tomato and C (+): infected tomato plants. P: infected pepper.

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Restriction fragment length polymorphism and Virtual RFLP

To distinguish phytoplasma isolate, a product of nested PCR (R16F2n/R16R2) amplified from pepper sample was digested with *Rsa*I, *Mse*I, *Taq*I, *Alu*I, *Cfo*I, *Hinf*I, *Hae*III, and *Hpa*II restriction enzymes (Fig. 2). Positive sample in nested PCR with R16F2n/R16R2 primer pair showed restriction profiles when subjected to RFLP analysis with 16srRNA and 8 restriction enzymes that were identical and referable to the profile of stolbur phytoplasma, belonging to 16SrXII-A ribosomal group (Fig. 2). For subgroup affiliation, the sequences were trimmed in virtual RFLP analysis.

Sequencing and phylogenetic analyses

PCR product obtained from the infected plant was directly sequenced, and the sequence was deposited in GenBank (MN877916.1). Based on the phylogenetic comparison of the 16SrRNA gene of phytoplasma obtained from symptomatic pepper with 41 phytoplasma reference strains of the genus "*Candidatus* Phytoplasma" from GenBank, it was revealed that the phytoplasma detected in pepper is closely related to the stolbur phytoplasmas (Fig. 3). Phylogenetic analysis indicated that this phytoplasma should be classified in the 16srXII-A subgroup. The 1250 bp PCR fragment sequence related to the 16SrRNA gene of pepper stolbur phytoplasma was compared with another reference phytoplasma in the NCBI database. The maximum identity was found with other phytoplasma isolates belonging to the 16SrXII group reported on pepper in different regions of the world such as *Candidatus* Phytoplasma solani isolate IG10-1 (MN398469.1); *Candidatus* Phytoplasma solani strain Sh1 (KC835139.1); Paper flower yellows phytoplasma strain PFY (JX128698.1); Iranian potato purple top phytoplasma (EU661607.1); 'Bois noir' phytoplasma strain CH-1 (HQ589193.1) with percentage similarities of 99.84, 99.84, 99.76, 99.68 and 99.68, respectively (Shimomoto *et al.,* 2019).

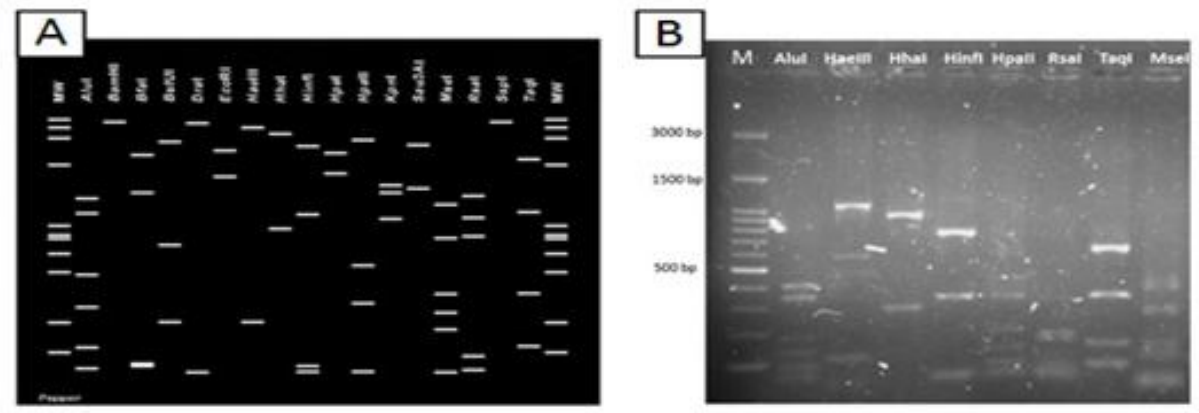

Figure 2 A. Virtual RFLP pattern of R16F2n/R2 PCR product sequence recognition sites for the following 17 restriction enzymes that were used in the simulated digestions: *RsaI, MseI, TaqI*, *AluI*, CfoI, *HinfI*, *HaeIII*, *HpaII, Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hha*I, *Kpn*I, *Rsai, Sspi* and *Sau3*AI. **B.** Restriction fragment length polymorphism of 16S rDNA amplified by nested-PCR using P1 / P7 followed by R16F2n / R2 primer pairs from the infected pepper plant. Lane M, DNA ladder. DNA products were digested using VIII restriction enzymes (*Hpa*II, *Taq*I, *Rsa*I, *Hinf*I, *Alu*I, *Rsa*I, *Cfo*I, *Mse*I) separated through a 1%agarose gel.

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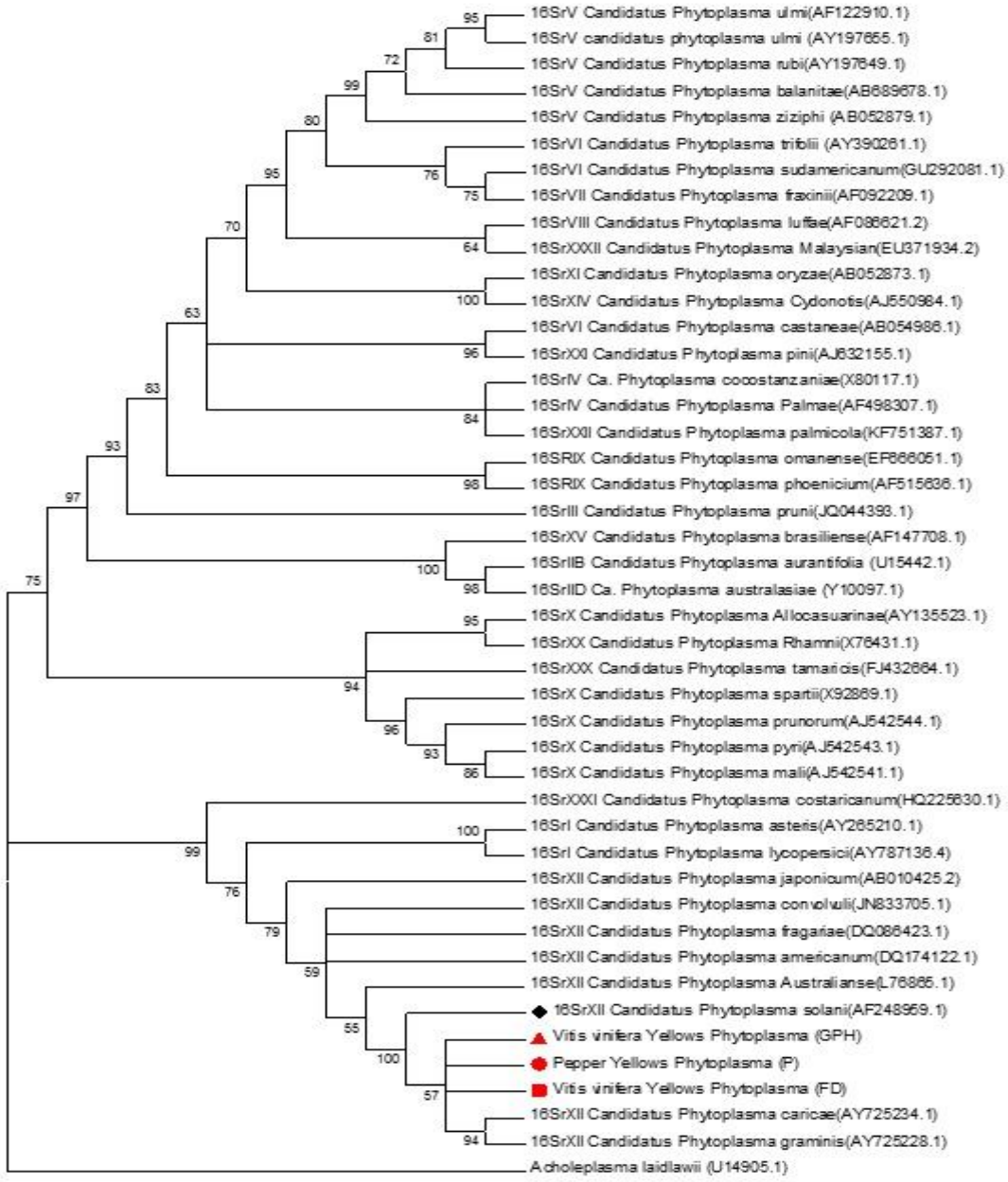

Figure 3 Phylogenetic tree of partial 16S rRNA gene sequences from pepper yellows phytoplasma isolate (marked by a red circle symbol) and 41 reference phytoplasma sequences (from different 16S rRNA groups), GenBank accession numbers shown in brackets. *Acholeplasma laidlawii* was used as an outgroup to root the tree. The tree was constructed by the neighbor-joining method.

Discussion

The phytoplasma strain related to 16SrXII-A subgroup was confirmed by nested PCR assay, partial sequencing of 16S rRNA gene, and *in vitro* and *in silico* RFLP analysis. Molecular analysis indicated that the 16S rRNA sequence of pepper Stolbur phytoplasma isolate shares 99.4% similarity with that of the *Candidatus* Phytoplasma solani reference strain (GenBank accession: AF248959). The stolbur phytoplasma, *Ca. P. solani*, was reported

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previously on pepper in Serbia (Mitrovic *et al.,* 2015), France (Cimerman *et al.*, 2009), Italy (Murolo *et al.,* 2010), Spain (Castro and Romero, 2002), Australia (Tran-Nguyen *et al.*, 2003) and Bosnia and Herzegovina (Delic *et al.,* 2016). Phytoplasma of the 16SrII group was identified in pepper with symptoms of yellowing, big bud, little leaf, and virescence in Iran (Faghihi *et al.,* 2016). The other phytoplasma groups, such as 16SrVI, 16SrI, 16SrII, 16SrIII, have been reported from several countries, including Iran, with different symptoms (Rao *et al.,* 2018). One of the most characterized phytoplasmas in Iran belonged to 16SrXII, causing devastating disease on other herbaceous and woody host plants (Siampour *et al.,* 2019). Stolbur has a broad host range, wide distribution, and various vectors that play a significant role in its epidemiology in Iran and worldwide (Maixner, 2006; Riedle *et al.,* 2008 Siampour *et al.,* 2019). The more affected hosts of the stolbur group provide more reservoirs for the phytoplasma and insect vectors. They may be an essential factor in the spread and increasing disease incidence. Observations and assays need to continue to detect and identify other hosts as potential sources of inoculum. More detailed assessments are required to determine important aspects of the disease's epidemiology caused by stolbur phytoplasma in Solanaceaeous crops, especially pepper. This is the first report of *Capsicum annuum* stolbur phytoplasma in Iran confirmed by nested PCR and RFLP assays.

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ردیابی مولکولی فیتوپلاسمایی از گروه 16SXII-A همراه با بیماری استولبور فلفل در ایران

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چکیده: فلفل یکی از سبزیجات مهم در ایران و به‌ویژه در شمال کشور می‌باشد. علائم مختلفی از استولبور شامل محدودیت در رشد، کوچکی و رنگ پریدگی برگ‌ها، قاشقی شدن برگچه‌ها، عقیمی و تغییر شکل میوه از نمونه‌های جمع‌آوری شده از مزرعه فلفل در استان قزوین مشخص شده است. استخراج DNA از رگبرگ میانی و دمبرگ برگ فلفل به‌روش CTAB انجام گرفت. فیتوپلاسما در نمونه‌های فلفل دارای علائم با استفاده از روش PCR (واکنش زنجیره‌ای پلیمراز) وکاربرد پرایمرهای P1/P7 و R16F2n/R16R2 ردیابی شد. توالی به‌دست آمده از ناحیه 16S rDNA در بانک ژن با شماره دسترسی MN877916.1 ثبت شد. براساس بررسی‌های فیلوژنتیکی ناحیه 16S rDNA، نتایج هضم آنزیمی قطعه حاصل از تکثیر با پرایمرهای R16F2n/R16R2 و نیز RFLP مجازی، عامل فیتوپلاسمایی همراه با بیماری استولبور فلفل متعلق به گروه 16SrXII و زیرگروه 16XII-A می‌باشد. طبق اطلاعات ما، این اولین گزارش از بیماری استولبور فلفل در ایران است.

واژگان کلید: فیتوپلاسما، فلفل، 16SrXII-A، استولبور

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