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Identification and distribution of *Tomato yellow leaf curl virus* TYLCV and *Tomato yellow leaf curl Sardinia virus* TYLCSV infecting vegetable crops in Morocco

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Leaf samples of 177 tomato plants were collected during 2006-2007 in tomato yellow leaf curl disease (TYLCD) infected fields, as well as 100 leaf samples of sweet pepper, common bean, zucchini and the wild species *Solanum elaeagnifolium*, in order to study the population structure and genetic variation of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato yellow leaf curl virus* (TYLCV) in Morocco. Molecular hybridization using specific probes and restriction of a genomic region corresponding to the coat protein gene were performed to differentially identify TYLCV and TYLCSV species. Both species were present in the infected plants from Southwestern and Northern Morocco analyzed in this study. This is the first report of the presence of TYLCD in Northwest area. 66% of the tomato samples and 37.8% pepper were infected, most of them with mixed infections. The rest of the species analyzed were virus free. Five of the six Moroccan haplotypes identified had previously been reported. We identified a new haplotype representing 76% of the TYLCSV infected samples collected in Agadir-Chtouka-Ait-Baha area. The genomic region corresponding to the CP gene was sequenced for 10 isolates. The TYLCV and TYLCSV sequences had 99% identity with those previously identified in Northern Morocco and of Murcia respectively.

Key words: *Tomato yellow leaf curl Sardinia virus* (TYLCSV), *Tomato yellow leaf curl virus* (TYLCV), molecular hybridization, population structure, genetic variation.

INTRODUCTION

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating diseases affecting cultivated tomato (*Solanum lycopersicum* L.) in tropical and subtropical regions. Symptoms of the disease include upward curling of leaflet margins, reduction of leaflet area, and yellowing of young leaves, as well as stunting and flower abortion. This disease is caused by several virus species belonging to the genus *Begomovirus*, family *Geminiviridae*.

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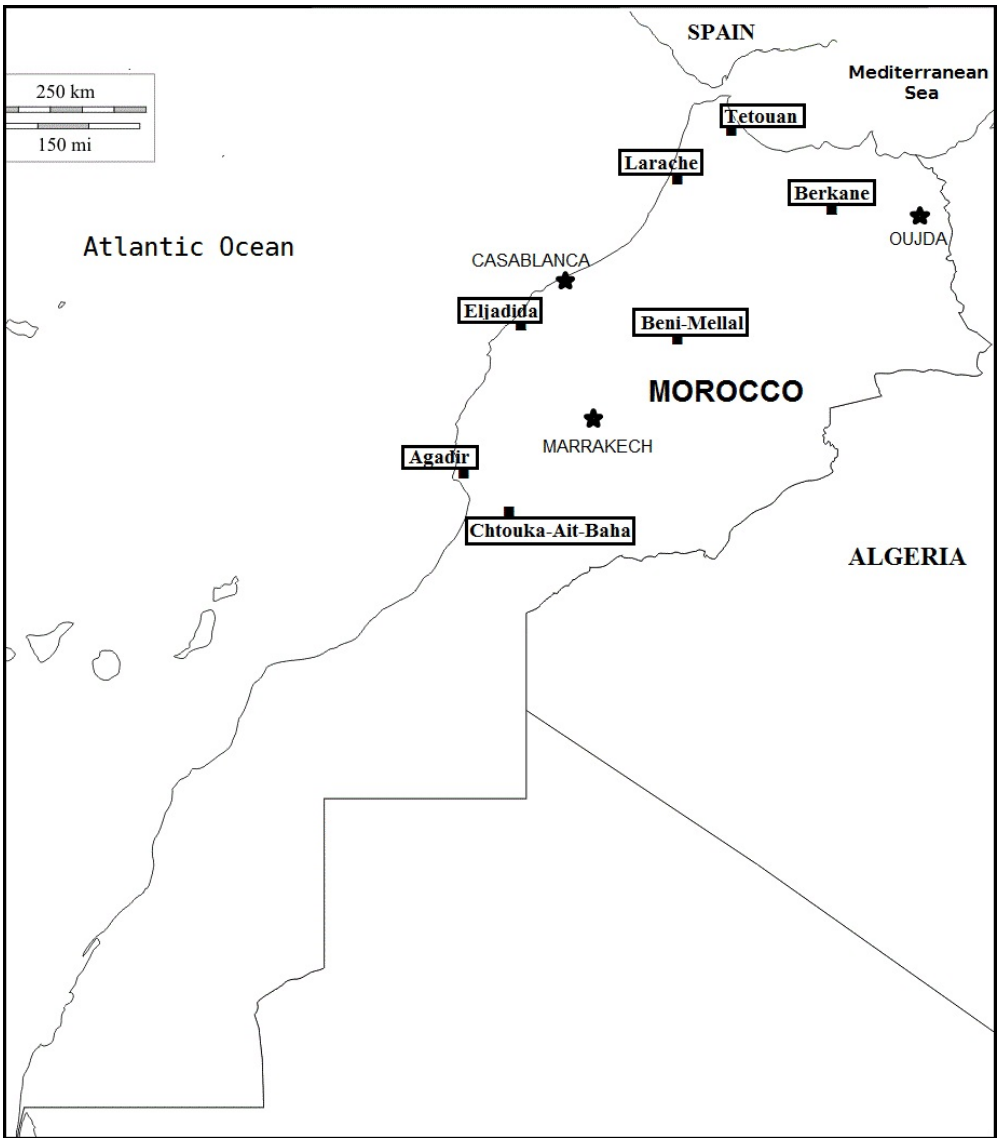

Figure 1. Geographic localization of analysed samples. Black shaded locations correspond to sampled areas.

They are transmitted by the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) in a persistent circulative manner. Presently, TYLCD is the most important tomato disease in the Mediterranean basin, causing serious economic losses in Morocco. Control of this viral disease is extremely complicated due to the high efficiency of vector transmission, as well as to the difficult eradication of the insect.

TYLCD was first identified in Morocco in 1996/1997 in surveys conducted by the Service for the protection of plants, in the region of El Jadida after importation of grafted tomato plants from the Netherlands by a farmer from this area (Peterschmitt et al., 1999a; Jebbour and Abaha, 2002) (Figure 1). Concretely, the species reported was the species form Middle East, *Tomato yellow leaf curl virus* (TYLCV); the isolate was closely related to the Dominican Republic isolate (Peterschmitt et al., 1999). The disease then spread to other tomato producer regions, particularly Berkane and Agadir (Jebbour and Abaha, 2002). Severe outbreaks of TYLCD occurred during summer and autumn 1999 in Agadir. The viral species identified were TYLCV and *Tomato yellow leaf curl Sardinia virus* (TYLCSV); the isolates corresponding to both species were in this case in closest relationship to isolates from Spain (Monci et al., 2000). TYLCD was the principal cause of the important losses registered in 1999 in the region of Agadir and reduction of 50% of the tomato cultivated area in El Jadida (Tahiri et al., 2007). The presence of both TYLCV and TYLCSV was subsequently reported in the Agadir region (Sedegui

et al., 2002). Analysis of samples harvested in 2001-2002 showed that infection of tomato crops was more common in the southwest than in the north (Tahiri et al., 2007). The sequence analysis revealed the existence of the Spanish strain of TYLCSV and of two genetically different strains of TYLCV. The Spanish origin of the TYLCSV isolate found in Morocco has been later confirmed (El Merach et al., 2007; Boukhatem et al., 2008). The degree of severity of the symptoms was evaluated by the dosage of viral DNA on the infected plants (Rotbi et al., 2010).

The exchange of plant material between countries facilitates the transfer of viral species (Hanafi, 2000). New isolates may have been introduced or the existing ones may have moved to other major growing areas, in the years since the latest studies in Morocco. The objectives of this study are identifying the geographical distribution of TYLCV and TYLCSV in tomato crops in Morocco, determining the incidence of these viral species in other vegetable crops, and studying the genetic diversity of the viral species TYLCV and TYLCSV in Morocco.

MATERIALS AND METHODS

Plant material

The sample recollection was carried out during 2006 and 2007 in 22 greenhouses and fields distributed on the most important areas of tomato production in Morocco (Figure 1). All tomato samples collected showed TYLCD symptoms, which varied from slight to severe. We also collected samples of the other cultivated and wild species with the aim of verifying the possible presence of the virus. Sweet pepper, zucchini and *Solanum elaeagnifolium* were symptomless while yellowing and curling of leaves was observed in common bean.

DNA extraction

Total DNA was extracted from dehydrated or frozen leaf samples using the CTAB method. Samples of 30 mg of dehydrated leaf tissue or 75 mg of frozen leaf tissue were ground and 450 µl of extraction buffer (2% CTAB, 20 mM EDTA, 100 mM Tris, 1.42 M NaCl) were added. Subsequently, samples were maintained at 65°C for 30 min. After adding 500 µl of chloroform:isoamyl alcohol (24:1), the mixture was centrifuged at 13400 × *g* for 10 min. The supernatant was recovered and one volume of cold isopropanol was added. After 10 min at -20°C, samples were centrifuged for 10 min at 13400 × *g*. The pellet was washed with 70% ethanol. After subsequent centrifugation, the pellet was resuspended in 50 µl of TE buffer (10 mM Tris, 1mM EDTA).

TYLCV and TYLCSV detection

Identification of the viral species was performed by dot blot hybridization and restriction of a genomic region corresponding to the coat protein gene.

Molecular hybridization

Aliquots of 5 µl of total extracted DNA from each sample and a 10-fold dilution were first denatured with 30 mM NaOH and 1 mM EDTA for 30 min at room temperature and charged on nylon positively charged membranes for hybridization. DNA was fixed on the membrane by UV crosslinking. Hybridization was carried out according to 'The DIG system user's guide for filter hybridization' (Roche Molecular Biochemicals) using digoxigenin-11-dUTP and chemiluminiscent detection. Membranes were pre-hybridized in standard hybridization buffer plus 50% deionized formamide for at least 1 h. Subsequent hybridization was done at 42°C overnight in fresh pre-hybridization solution containing 20 ng of denatured probe per ml. The probes employed represented the intergenic region of Spanish isolates belonging to TYLCV (YLCV-Mld[ES:72:97]) and TYLCSV (TYLCSVES[ES:Alm2:92]) species (kindly supplied by E.R. Bejarano, Universidad de Málaga, Spain). The probes were labelled by incorporation of digoxigenin-11-dUTP during PCR. One replicate of each membrane was hybridized with each probe, respectively. Washing steps and incubation with antibody were done according to manufacturer's instructions. Detection was carried out with CSPD and direct exposition to CCD camera (Intelligent Dark Box-II, Fujifilm, and Tokyo, Japan).

DNA typing

General primers of the *Begomovirus* genus were used to amplify the genomic region comprised of the coat protein gene (CP) of TYLC viruses. The primers were:

TY-1(+): 5'-GCCCATGTA(T/C)CG(A/G)AAGCC-30 and TY-2(-): 5'-G(A/G)TTAGA(A/G)GCATG(A/C)GTAC-3' (Accotto et al., 2000).

PCR conditions were 30 cycles of 1 min at 94°C, 1 min at 48°C and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. Final volume of PCR was 25 µl containing: 100 ng of template DNA, PCR buffer, 2 mM of $MgCl_2$, 100 µM of each dNTP, 0.4 µM of each primer, and 1 U of Taq polymerase (Roche, Spain). PCR products were digested with *Ava*II (TAKARA BIO INC.), which was predicted to cut TYLCSV differently from TYLCV. Digested DNA was separated by 2% agarose gels and stained with Ethidium Bromide.

PCR products were also digested with the restriction endonucleases used by Font et al. (2007): *Hae*III, *Hpa*II, *Rsa*I, *Taq*I and *Hin*fI (TAKARA BIO INC.), and the resulting DNA fragments were separated by electrophoresis in 3% agarose using 0.5 × TBE buffer. DNA fragments were visualized under UV light after Ethidium Bromide staining. A sample representative of each restriction pattern, obtained with all five endonucleases, was considered to define a haplotype and was sequenced and aligned using the tool nucleotide BLAST of NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

RESULTS

Identification of TYLCV and TYLCSV species

Molecular hybridization and restriction of a genomic region corresponding to the coat protein gene were successfully used to identify the species TYLCV and TYLCSV in infected samples of some crops collected in Morocco. The presence of both viral species in some areas of single and mixed infections in tomato and pepper plants was confirmed.

For dot-blot experiments, specific probes to TYLCV and TYLCSV were used. In both cases good signal intensity with the original samples and with the 10-fold dilution were obtained. In no case did healthy controls produce

Table 1. Number of infected samples with each species or with mixed infection as shown by molecular hybridization and PCR.

Crop	Origin	Number of analysed samples	Number of positive samples											
			Molecular hybridization						PCR					
			TYLCV	%	TYLCSV	%	Mixed	%	TYLCV	%	TYLCSV	%	Mixed	%
Tomato	Berkane	40	17	42.5	0	0	15	37.5	7	17,5	1	2.5	5	12.5
Tomato	Larache	53	6	11.3	0	0	10	18.9	-	-	-	-	-	-
Tomato	Tetouan	18	18	100	0	0	0	0	-	-	-	-	-	-
Tomato	Agadir	10	6	60	0	0	2	20	10	100	0	0	0	0
Tomato	Chtouka	56	11	19.6	7	12.4	28	47.4	19	33.9	22	39.2	7	12.5
Total tomato		177	58	33	7	4	55	31	36	20.3	23	13	12	8.3
Pepper	Berkane	10	0	0	0	0	0	0	0	0	0	0	0	0
Pepper	Larache	29	3	10.3	0	0	8	27.5	-	-	-	-	-	-
Pepper	Tetouan	6	0	0	0	0	6	100	-	-	-	-	-	-
Total pepper		45	3	7	0	0	14	31.1	0	0	0	0	0	0
Common bean	Berkane	10	0	0	0	0	0	0	0	0	0	0	0	0
Common bean	Agadir	11	0	0	0	0	0	0	0	0	0	0	0	0
Total bean		21	0	0	0	0	0	0	0	0	0	0	0	0
Zucchini	Tetuan	5	0	0	0	0	0	0	0	0	0	0	0	0
Solanum elaeagnifolium	Beni-Mellal	29	0	0	0	0	0	0	0	0	0	0	0	0

a signal.

Tomato showed the highest infection level (Table 1): 68% of tomato samples were infected, 33% corresponding to TYLCV, 4% to TYLCSV and 31% to mixed infections. In the case of pepper where 38% of samples are infected, most of them with mixed infections. Single infection with TYLCSV was not detected in pepper. The rest of the samples analyzed were free from these viruses. The percentage of infection varied according to the geographical origin. The highest percentage of infected plants corresponded to Tetouan, followed by Berkane, Agadir and Chtouka (Table 1).

Amplification of the coat protein gene with general primers for *Begomovirus*, followed by restriction with *Ava*II to differentially identify TYLCV and TYLCSV confirm the results of molecular hybridization. The controls (based on hybridization results) produced the expected patterns, that is, three bands of 360, 150 and 68 pb for TYLCSV, and two bands of 302 and 277 pb for TYLCV; the five expected bands were present for mixed infections. The percentages of infection obtained by PCR for the samples collected in Berkane, Agadir and Chtouka were consistently lower than the ones obtained using molecular hybridization (Table 1). In contrast, TYLCV was detected in the 100% of analyzed samples from Agadir. No amplification was obtained from the samples collected in Larache and Tetouan, in spite of the modifications performed on the PCR protocol. Samples of sweet pepper, bean, and squash were negative.

Haplotypes differentiation

PCR products obtained from 62 of the TYLCD infected tomato plant were digested with five endonucleases to characterize the virus isolates (Figure 2). The resulting DNA fragments showed

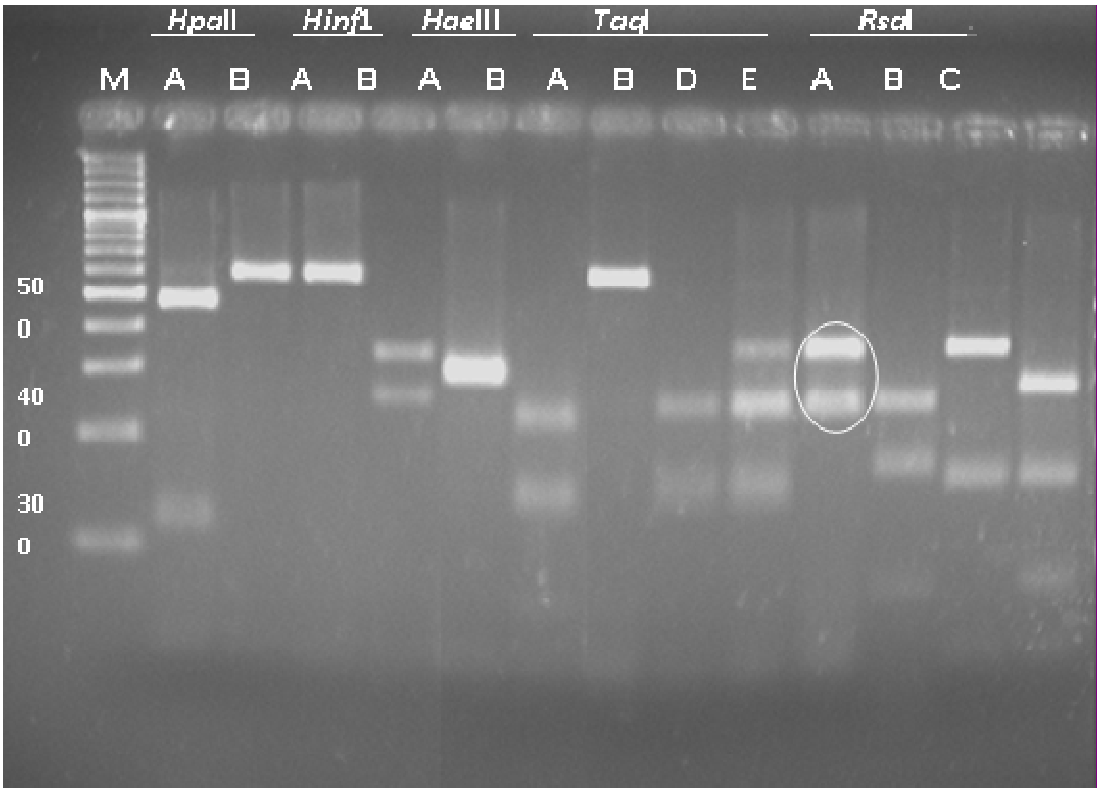

Figure 2. 3% agarose gel electrophoresis of CP PCR product digested with the restriction endonucleases *Hae*III, *Hpa*II, *Rsa*I, *Taq*I and *Hinf*I (Fermentas) corresponding to haplotypes identified from 51 Moroccan isolates of TYLC virus. Haplotypes nomenclature according to Font (2003). The new haplotype is indicated with a circle. M: 100 pb GeneRuler™ DNA Ladder (Fermentas).

Table 2. Restriction pattern of haplotypes of the coat protein gene of tomato yellow leaf curl viruses from Morocco. We respected the Font (2003) nomenclature.

Region	Species	Restriction pattern						Frequency (%)
		*Hpa*II	*Hinf*I	*Hae*III	*Taq*I	*Rsa*I	Haplotype	
Berkane	TYLCV	B	A	B	B	B	VI	8 (1/ 13)
	TYLCV	B	A	B	B	C	X	23 (3/13)
	TYLCV	B	B	B	B	B	IV	30.7 (4/13)
	Mixed	-	-	-	-	-	-	38 (5/13)
Chtouka-Ait-Baha	TYLCV	B	B	B	B	B	IV	40.5 (17/42)
	TYLCV	B	C	B	B	B	XXI	2 (1/42)
	TYLCSV	A	A	A	E	A	**XXII**	38 (16/42)
	TYLCSV	A	A	A	A	A	I	9.5 (4/42)
	Mixed	-	-	-	-	-	-	9.5 (4/42)
Agadir	TYLCV	B	B	B	B	B	IV	100 (7/7)

13 polymorphic restriction sites. Eight different haplotypes were identified, 6 belonging to TYLCV species and 2 belonging to TYLCSV (Table 2). There was a high similarity between Spanish and Moroccan isolates of TYLCV and TYLCSV. Three out of the four TYLCV Moroccan haplotypes detected in this study had been previously identified in Spain (Font, 2003). The dominant TYLCV haplotype was the IV, representing 49 and 30.7% in the South West and the North East, respectively. One of the TYLCSV Moroccan haplotypes had also been previously identified in Spain (Font, 2003). However, a new restriction pattern after digesting with

Table 3. Comparison of identified haplotypes of 64 Moroccan isolates of the tomato yellow leaf curl virus with the described isolates in the database GenBank.

Origin	Species	Haplotype (Font accession, 2007)	% of homology (accession NCBI)	Number of sequenced samples
Berkane	TYLCV	VI (DQ058099)	-	0
	TYLCV	X (DQ058103)	99 (AF071228) TYLCV-Mld[ES:72:97]	1
	TYLCV	IV (DQ058097)	99 (AM409201 ; DQ503437)	2
	Mix		97 (EF625894)97 (AF071228) TYLCV-Mld[ES:72:97]	2
Chtouka-Ait-Baha	TYLCV	IV (DQ058097)	99 (EF060196) TYLCVIL[MO:Ber:05]	
	TYLCV	XXI	-	
	TYLCSV	XXII	99 (Z25751, TYLCSVES[ES:Mur1:92] AF271234) TYLCMalV-[ES:421:99]	3
	TYLCSV	I (DQ058094)	99 (DQ058094)	1
Agadir	TYLCV	IV (DQ058097)	99 (EF060196) TYLCVIL[MO:Ber:05]	1

*Taq*I was identified in TYLCSV samples collected in the province of Chtouka-Ait-Baha. All samples in which this pattern was observed corresponded to the same haplotype. The frequency of haplotype, named XXII, was remarkable, representing 38% of TYLCSV haplotypes identified in southwestern Morocco (Chtouka-Ait-Baha). In all geographic areas surveyed (South West and the North East), the structure of the virus population comprised a predominant haplotype and a few haplotypes with very low abundance.

Sequencing of the CP fragment of TYLCV haplotypes

One sample representative of each of the identified haplotypes was sequenced. All the sequences of the Moroccan haplotypes causing TYLCD belong to TYLCV and TYLCSV species. The isolates identified in the work here reported had 99% identity with those corresponding to the same haplotypes described by Font et al. (2007) (Table 3).

We sequenced a region of 580 pb of the CP of one representative of each of the identified haplotypes. We confirmed all the Moroccan haplotype sequences as TYLCV and TYLCSV species, being the percentages of identity higher that 90% in all cases. By sequences alignment we confirmed that the sequences of haplotypes I, IV, and X identified in Moroccan samples correspond perfectly to those described in Spain (Font et al., 2007) with a 99% of identity. The same grade of similarity was revealed when comparing each two of the obtained sequences corresponding to the same haplotype.

DISCUSSION

Molecular hybridization and PCR analysis performed on samples of different horticultural crops collected in Morocco allowed the identification of TYLCV and TYLCSV viruses in the main areas of tomato production. Tomato was the species with the highest infection percentage. However, a part of the samples tested were negative for TYLCV and TYLCSV with the two detection methods used despite the plants exhibited typical TYLCD symptoms. The same result was found by Tahiri in 2007 where 21 out of 54 samples resulted negative for begomovirus detection. As stated by Tahiri et al. (2007) it could be due to the presence of TYLCV like symptoms induced as a result of infection by another pathogen or by a physiological stress. Alternatively, it could also be

caused by too low concentration of the virus or an uneven distribution of TYLCV/TYLCSV in the plant preventing its detection by PCR.

The presence of TYLCV and TYLCSV in tomato in the Southwest and the Northeast of Morocco was in agreement with previous reports (Peterschmitt et al., 1999; Monci et al., 2000; Tahiri et al., 2007). Our results show higher levels of infection in Berkane and similar infection in Agadir and Chtouka. We have also noted a higher decrease in the percentage of infections by TYLCSV in all areas compared with the results obtained by Tahiri et al. (2007) except Chtouka, which stayed stable. The decrease of TYLCSV was also reported by Sánchez-Campos et al. (1999) in Spain. We note also an important increase in the level of mixed infections. This is a worrying scenario as the appearance of recombinants is very high in these conditions. In fact, recombinants from TYLCV and TYLCSV were also detected by Monci et al. (2002) and García-Andrés et al. (2006) appearing in some cases new pathogenic characteristics.

About other crops, only 17 of the 45 (38%) pepper samples are positive. This percentage was a little higher than the one found by Morilla et al. (2005), who detected TYLCV infection in 6% of the pepper plants and in about 50% of the tomato plants tested. Infection of pepper was no detected by Tahiri et al. (2007). All samples of bean, zucchini and the wild species *Solanum elaeagnifolium* were negative. Although pepper is a host for TYLCV, transmission of the virus from an infected pepper plant into a tomato plant is only possible under laboratory conditions. As stated by Morilla et al. (2005), this suggests that pepper is a dead end in the epidemiological cycle of this virus. However, it cannot be completely ruled out that pepper may serves as an inefficient reservoir when vector populations are extremely high. In contrast, bean is a host of TYLCV that from can be transmitted to tomato by the vector *Bemisia tabaci*. Though in this work we have not detected bean infected samples, it would be convenient to continue sampling in order to detect as soon as possible its appearance and to take the appropriated preventive measures.

We reported for the first time infected fields in Tétouan and Larache areas. This would be kept in mind and continuous monitoring would be conducted as they are important areas of cultivation of tomato industry. The molecular characterization of the viruses has been conducted digesting a fragment of 580 pb of the CP with five restriction enzymes. This method has proved to be useful for the characterization of the TYLCV and TYLCSV isolates present in Morocco and their comparison among them and those available in databases. All isolates identified in Morocco except the XXII had been previously detected in Spain, being the frequency of this new haplotype remarkably. It would be necessary to continue with the sampling of infected samples to monitor the spread of this new haplotype as well as to check its pathogenic characteristics and aggressiveness. A high similarity between the sequence diversity of TYLCV and TYLCSV of Moroccan and Spanish isolates has been found. Five of the six Moroccan haplotypes identified by RFLPs analysis were described in Spain (Font 2003), 4 corresponding to TYLCV species and 1 to TYLCSV species.

We identified a new haplotype (Table 1) of TYLCSV in Southwestern part of the country and its frequency was the 38% of the TYLCSV infected samples analyzed were collected from Chtouka-Ait-Baha zone. The major haplotype of TYLCV species was the IV with 49 and 31% in the Southwest and the Northeast of the country respectively. In all geographic sampled regions, the structure of virus population was composed of a predominant haplotype and a few other haplotypes with very low abundance. Then, we demonstrate the validity and efficiency of the RFLPs method used in this work for identification and characterization of TYLCD Moroccan haplotypes. The new haplotype nucleotide sequence had the highest homology with a TYLCSV isolate from Murcia "Z25751" suggesting that it was introduced from Spain.

By comparison with the results published up to 2013, accessions AF071228, Z25751 and EF060196 of TYLCV are the most similar to the ones identified in Morocco (Peterschmitt et al., 1999; Tahiri et al., 2007; Boukhatem et al., 2008). This finding supports the hypothesis that the disease was introduced in Morocco in same time that was detected in Spain. However probable that more than one introduction of TYLCV occurred in Morocco according to the observations made by Peterschmitt et al. (1999) who stated that in Morocco, TYLCV symptoms were observed during the 1996 to 1997 growing season, following importation of grafted tomato plants from the Netherlands by a farmer from the Casablanca region. Given the high rate of vegetal material exchange, it is frequent the transmission of vectors and diseases across countries and continents as has been evidenced in this work.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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