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Identification of Biochemical and Molecular Markers in *Tomato Yellow Leaf Curl Virus* Resistant Tomato Species

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A B S T R A C T

Tomato yellow leaf curl virus (TYLCV) is devastating to tomato (*Solanum lycopersicum*) crops and resistant cultivars are highly effective in controlling the disease. Breeding programmes aimed at producing TYLCV-resistant tomato cultivars have utilized resistance sources derived from wild tomato species. So far, all reported breeding programmes have introgressed TYLCV resistance from a single wild tomato source. Here, we tested degree of resistant different nine tomato species to TYLCV. Results showed that *S. chilense*, *S. peruvianum*, *S. hirsutum* and *S. minutum* appeared resistant to TYLCV. On the other hand, the maximum PPO and POD isozyme activities were recorded in the susceptible plants of *S. penneleii* (15) unique isoforms, while the minimum in the resistant plants of *S. minutum* (one). Random amplified polymorphism DNA-polymerase chain reaction (RAPD-PCR) using five primers revealed the differences between the TYLCV resistant wild tomato species and the healthy control, thus the TYLCV resistant *S. hirsutum* revealed the highest number with 14 unique markers, followed by *S. cheesmaniae* with 11 markers, while *S. chilense* and *S. peruvianum* revealed six markers. These polymorphic fragments are valuable for future use in tomato breeding programs, as marker assisted selection by transfer TYLCV-tolerance genes from a wild green-fruited tomato species to a cultivated line.

Introduction

Tomato production worldwide is under the constant threat of *Geminiviruses* (genus: *Begomovirus*, family: *Geminiviridae*) transmitted by the whitefly *Bemisia tabaci*. Some *Begomoviruses* infecting tomato, such as *Tomato yellow leaf curl virus* (TYLCV) have a single DNA-A like genomic component (monopartite) (Navot et al., 1991). TYLCV is a major constraint for open field tomato production in many regions of the world. The disease is induced by a number of *Begomovirus* species and strains, among them, TYLCV, widely spread worldwide (Moriones and Navas-Castillo, 2000; Fauquet and Stanley, 2005). Management of TYLCV usually consists in spraying large amounts of insecticides to control the population of the whitefly virus vector. Abuse has greatly contributed to the development of pesticide-resistant *B. tabaci* populations (Palumbo et al., 2001).

Classical breeding for resistance first involves the identification of resistance sources, which are often found in wild germplasm unadapted to agriculture; this is followed by the introgression into cultivars via phenotypic selection of resistant progeny. Sources of resistance to TYLCV have been discovered in wild tomato species, including *Solanum pimpinellifolium*, *S. peruvianum*, *S. chilense*, *S. habrochaites* and *S. cheesmaniae* (Ji et al., 2007a). Thus, breeding efforts to develop TYLCV resistance have been largely based on transfer of genes from these wild *Solanum* species. The type of resistance, the availability of the infected vectors and the presence of different viruses causing TYLCV make classical selection slow and difficult because phenotypic analyses of partial resistance or tolerance traits are often of limited utility. Marker-assisted breeding overcomes such problems focusing on the direct selection of genomic loci underlying the trait.
This research focused on screening wild tomato lines resistance to tomato yellow leaf curl virus. In addition, identify molecular marker (RAPD-PCR) linked to the resistance loci from these wild tomato accessions that can be used for marker-assisted selection in breeding programs.

Material and Methods

Plant material

Eight wild *Solanum* spp used in this study are listed in (Table 1) obtained from (Centre for Genetic Resources, The Netherlands) and one tomato cultivar Alissa.

Virus and whitefly maintenance

The Egyptian isolate of TYLCV supplied from (Virology laboratory, Faculty of Agriculture, University of Ain shams). TYLCV were maintained in susceptible tomato (cv. Castle Rock) in an insect-proof greenhouse. Whitefly colonies (*B. tabaci*, biotype B) were reared on cotton plants (*Gossypium hirsutum*) grown in muslin-covered cages maintained within an insect-proof greenhouse.

TYLCV inoculation

Adult whiteflies were provided a 48-h acquisition access period on TYLCV-infected tomato source plants, after which they were provided a 48-h inoculation access period (IAP) on tomato test plants (10 plants per species), 17–22 days after sowing. To ensure 100% infection, the plants were inoculated with 50 whiteflies per plant. Control, non-inoculated plants of the same genotypes were exposed to non-viruliferous whiteflies for a 48-h IAP. Following the IAP, whiteflies were discarded by treating plants systemically with imidacloprid. The plants were raised for 3-4 weeks in an insect-proof greenhouse at 26–32ºC prior to transplanting to the field.

Detection of TYLCV by Tissue blot immunoassay (TBIA)

Tissue blots were prepared essentially as described by (Lin et al., 1990). Prints were made from leaf petioles, vascular cores of petioles and bark samples were trimmed to an appropriate size for blotting. A smooth fresh cut was made with a razor blade and the cut surface was pressed gently and evenly to the membrane. After the membrane was imprinted with the tissue samples and dried, it was usually placed in a solution of 1% Bovine Serum Albumin (BSA) and Phosphate Buffer Saline (PBS) and incubated for 1 h at 25ºC to block any remaining protein binding sites. The treated membrane was placed in the virus specific antiserum diluted in PBS 1:500 and then incubated for 1 h at room temperature with gently shacking. The membrane was washed three times with PBS-Tween (PBST) at 5 min interval. The goat anti rabbit immunoglobulin-alkaline phosphate conjugate (Sigma A 4503) dilution 1:1000 in conjugate buffer {PBST + 2% Polyvinylpyrrolidone (PVP) + 0.2% Ovalbumin)} was added to the membrane and incubated for 1 h at room temperature. The membrane was washed three times with PBS-Tween at 5 min interval. The substrate solution (Nitro Blue Tetrazolium and 5-bromo 4-chloro 3-indolyl phosphate) was added and incubated for 5 min at room temperature. After the color appeared, the membrane was rinsed quickly with H_20 then air-dry.

Polyphenol oxidase (PPO) isozymes extraction

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among resistant and susceptible tomato plants compared to the control using polyphenol oxidase isozymes according to (Stagemann et al., 1985). 500 mg fresh leaves were homogenized in liquid N_2 and 100 µl of 0.2 M Phosphate buffer was added (pH 7.0 was adjusted by Potassium Phosphate, monobasic) and 10 µl of 2-Mercaptoethanol before centrifugation at 14000 rpm for 15 min at 4°C. The supernatant was stored at a temperature of -20°C until isozyme analysis. Polyphenol oxidase isozymes were detected according to (Baaziz et al., 1994), in which the gel was immersed in a solution containing 0.1% 1-dihydroxyphenyl alanine solubilized in 0.05 M phosphate buffer pH 7.5. Relative band mobility was measured in relation to the dye front and indicated by Rf values.

Peroxidase (POD) isozymes electrophoresis

Peroxidase isozymes were analyzed using the native polyacrylamide gel electrophoresis (native-PAGE) 10%, according to (Vallejos, 1983; Mahfouze et al., 2012). The gel was run for 2 h at 10ºC and 30 mA in a vertical electrophoresis unit. POD-isozymes were detected by incubating the gels for 5-20 min in a reaction mixture containing 0.5 mM benzidine hydrochloride and 10 mM H_2O_2 in 0.05 M acetate buffer, pH 4.9. Peroxidase isozymes were designated by their migration position (mm of the origin line) on the gel.

DNA extraction

Young leaves of TYLCV resistant tomato species and the healthy control were collected and soaked in liquid nitrogen for DNA extraction using the 2% CTAB method modified by Agrawal et al., (1992).

Random amplified polymorphism DNA-polymerase chain reaction (RAPD-PCR) analysis

A total of five primers sequenced in (Table 2) were used to amplify DNA (manufactured by Bioneer, New technology certification from ATS Korea). The total reaction mixture was 15 µl contained 10× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mixed, 10 pmol primer, 1.25 U *Taq* polymerase and about 150 ng genomic DNA. DNA amplification was obtained through 35 cycles in a DNA thermal cycler. The temperature profile was as follow: denature temperature 94°C

for 30 sec.; annealing temperature 45°C for 1 min; and extension temperature 72 ºC for 1 min, final extension at 72ºC for 5 min.

Amplification product analysis

The amplified DNA (15 µl) for all samples was electrophoresed on 1% agarose containing ethedium bromide (0.5 µg/ml) in 1x TBE buffer (89 mM Tris-HCl, 89 mM Boric acid, 2.5 mM EDTA, pH 8.3) at 75 constant volt, and determine with UV transilluminator. The size of each fragment was estimated with reference to a size marker of 10 Kb DNA ladder (BioRoN, Germany).

Gel analysis

Gels were analyzed by UVI Geltec version 12.4, 1999-2005 (USA).

Results and Discussion

TYLCV symptoms

Thirty days following inoculation with TYLCV, the tested plants were assayed for TYLCV-induced symptom severity during the growth period. There were fundamental differences both in the onset and the degree of symptom development among the different tomato species. Plants of the susceptible *S. pimpinellifolium* were the first to produce symptoms, which began with a crinkle, vein clearing and leaf curl, *S. glandulos* appeared yellowing of the leaves; *S. penneleii* showed vein banding, curling of young leaves and crinkle, while *S. esculentum* var Hybrid Alissa F1 exhibited mild mosaic and leaf curl (Table 3). On the contrary, other species such as *S. chilense, S. peruvianum, S. hirsutum, S. minutum* and *S. cheesmaniae* were completely symptomless when compared with the non-inoculated control plants (Table 3). The presence of TYLCV in leaves was tested by TBIA (Fig. 1). These results were in an agreement with Bian et al., (2007) who mentioned that plants defense against pathogens involves a range of mechanisms. A well-characterized resistance mechanism known as the gene-for-gene response is governed by specific interactions between the pathogen avirulence (avr) gene and the corresponding resistance (R) gene of the plant (Dangl and Jones, 2001). A passive mechanism allowing plants to escape pathogen attack is the lack of a specific host factor required by the virus for its replication or spread (Diaz-Pendon et al., 2004). Polston and Anderson (1997) who found that whitefly-transmitted *Begomoviruses*, such as TYLCV and *Tomato mottle virus* (ToMoV), cause great losses to tomato production in the world. To date, no resistance has been found in the *Solanum lycopersicum* germplasm although some tomato varieties have been reported to be less susceptible than others during severe epidemics (Laterrot, 1993). However, resistance to TYLCV has been found in numerous tomato wild species, including *S. pimpinellifolium, S. peruvianum, S. chilense, S. habrochaites*, and *S. cheesmaniae* (Scott et al., 1995; Vidavsky and Czosnek, 1998). *S. chilense* accession LA1969 showed the highest level of resistance among the 23 accessions representing five tomato species based on symptom expression and virus detection criteria (Zakay et al., 1991). However, resistance appears to be controlled by one to five genes depending on the plant source, therefore, several strategies have been used by plant breeders to produce TYLCV-resistant plants incorporating tolerance or resistance gene(s) from the related wild species of tomato into cultivated backgrounds. Resistance to TYLCV in *S. chilense* is controlled by the major gene Ty-1, which exhibits a reduction of the virus titres and long-distance movement of the virus in the plant (Michelson et al., 1994).

Polyphenol oxidase (PPO) and peroxidse (POD) isoforms

PPO analysis displayed a total of 12 bands at different Rf values varying from 0.104 to 0.899, whereas 10 bands were polymorphic at 83.33% polymorphism, and the two bands at Rf values (0.644 and 0.711) were monomorphic among the TYLCV resistant, susceptible tomato plants and the healthy control (Fig. 2). On the other hand, POD profiles appeared 13 bands at Rf values ranging from 0.207 to 0.928, thus 11 bands were polymorphic with 84.62% polymorphism, two bands were commonly among the TYLCV resistant, susceptible tomato plants and the healthy control (Fig. 3). The maximum PPO and POD activities were observed in the susceptible plants of *S. penneleii* (15) markers, followed by the resistant plants of *S. cheesmaniae* (6), then *S. hirsutum* (5), while the minimum PPO and POD activities showed in the resistant plants of *S. minutum* (one) (Table 4). The differences in bands in TYLCV inoculated wild tomato species compared with the healthy control plants indicate the role of PPO and POD in TYLCV resistance. Enzymes control biochemical reactions, and their syntheses are under the control of specific gene, any change in the activity of an enzyme would reflect the pattern of gene expression and corresponding metabolic events in the cell (Neog et al., 2004). Hence, enzymes can be used as tools to study the induced responses of plants showing disease symptoms at the biochemical level. Differences in the isozymes binding pattern are due to, variation in the amino acid content of the molecule, which in turn is depend on the sequence of nucleotide in DNA. Different bands obtained indicate different electrophoretic metabolites of the isozymes, which are coded by different alleles or separate genetic loci. Therefore, such studies are useful in identification and characterizing resistance in tomato caused by TYLCV. In addition, phenol-oxidizing enzymes such as POD and PPO are associated with many diseases (Pegg, 1985)**.** Nadlong and Sequeira (1980) suggested that the increased POD-activity following virus infection was a reflection of physiological changes associated with, but not responsible for, induced resistance whereas up-regulated POD might be responsible for growth reductions and malformations in virus-infected plants. Peroxidase plays an integral part in the biosynthesis of plant cell wall components viz., lignin, suberin and

cross-linked extension (Lamport et al., 1986). The lignifications and wall thickening are part of defense response to pathogens. Besides these, peroxidases also play an important role in one of the earliest observable event of plants defense response i.e., oxidative burst (Wojtaszek, 1997). Earlier Bose & Rajan (2000) indicated the possibility of using peroxidase isozyme as markers for resistant and moderately resistant varieties of tomato. Polyphenol oxidase catalyzing the oxygen dependent oxidation of phenols to quinines are ubiquitous among angiosperms and assumed to be involved in plant defense against pests and pathogens (Yedidia et al., 2003). Moshe et al., (2012) who mentioned that reactive oxygen species (ROS) scavenging mechanisms in plants involve enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). SODs act as the first line of defense against ROS, dismutating superoxide to H_2O_2. APX, GPX, and CAT subsequently detoxify H_2O_2. Most anti-oxidative enzymes were detected in TYLCV infected tomatoes. SOD_S, APX, thioredoxin peroxidase, ferredoxin-nitrite reductase were more abundant in susceptible then in resistant plants. Plant thioredoxins are the key factors in oxidative stress response.

Table 1. Wild and cultivated tomato species.

No.	Accession	No.	Accession
1	*S. pimpinellifolium* CGN 15812	6	*S. hirsutum* CGN 24035
2	*S. glandulos* CGN 15803	7	*S. cheesmaniae* CGN 24038
3	*S. chilense* CGN 1553	8	*S. minutum* CGN 15815
4	*S. penneleii* CGN 15533	9	*S. esculentum* var Hybrid Alissa F1
5	*S. peruvianum* CGN 17047		

Table 2. The sequences of the five used primers were as follows.

Primer	Sequence
Primer-1	GTT TCG CTC C
Primer-2	AAC GCG CAA C
Primer-3	CCC GTC AGC A
Primer-4	CCA CAG CAG T
Primer-5	AAG CCC GAG G

Table 3. TYLCV symptoms on *Solanum* species.

Tomato species	Symptoms	DBIA
S . pimpinellifolium	C, VC, SL, LC	+
S . glandulos	Y	+
S . chilense	NS	+
S . penneleii	VB, LC, C	+
S . peruvianum	NS	+
S . hirsutum	NS	+
S . cheesmaniae	NS	+
S . minutum	NS	+
S . esculentum var Hybrid Alissa F1	MM, LC	+

C=Crinkle, MM= Mild mosaic, NS= No symptoms, VC=Vein clearing, SL= Small leaves, LC= Leaf curl.

Identification of DNA markers associated with TYLCV resistance

Five of the RAPD-PCR primers were used to find markers for TYLCV-resistant wild tomatoes species, these primers revealed polymorphism depending on bands number, and level delectable polymorphism between the controls, and tomato plants resistant to TYLCV (Figure 4 and table 5). A total of 94 scorable amplified DNA fragments were amplified of which 44 monomorphic, and 50 bands were polymorphic resulting in a polymorphism of 53.19%. The number of bands amplified per primer varied between 13 (primer-4), and 25 (primer-5). The extent of polymorphism per primer ranged from 44.44% (primer-3) to 61.54% (primer-4). Among the 50 polymorphic bands, 26 bands were unique markers to the resistance of TYLCV with a total average of 27.66%. It is interesting to note that, the four tomato species were varied considerably in their TYLCV-resistance markers using the five primers, wherease *S. hirsutum* revealed the highest number with 14 markers, followed by *S. cheesmaniae* with 11 specific bands, while *S. chilense* and *S. peruvianum* revealed six unique markers (Table 5). RAPD marker tightly linked to disease resistance genes can be used for multiple screening of genotypes in breeding programs without resorting to inoculations with the pathogens. The availability of markers for the major disease resistances in tomato and the efforts directed at mapping additional genes will undoubtedly have an impact on the performance of future tomato varieties. In breeding program aided by molecular markers we were able to map and transfer TYLCV-tolerance genes from a wild green-fruited tomato species to a cultivated line. Anbinder et al., (2009) who mentioned that five major loci (*Ty-1* through *Ty-5*) from wild tomato species associated with resistance to TYLCV and related *Begomoviruses* have been identified so far and mapped on tomato chromosomes using such markers. The RAPD markers mapped to these resistance regions were used to screen more advanced resistant breeding lines in search for tightly linked markers, which were then converted to sequence characterized amplified region (SCAR) markers (Ji and Scott, 2005). The presence of both Ty-1 and Ty-3 genes in a single genotype most likely offers the highest resistance to TYLCV, which might be the case for some of the present commercial hybrids in the market.

Table 4. PPO and POD isozyme markers of the tomato species under TYLCV infection.

No.	Rf	*S. pimpinellifolium*	*S. glandulos*	*S. chilense*	*S. penneleii*	*S. peruvianum*	*S. hirsutum*	*S. cheesmaniae*	*S. minutum*	*S. esculentum* cv. Alissa
Polyphenol Oxidase (PPO)										
1	0.104				+	+				
2	0.185				+	+				
3	0.313				+					
4	0.359				+					
5	0.456				+					
6	0.540				+					
7	0.778						+			
8	0.829		+		+		+	+		
9	0.899						+			
Total=9		0	1	0	7	2	3	1	0	0
Peroxidase (POD)										
1	0.207		+		+			+		+
2	0.276		+		+			+		+
3	0.379				+					
4	0.426				+					
5	0.473				+				+	+
6	0.527				+					
7	0.574				+					
8	0.652							+		
9	0.809						+	+		
10	0.871		+		+			+		
11	0.928						+			
Total= 11		0	3	0	8	0	2	5	1	3
Total= 20		0	4	0	15	2	5	6	1	3

Table 5. RAPD-PCR amplified bands, polymorphic bands and markers of TYLCV resistant tomato species using five primers.

Primer name	Polymorphism (P)			No. of unique markers with size (bp)		%	RAPD markers for resistance to TYLCV			
	Total	PAF	%				*S. chilense*	*S. hirsutum*	*S. cheesmaniae*	*S. peruvianum*
					880			+		+
1	15	9	60	5	750	33.33		+	+	
					150					+
					100					+
					90			+	+	
					1750			+		
2	23	13	56.52	6	1450	26.09		+		
					1100			+	+	+
					400				+	
					350				+	
					150					+
					1500			+	+	
3	18	8	44.44	7	1410	38.89		+	+	
					1200			+	+	
					880				+	
					550			+	+	
					490		+			
					390		+		+	+
					310		+			
4	13	8	61.54	3	305	23.08	+			
					280		+			
					1380			+		
5	25	12	48	5	950	20		+		
					940			+		
					785		+			
					477			+		
Total	94	50	53.19	26		27.66	6	14	11	6

P: number of polymorphic bands.
+: presence of marker band.

1- *S. pimpinellifolium*	2- *S. glandulos*	3- *S. chilense*	4- *S. penneleii*	5- *S. peruvianum*
6- S. hirsutum	7- *S. cheesmaniae*	8- *S. minutum*	9- *S. esculentum* cv. Alissa	10- positive control

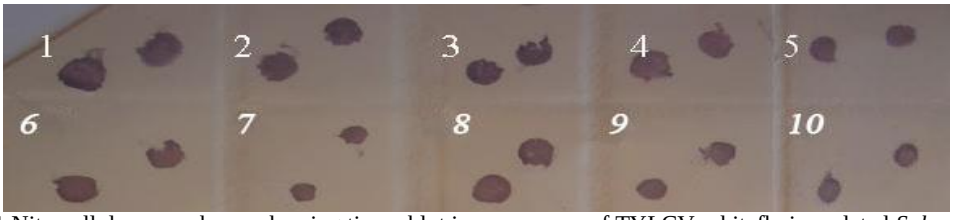


Figure 1.Nitrocellulose membrane showing tissue blot immunoassay of TYLCV-whitefly inoculated *Solanum* spp.

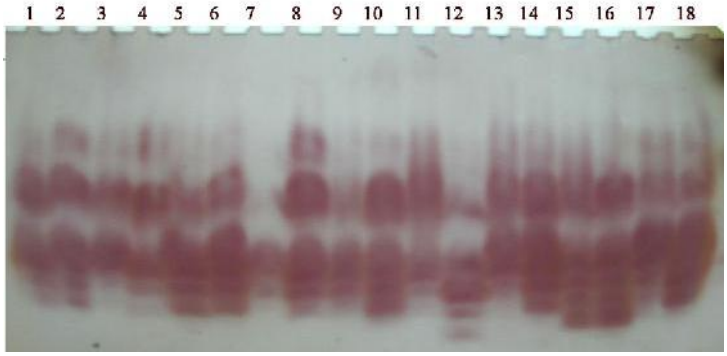


Figure 2. PPO isoforms profile in the resistant and susceptible *Solanum* spp induced by TYLCV compared to the control. Lanes 6, 10, 12, 14 and 16 TYLCV resistant *S. chilense, S. peruvianum, S. hirsutum, S. cheesmaniae,* and *S . minutum,* respectively. Lanes 2, 4, 8 and 18 TYLCV susceptible *S. pimpinellifolium, S. glandulos, S. penneleii* and *S. esculentum* var Alisa, respectively. Lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17 the healthy control *S. pimpinellifolium, S. glandulos, S. chilens*e, *S. penneleii, S. peruvianum, S. hirsutum, S. cheesmaniae, S. minutum,* and *S. esculentum* var Alissa, respectively.

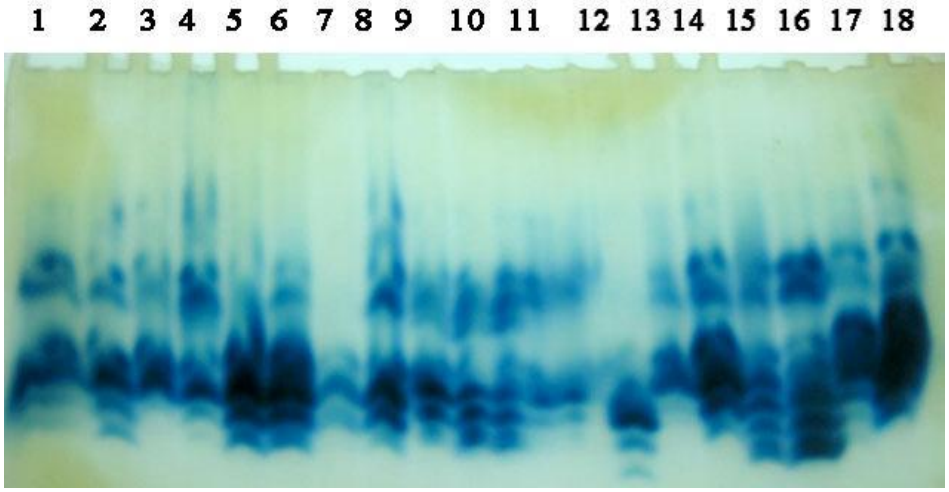
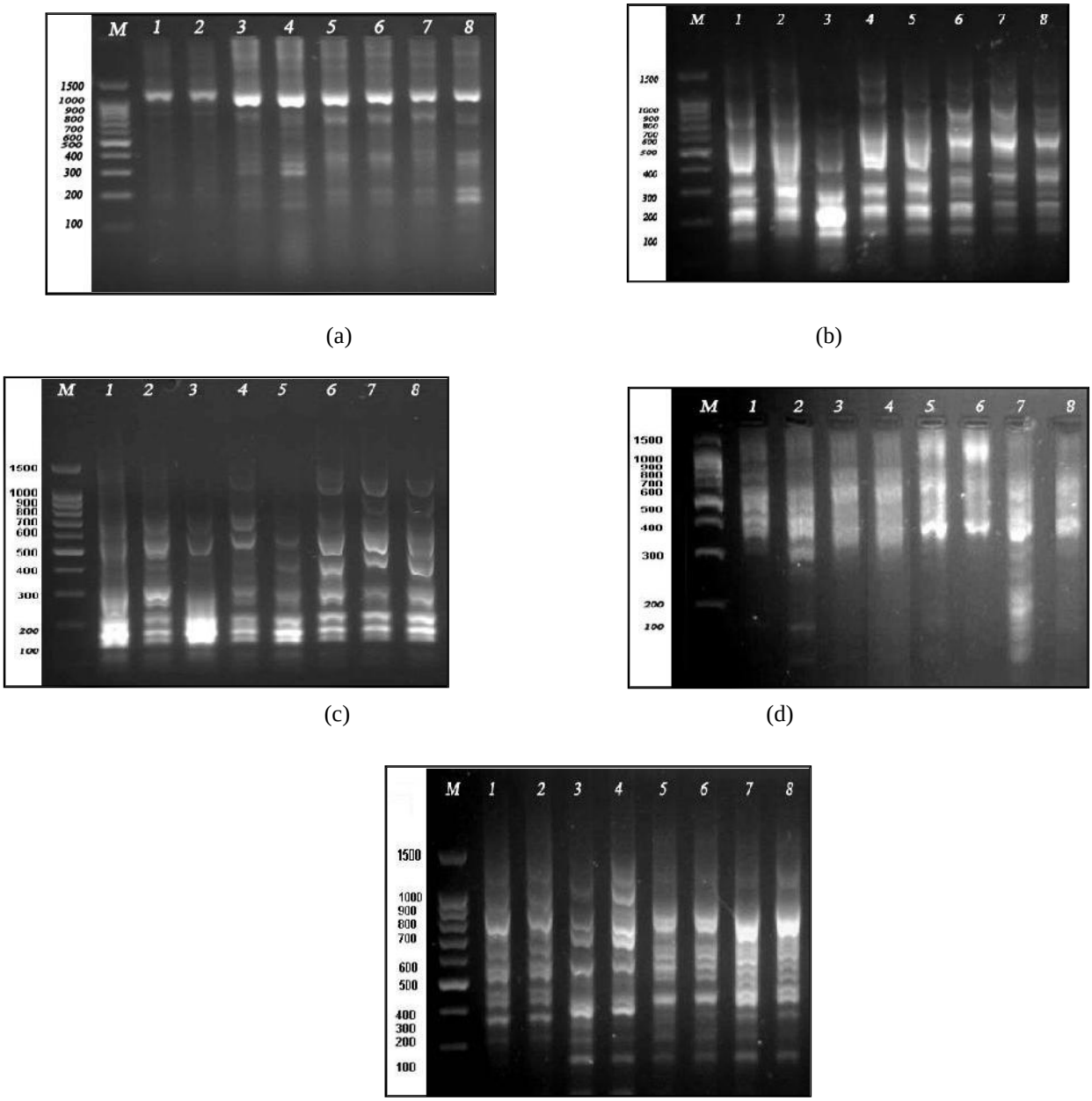


Figure 3. POD isoforms profile in the resistant and susceptible *Solanum* spp induced by TYLCV compared to the control. Lanes 6, 10, 12, 14 and 16 TYLCV resistant *S. chilense, S. peruvianum, S. hirsutum, S. cheesmaniae,* and *S . minutum,* respectively. Lanes 2, 4, 8 and 18 TYLCV susceptible *S. pimpinellifolium, S. glandulos, S. penneleii* and *S. esculentum* var Alisa, respectively. Lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17 the healthy control *S. pimpinellifolium, S. glandulos, S. chilense, S. penneleii, S. peruvianum, S. hirsutum, S. cheesmaniae, S . minutum* and *S. esculentum* var Alissa, respectively.



(a) (b)

(c) (d)

(e)

Figure 4. RAPD-PCR profiles generated by primers (a) 1, (b) 2, (c) 3, (d) 4 and (e) 5. Lane M = 100 bp DNA ladder plus, lanes 2, 4, 6 and 8 TYLCV resistant *S. chilense*, *S. hirsutum*, *S. cheesmaniae* and *S. peruvianum*, respectively, compared with the healthy control *S. chilense*, *S. hirsutum*, *S. cheesmaniae* and *S. peruvianum* (lanes 1, 3, 5 and 7), respectively.

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