Biological, serological and molecular typing of potato virus Y (PVY) isolates from Tunisia

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ABSTRACT. In Tunisia, potato virus Y (PVY) currently presents a significant threat to potato production, reducing tuber yield and quality. Three hundred and eighty-five potato samples (six different cultivars) collected in autumn 2007 from nine regions in Tunisia were tested for PVY infection by DAS-ELISA. The virus was detected in all regions surveyed, with an average incidence of 80.26%. Subsequently, a panel of 82 Tunisian PVY isolates (PVY-TN) was subjected to systematic biological, serological and molecular typing using immunocapture reverse-transcription polymerase chain reaction and a series of PVY^{OC}- and PVY^N-specific monoclonal antibodies. Combined analyses revealed ~67% of PVY^{NTN} variants of which 17 were sequenced in the 5'NTR-P1

region to assess the genetic diversity and phylogenetic relationship of PVY-TN against other worldwide PVY isolates. To investigate whether selective constraints could act on viral genomic RNA, synonymous and non-synonymous substitution rates and their ratio were analyzed. Averages of all pairwise comparisons obtained in the 5'NTR-P1 region allowed more synonymous changes, suggesting selective constraint acting in this region. Selective neutrality test was significantly negative, suggesting a rapid expansion of PVY isolates. Pairwise mismatch distribution gave a bimodal pattern and pointed to an eventually early evolution characterizing these sequences. Genetic haplotype network topology provided evidence of the existence of a distinct geographical structure. This is the first report of such genetic analyses conducted on PVY isolates from Tunisia.

Key words: PVY^{NTN}; Serotyping; Biotyping; Immunocapture RT-PCR; Sequencing; Genetic and phylogenetic analysis

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an important crop grown over ~23,500 ha, and with an estimated production of 385,000 tons in Tunisia (FAOSTAT Database Results, 2014). It is grown as three distinct, though partly overlapping, season crops, where the main and late seasons are the most important with regard to acreage and production but differ in that the late season seeds are mostly self-produced, thus with substantial levels of virus (Khamassy, 1999). Spunta is the prevalent cultivar (75% of the planted acreage) compared to the others cultivated in Tunisia, such as Nicola, Liseta, Atlas, Safrane, and Pamina (Khamassy, 1999). Viral diseases are the major cause of poor seed quality and low yield. Potato virus Y (PVY) is reported to be one of the most detrimental potato viruses responsible for the major yield losses worldwide (Gray et al., 2010) and in Tunisia (Boukhris-Bouhachem et al., 2010; Djilani-Khouadja et al., 2010). The type-species is of the genus *Potyvirus* in the family Potyviridae, and this virus is a single-strand, positive-sense genomic RNA of ~9.7 kb. Within a suitable host, the PVY RNA genome is translated into a large precursor polyprotein, which is cleaved into 10 mature proteins (Berger et al., 2005). PVY has a large diversity of strain groups and genetic variants within strains, linked to its biological, serological and molecular properties (Singh et al., 2008; Karasev et al., 2011). According to the reaction of potato cultivars carrying different resistance genes and *Nicotiana tabacum*, potato isolates of PVY are classified into five strain groups: PVY^O, PVY^C, PVY^N, PVY^Z, and PVY^E (Galvino-Costa et al., 2012). According to their sequences, isolates of the PVY^N strain group fall into two genetic subgroups, which are the European (PVY^N) and North American (NA-PVY^N) (Chikh Ali et al., 2010). Among these strain groups and subgroups, PVY^O and PVY^N are the most frequent strains in potato, whereas PVY^C is not common in potato fields and has less economic importance (Kerlan et al., 2011). Genomic recombination plays a significant role in PVY evolution and has led to the emergence of new genotypes/strains including the recombinant PVY^{NTN}, PVY^{NW} and PVY^{NTN}-NW (Chikh Ali et al., 2010). PVY^{NTN} variant has become common in potato fields in most potato production areas (Glais et al., 2005; Kerlan et al., 2011) causing potato

tuber necrotic ringspot disease (PTNRD) and was reported to be able to overcome the field resistance of potato cultivars (Van den Heuvel et al., 1994). The destructive effects of this threatening disease are a particular problem in consumption and seed potato production, as it makes tubers of susceptible cultivars unmarketable as fresh potato and since infected seed lots submitted for certification are rejected (Beczner et al., 1984). This disease is now well-known in Tunisia (Boukhris-Bouhachem et al., 2010) and in most countries cultivating potatoes (Kerlan et al., 2011). Several DNA-based molecular tools have been developed as an alternative to serological assays to distinguish between PVY^{N-W} and PVY^O isolates (serotype O) and between PVY^{NTN} and PVY^N isolates (serotype N) (Singh et al., 2008). Immunocapture RT-PCR was used in this study for specific detection of PVY^{NTN} isolates on the basis of the nucleotide sequence polymorphism observed in the 5'NTR/P1 region (Glais et al., 2005). Moreover, studies of the genetic structure of populations of plant viruses are important for understanding the evolution of virus/host interactions and the geographic distribution of viruses (Gibbs and Ohshima, 2010).

In this context, surveys of potato plants were undertaken in this study, and PVY strain and variant diversity was identified for different growing areas in Tunisia. Consequently, the 5'NTR-P1 nucleotide sequences of Tunisian PVY isolates (PVY-TN) were obtained and genetic structure of PVY isolates was determined. The pairwise mismatch distribution was combined to the network shape, and the statistical neutrality test was performed to look for a possible population expansion. The phylogenetic relationships of PVY-TN isolates with other worldwide PVY isolates from different countries were discussed to determine whether they represent a novel type or share common origins with strain variants reported elsewhere. Lastly, the correlation between these partial nucleotide sequences and the collection site of PVY isolates was considered.

MATERIAL AND METHODS

Surveys

Surveys were carried out in late season crops (autumn 2007) in several fields of nine regions in the Northern and Central Tunisia belonging to different bioclimatic zones (Emberger, 1966): Bizerte (humid), Cap Bon (subhumid), Manouba (subhumid), Jendouba (subhumid), Kairouan (very arid), Monastir (subsemiarid), Mahdia (subsemiarid), Sidi Bouzid (arid), and Gafsa (arid) (Figure 1). Regions were 60 to 230 km apart and the distance between sites within regions varied from 1 to 5 km (Figure 1). A total of 385 leaf samples were collected from six potato cultivars (Spunta, Nicola, Atlas, Pamina, Gredine, and Belle de Fontenay) showing symptoms associated with virus infection (mosaic, dwarfing, mottle, yellowing, necrosis, and/or rugosity). The presence of aphids and weeds was especially noted in Bizerte and Monastir.

Standard viral isolates

For biological, serological and molecular characterization, 5 standard isolates maintained on *N. tabacum* cv. Xanthi and representing the main strains and variants of PVY, were used in each experiment: PVY^N-605 (accession No. X97895), PVY^{NTN}H (accession No. M95491), PVY^{NTN}NZ (accession No. AM268435), PVY^N-W-iP (accession No. AJ889868), and PVY^O-139 (accession No. UO9509).

Figure 1. Geographic origin of the 385 collected leaf potato samples. Designation in brackets indicates numbers of collected samples in each region.

For phylogenetic analysis, nine nucleotide PVY sequences retrieved from the GenBank database (11627-12, NTNTK1, HN1, L26, Ditta, Satina, GBVC, NIB-NTN, and NTNH) were selected and included in this study (Table 1).

Table 1. Origin and characteristics of PVY sequence isolates.

Full name of isolate	Origin	Country		Strain or variant ^a	Accession No.	Corresponding haplotype
11627-12	<i>S. tub</i>	United Kingdom		NTN	KC634007	Hap 18
NTNTK1	<i>S. tub</i>	Japan		NTN	AB711146	Hap 18
HN1	<i>S. tub</i>	China		NTN	HQ631374	Hap 19
L26	<i>S. tub</i>	USA		NTN	FJ204165	Hap 20
Ditta	<i>S. tub</i>	Poland		NTN	AJ890344	Hap 21
Satina	<i>S. tub</i>	Germany		NTN	AJ890347	Hap 22
GBVC	<i>S. tub</i>	Belgium		NTN	JQ969035	Hap 23
NIB-NTN	<i>S. tub</i>	Slovenia		NTN	AJ585342	Hap 24
NTNH	<i>S. tub</i>	Hungary		NTN	M95491	Hap 6
Tunisian isolates			Bioclimate			
BS2	<i>S. tub</i> (Pamina)	Tunisia (Jendouba)	Sub-humid	NTN	KJ713153	Hap 1
BS7	<i>S. tub</i> (Spunta)	Tunisia (Jendouba)	Sub-humid	NTN	KJ713154	Hap 2
BS10	<i>S. tub</i> (Spunta)	Tunisia (Jendouba)	Sub-humid	NTN	KJ713155	Hap3
BS13	<i>S. tub</i> (Spunta)	Tunisia (Jendouba)	Sub-humid	NTN	KJ713156	Hap 3
BS14	<i>S. tub</i> (Nicola)	Tunisia (Jendouba)	Sub-humid	NTN	KJ713157	Hap 4
BS17	<i>S. tub</i> (Nicola)	Tunisia (Jendouba)	Sub-humid	NTN	KJ713158	Hap 5
KA1	<i>S. tub</i> (Nicola)	Tunisia (Kairouan)	Upper arid	NTN	KJ713159	Hap 17
KA5	<i>S. tub</i> (Nicola)	Tunisia (Kairouan)	Upper arid	NTN	KJ713160	Hap 16
KA8	<i>S. tub</i> (Atlas)	Tunisia (Kairouan)	Upper arid	NTN	KJ713161	Hap 15
KA10	<i>S. tub</i> (Spunta)	Tunisia (Kairouan)	Upper arid	NTN	KJ713162	Hap 14
KA11	<i>S. tub</i> (Spunta)	Tunisia (Kairouan)	Upper arid	NTN	KJ713163	Hap 13
CH6	<i>S. tub</i> (Spunta)	Tunisia (Mahdia)	Under semi-arid	NTN	KJ713164	Hap 12
MJ2	<i>S. tub</i> (Spunta)	Tunisia (Manouba)	Sub-humid	NTN	KJ713165	Hap 11
MJ11	<i>S. tub</i> (Spunta)	Tunisia (Manouba)	Sub-humid	NTN	KJ713166	Hap 10
MJ13	<i>S. tub</i> (Spunta)	Tunisia (Manouba)	Sub-humid	NTN	KJ713167	Hap 9
MJ15	<i>S. tub</i> (Nicola)	Tunisia (Manouba)	Sub-humid	NTN	KJ713168	Hap 8
RJ4	<i>S. tub</i> (Spunta)	Bizerte (Ras Jebel)	Humid	NTN	KJ713169	Hap 7

^aGrouped according to the molecular structure, NTN: PVY^{NTN} (European or recombinant type); designation in brackets in column 2 indicates the potato cultivar. Designation in brackets in column 3 indicates the city *S. tub*: *Solanum tuberosum*.

PVY diagnosis

Detection of PVY infection in potato samples was conducted by double antibody sandwich-ELISA (DAS-ELISA) (Clark and Adams, 1977) using anti-PVY polyclonal antisera (Bioreba, Basel, Switzerland) with microtiter plates (Nunc-immuno plate MaxiSorp surface, Polylabo) Absorbance of alkaline phosphatase reaction end-product was read at 405 nm using a microplate reader (Molecular Devices, Palo Alto, CA, USA).

Biotyping and serotyping analyses

All PVY isolates were further analyzed for strain infection by DAS-ELISA using anti-PVY^N and anti-PVY^{O-C} (Bioreba, Basel, Switzerland) specific monoclonal antibodies.

PVY infection of some potato samples was confirmed by mechanical inoculation onto *N. tabacum* cv. Xanthi plantlets in an insect-proof greenhouse with controlled conditions (20°-23°C). Symptoms in tobacco plantlets were observed daily and recorded at 18-20 days post-inoculation. PVY^N strain is known to induce veinal necrosis with smaller distorted leaves and dwarfing of the whole plant in tobacco cv. Xanthi; whereas PVY^O and PVY^C induce only vein banding or interveinal clearing without any necrosis, crinkling or dwarfing (Kerlan et al., 2011). Tobacco samples were analyzed by DAS-ELISA using the above specific monoclonal antibodies.

Molecular analyses

Immunocapture-RT-PCR amplification and sequencing

PVY amplification was performed by immunocapture RT-PCR as modified by Nolasco et al. (1993) and as previously described by Glais et al. (2005) and Djilani-Khouadja et al. (2010). The specific detection of PVY^{NTN} variants was performed using the FR2000/F2-d primer pair based on the nucleotide sequence polymorphism observed in the 5'NTR/P1 region (Glais et al., 2005). The expected amplification fragment from this primer combination is 815 bp in length. Amplified products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and photographed under UV light.

Sequencing reactions were carried out directly with purified PCR products (Qiaquick PCR purification kit, Qiagen France) with the same FR2000/F2-d primer pairs and analyzed in both strands on an ABI Prism 310 Automated DNA-377 sequencer (Applied Biosystems, Paris, France). Sequencing was performed for seventeen Tunisian isolates belonging to five geographical regions: Ras Jebel, Manouba (extreme north), Jendouba (north), Mahdia (Sahel), and Kairouan (central), and collected from four potato cultivars: Pamina, Spunta, Nicola, and Atlas (Table 1).

Sequence analysis

The whole dataset was first aligned using MEGA5 version (Tamura et al., 2011) and then corrected manually. The identity of PVY sequences were verified by BLAST. Sequences of all the Tunisian PVY isolates were submitted to NCBI GenBank and the relative accession Nos. were obtained (Table 1). The sequences were aligned using the ClustalW package in

the MEGA5 program (Tamura et al., 2011). Aligned sequences were analyzed with the DnaSP software version 5.10.01 (Librado and Rozas, 2009) to estimate polymorphism indices and past demographic history. Indices of haplotype diversity (Hd) (Nei and Tajima, 1983), pairwise estimates of nucleotide diversity (Pi) (Jukes and Cantor, 1996), site types (variable, conserved, singleton, and parsimony informative), and number of indels were used to determine genetic diversity between Tunisian PVY sequences. Indels were treated as missing data in each analysis and transition/transversion ratio (ts:tv) was obtained. For each sequence, length and proportion of GC and AT contents were estimated. The averages of pairwise nucleotide differences (K) and minimum number of recombination events (Rm) were also estimated. Thereafter, a codon-by-codon XY-plot graph was drawn for the 5'NTR/P1 nucleotide sequence on the basis of synonymous (dS) and non-synonymous (dN) substitution using the SNAP analysis program available from the HIV database (<http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html>).

Selective neutrality was tested by both Tajima's D (Tajima, 1989) and Fu and Li's D* and F* methods (Fu and Li, 1993). Demographic parameters were assessed using the distribution of pairwise sequence differences (mismatch distribution) of Rogers and Harpending (1992) using the program DnaSP software version 5.10.01 (Librado and Rozas, 2009).

Phylogenetic analysis

Phylogeny reconstruction was performed using neighbor joining (NJ) methods based on the maximum likelihood (ML) approach (Saitou and Nei, 1987) applying the MEGA5 software (Tamura et al., 2011). The Tamura-Nei model of substitution was used for nucleotide sequences and both the ts:tv and the shape parameter (α) of the distribution of substitution rate variation between sites (discrete gamma distribution with eight categories) were estimated during the tree reconstruction. The unrooted NJ tree was constructed with 1000 bootstrap replicates using MEGA version 5 (Tamura et al., 2011). The genetic relationship of the detected haplotypes was graphically displayed by the network program NETWORK version 4.6.1.0 (Bandelt et al., 1999).

Correlation analysis

Pairwise correlations between genetic and geographical distances were assessed by the Mantel test (Mantel, 1967) using the TFPGA 1.3 program (Miller, 1997). This method makes it possible to compare two distance matrices which are obtained independently by calculating an association coefficient (z) using the following statistic: $z = \frac{1}{n} \sum_{i \neq j} x_i y_j$ for $i \neq j$, where i and j are row and column indices, and where significance of z is assessed by a permutation test (10000 random permutations within one of the matrices). The significance of correlation was estimated at $P < 0.05$.

RESULTS

PVY incidence

Three hundred and eighty-five potato leaf samples were subjected to serological DAS-ELISA. The virus was detected in all surveyed regions, and results gave evidence that PVY is a virus of great concern for potato production in Tunisia with an incidence up to 80%. Indeed,

309 (80.26%) reacted with polyclonal anti-PVY antibodies and the infection rates ranged from 57.7% (Sidi Bouzid) to 100% (Cap Bon, Kairouan and Monastir) (Figure 2). Thus, all positive samples were largely geographically distributed in Tunisia except for the central part of the country (Figure 2).

Figure 2. PVY incidence estimation (%) by DAS-ELISA according to the geographic origin.

Biological and serological typing of PVY

A set of ten PVY isolates collected from each region was inoculated in tobacco plantlets. Successful infection occurred for only 82 of the 90 PVY isolates inoculated. Eighty (97.6%) of 82 inoculated plantlets of *N. tabacum* cv. Xanthi displayed vein necrosis, interveinal chlorosis and leaf distortion associated with plant dwarfing, as did standard PVY^N and PVY^{NTN} isolates. PVY^O-type symptoms, i.e., typical mottle without distortion of the tobacco leaves, were obtained after of two potato samples.

Positive reactions with both anti-PVY^{O-C} and PVY^N antibodies were obtained with twenty (24.4%) PVY isolates that induced necrotic symptoms in tobacco, whereas the other 60 (73.2%) isolates only reacted with anti-PVY^N antibodies. Plantlets from two inoculated potato samples (2.4%) showing PVY^O-type symptoms reacted with anti-PVY^{O-C} antibodies but not anti-PVY^N antibodies.

Combination of both biological and serological testing showed that 20/82 isolates were infected by a mixture of PVY^{O-C} (or PVY^{NW}) and PVY^N (or PVY^{NTN}) isolates and 2/82 samples were infected by PVY^{O-C} (or PVY^{NW}) isolates, while 60/82 were infected by either PVY^N or PVY^{NTN} isolates.

Molecular typing of PVY

Immunocapture RT-PCR amplification and PVY diversity pattern

Immunocapture RT-PCR was used to determine the genotype of the 60 PVY isolates of the serotype N, by amplifying the 5'NTR/P1 region. On the basis of the 5'NTR/P1 polymorphism region, a PCR product of the expected size (around 800 bp) was obtained from 55 isolates of the PVY^N serotype (91.7%). The same size product was amplified from the standard isolates PVY^{NTN}-H and PVY^{NTN}-NZ but not from the isolates PVY^O-139, PVY^N-605 and PVY^NWi-P.

Combination of biological, serological and molecular testing showed that from the 82 PVY isolates, two were from the PVY^{O-C} (or PVY^{NW}) isolate group (2.4%), five from the PVY^N isolate group (6.1%), fifty-five from the Y^{NTN} strain (67.1%) and twenty from the coinfecting PVY^{N+O-C} isolates (24.4%) (Figure 3).

Figure 3. Spectrum of PVY diversity on potato in Tunisia. All infection percentages were assessed from the 82 PVY isolates (number of virus infected plants/82).

Nucleotide composition and genetic diversity

Sequence alignment of PVY sequences resulted in a matrix of 763 characters (13% of the total PVY genome) with 726 conserved sites. Among the 37 variable sites, 8 were parsimony-informative characters and 29 were singleton variable sites.

Analysis of PVY sequences showed that the nucleotide composition was: 31.3% for adenine (A), 23.1% for thymine (T), 21.2% for cytosine (C), and 24.4% for guanine (G). The ts:tv ratio was recorded for all bases: $R = 5.52 > 1$. This result confirms the highest level of transition overall.

A sequence variation in the virus has been detected. In fact, 16 haplotypes were observed for the 17 isolates studied. The Hd and Pi values were estimated for Tunisian PVY sequences at 0.993 and 0.0086, respectively. In addition, the average differences between pairs of nucleotides (K) was calculated (6.574) showing a high level of polymorphism. Furthermore, minimum recombination event ($R_m = 3$) was noted (Table 2).

Analysis of synonymous and non-synonymous substitution

To investigate whether selective constraints could act on viral genomic RNA, dS and dN rates were computed for the 5'NTR-P1 sequenced region. Averages of all pairwise comparisons were obtained: $dS = 0.0236$, $dN = 0.0078$ and $dN/dS = 0.3305$. With $dN/dS < 1$, the 5'NTR-P1 region allowed more synonymous changes, suggesting selective constraint acting at this region. Consequently, a codon-by-codon XY-plot graph was drawn for this nucleotide sequence on the basis of synonymous and non-synonymous substitution using the

Table 2. Sequence polymorphism and divergence from viral PVY 5'NTR-P1 region.

	Tunisian sequences
Number of analyzed sequences	17
%GC	45.6
Variable sites	37
Conserved sites	726
Parsimony informative sites	8
Singleton variable sites	29
Transition/transversion ratio	5.52
Haplotypes	16
Nucleotide diversity	0.00862
Haplotype diversity	0.993
Average number of nucleotide differences	6.574
Minimum recombination events	3
Fu and Li's D*	-2.47584**
D	-1.64533 ^{NS}

**Significant ($P < 0.05$). NS: not significant, $P > 0.05$. D: Tajima's neutrality test.

SNAP program (Figure 4). Both dS and dN substitutions started with a low constant rate at the start codon of the sequenced region. The dN mutation rate was higher than that of the dS one, up to codon 150 (Figure 4) showing a constant rate of dN substitution mutations up to position 150. Conversely, these two rates were inverted from codon 200. We also observed that both synonymous and non-synonymous substitutions were identically constant between positions 150 to 200. Furthermore, indel mutation rates increased at the beginning and the end of the sequenced region, and were constant from codon 25 to 250 (Figure 4).

Figure 4. Cumulative incidences of synonymous (dS) and non-synonymous (dN) mutations estimated at a specific codon position. Black curves: dS mutations; gray curves: dN mutations.

Selective neutrality tests

For the selective neutrality test, our results showed significant negative values obtained for Fu and Li's D^* and F^* test and negative and non-significant value for Tajima's D test (Table 2). Therefore, they supported the selection of an excess of singletons. This test provided evidence that Tunisian PVY virus sequences have been undergoing recent expansion (Table 2).

Mismatch distributions

The resulting profile exhibited a bimodal domain, which can be explained by the co-existence of two groups, separated by a geographical barrier. The multimodal aspect of mismatch did not seem to confirm recent demographic expansion of PVY sequences but an early evolution seemed to characterize these sequences (Figure 5).

Figure 5. Mismatch distribution of the pairwise among Tunisian isolates.

Genetic relationships

Nucleotide homology between Tunisian isolates ranged from 99.98 to 100% identity (BS10 and BS13). The comparison of the Tunisian PVY nucleotide sequences with each of the PVY sequences from databases showed that they shared 99.97 to 99.99% identity.

Accordingly, the phylogenetic NJ tree reconstructed from all the 26 nucleotide sequences (Tunisian and worldwide PVY sequences) allowed the distribution of the isolates into three groups independently of their geographical origin (Figure 6). Group I encompassed one Tunisian isolate (BS14) collected from the Nicola cultivar and the Hungarian isolate (M95491). Group II was composed of the Belgium isolate (JQ969035) and seven Tunisian isolates originating from the Spunta cultivar and collected in three different regions. The remaining isolates clustered in group III consisting of nine Tunisian isolates from different sites and potato cultivars and seven isolates originating from diverse continents. The Belgian

isolate (JQ969035) and the Tunisian isolate RJ4 clustered together and measured the same branch lengths. It is worth noting that Tunisian isolates did not cluster all together in the phylogenetic tree, rather they were distributed into three clusters. Correlation between potato cultivar origin and the branching pattern was noted except for group III (Figure 6).

Figure 6. Unrooted neighbor-joining phylogenetic tree based on 5'NTR-P1 region of PVY isolates. Bootstrap analysis was applied using 1000 bootstrap samples. Bootstrap percentages at nodes are reported. The Tunisian PVY isolates are underlined. Roman numerals in brackets indicate numbers of group isolates. Potato cultivar origin of each Tunisian isolate is indicated in brackets.

Haplotype occurrence and spatial network

To confirm the relationships between isolates, an unrooted phylogenetic network based on variation of the inferred haplotypes resulting from the 5'NTR-P1 region was constructed (Figure 7). As can be seen, the network was represented by 24 haplotypes with one median vector (mv) corresponding to the intermediate evolutionary step. This mv schematized by a dark black node was not collected in our survey. As can be seen, no reticulations placed in the network were lozenge-shaped. Thus, no recombination events separating the corresponding virus sequence was possible. The central haplotype H18 was considered the major haplotype class corresponding to 2 sequences from the United Kingdom and Japan (Table 1). This haplotype was inferred as the common ancestral one on the basis of both frequency and position. From H18, ten haplotypes diverged from which seven were Tunisian isolates originating from different geographical sites and collected from different potato cultivars. Thus, the haplotype

network topology displayed a distinct geographical structure since H18 seemed to be connected to different geographical haplotypes. The H3 regrouped BS10 and BS13 Tunisian sequences collected from the same region and potato cultivar, constituted the second main haplotype from which the remaining isolates diverged. In contrast to other Tunisian isolates, H10 (MJ11) and H13 (KA11), collected from two different regions, and the Hungarian isolate of haplotype H6 showed the highest level of mutations (7, 6 and 8, respectively) (Figure 7). The lack of a geographical structure observed here indicated a pattern of PVY global expansion that was due to the pathogenicity of the haplotypes. The most frequent haplotype was probably the most stable within a heterogeneous population of closely related variants.

Figure 7. Evolutionary haplotypic network on the basis of the 17 Tunisian sequences with the nine GenBank-imported sequences. Branch length is proportional to the number of occurring mutations. Haplotypes designations are indicated in Table 1, Nodes are proportional to haplotype frequencies, Underlined haplotypes in bold correspond to Tunisian isolates and Numbers indicate nucleotide position submitted to mutations. Half-filled circle: haplotype corresponding of two isolates. Open circle: haplotype corresponding to one isolate.

Correlation analysis

The correlation between the 5'NTR-P1 nucleotide sequences and the geographical origin of the Tunisian PVY isolates was estimated by the Mantel test. Results showed a positive but not significant correlation ($r = 0.095$, $P > 0.05$) between the two matrices analyzed (Figure 8).

Figure 8. Spatial representations of Mantel distogram (a) and correlogram (b).

DISCUSSION

This paper presents a series of experiments that started with the occurrence and distribution of PVY in Tunisia. Consequently, biological, serological and molecular typing of PVY-TN was determined to assess their strain variant type, genetic structure and phylogenetic relationship with worldwide PVY isolates retrieved from the GenBank database.

Here, preliminary results indicating the high prevalence of PVY (up to 80%) are in close agreement with the situation reported in numerous worldwide potato growing areas (Gray et al., 2010), especially in Northern Africa (Boukhris-Bouhachem et al., 2010; Djilani-Khouadja et al., 2010). All positive samples were largely geographically distributed in Tunisia except for the central part of the country (Sidi Bouzid), which showed the lowest rate. This could be due to climate and seems to be in accordance with other works on seed potatoes in Tunisia (Boukhris-Bouhachem et al., 2010). Several factors may have contributed to the widespread distribution of PVY in Tunisia, which is one of the largest producers of ware potatoes in North Africa. Indeed, it is currently dependent on seed potato importation necessary for subsequent multiplication and propagation in certified seed potato fields. Thus, large volumes of imported seed potatoes may certainly carry a risk of introduction of potato pathogens, including the new recombinant PVY strains. The main sources of seed potatoes imported by Tunisian producers are Holland and France (Khamassy N, personal communication), where a wide range of PVY types have been reported since the 1980s (Kerlan et al., 2011). Furthermore, the absence of local well-established systems for multiplication and distribution of virus-free seed potatoes of high quality, the presence of alternate PVY hosts (weeds or crops) and the large range of aphid species present in Tunisia and able to transmit this virus (Boukhris-Bouhachem et al., 2010) will also contribute to PVY distribution. Furthermore, our data clearly showed that the PVY isolates were mainly necrotic PVY^N strains (~73.2%). This is in agreement with other studies in many countries (Kerlan et al., 2011).

Consequently, the analysis by immunocapture RT-PCR of the 5' terminal region of the genome made it possible to separate NTN from the rest of PVY^N. This provides host-independent criteria to classify PVY isolates, which can be applied in variability and epidemiological studies (Glais et al., 2005). Such an approach pointed to the spread and predominance of the necrotic PVY^{NTN} variants (~ 67%) in Tunisian potato crops compared to ordinary PVY^{O-C} strains, similar to what was reported in Europe, North Africa (Djilani-Khouadja et al., 2010) and South America (Ávila et al., 2009). On the contrary, PVY^O was recently shown to be the most prominent strain infecting potato crops in North America followed by the PVY^{NTN} strain (Gray et al., 2010). This high frequency of the PVY^{NTN} variant in Tunisia, causal agent of PTNRD (Beczner et al., 1984), is not correlated with a significant presence of this disease in Tunisia. This could be due to the fact that the cv. Spunta, widely cultivated in Tunisia, is tolerant to this disease, although susceptible to PVY, i.e., it does not express typical tuber necrosis symptoms under natural conditions of infection (Kerlan C, unpublished results). However, this PVY^{NTN} predominance should be considered a significant risk for growers planting cultivars susceptible to PTNRD, such as Nicola. Recent outbreaks of this disease in the last twelve years have occurred worldwide (Kerlan et al., 2011).

The increased incidence of PVY^{NTN} in potato production across the country noted here is of great concern. It is of considerable importance to highlight mechanisms and factors contributing to virus epidemiology and to establish effective strategies for viral control. Thus, partial genomic sequences of amplified 5'NTR/P1 region were directly determined

for seventeen Tunisian isolates belonging to different geographical origins, extreme north (Bizerte), north (Manouba and Jendouba), Sahel (Mahdia) and center (Kairouan), and collected from four potato cultivars: Pamina, Spunta, Nicola, and Atlas (Table 1). The information they provide about the changes that have occurred during worldwide evolution, the migration in PVY populations and the phylogenetic relationship with worldwide isolates was discussed.

Studies of the genetic structure of populations of plant viruses are important for understanding the evolution of virus/host interactions and the geographical distribution of viruses (Gibbs and Ohshima, 2010). There are many reports on the genetic structure of potyvirus populations, notably those on PVY (Ogawa et al., 2008; Karasev et al., 2011). These reports showed that virus populations were shaped by selection, founder effects and genetic recombination. Similarly, we attempted here to understand the genetic structure of the Tunisian PVY isolates. Nucleotide composition and genetic diversity of the 5'NTR/P1 region showed a high level of polymorphism consisting on nucleotide substations (transition) overall (Table 2). Furthermore, the rate of the identical sites was correlated with the pairwise identities, showing high conservation sites. This result confirms the efficiency of the 5'NTR-P1 region as a barcode. It also supports the high level of polymorphism characterizing Tunisian crops. All the sequences analyzed showed a low Rm value ($R_m = 3$). This makes such an event an implausible mechanism for the generation of the observed Hd (Table 2). Sequence polymorphism devoted to the 5'NTR-P1 region may be associated with the evolution that has allowed mutations rather than recombination to accumulate within this viral segment. PVY isolates propagate as a mixture of closely related mutants termed viral quasi species (García-Arenal et al., 2001).

To understand the selective determinants of sequence variation patterns, dN/dS rates were analyzed. Results showed the lower rates of dN/dS ($dN/dS < 1$), suggesting selective constraint against non-synonymous mutations acting at this region (García-Arenal et al., 2001). A dS or silent mutation does not increase the likelihood of a change in the structure and may not affect protein function and hence the determinants of pathogenicity of the virus (Kimchi-Sarfaty et al., 2007). Mutations that can be a threat to the functionality of the viruses are eliminated in the virus replication cycles creating a process of “purification” leading to the conservation of involved sequences (Dake et al., 2010). Thus, this region would accumulate dS mutations, described as neutral, along the coding sequences (Lopez et al., 2002). It has long been accepted that dS mutations are neutral until the last two decades, when it was realized that in some populations, especially in highly expressed genes, these mutations do not affect synonymous codons in a random manner. It would appear that this type of mutation would be able to affect the abundance of the protein, disrupting splicing and transport of mRNA while altering its stability (Goymer, 2007). The high genetic stability observed when a virus is in its natural host (host for which it is well adapted) (Koenig and Lennefors, 2000) suggests that it is the action of the stabilizer on the selection of the viral genome that maintains this balanced state. The best example of genetic stability of viral populations is the analysis of European and Australian isolates of turnip yellow mosaic virus, which showed only 3-5% of nucleotide differences (mostly synonymous mutations) between these two populations, which nevertheless diverged about 14,000 years (Keese et al., 1989). Structural and functional constraints on viral populations therefore tend toward temporal and geographical stability of viral populations and may then impose limits to the adaptation of viral populations.

To understand the demographic history of the viral PVY populations, phylogenetic analysis of the sequenced region was performed. The relationship between haplotype network,

Tajima's D neutrality test and mismatch distribution of pairwise differences can provide insights into population genetic structure. The relationship between H_d and P_i can help to determine the population phylogenetic pattern. In this study, H_d was high (0.993) in contrast to P_i value (0.00862). This could be explained by the occurring mutations. This large number of closely related haplotypes may indicate a signature of a rapid population expansion (Lévy-Hartmann et al., 2012). The Tajima's D neutrality test provides consistent information related to population expansion, genetic hitchhiking and purifying selection (Holsinger et al., 2010). Here, the negative Tajima's D test predicts an excess of rare alleles (Schmidt and Pool, 2002) and indicates that PVY evolves into stable population's size, strongly suggesting a recent population expansion of PVY isolates. This may be explained by the good adaptation of Tunisian isolates to their endemic areas. Pairwise mismatch distribution was estimated and a bimodal pattern was revealed for Tunisian PVY isolates. This bimodal distribution with a distinct separation between the two observed modes corresponds to two PVY populations separated by a geographical barrier (Mora et al., 2007). This does not seem to confirm recent demographic expansion of PVY sequences, but an early evolution seems to characterize these sequences. These findings were correlated to the haplotype network, showing the clustering of PVY isolates into two distinct subgroups, regardless of their geographic and potato cultivar origin. In the same prospect, the seventeen Tunisian partial-genome sequences were subjected to a phylogenetic analysis against nine PVY sequences from GenBank. Sequence analysis revealed a strong identity between Tunisian isolates. In contrast, previous analyses at the 5' end have revealed high variability between PVY^N field isolates (Blanco-Urgoiti et al., 1998). It is worth noting that Tunisian isolates were distributed independently of geographical origin. However, a correlation between potato cultivar origin and the branching pattern seems to exist. Furthermore, the Belgian isolate (JQ969035) and the Tunisian isolate RJ4 clustered together and showed the same branch length. This could be due to the fact that Tunisia imports a small amount of potato seed from Belgium. Additionally, Nie and Singh (2002) showed that sequence comparison and phylogenetic analysis of the 5'-UTR and P1 region indicated that PVY^N isolates from the European Union and North America formed their own separate groups. Intra-group sequence identity for all except one was over 98%, as opposed to the inter-group identity of 90%. On the other side, the PVY^{NTN} isolates from the European Union and North America clustered with their respective PVY^N isolates. This indicates a possible evolution of PVY^{NTN} isolates from the PVY^N isolates of a geographical region (Nie and Singh, 2002). Thus, sequencing of the P1 gene and use of the competitive RT-PCR approach could be applicable for determining the possible origin of new occurrences of PVY^{NTN} from other geographical regions.

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