Genetic Variation Among Four Local Isolates of *Oidium neolycopersici,* One of the Causal Agents of the Powdery Mildew Disease of Tomato Using Inter Simple Sequence Repeats (ISSR) Technique

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Abstract

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Oidium neolycopersici is one of the tomato powdery mildew pathogens in greenhouses. This study aimed to determine the genetic relatedness and pathogenicity of four local isolates (M8, M10, G12, and R12) of *O. neolycopersici* using the Inter Simple Sequence Repeats (ISSR) technique. The DNA of the isolates was amplified using 23 ISSR primers. The results obtained showed that there was no significant variation in pathogenicity among the tested isolates. Only 11 primers gave polymorphism among the studied isolates, yielding a total of 191 bands; 58 of which were polymorphic, and the polymorphism rate was 31%. The number of polymorphic bands ranged between 1 band with the primer A8 to16 bands with the primer E11, with an average of 5.2 bands per primer. Genetic divergence values were low in general, ranging between 0.05 and 0.26, which means high genetic relatedness between the isolates. The phylogenetic tree was divided into two main clusters: the first included the two isolates M10 (Alkhrab region) and M8 (Alademah region), which had a 95% genetic similarity. The second included G12 and R12 isolates with a high degree of relatedness (92%), but lower than the previous ones, even though the two isolates were collected from the same region (Matn Abu Raya). The results revealed that ISSR technique was effective in detecting diversity and genetic relatedness among *O. neolycopersici* isolates.

Keywords: *Oidium neolycopersici,* powdery mildew, genetic variation, tomato, ISSR.

Introduction

The tomato plant, *Solanum lycopersicum* L. is one of the most important and widely consumed vegetable crops in the world. It is commonly grown in greenhouses and open fields (Gebhardt, 2016). Protected tomato plants are infected with many fungal diseases, such as blights, gray mold, and powdery mildew. *Oidium neolycopersici* L. Kiss sp. Nov (2001) is one of the powdery mildew causatives that considered destructive of tomato cultivation in greenhouses with more than 50% loss in production (Cerkauskas & Brown, 2015; Getinet, 2021; Lebeda *et al*., 2014; Lian *et al*., 2020; Wang *et al*., 2023).

The necessity of determining the degree of relatedness or understanding the genetic diversity of plant pathogens stems from the risk of these pathogens evolving or being subject to mutations, and thus the possibility of forming strains that are aggressive or resistant to chemical pesticides which are widely used in high and random doses by farmers. On the other hand, determining genetic diversity helps to understand the biology of the pathogen which led to allowing for the development of effective measures to control it, such as employing resistant plant genes or selecting pesticides and establishing appropriate doses (Amaradasa & Everhart, 2016; Ellwood *et al*., 2019; Mcdonald & Linde, 2002). Furthermore, investigating the isolates pathogenicity and virulence helps to detect the efficacy of resistance genes utilized in breeding and producing disease-resistant plant genotypes (Liu *et al*., 2015a). Several molecular indicators are known to be more accurate in determining the degree of relatedness between isolates (O˘guz & Karakaya, 2021). ISSR indicators are characterized by being fast, simple, and relatively cheap, and they require small amounts of the organism's DNA (Sarwat, 2012). ISSR indices have been used in many studies to determine the genetic diversity of isolates of fungal species causing plant diseases, such as *Fusarium* isolates and *Alternaria alternata* (Fr) Keissl. (1912) on tomato (Adss *et al*., 2022; Akbar *et al*., 2018; Mohammadi & Bahramiki, 2019; Rampersad, 2021; Thangavelu *et al*., 2012; Troncoso-Rojas *et al*., 2013). Liu *et al*. (2015a) found that ISSR and sequence-related amplified polymorphism (SRAP) indicators were effective in revealing genetic diversity among *Blumeria graminis f. sp. tritici* emend. É. J. Marchal isolates, the causal agent of powdery mildew on wheat, as the isolates showed great genetic variation within the different regions in China, whereas the isolates from the same region were genetically identical, but varied in virulence. Parthasarathy *et al*. (2017) also utilized ISSR to investigate genetic diversity among *Erysiphe pisi* Dc. (1821) isolates. Cieplak *et al*. (2022) discovered that ISSR indices were beneficial in detecting genetic variety among isolates of the species *Blumeria graminis* f. sp. *avenae* Em. Marchal, the powdery mildew on oats, as these indicators outperformed the SCoT (Start Codon Targeted) indicators by giving greater polymorphism.

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There has been no research on the use of this indicator to determine the degree of relatedness among *O. neolycopersici* isolates. Rather, the internal transcribed spacer (ITS) indicator was used to assess genetic diversity (Hirata *et al*., 2000; 2001; Kiss *et al*., 2005). Jankovics *et al*. (2008) also found a match between *O. neolycopersici* isolates collected from different geographical locations using the same index, whereas AFLP analysis revealed significant genetic diversity by identifying different genotypes. Thus, the ITS index was considered to have narrow limits in fully characterizing the genetic diversity of powdery mildew diseases. Therefore, it was necessary to use more accurate molecular techniques to assess genetic variation (Kovács *et al*., 2011; Lücking *et al*., 2020). *O. neolycopersici* was reported earlier in Syria on protected tomatoes in some regions of Tartous Governorate (Alio *et al.*, 2023). Later, Alio *et al*. (2024) found a high degree of genetic relatedness between *O. neolycopersici* isolates using ITS technology, where two isolates (M10 and M8) were 100% identical, as were G12 and R12 isolates, and the two mentioned groups of isolates differed in only one nucleotide position within the ITS region and in six amino acids. This study attempted to confirm the results of the ITS index that was utilized in the aforementioned study by assessing the degree of genetic relatedness among those four isolates using ISSR technology.

Materials and Methods

***Oidium neolycopersici* isolates**

Four isolates (M8, M10, G12, and R12) investigated in this study were obtained from a previous study through a field survey conducted in the 2019 and 2021 seasons in Tartous Governorate, Syria (Alio *et al*., 2023), and described morphologically and molecularly using ITS technology and registered in the GenBank database NCBI (Alio *et al*., 2024) (Table 1). The isolates were maintained on the tomato cultivar Mandalon under greenhouse conditions. Infected leaf samples of each isolate were then dried in order to use in DNA extraction.

Pathogenicity test

The pathogenicity of the four isolates was studied on hybrid tomato (Mandaloun) at the fourth true leaf stage under greenhouse conditions (26±4°C). Four replicates of four tomato plants were allocated for each isolate. Inoculation suspension was prepared at a concentration of 3×10^4 spores/ml, and it was sprayed after preparation directly in the early morning on healthy plants until completely wet. The inoculum was sprayed onto the upper side of plant leaves. The inoculated plants were covered with plastic bags for 24 hours. Subsequently, symptoms of infection were monitored for each isolate 7–14 days after inoculation. The severity of infection was measured based on a 0-4 scale as follows: 0 = no visible growth/healthy, 1 = infected area less than 5% of leaf area, 2 = infected area 5-20% of leaf area, 3 = the area infected with the disease from 21–50% of the leaf area; 4 = the area infected with the disease is more than 50% of the leaf area, and the disease index was calculated by the following equation:

$$\text{Disease index (DI) (\%)} = \frac{\Sigma(a.b).100}{N.4}$$

where a= number of infected leaves, b= numerical values of symptoms severity, N= total number of leaves, 4= maximum scale point (EPPO,1998). The results were analyzed statistically using the Genestat7 program, and the least significant difference (LSD) at P=0.05. The infected leaves of each isolate were air-dried and stored until further use.

DNA extraction

DNA was extracted from the dried infected tomato leaves with the four isolates (M8, M10, G12, R12) using the techniques outlined earlier (Liu *et al*., 2015b; Lodhi *et al*., 1994), with some modifications. The DNA extraction was performed at the Atomic Energy Commission laboratories in Syria according to the following steps:

Infected tomato leaves with the four isolates were placed individually in a 2 ml tube, with 1 ml of Chelex-100 solution. The tubes were incubated at 94 °C for half an hour, and then centrifuged at 13,000 rpm for 20 min. The filtrate was transferred to a new 2 ml tube. Each tube received 1 ml of 2% CTAB extraction solution and was placed in a water bath set to 65 °C. The tubes were shaken on a Vortex shaker before being placed in a water bath at 60°C for 30 min. with the tubes shaken every 15 min. Each tube received 850 microliters of chloroform/isoamyl alcohol (24:1) solution. The tubes were manually swirled for 5-10 min before being frozen at -20°C for 10 min. The tubes were then centrifuged at 13,000 rpm for 20 min, so that the upper liquid phase (the filtrate containing the DNA) was taken and transferred to a new tube with absolute alcohol, and placed in a freezer at -20°C for 10 min. Tubes were centrifuged at 13,000 rpm for 20 min. The liquid phase was discarded, and 0.5-1 ml of cold 70% alcohol was added to the DNA precipitate. The tubes were gently inverted multiple times to separate the DNA globulus from the bottom. To remove alcohol, the tubes were placed in a sterile tent at 37°C for 10 min. Then, 90-100 µl of sterile distilled water was added and gently mixed.

The extracted DNA quality was assessed using a horizontal electrophoresis apparatus (Apelex-France) by combining 10 µl of each sample with 5 µl of Luding's dye in 0.8% agarose gel and TBE electrophoresis solution in the presence of standard DNA (DNA molecular ladder). The voltage was set to 80 volts for two hours, and the gel was photographed by exposing it to UV radiation (CAMAG Reprostar 3, Switzerland).

Table 1. Source of *O. neolycopersici* isolates used in this study.

Isolates	Accession number	Height above sea level (m)	Location (Longitude E, Latitude N)
M8	OM921389	Less than 100	Adimeh (35°9′4″N 35°55′41″E)
M10	OM921390	Less than 100	Al-Kharab (35°4′30″N 35°53′53″E)
R12	OM921393	365	Maten Abo Rya (35°02'20.4"N 35°56'04.4"E)
G12	OM921392		

The DNA concentration was measured by a spectrophotometer (GeneQuant) from Amersham Biosciences. Absorbance readings were taken at 260 and 280 nm wave length, and the concentration was calculated using the following equation (Maniatis *et al*., 1982):

DNA concentration (ng/µl) = O.D. units at 260 nm×50× dilution factor/1000

The purity of DNA was measured by determining the ratio of the absorbance (optical density) readings between the two wavelengths OD_{260}/OD_{280} (Sambrook *et al*.,1989).

DNA replication

An Eppendrof-Mastercycler was used to execute the PCR reaction with a final volume of 25 µl for each sample, including 50 ng of DNA, PCR Master solution (Generi Biotech), and 5 µM of each primer (Invitrogen and Macrogen). A set of 23 primers were used (Table 2). The amplification thermocycling program is on cycle 94℃ for 5 min, then 40 cycles include 94℃ for 30 sec, 50℃ for 30 sec and 72℃ for 30 sec and final extension at 72℃ for 7 min. The reaction products were placed at 4°C, then were separated on a 1.8% agarose gel (Q-BIOgene Company) in a 0.5X TBE buffer solution, with ethidium bromide (Fluka Company) added to detect the DNA bands through ultraviolet radiation, and a DNA molecular Ladder (Universal) (Kapa Biosystems) was used to indicate the size of amplified fragments.

Table 2. ISSR primers used in the study (primer annealing temperature was 50ºC).

S. no	Primer	Nucleotide sequences 5'-3'
1	HB9	GTGTGTGTGTGTGG
2	HB10	GAGAGAGAGAGACC
3	HB12	CACCACCACGC
4	UBC825	ACACACACACACACT
5	UBC826	ACACACACACACACACC
6	UBC857C	ACACACACACACACACCTGC
7	E3	GAGAGAGAGAGAGAGAT
8	E5	TCTCTCTCTCTCTCTCTCC
9	E8	GAGAGAGAGAGAGAGAG
10	E11	GACAGACAGACAGACA
11	IG-3	GAGGGTGGAGGATCT
12	IG-09	AGAGAGAGAGAGAGAGC
13	IG12	GAGAGAGAGAGAGAGAC
14	IG-13	GAGAGAGAGAGAGAGAA
15	A6	CACACACACACARY
16	A8	CACACACACACARM
17	A25	CACACACACACAK
18	A30	AGCAGCAGCAGCR
19	A40	AGCAGCAGCAGCK
20	B10	CAGCAGCAGCAGCAG
21	C24	CTCTCTCTCTCTCTCTTG
22	C25	GTGGTGGTGGC
23	D8	AGCAGCAGCAGCS

Results and discussion

Isolates Pathogenicity test

The pathogenicity of the *O. neolycopersici* isolates (M8, M10, G12 and R12) on Mandaloun tomato hybrid was investigated, and isolate R12 had the highest severity, one week after inoculation (36.8%). Two weeks after inoculation, the degree of infection increased for all tested isolates, and isolate R12 showed the highest severity (61.7%). There were no significant variations in pathogenicity among isolates during the first and second week after inoculation (Table 3). Because there has been insufficient research on the variance in the pathogenicity of *O. neolycopersici* isolates on the same tomato variety, it is required to investigate the pathogenicity of additional hybrids or specific varieties. This what Lebeda & Mieslerová (2002) reported, with British isolate which had a high pathogenicity to specific tomato varieties than the Czech, German, and Dutch isolates. Kashimoto *et al*. (2003) mentioned that the Japanese isolate KTP-01 was aggressive against a tomato cultivar cultivated in the Netherlands. Xiang *et al*. (2020) observed no significant differences in pathogenicity among *Podosphaera xanthii* (Castagne) U. Braun & N. Shishkoff isolates, the causal agent of powdery mildew on cucumber, collected from different countries. Also, Cieplak *et al*. (2022) studied the oats powdery mildew, the pathogenicity and virulence of isolates collected from one country were similar.

Table 3. The pathogenicity of *O. neolycopersici* isolates on tomato following artificial inoculation.

Isolate	Severity, one week after inoculation	Severity, two weeks after inoculation
M8	34.2 a*	60.0 a
M10	35.7 a	60.1 a
R12	36.8 a	61.7 a
G12	36.2 a	59.9 a
$LSD_{0.05}$	2.8	7.4

*Values are the average of four replicates. Means followed by the same letters in the same column are not significantly different at P=0.05.

DNA extraction efficiency and purity

The DNA molecules appeared in the upper part of the gel near the sample cells in the form of an unbroken band, which indicates a high molecular weight and the DNA was not subjected to fragmentation during the extraction process. Thus, the extraction process was appropriate, and the DNA band appeared intact and pure on the gel (Figure 1). The concentration ranged between 5.7 and 9.1 ng/µl, and the purity indicator of the samples ranged between 1.3 to 1.6 (Table 4). The size of the resulting bands ranged between 10 to 1000 base pairs.

Polymorphism obtained from ISSR application

The DNA of the four isolates was amplified using 23 primers for internal simple sequence repeats (ISSR). The amplification products of the different primers were studied

by comparing the resulting paths among them. Bands were counted based on the presence of the band (1) or its absence (0), and only clear bands that were repeated in two tests were considered (Figure. 2). Only 11 primers gave polymorphism among the studied isolates, yielding a total of 191 bands (Table 5), 58 of which were polymorphic, and the polymorphism ratio was 31%. The number of total bands ranged from eight bands with primers E8 and IG-3 to 24 bands with primers HB12, E11 and A40, with an average of 17 bands per primer. The number of polymorphic bands ranged between 1 band to16 bands, with an average of 5.2 bands per primer. Primer E11 gave the highest number of polymorphic bands (16 bands) with a polymorphism of 67%, followed by primers HB12 and D8 with 12 and 7 bands, respectively, and polymorphism of 50% and 37%, respectively. Primer A8 gave the lowest number of polymorphic bands (1 band) with a polymorphism of 5%, and the rest of the primers gave 2-4 bands with variable polymorphisms rate (11-50%).

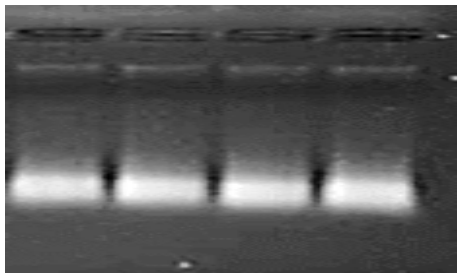

Figure 1. DNA quality of four tested isolates on agarose gel.

Table 4. Concentration and purity of the DNA extracted from the four fungal isolates.

Isolate	DNA (ng/µl)	Purity indicator
G12	5.7	1.6
M10	6.6	1.3
M8	9.1	1.4
R12	6.1	1.6

Purity indicator is the ratio A_{260}/A_{280}

To determine the degree of genetic relatedness between the studied isolates based on the number of shared amplification products, a matrix of percent disagreement values (PDV) was created using the UPGMA method and the STATISTICA6 program. High matrix values indicated the presence of genetic variation between the isolates. It was clear (Table 6) that the lowest value of genetic divergence was 0.05 between the two isolates M10 and M8, and this indicates genetic closeness between these two isolates, whereas the highest value of genetic divergence was 0.26 between the two isolates G12 and M10, followed by 0.25 between the two isolates G12 and M8, which indicates a low genetic variation between them.

The degree of genetic relatedness between the isolates appeared clearly through the cluster analysis resulting from the use of ISSRs data based on the incompatibility ratios (Figure 3). The phylogenetic tree was divided into two main clusters: the first included the two isolates M10 (Alkhrab region) and M8 (Alademah region), which had a 95% genetic similarity. The second included G12 and R12 isolates with a high degree of relatedness (92%), but lower than the previous isolates, even though the two isolates were collected from the same region (Matn Abu Raya). Few previous studies have shown that the isolates of powdery mildew species obtained from the same host plant and from close geographical locations were identical when using the ITS index (Hirata *et al*., 2000; 2001; Kiss *et al*., 2005). Although the number of the isolates in this study were low, and the geographic locations from which they were collected were close, it was found that there was low genetic variation between the two groups of isolates (M10, M8) and (G12, R12). This is consistent with the study of Alio *et al*. (2024), where the two isolates M10 and M8 were 100% identical, as were the G12 and R12 isolates using the ITS technique. Such results indicate agreement between the use of the two indicators (ITS and ISSR), and their efficiency in determining the degree of relatedness between *O. neolycopersici* isolates.

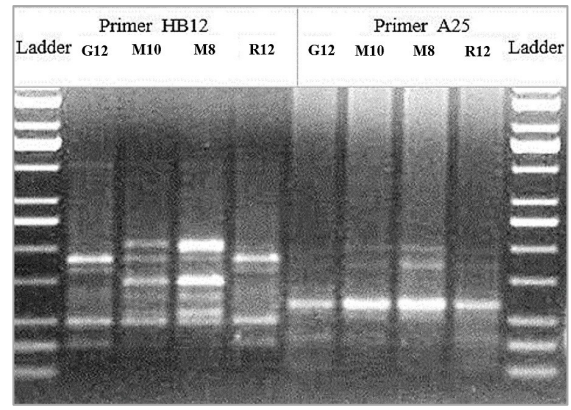

Figure 2. ISSR- PCR DNA amplification using HB12 and A25 primers.

Table 5. Total number of bands and the number of polymorphic bands obtained from the *O. neolycopersici* tested isolates.

Primers	No. of amplified fragments			Polymorphic/ total bands (%)
	Total	Poly-morphic	Mono-morphic	
HB12	24	12	12	50
UBC826	18	2	16	11
E3	14	2	12	14
E8	8	4	4	50
E11	24	16	8	67
IG-3	8	4	4	50
A8	21	1	20	5
A30	15	2	13	13
A40	24	4	20	17
C24	16	4	12	25
D8	19	7	12	37
Sum	191	58	133	-
Average	**17**	**5.2**	**12**	**31**

Table 6. Matrix of the ratios of PDVs resulting from the ISSRs technique among the studied genetic polymorphism of the four fungal isolates.

Fungal isolate	G12	M10	M8	R12
G12	0.00			
M10	0.26	0.00		
M8	0.25	0.05	0.00	
R12	0.08	0.24	0.25	0.00

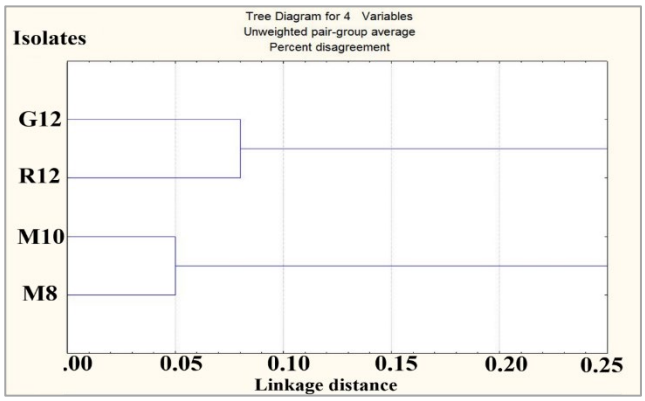

Figure 2. A dendrogram shows the phylogenetic relationship among the four *O. neolycopersici* isolates based on ISSRs analysis of the percent disagreement values (PDV) of UPGMA.

The similarity in pathogenicity and the presence of a high degree of genetic relatedness indicate slow development within the population of this species. In addition, the absence of the sexual phase of *O. neolycopersici* may be the reason behind the absence of genetic variation between isolates representing its asexual phase, as it is known that the sexual phase is responsible for different genetic mechanisms in genetic recombination within the species (Lebeda *et al*., 2017). The use of low number of isolates in this study does not give a credible information on the genetic variability of this pathogen in Syria. Therefore, to better understand genetic variability within this pathogen, many more isolates of the pathogen need to be analyzed in future studies.

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الملخص

عليو، نهى، صباح المغربي ونزار معلا. 2026. الكشف عن التباين الوراثي بين أربع عزلات محلية للنوع *Oidium neolycopersici* أحد مسببات البياض الدقيقي على البندورة/الطماطم في الزراعة المحمية، باستخدام تقانة التتابعات الداخلية البسيطة المتكررة (ISSR). مجلة وقاية النبات العربية، 44(1):119–125. https://doi.org/10.22268/AJPP-001378

يعدّ النوع *Oidium neolycopersici* أحد مسببات البياض الدقيقي على البندورة/الطماطم في البيوت المحمية. هدفت هذه الدراسة إلى الكشف عن القدرة الإمراضية والقرابة الوراثية لأربع عزلات محلية (M8، M10، G12 و R12) تتبع لهذا النوع باستخدام تقانة التتابعات الداخلية البسيطة المتكررة (ISSR)، حيث تمّ تضخيم الـ DNA للعزلات باستخدام 23 بادئة للـ ISSR. أظهرت النتائج عدم وجود فروق معنوية بين العزلات من حيث القدرة الإمراضية، وأعطت 11 بادئة فقط تعددية شكلية بين العزلات، حيث بلغ عدد الحزم الكلية الناتجة 191 حزمة، منها 58 حزمة كانت ذات تعددية شكلية وبنسبة تباين 31%. تراوح عدد الحزم ذات التعددية الشكلية بين الحزمة الواحدة مع البادئة (A8) إلى 16 حزمة مع البادئة (E11)، بمتوسط قدره 5.2 حزمة لكل بادئة. كانت قيم التباعد الوراثي بين العزلات منخفضة عموماً، وتراوحت بين 0.05 و 0.26، الأمر الذي يدل على درجة قرابة عالية بين العزلات. انقسمت شجرة القرابة الوراثية إلى عنقودين رئيسين: انفردت العزلتان M10 (منطقة الخراب) و M8 (منطقة العديمة) في العنقود الأول بدرجة قرابة وراثية عالية 95%، وضمّ العنقود الثاني العزلتين G12 و R12 بدرجة قرابة عالية أيضاً، ولكن بنسبة أقل من العنقود الأول (92%) على الرغم من أن العزلتين من المنطقة نفسها (متن أبو ريا). أشارت النتائج إلى كفاءة مؤشرات ISSR في إظهار التنوع والقرابة الوراثية بين عزلات النوع *O. neolycopersici*.

كلمات مفتاحية: *Oidium neolycopersici*، البياض الدقيقي، القرابة الوراثية، البندورة/الطماطم، ISSR.

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