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Research Article

Characterization of phytoplasmas associated with tomato big bud disease, using single-tube nested PCR

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Abstract: Tomato is an important vegetable crop in Iran. Recently symptoms associated with phytoplasma disease were observed in tomato cultivars in Karaj vicinity. The phytoplasmas were detected and characterized using single-tube nested PCR and RFLP. The universal primer pairs amplified the target DNA with expected sizes from symptomatic samples in direct PCR and single-tube nested PCR. The patterns of RFLP analysis obtained from plants with symptoms of yellowing and showing a bushy aspect corresponded to the profile of tomato big bud phytoplasma–subgroup 16SrI, and those detected from plants with symptoms of shoot proliferation and swollen and virescent buds belonged to tomato big bud phytoplasma–subgroup 16SrVI. Mobeen was the most susceptible cultivar with 23% natural infection, and Alex, Raha, Sadeen 21, and Sadeen 95 cultivars showed no symptoms of disease. Lack of symptoms in the latter cultivars might be due to inefficient transmission of phytoplasma by vectors or their resistance or tolerance to disease.

Keywords: tomato, Phytoplasma, cultivar, single-tube nested PCR

Introduction

Iran is among the seven top producers of tomato (*Solanum lycopersicum*) in the world with an approximate annual production of 4,826,396 tons (FAOSTAT, 2009). Phytoplasma infections of tomato have been reported from several areas in the world (Del Serrone *et al*., 2001; Lee *et al*., 1998; Gibb *et al.*, 1996; Jones *et al*., 2005; Shaw *et al*., 1993). Previous studies have indicated that phytoplasma disease of the tomato is genetically diverse (Santos-Cervantes, 2008) and belongs to different phytoplasma groups.

Phytoplasmas are phytopathogenic bacteria without cell wall that are limited to plant phloem. They were identified based on symptomatology, 4,6-Diamidino-2-phenylindole (DAPI), fluorescence microscopy, serological methods, PCR, and nested PCR (Cousin and Boudon-Padieu, 2001). DNA based molecular techniques such as PCR-RFLP and sequence analyses have been used to identify and classify phytoplasma strains (Gundersen *et al*., 1996; Lee *et al*., 2000; Schneider *et al.*, 1993). Phytoplasma have low concentration in tissues, so it is necessary to use nested PCR to obtain high sensitivity. The second round of amplification significantly increases risk of contamination (Olmos *et al.,* 1999), and false positive results are obtained. In this research, single-tube nested PCR was used

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as an easier, cheaper and faster method for the detection of phytoplasma infections.

Symptoms of tomato big bud have been previously reported from tomato fields in the Bushehr (Salehi *et al*., 2014), Fars (Salehi and Izadpanah, 1992), Khorasane Razavi (Jamshidi *et al.,* 2011), Ardabil, Isfahan (Rashidi *et al.,* 2006) and Lorestan (Dehghani and Salehi, 2011) provinces, but there is little information on phytoplasma groups infecting tomatoes in Iran.

Phytoplasma disease symptoms were observed in 21 tomato cultivars in experimental field in Alborz (Karaj) province. The aims of the present study were to identify and classify the phytoplasma associated with various symptoms in tomato fields using single-tube nested PCR (STNP).

Materials and Methods

During the summer of 2013 surveys were carried out in experimental fields of Alborz province growing 21 tomato cultivars including, Alex, Super Luna, Super Beita, Meridian 586, IDOL, EM Reina, Sadeen 95, Sadeen 21, Hormoz, Hengam, Matin, Mobin, Raha, Toro, Sun 6108, Sun 6189, Reddex, Selena, Tiyon, Nahid, Paradis. Cultivars were planted in a randomized complete block design with three blocks. Each plot consisted of two rows of seven meters length and one meter apart. Fifteen plants were planted per row.

All Plants were visually inspected for the presence of symptoms related to phytoplasma disease in the field and number of infected plants in each cultivar was recorded.

Hand sectioned symptomatic tissues were stained with Dienes staining and phytoplasmas localized among phloem cells were observed under a light microscope (Musetti, 2013).

DNA was extracted from fresh midribs of plant with symptoms, using a CTAB procedure (Zhang *et al*., 1998)

Direct PCR with two primer pairs P1/P7 and P1/Tint was done in a 25 µl reaction mixture consisting 25 ng template DNA, 300 µM dNTPs, 1x PCR buffer, 4 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and 0.8 µM of primers P1/P7 (Deng and Hiruki, 1991) or P1/tint (Smart *et al*., 1996). The temperature profile for PCR amplification was 94 ºC pre-denaturation for 4 min followed by 40 cycles of denaturation at 94 ºC for 1 min, annealing at 54 ºC for 1 min and extension at 72 ºC for 1 min, and final extension at 72 ºC for 7 min

Single tube nested PCR (STNP) was done in a 50 µl reaction volume containing 50 ng template DNA, 0.4 mM of each dNTPs, 1x PCR buffer, 3 mM $MgCl_2$, 3 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and 0.1 µM of each primer P1/P7 and 0.3 µM of each primer R16F2n/R16R2.

The temperature profile for PCR amplification was 94 ºC pre-denaturation for 4 min followed by 40 cycles of denaturation at 94 ºC for 35s, annealing at 53 ºC for 35s and extension at 72 ºC for 45s, and final extension at 72 ºC for 5 min.

Infected Potato, lime and sesame plants were used as positive controls for studying reliability of STNP.

Restriction fragment length polymorphism (RFLP)

For RFLP analysis, 10µl of PCR products were digested with endonuclease *Hpa*II restriction enzyme for R16F2n/R16R2 amplicons following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). Digested fragments were separated on 3% agarose gels, stained by gel red and visualized under an UV transilluminator. RFLP patterns were compared with those previously published, obtained from phytoplasma reference strains (Lee *et al*., 1998).

Results

In visual inspection, two main types of symptoms were distinguished in the field. Type1 consisted of shoot proliferation, swollen and virescent buds, and purple vein; Type 2 symptoms were yellowing, development of lateral shoots which gave the plant a bushy appearance with few virescent buds. (Fig. 1).

Phytoplasmas were detected in 51 plants of different tomato cultivars; however, no infection was detected in the experimental fields of Alex, Raha, Sadeen 21, and Sadeen 95 in Alborz Province of Iran (Table 1).

Phytoplasma infected cells were observed as purple spots scattered in sieve tubes, using Dienes' staining that verified the presence of phytoplasma in symptomatic tomato plants. (Fig. 2).

The universal phytoplasma primer pairs R16F2n/R16R2 in STNP amplified the target DNA from all tomato samples showing symptoms, but no amplification product was obtained from asymptomatic, healthy plants. Direct PCR assay with the primer pairs P1/P7 and P1/Tint amplified fragments of approximately 1.8 kb and 1.6 kb, respectively (Fig. 3). Amplified products with expected sizes were obtained from phytoplasma-infected potato, lime, sesame plants as positive controls in both direct PCR and STNP techniques.

RFLP analysis conducted on R16F2n/R16R2 products with restriction enzyme *Hpa*II (Fig. 4) revealed different banding patterns from amplified products obtained from plants with the two types of symptoms. The RFLP patterns obtained from plants with type 2 symptoms could be superimposed to the profile of tomato big bud phytoplasma–subgroup 16SrI, and phytoplasma detected from plants with type 1 symptoms belonged to the tomato big bud phytoplasma–subgroup 16SrVI (Lee *et al*., 1998).

Discussion

Understanding of the presence, incidence of phytoplasmas and epidemiology of the disease has greatly progressed in the last decade due to the development of sensitive methods (Weintraub and Jones, 2010). In the current study, single tube-nested PCR assay reliably detected phytoplasmas. Many researchers have reported using nested PCR for the detection of phytoplasmas (Gundersen *et al*., 1996; Heinrich *et al*., 2001). Nested PCR increases sensitivity, but two steps of nested PCR greatly increase the chance for contamination leading to false positive results. Single-tube nested PCR has resolved these problems, and it is faster and cheaper compared with the two-step nested PCR. To our knowledge, this is the first report of using universal primers in a single-tube reaction. The procedure detected phytoplasma in potato, tomato, lime and sesame; thus, it may be suitable for broad spectrum phytoplasma detection.

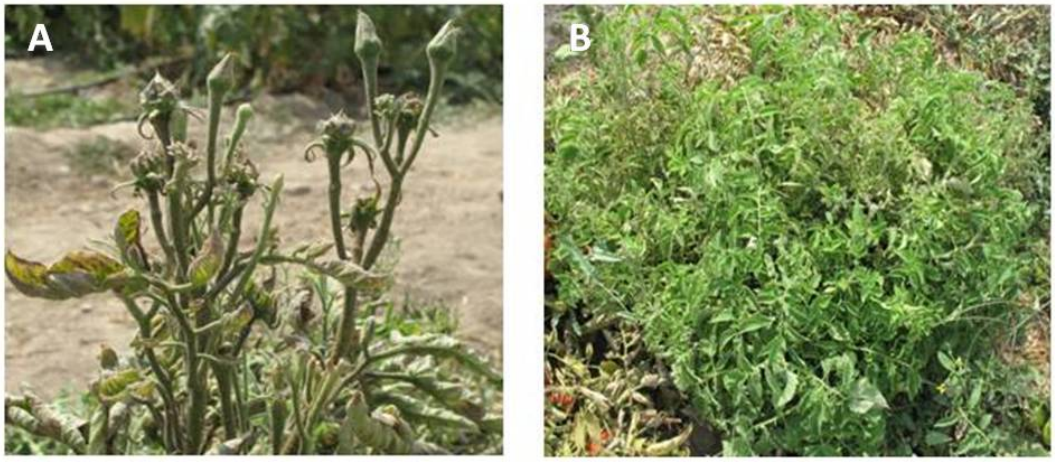

Figure 1 Symptoms of tomato plants infected by phytoplasmas. A: shoot proliferation, swollen and virescent buds, purple vein, B: plant yellowing, lateral shoots develop giving the plant a bushy aspect with few virescent and swollen buds.

Table 1 Occurrence of Phytoplasma in symptomatic tomato cultivars maintained in Karaj.

Code	Cultivar	Infected plant in each plot	Symptoms Type[1]	Phytoplasmas group
1	Mateen	3	1, 2	16SrI/16SrVI
2	Mobeen	7	1	16SrVI
3	San 6189	5	1	16SrVI
4	Selena	7	1	16SrVI
5	Tara-Nahid	1	1	16SrVI
6	Turo	2	1	16SrVI
7	Hengam	1	1	16SrVI
8	Meridian586 (Luck 85)	2	1	16SrVI
9	Tion	3	1	16SrVI
10	Pardis	2	2	16SrI
11	Hormoz	1	1	16SrVI
12	EM Reina (Lucky)	3	1	16SrVI
13	Super Beita	4	1	16SrVI
14	Super Luna	3	1	16SrVI
15	IDOL (Minoo)	2	1	16SrVI
16	San 6108	4	1	16SrVI
17	Redox	1	1	16SrVI
18	Raha	0	-	-
19	Sadeen 21	0	-	-
20	Sadeen 95	0	-	-
21	Alex	0	-	-

[1] Type 1 symptoms: shoot proliferation, swollen and virescent buds, purple vein and Type 2 symptoms: plant yellowing, lateral shoots develop giving the plant a bushy aspect with few virescent and swollen buds.

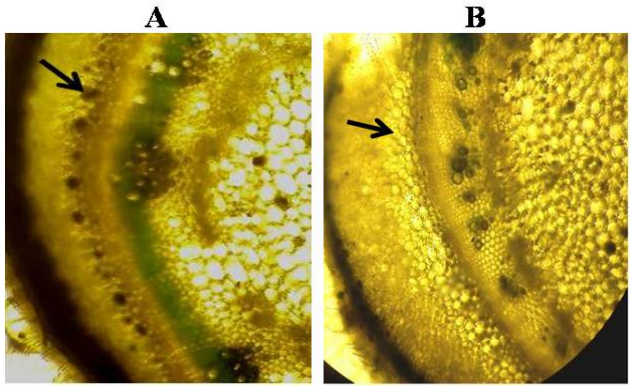

Figure 2 Micrograph of hand cut stems of tomato stained with Dienes stain. Phloem cells (arrows) are stained in section obtained from infected plant (A), section of healthy plant with unstained phloem (B).

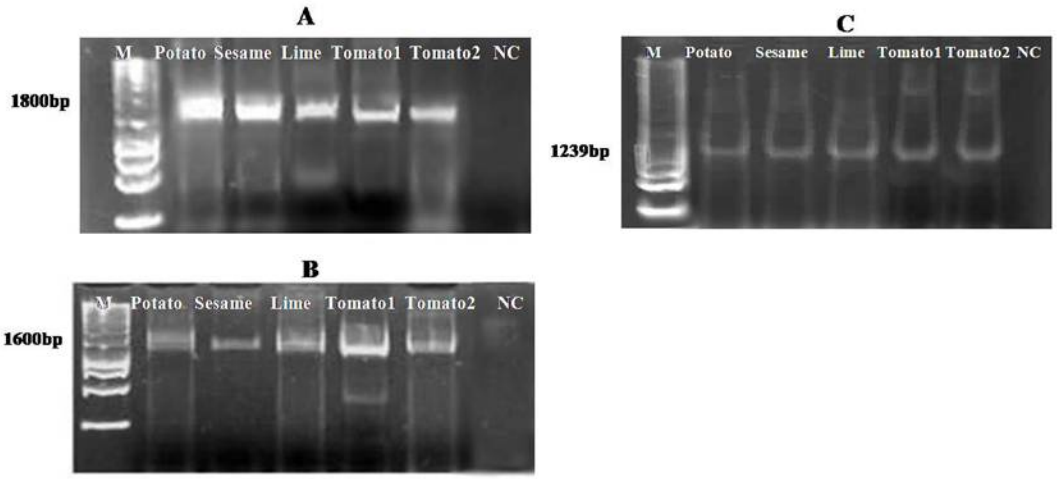

Figure 3 PCR detection of phytoplasma from tomato cultivars with two types symptoms in Direct PCR, using P1/P7 (A) and P1/Tint (B) and PCR products of single-tube nested PCR, with primer pairs P1/P7 and R16F2*n*/R16R2 (C).Phytoplasma- infected samples of potato, lime and sesame were used as positive controls. M: 1kb DNA Size Marker, NC: negative control.

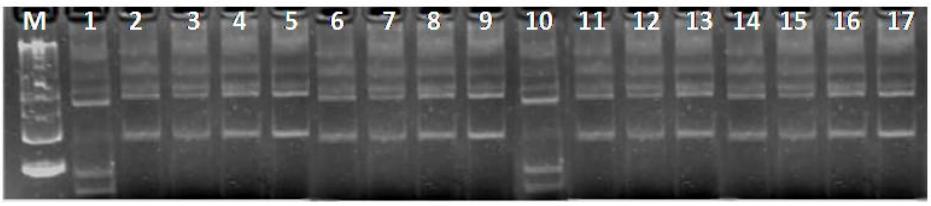

Figure 4 *Hpa*II restriction profiles of phytoplasma ribosomal DNA amplified from tomato cultivars using universal primers R16F2n/R16R2. M: 1kb Ladder.

Our observations indicated extensive spread of phytoplasma infection in tomato fields in Alborz province, and preliminary results were obtained regarding the etiology of this disease in different tomato cultivars. Samples collected from 21 tomato cultivars showed that the typical symptoms are strongly associated with the presence of tomato big bud, as reported previously in other provinces of Iran (Dehghani and Salehi, 2011). The dispersion of disease around the field can be taken as an indication that vectors are responsible for the introduction of disease into the field (Shaw *et al*., 1993). Since no special management practices have been adopted by growers to prevent the transmission of phytoplasma by vectors, the incidence of disease had increased.

PCR positive samples of tomato belonged to different phytoplasmas 16Sr groups based on the ribosomal gene analysis. In fact, RFLP patterns identified two different phytoplasma subtypes corresponding with those reported by Anfoka *et al*. (2003), 16SrVI (Clover proliferation group), and by Santos-Cervantes *et al*. (2008), 16SrI (Aster yellows group). The results of our study indicate that 89% of phytoplasmas present in tomato samples belonged to 16SrVI; but less than 11% belonged to 16SrI group which were observed only in the Pardis and Mateen cultivars. On the basis of the RFLP analysis, nucleotide sequence, and phylogenetic analysis, phytoplasmas associated with tomato diseases in various countries have been classified in different 16SrDNA groups: III (Del Serrone *et al*., 2001), VI (Anfoka *et al*., 2003), I, V, XII (Del Serrone *et al*., 2001), and II (Dong *et al*., 2013). This indicates that phytoplasma diseases of the tomato are genetically diverse (Santos-Cervantes, 2008).

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تشخیص فیتوپلاسمای همراه بیماری تورم گوجه‌فرنگی با استفاده از PCR آشیانه‌ای یک مرحله‌ای

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چکیده: گوجه‌فرنگی یکی از مهم‌ترین سبزیجاتی است که در ایران کشت می‌شود. اخیراً در مزارع شهرستان کرج علائم بیماری فیتوپلاسمایی در ارقام مختلف گوجه‌فرنگی مشاهده شده است. فیتوپلاسمای عامل بیماری به‌وسیله PCR آشیانه‌ای یک مرحله‌ای و آزمون RFLP ردیابی و شناسایی شد. پرایمرهای عمومی P1/P7، P1/Tint و R16F2n/R16R2 در آزمون‌های PCR آشیانه‌ای یک مرحله‌ای و PCR استاندارد، قادر به تکثیر DNA هدف فیتوپلاسما، از گیاهان آلوده بودند. براساس الگوی RFLP باند تکثیر شده از گیاهان با علائم زردی و ظاهر انبوه، فیتوپلاسمای عامل بیماری تورم گوجه‌فرنگی متعلق به گروه 16SrI است و در گیاهان با ظاهر جارویی، تورم جوانه و به رنگ سبز غیر طبیعی ، فیتوپلاسمای جدا شده متعلق به گروه 16SrVI بود. رقم مبین به‌عنوان حساس‌ترین رقم با آلودگی طبیعی ۲۳ درصد مشخص گردید. ارقام آلکس، رها، سادین ۲۱ و سادین ۹۵ علائمی از آلودگی نشان ندادند که احتمالاً عدم ظهور بیماری در این ارقام به‌دلیل وجود اختلال در انتقال فیتوپلاسما به‌وسیله ناقل و یا به‌دلیل وجود تحمل یا مقاومت میزبان به بیماری می‌باشد.

واژگان کلیدی: گوجه‌فرنگی، فیتوپلاسما، رقم، PCR آشیانه‌ای یک مرحله‌ای