Original Article

بررسی مقاومت ژنوتیپ‌های گوجه‌فرنگی نسبت به پژمردگی ورتیسیلیومی و فوزاریومی با استفاده از نشانگرهای مولکولی

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چکیده

سابقه و هدف: بیماری‌های پژمردگی ورتیسیلیومی و فوزاریومی دو بیماری مهم در گوجه‌فرنگی می‌باشند و تولید گوجه‌فرنگی را در دنیا محدود می‌کنند. کاشت ارقام مقاوم گوجه‌فرنگی بهترین روش برای کنترل این بیماری ها می‌باشد. نشانگرهای مولکولی که به ژن‌های مقاومت متصل می‌شوند برای توسعه برنامه‌های اصلاحی مفید هستند. این مطالعه با هدف شناسایی ژنوتیپ‌های مقاوم به پژمردگی ورتیسیلیومی با استفاده از نشانگرهای اختصاصی آلل و شناسایی ژنوتیپ‌های مقاوم به پژمردگی فوزاریومی به وسیله نشانگر CAPS انجام شد.

مواد و روش ها: این پژوهش به صورت مقطعی-توصیفی بر روی ۳۵ هیبرید و رقم تجاری گوجه‌فرنگی تهیه شده از شرکت فلات انجام شد. DNA نمونه ها با روش CTAB استخراج شدند. سپس واکنش زنجیره‌ای پلی مراز با استفاده از نشانگرهای مولکولی انجام گردید. به منظور شناسایی ژنوتیپ های مقاوم به پژمردگی فوزاریومی، از روش PCR-RFLP با استفاده از آنزیم‌های برشی RsaI و FokI استفاده شد. درنهایت نتایج با آزمون بیماریزایی تایید شدند.

یافته ها: از مجموع ۳۵ ژنوتیپ مورد بررسی، ۸۳ درصد ژنوتیپ ها نسبت به پژمردگی ورتیسیلیومی مقاوم و ۱۷ درصد حساس بودند. از طرفی ۴۶ درصد از ژنوتیپ ها نسبت به پژمردگی فوزاریومی مقاوم و ۵۴ درصد حساس بودند. ژنوتیپ هایی که تولید باند اختصاصی مقاومت به بیماری پژمردگی ورتیسیلیومی کردند، دارای ژن مقاومت Ve-1 بودند. همچنین ژنوتیپ هایی دارای باندهای اختصاصی مقاومت به بیماری پژمردگی فوزاریومی، ژن مقاومت I-2 را داشتند.

نتیجه گیری: با کاشت ژنوتیپ های مقاوم یافت شده در این تحقیق در مناطق آلوده می توان این دو بیماری را بدون استفاده از قارچ کش ها کنترل نمود.

واژگان کلیدی: پژمردگی فوزاریومی، پژمردگی ورتیسیلیومی، نشانگر مولکولی، ژن I-2، ژن Ve-1.

دریافت مقاله: دی ماه ۹۵   پذیرش برای چاپ: بهمن ماه ۹۵

٭) آدرس برای مکاتبه: تاکستان، دانشگاه آزاد اسلامی، گروه گیاه پزشکی

تلفن: ۰۲۱۴۴۶۴۶۳۱۸

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Original Article

Assessment of tomato genotypes resistance to Verticillium and Fusarium wilt diseases using molecular markers

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Abstract

Background & Objectives: Fusarium and Verticillium wilts are two major fungal wilt diseases of tomato that have restricted its production worldwide. Culturing resistant tomato cultivars is the best way to control such diseases. Molecular markers linked to resistance genes would be useful for improving tomato breeding programs. In this study allele, specific markers and cleaved amplified polymorphic sequences (CAPS) markers were used to identify tomato genotypes that are resistant to Verticillium wilt and Fusarium wilt, respectively.

Materials & Methods: This cross-sectional study was carried out on 32 tomato hybrids and commercial varieties provided from Falat company. DNA was extracted using cetyl-trimethyl ammonium bromide (CTAB) method. Then, polymerase chain reaction (PCR) was performed using molecular markers. To detect Fusarium wilt resistant varieties, PCR-RFLP was down using *Rsa*I and *Fok*I restriction enzymes. The results were confirmed by pathogenicity test.

Results: Out of 35 tomato genotypes, 83% were resistant to Verticillium wilt, while 17% were sensitive. Also, 46% of genotypes were resistant to Fusarium wilt, while 54% were sensitive. Genotypes that showed resistance to Verticillium and Fusarium wilts possessed *Ve*1 and *I*-2 genes, respectively.

Conclusion: Planting resistant genotypes in infected areas can control fungal diseases such as Verticillium and Fusarium wilts, without using any fungicides.

Keywords: Fusarium wilt, Verticillium wilt, Molecular marker, *I*-2 gene, *Ve-1* gene.

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop after potato (1). Total tomato world production is 152.9 million tons, with a value of $74.1 billion (FAOSTAT Database, 2009). Iran is ranked as the sixth tomato producer in the world (2). Two major fungal wilt diseases of tomato are Fusarium and Verticillium wilts. Verticillium wilt causes serious losses in the field (3).

Verticillium dahliae Kleb., *Verticillium alboatrum* Reinke, and Berthold are the major causal agents of this disease (3). *Fusarium oxysporum* f. sp. *lycopersici* (FOL) causes this

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disease only in plants of the genus *Lycopersicon,* and inhabits most tomato growing regions worldwide, causing tomato production yield losses (4).

The most practical means of controlling Fusarium and Verticillium wilts is the use of resistant varieties. Use of host resistance is an effective and environmentally friendly method to control such diseases. Verticillium wilt resistance is conferred by the *Ve* gene. Nowadays, the *Ve* gene is generally present in modern commercial tomato hybrids (5).

The *Ve* gene was reported to confer resistance to this disease, and is mapped to chromosome 9th of tomato (6). Kawchuk et al. identified a co-dominant random amplified polymorphic DNA (RAPD) marker associated to the *Ve* gene and subsequently they developed co-dominant and allele specific sequence characterized amplified region (SCAR) markers linked to *Ve* using the sequences from the RAPD marker (7, 8).

Furthermore, the two genes, *Ve*1 and *Ve*2 were cloned and their sequences were stored in GenBank (6). Based on the sequences of the two genes, Acciarri et al. developed allele specific PCR-based markers for *Ve*1and *Ve*2 and reported a cleaved amplified polymorphic sequences (CAPS) marker for *Ve*2 (5).

Recently, Kuklev et al. also reported a CAPS marker developed from the cloned homologies of *Ve*1 and *Ve*2 and showed polymorphism at this locus among resistant and susceptible plants (9). Fradin et al. showed that *Ve*1, but not *Ve*2, provides resistance in tomato against race 1, but not race2, strains of *V. dahliae* and *V. albo-atrum,* and recognized that *Ve1*and *Ve2* were the two sections of *Ve* gene (10, 11).

SNPs have been discovered and verified in tomato by Labate and Baldo (12) and Yang et al. (13). It will be useful to discover and validate all SNP markers between the resistant and susceptible alleles of *Ve*1 and *Ve*2 genes (11).

The gene *I*-2 confers resistance to FOL race 2. A CAPS marker, designated $TAO1_{902}$, which is linked to the *I*-2 gene, is useful for marker-assisted selection in tomato (4). A molecular marker linked to resistance would be useful for tomato improvement programs. Marker assisted selection (MAS) has been widely and successfully used to identify and select specific genes or combine multiple resistance genes in tomato breeding (14).

Single nucleotide polymorphism (SNP) has become one of the most useful molecular markers in genome mapping, association studies, diversity analysis, and tagging of economic important genes in plant genomics because of their abundance and automated high-throughput genotyping. They are the most cost-effective genetic markers currently available (15).

The objective of this study was to identify tomato varieties and hybrids that are resistant to Verticillium and Fusarium wilts and posses *Ve* and I-2 genes, respectively.

Materials and Methods

Plant materials

Thirty five tomato genotypes including commercially available cultivars and hybrids were used in this cross-sectional study. The seeds were provided from Falat agricultural company. Early Urbuna Y cultivar was used as a susceptible control and Kia hybrid was used

Table 1. Primers that used in this study.

Primer name	Primer Sequence	Tm (°C)	size (bp)	References
V1LeO2newF	5'-GTTACATGCAATCTCTTTGG-3'	52	1001 bp	8
ASAVe1_2R	5'-AATTAATGTGGACAAGCTCTG-3'			
ASAVe1_2F	5'-AGCTCATTTTGAAGGACTCTA-3'	61	696 bp	8
V1LeO2newR	5'-AGAGTAGTCCACATAGATGG-3'			
TAO1 F	5'-GGGCTCCTAATCCGTGCTTCA-3'	63	902 bp	6
TAO1 R	5'-GTGGAGGATCGGGTTTGTTTC-3'			

as a resistant control to Fusarium wilt.

Extraction of total genomic DNA from plant tissues

Total DNA was extracted from fresh four stage leaves of greenhouse grown plants using a slightly modified CTAB method described by Ausubel et al. (16). Approximately 70 mg of frozen tissue was grounded with a pestle dipped in liquid nitrogen till a fine powder was obtained. Successively, 700 µl of pre-heated (62 °C) CTAB extraction buffer was added to the pulverized plant material. The microtubes were vortexed for a few seconds and incubated at 62 °C for 30 min. Then 1 µl proteinase K (20mg/ml) was added and incubated at 37 °C for 30 min. 600 µl of chloroform/isoamyl alcohol (24:1) was added and the mixture and the reaction tubes were vigorously shaken. The extract was centrifuged for 10 min at about 15000 g at 4 °C. The supernatant was transferred to new reaction tubes. 2 µl RNase (10mg/ml) was added and the tubes were incubated at 37 °C for 30 min. Equal volume of chloroform/isoamyl alcohol (24:1) was added and the mixture and the reaction tubes were vigorously shaken. The extract was centrifuged for 10 min at about 15000 g at 4 °C. The supernatant was transferred to new reaction tubes where 700 µl of 100% cold isopropanol was added and incubated at -20 °C for 20 min.

The extract was centrifuged for 10 min at about 13000 g at 4 °C. Then supernatant was carefully discarded. DNA pellet was rinsed twice with 70% ethanol and dissolved in 50 µl of distilled water.

Allele-specific PCRs for the presence of Ve gene

The two allele-specific amplification (ASA) primers that were used to discriminate the susceptible allele from the resistant one have been designed by Acciarri et al. (5). Each ASA PCR reaction was conducted separately, using one ASA primer for the detection of each allele. The sequences of primers and amplicons size is shown in Table 1.

The reaction condition is presented in Table 2. PCR conditions for ASA-*Ve* allele (for resistant genotypes) were as follow:150 s at 94 °C as initial denaturation step, 45 s at 94 °C, 30 s at 52 °C, 120 s at 72 °C (30 times), 180 s at 72 °C. The PCR conditions for ASA-*ve* allele (for susceptible genotypes) were as

Table 2. Concentration of reagents used in PCR reaction.

	ASA PCR reaction in 25 µL volume	TAO1 PCR reaction in 25 µL volume
PCR buffer (10X)	2.5	2.5
$MgCl_2$ (50 mm)	0.85	1
dNTP (10 mm)	0.5	0.5
Primer (each) (10 pmol/ml)	1	0.5
Taq DNA polymerase (5 unit/ml)	0.2	0.2
DNA(40 ng)	2	2

follow: 150 s at 94 °C as initial denaturation step, 45 s at 94 °C, 30 s at 61 °C, 120 s at 72 °C (30 times), 180 s at 72 °C using an Eppendorf Thermal Cycler Mastercycler Gradient (Germany).

Polymerase chain reaction with CAPS marker for the presence of I-2gene

The CAPS marker that was used to discriminate the susceptible allele from the resistant one was originally designed by Staniazsek et al. (4). The sequence of CAPS primers (TAO1) and amplicon size is shown in Table 1. The PCR parameters were as follows: 94°C for 60 s followed by 40 cycles of 93 °C for 15 s, 63 °C for 20 s, 72 °C for 60 s and a final extension time of 5 min at 72 °C, using an Eppendorf Thermal Cycler Mastercycler Gradient (Germany). PCR product was digested at 37 °C in the presence of 1 U of *Fok* I and *Rsa* I restriction enzymes using the standard buffers (Fermentas, Germany).

Pathogenicity test to assay Verticillium wilt

To compare the pathogenic reactions of *Verticillium* isolates, Early Urbana Y cultivar was selected that was susceptible to *Verticillium* (17). Tomato seedlings were grown in mixture of peatmoss, vermiculate and perlite (1:1:1, v/v/v). Fourteen *Verticillium* isolates, obtained from tomato field soil, were

used. The code and origin of the isolates is shown in Table 3. *Verticillium* strains were cultured at 25 °C for eight days in malt extract broth on a rotary shaker having 150 rpm. The cultures were filtered through sterile cheese cloth, and successively centrifuged at 2160 g for 10 min to isolate the conidia. Conidia were resuspended in sterile distilled water to a concentration of 2×10^6 ml^{-1} (18). Thirty-day old plants were inoculated by immersing the roots for 20 min in the conidial suspension. The inoculated seedlings were trans-planted in the same type of soil. Three replications of four plants were used. The plants were kept at 21–23 °C. Daylight was supplemented by fluorescent lights to provide a 12 h day length (19). Disease symptoms were recorded approximately every 10 days for about 40 days after inoculation. Disease severity on leaves (chlorosis, wilting, and necrosis) and stem (dwarfing, adventitious roots) was assessed following a method of Acciarri et al. (17).

In brief, an arbitrary disease index was scored on a scale ranging from 0 (healthy plant) to 5 (unproductive plant, defoliation higher than 76%) through the following intermediate index criteria: 1= asymmetry and/or chlorosis on 1–3 leaves; 2= chlorosis on 50% of the leaves; 3= dwarfing, emerging adventitious roots, 50% defoliation; 4= dwarfing, developed adventitious roots, 51–75%. To compare the

Table 3: Code and origin of Verticillium isolates that used in this research.

Code of *Verticillium* isolate	Collection region	Code of *Verticillium* isolate	Collection region
Ve1	Tehran	Ve8	Fars
Ve2	Tehran	Ve9	Fars
Ve3	Tehran	Ve10	Fars
Ve4	Tehran	Ve11	Isfahan
Ve5	Fars	Ve12	Isfahan
Ve6	Fars	Ve13	Kerman
Ve7	Fars	Ve14	Kerman

reactions of tomato varieties and hybrids to Verticillium wilt, high virulent monoconidial *Verticillium* strains (V4 and V7) from tomato was used to screen the germplasm set.

Thirty five tomato genotypes were inoculated with method that described previously. Symptoms were recorded every 10 days for about 40 days following inoculation. Disease index was scored on a scale ranging from 0 to 5 according to the modified method of Acciarri et al. (17).

Pathogenicity test to assay Fusarium wilt
Disease test was performed two times. To compare the pathogenic reactions of *F. oxysporum* isolates, Early Urbana 111 cultivar was selected that was susceptible to FOL races. Tomato seedlings were inoculated at the two-leaf stage. Code, races and origin of the isolates is shown in Table 4. Plants were inoculated by soaking their roots in a freshly prepared spore suspension, (or, in the case of control seedlings, water) for 1 min and replanted to pots (9 cm in diameter). Seedlings were maintained at 25°C under 12 h of fluorescent light per day. After 3 weeks, they were uprooted and the lower stem and tap root were longitudinally sectioned for the examination of internal tissues. Each plant was rated on a scale of 0-4 as follows: 0= healthy plants; 1= < 25% vascular discoloration; 2= 26 -50% vascular discoloration; 3= wilting with

51-75% vascular discoloration; and 4= 76-100% vascular discoloration or death. To compare tomato varieties reactions to FOL races isolates, one isolate was selected from each race with the highest pathogenicity. Plants were inoculated with method that described previously. At 3 weeks post-inoculation, plants were visually rated for disease symptom development on vertical and horizontal sections of roots and hypocotyls.

Results
Amplification with ASA-Ve primers
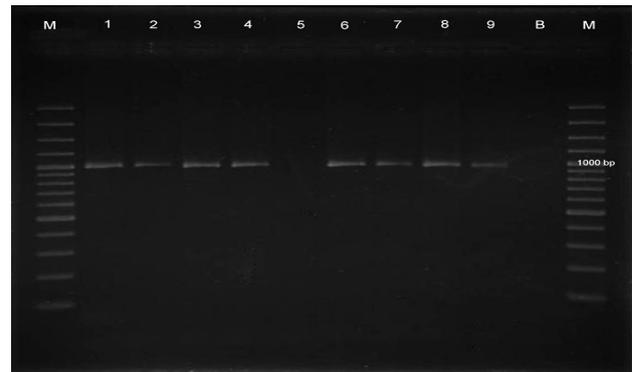Amplification with ASA-*Ve* primers gives a specific marker (1001 bp) linked to *Ve*1 gene

Fig. 1: *Ve*1 polymorphism detected on a standard agarose gel with 9 genotypes. 1: Kia (resistant control); 5: EarlyUrbana Y (susceptible control); lanes 2-4 and 6-9 genotypes were tested. 2: Caligen, 3: Matin, 4: Fani, 6: Eden, 7: Pulad, 8: CH-Falat and 9: Sun Seed. B: Blank and M: Molecular weight standard: 100 bp Plus DNA Ladder SM0323 (Fermentas). (*Ve*-allele specific marker fragment size 1001 bp).

Table 4. Code, races and origin of *Fusarium* isolates that used in this research.

Code of *Fusarium* isolate	Race of *FOL*	Collection region	Code of *Fusarium* isolate	Race of *FOL*	Collection region
F4	Race2	Tehran	F23	Race3	Fars
F5	Race2	Tehran	F24	Race1	Fars
F6	Race3	Tehran	F27	Race1	Fars
F7	Race3	Tehran	F34	Race2	Fars
F12	Race1	Fars	F13	Race3	Fars

in resistant genotypes. In this study 29 of the 35 samples (83%) revealed the 1001 bp marker (Fig. 1), so they were determined to be *Ve* positive (Table 5). Susceptible genotypes did not produce bands with ASA-*V* primers.

Amplification with ASA-ve primers

Amplification with ASA-*ve* primers gives a specific marker (696 bp) linked to *ve2* gene in susceptible genotypes. In this study 6 of the 35 samples (17%) revealed the 696 bp marker (Fig. 2) so they were determined to be *Ve* negative (Table 5). Resistant genotypes did not produce bands with these primers.
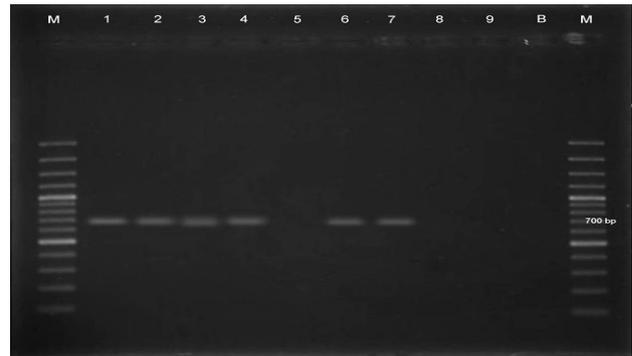
Fig. 2: *Ve*2 polymorphism detected on a standard agarose gel with 7 genotypes. 1: Early Urbana Y (susceptible control); 5: Kia (resistant control); 2-4, 6 and 7 genotypes were tested. 2: Booshehr, 3: Isfahan, 4: Fars, 6: Kermen, 7: Tehran. B: Blank and M: Molecular weight standard: 100 bp Plus DNA Ladder SM0323 (Fermentas). (*ve*-allele specific marker, fragment size 696 bp).

Table 5. Response of tomato cultivars and hybrids to allele specific markers.

Cultivars or F1 hybrids	Response to ASA-*Ve* primers	Response to ASA-*ve* primers	Resistance/Susceptibility
Cal JN	+	-	R
Cal JN 3	+	-	R
Caligen	+	-	R
Chef	+	-	R
CH-Falat	+	-	R
Early Urbana 111	+	-	R
Early Urbana Y	-	+	S
Falat Y	+	-	R
Karoon	+	-	R
King Ston	+	-	R
Matin	+	-	R
Navid-3	+	-	R
Peto early 84	+	-	R
Peto Rock	+	-	R
Sun Seed	+	-	R
Super Queen	+	-	R
Super 2270	+	-	R
Hyb. Comodoro	+	-	R
Hyb. Eden F1	+	-	R
Hyb. Fani	+	-	R
Hyb. Filos F1	+	-	R
Hyb. Firenze (PS8094)	+	-	R
Hyb. Kia F1	+	-	R
Hyb. Petopride II	+	-	R
Hyb. Petopride 5	+	-	R
Hyb. Petopride 6	+	-	R
Hyb. PS6515	+	-	R
Hyb. Pulad	+	-	R
Hyb. Queenty	+	-	R
Hyb. Speedy	+	-	R
Booshehr (Landrace)	-	+	S
Isfahan (Landrace)	-	+	S
Fars (Landrace)	-	+	S
Kerman (Landrace)	-	+	S
Tehran (Landrace)	-	+	S

(+) presence of marker; (-) absence of marker; R, resistant; S, susceptible; Hyb., hybrid

Amplification with CAPS primers and restriction with Fok I and Rsa I

Amplification with TAO1 primers revealed the 902 bp fragment for 35 varieties (Fig. 3). A 902-bp-long fragment of the TAO1 marker was found to be polymorphic in resistant tomato lines (4). This shows that these plants have the resistance gene (*I*-2). But, in order to understand the genotypic structure better and to determine whether they are homozygous or heterozygous, further analysis was carried out by digestion of the PCR products with *Fok* I and *Rsa* I restriction endonuclease. After digestion with *Fok* I, some samples revealed 390 and 410 bp fragments as reported by Staniazsek et al. (4).

These fragments show that both parental alleles of *I*-2 gene are present in the sample, thus the lines were considered to be from a homozygous resistant plant. DNA polymorphism was revealed following digestion of the amplicons with restriction enzymes *Rsa* I and *Fok* I. A *Rsa* I-digested fragment of 500 bps length, and two restriction fragments of 390 bps and 410 bps

for *Fok* I digestion of $TAO1_{902}$ were revealed in the homozygous-resistant plants (Fig. 4 and 5). Restriction products were analyzed in 35 plants and the *I-2* specific restriction fragments were detected in all 16 resistant plants, but not in the 19 susceptible plants (Table 6).

The co-dominant marker $TAO1_{902}$ directly recognizes the homozygote and heterozygote classes in F2 progeny, making the selection process for *F. oxysporum* f. sp. *lycopersici* resistance more precise compared to phenotypic selection in segregating populations (6). Full list of the plants is given in Table 2 showing the positive and negative results.
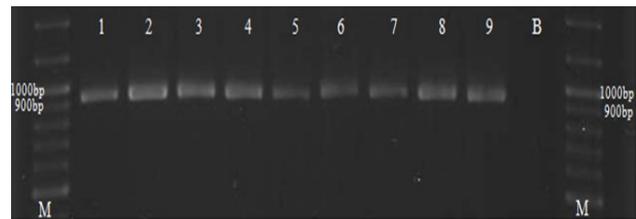

Fig. 3: PCR products amplified with TAO1 primer. All of the samples show 902 bp. bands. 1: Caligen, 2: Queenty, 3: Matin, 4: Fani, 5: CH-Falat, 6: Eden, 7: Pulad, 8: Early Urbana 111, 9: Super 2270, M: Marker.
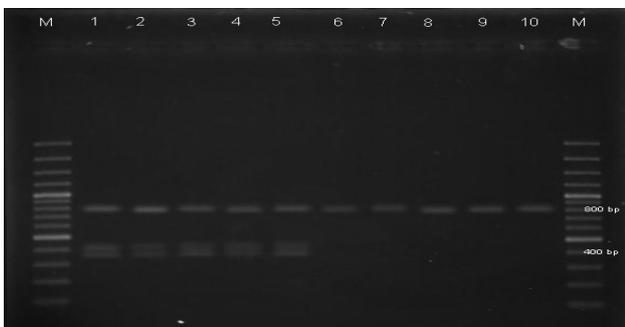

Fig. 4: PCR products digested with *Fok* I restriction endonuclease. 1-5 are resistant varieties and 6-10 are susceptible varieties. 1: Sunseed, 2: Matin, 3: Fani, 4: Caligen, 5: Petopride II, 6: Karoon, 7: Chef, 8: pulad, 9: Eden, 10 Early urbana Y, : M: Marker.
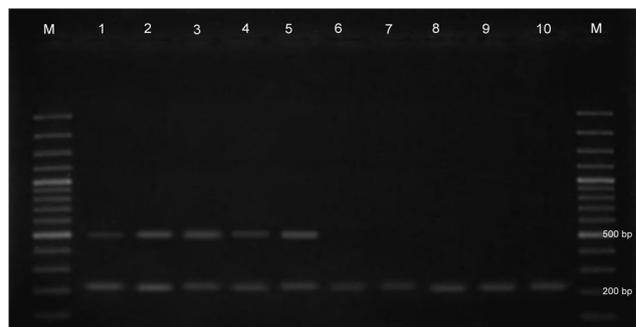

Fig. 5: PCR products digested with *Rsa* I restriction endonuclease. 1-5 are resistance varieties and 6-10 are susceptible varieties. 1: Sunseed, 2: Matin, 3: Fani, 4: Caligen, 5: Petopride II, 6: Karoon, 7: Chef, 8: pulad, 9: Eden, 10 Early urbana Y, M: Marker.

Isolation of Verticillium from soil and the pathogenicity test

Fourteen *Verticillium* isolates were obtained from tomato field soil. After these isolates were tested on susceptible genotype Early Urbana Y, the two isolates with the highest disease symptoms were selected to assess the reactions of tomato varieties and hybrids to Verticillium wilt. In this pathogenicity test, disease index for infected plant was between 2 to 4.25 (Table 7).

Tomato varieties that showed resistance amplicons with ASA-*Ve* primers had no disease symptoms on tomato plants. The varieties that showed susceptible amplicon with ASA-*ve* primers produced disease symptoms, with different disease indices following four weeks of inoculation (Table 7) (17).

Pathogenicity test to assay Fusarium wilt

Tomato varieties that showed resistant amplicons with CAPS marker produced no symptoms on tomato plants, while varieties which did not show resistance band produced disease symptoms, with different disease indices (Table 7).

Table 6: Distribution of the restriction fragments of the CAPS marker TAO1$_{902}$ in tomato cultivars, and F1 hybrids.

Cultivars or F1 hybrids	Response to *FOL*	TAO1 restriction fragments			
		Rsa I		Fok I	
		A	B	C	D
Peto Rock	R	+	+	+	+
Sunseed	R	+	+	+	+
Cal JN	R	+	+	+	+
Cal JN 3	R	+	+	+	+
Caligen	R	+	+	+	+
Hyb. Petopride 5	R	+	+	+	+
Hyb. Queenty	R	+	+	+	+
Hyb. Comodoro	R	+	+	+	+
Matin	R	+	+	+	+
Hyb. Petopride II	R	+	+	+	+
Hyb. Speedy	R	+	+	+	+
Navid-3 (N.3)	R	+	+	+	+
Hyb. Fani	R	+	+	+	+
Hyb. Firenze (PS8094)	R	+	+	+	+
Chef	S	-	+	-	+
Falat Y	S	-	+	-	+
Peto Early 84	S	-	+	-	+
CH-Falat	S	-	+	-	+
Super Queen	S	-	+	-	+
Hyb. PS 6515	S	-	+	-	+
Karoon	S	-	+	-	+
Hyb. Eden F1	S	-	+	-	+
Hyb. Pulad	S	-	+	-	+
Hyb. Petopride 6	S	-	+	-	+
Early Urbana 111	S	-	+	-	+
King Stone	S	-	+	-	+
Super 2270	S	-	+	-	+
Early urbana Y	S	-	+	-	+
Hyb. Kia F1	R	+	+	+	+
Hyb. Filos F1	R	+	+	+	+
Kerman (Landrace)	S	-	+	-	+
Tehran (Landrace)	S	-	+	-	+
Booshehr (Landrace)	S	-	+	-	+
Isfahan (Landrace)	S	-	+	-	+
Fars (Landrace)	S	-	+	-	+

FOL, Fusarium oxysporum f. sp. *lycopersici*; A, *Rsa* I–restriction fragment of 500 bp; B, *Rsa* I–restriction fragment of 220 bp; C, *Fok* I restriction fragments of 390 and 410 bp; D, *Fo*k I-restriction fragment of 800 bp; +, presence of marker; -, absence of marker; R, resistant; S, susceptible.

Discussion

Molecular markers have been widely used in plant breeding for phylogenetic studies, comparative genomics, mapping of genes or quantitative trait locus (QTL) and marker assisted selection (MAS) (20). Collard and Mackill (21) described four fundamental advantages of marker-assisted selection (MAS) as compared with conventional phenotypic screening based breeding: (i) MAS is simple, cost-effective, time–saving, and facilitates a non-destructive assay.

(ii) Selection can be carried out at any growth stage from seed to maturity. (iii) It facilitates differentiation of homozygous plants from heterozygous ones in backcross, bulk and pedigree breeding methods, thus facilitating early generation selection of superior recombinants, particularly for those traits controlled by recessively inherited genes. (iv) Screening can be done even without having the incidence of pests/disease. Molecular markers including RFLP, AFLP, RAPD, SCAR, CAPS and SNP have been

Table 7: Determination of disease index (DI) in tomato cultivars, and F1 hybrids to *FOL* races and verticillium isolates.

Cultivars or F1 hybrids	Race 1 of ROL	Race 2 of ROL	Race 3 of ROL	Verticillium
Peto Rock	0	0	0	0
Sunseed	0	0	0	0
Cal JN	0	0	1	0
Cal JN 3	2	0	1	0
Caligen	2	0	2	0
Hyb. Petopride 5	0	0	0	0
Hyb. Queenty	2	0	0	0
Hyb. Comodoro	0	0	0	0
Matin	0	0	4	0
Hyb. Petopride II	0	0	0	0
Hyb. Speedy	0	0	0	0
Navid-3(N.3)	1	0	1	0
Hyb. Fani	1	0	0	0
Hyb. Firenze	0	0	0	0
Chef	0	3	1	0
Falat Y	1	2	1	0
Peto Early 84	3	2	2	0
CH-Falat	3	4	2	0
Super Queen	0	2	1	0
Hyb. PS 6515	0	1	1	0
Karoon	1	3	2	0
Hyb. Eden F1	0	2	1	0
Hyb. Pulad	2	2	1	0
Hyb. Petopride 6	2	3	2	0
Early Urbana 111	3	4	3	0
King Stone	4	2	3	0
Super 2270	2	3	4	0
Early urbana Y	3	4	1	4
Hyb. Kia F1	0	0	0	0
Hyb. Filos F1	0	0	0	0
Kerman (Landrace)	3	2	2	3
Tehran (Landrace)	2	3	2	2.75
Booshehr (Landrace)	4	2	3	4.25
Isfahan (Landrace)	3	3	1	3.25
Fars (Landrace)	3	4	2	3.75

identified and successfully used in selecting disease resistance in tomato, but most of them are linked to target genes of disease resistance (14). With more genes being cloned and sequenced, the sequence-derived PCR-based and SNP markers will be found easily for specific gene/allele. The most abundant molecular markers in plant and animal genomes are SNPs, which constitute single base-pair variations in the DNA sequence.

They are becoming the marker of choice in plant breeding, as they are easily prone to automation and high-throughput. Consequently, SNP- based genomic resources for crop plant species are growing fast (21). SNPs are the most abundant source of variation in the genome for both intergenic and intragenic regions. They therefore represent a valuable basis for the development of molecular markers to identify polymorphisms among closely- related lines. Previous studies have suggested that DNA markers developed from intragenic regions tend to cluster in heterochromatic portions of chromosomes, while those derived from intergenic regions disperse along entire chromosomes (22, 23).

Therefore, SNPs, especially those located in intragenic regions, are expected to distribute randomly along the whole genome. In addition, novel techniques based on the DNA microarray method allow high-throughput SNP genotyping. For these reasons, SNP markers derived from intragenic regions are the most informative markers for genome-wide genetic analysis in intraspecific tomato populations (24). As the most abundant and stabile form of genetic variation in most organism genomes, SNPs are more suitable for genotyping markers

compared to the conventional markers such as RFLP, AFLP and SSR. With the development of bio-technology, SNPs are becoming favored genetic markers cloning (22), for the study of evolutionary conservations between different species (25), and the detection of risk-associated alleles linked to diseases (26). CAPS markers are co-dominant; they can be easily detected by agarose gel electrophoresis and in the case of recognizing SNP causing a change in the phenotype, they are treated as "perfect" markers (27).

By means of molecular markers linked to the trait of interest, selection can be performed at an early seedling stage of development, and the Verticillium-resistant genotypes can be easily distinguished. The use of molecular markers tightly linked to resistance is particularly important in the selection for Verticillium wilt resistance.

In fact, Verticillium wilt symptoms are not easy to estimate and their expression is strongly influenced by environmental conditions. The *Ve*1 and *Ve*2 genes are closely linked and usually inherited as a single unit, and no recombination event has been described so far. In tomato, these two genes do not confer resistance to *V. dahliae* race 2, thus new genes to control this highly virulent race are needed (5). Molecular markers have been used successfully to identify tomato accessions with resistant genes to Fusarium wilt (28-30).

This process of identification is crucial in the development of tomato varieties that are resistant to the highly destructive pathogen, *Fusariumoxysporum* f. sp. *lycopersici* is responsible for the vascular wilt of tomato.

Developing resistant varieties is the most environmentally sound and effective strategy for the management of the disease (30).

Popoola et al. identified some Nigerian accessions of tomato containing homozygous resistant *I*-2 gene using CAPS marker TAO1 (29). In Nigeria, fifty Nigerian accessions of tomato were screened with the two CAPS markers for resistant *I*-2 gene. Restriction fragments from the two markers indicated that 11 accessions were homozygous resistant to *Fol* (31).

Hansona et al. described a three-year selection process, and the sequence of field, lab, greenhouse and molecular marker protocols applied by AVRDC-The World Vegetable Center (AVRDC) to a segregating population, which led to the development of fresh market tomato lines resistant to late blight, tomato yellow leaf curl disease, bacterial wilt, Fusarium wilt, gray leaf spot, and Tobacco mosaic virus (TMV) (32).

Conclusions

In this study 35 tomato varieties were screened for *Ve* and I-2 resistance genes using specific molecular markers. The results revealed that 29 genotypes were resistant to Verticillium wilt in which 16 genotypes showed resistance due to the presence of *I*-2 gene which confers resistance to *F. oxysporum* f. sp. *lycopersici* race 2.

Sixteen tomato genotypes were resistant to both *Fusarium* and *Verticillium* wilts. High reliance on fungicides poses health hazards to farmers, the environment, and consumers and also can substantially increase production costs, which increase farmer financial risks and pass the accrued higher costs to consumers.

Resistant cultivars are among the cheapest, simplest, and most environmentally safe ways to manage disease. Marker-assisted screening had reduced a bulk of genetic material to manageable size which can be taken to the field for phenotypic or bioassay confirmation (29).

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