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Full Length Research Paper

Potato virus surveys and wide spread of recombinant PVY^{NTN} variant in Central Tunisia

I. Larbi[1#], F. Djilani-Khouadja[1#], N. Khamassy[2] and H. Fakhfakh[1]*

[1]Laboratoire de Génétique Moléculaire, Immunologie et Biotechnologie, Faculté des Sciences de Tunis, Campus Universitaire El Manar, 2092 Tunis, Tunisia.
[2]Laboratoire d'horticulture Programme pomme de terre, Institut National de Recherche Agronomique de Tunisie (INRAT), 2080 Ariana, Tunisia.

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Surveys in the late season crops of Central Tunisia, one of the main potato growing areas, revealed the presence of the six most economically devastating viruses: potato leaf roll virus: PLRV: (*Polerovirus*), potato virus S (PVS) and M (PVM) (*Carlavirus*), potato virus X (PVX: *Potexvirus*), potato virus A (PVA) and Y (PVY) (*Potyvirus*) with large differences in incidence. Infection rates at harvest, assessed by serological test ranged from 0.5% (PVM) to 71% (PVY). The characterization of PVY variability was analyzed with a combination of serotyping, indexing on tobacco and reverse transcription polymerase chain reaction (RT-PCR) tests targeting 2 genomic regions (5'NTR/P1 and CP/3'NTR). Serological analysis of the collected samples revealed dominance of the PVY^{N} group (88.2% of the total number of singly PVY positives). Furthermore, molecular typing of strains revealed that 73.3% of the PVY^{N} group were typical PVY^{NTN} variants with a recombination junction in CP/3'NTR region for 94.4% of them, whereas no recombination junction was identified in the genome of the isolates belonging to the PVY^{N} group. To our knowledge, this is the first report of the occurrence of the PVY^{NTN} variant and its high incidence in late season potato growing areas of central Tunisia.

Key words: Diagnosis, immunocapture reverse transcription polymerase chain reaction (RT PCR), potato viruses, potato virus Y (PVY) diversity, Central Tunisia.

INTRODUCTION

In Tunisia, potato produces each year three types of marketable potatoes: early, main and late season crops. Spunta is the prevalent cultivar, accounting for 80% of the late-season potatoes and other cultivars such as Nicola, Atlas, Pamina, Liseta and Safrane have been recently introduced (Economic balance, 2008). Around 20% of late potato crops are planted from local certified seed potatoes provided by GIL (Groupement Interprofessionnel des Légumes: Interprofessional Organization for Vegetables) and around 80% are produced from mother tubers saved by farmers from tubers harvested in the previous main-season. These farm-saved potato seeds are known to have significant levels of virus that cause severe damage on potato crops worldwide such as lowered quality due to internal damage to tubers (Khamassy, 1999).

In the field, potato is frequently infected by several viruses during the growing season (Mc Donald, 1984), which reduces yield and impairs tuber quality. Thus, viruses constitute a permanent threat for seed potato growers because most of them induce diseases that are systemic and transmitted through seed tubers. Potato leaf roll virus (PLRV), potato virus X (PVX), A (PVA), Y (PVY), S (PVS) and M (PVM) are known to be the most damaging to potato crops worldwide. Leaves, stems, and tubers may exhibit different necrotic patterns. Tubers can be affected in their size, number, aspect, and content. Up to 90% of yield losses can be registered depending on virus, strain, and time of infection. Lastly viruses inducing

*Corresponding author. E-mail: Hatem.fakhfakh@fsb.rnu.tn. Tel/Fax: 216 70 860 432.

[#]Authors have contributed equally to this work.

tuber necrosis can make tubers totally unmarketable (Kerlan, 2008). However, PVY is rapidly becoming the most widespread and economically destructive virus affecting potato seed and commercial production crops in all potato growing regions in the world (Valkonen, 2007). In Tunisia, it was identified as a major problem in seed potato multiplication and production causing approximately 35% yield losses in crops planted from local self-produced potato seed (Khamassy, 1999).

Type member of genus *Potyvirus,* family *Potyviridae* (Berger et al., 2000), it is transmitted by aphids in a non-persistent manner (Sigvald, 1984) and has a single-stranded, positive-sense RNA genome of about 9.7 kb in length that encodes a single polyprotein which upon maturation is cleaved into ten proteins (P1, Hc-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, Nib and CP). Furthermore, PVY causes significant losses in many other crops, mainly in the *Solanaceae* family: tobacco, tomato, pepper and numerous self-propagating plants. It is non-persistently transmitted by more than 50 aphid species (Robert et al., 2000; Ragsdale et al., 2001).

The biological, serological and molecular diversity of PVY depends on the distinct host specificity among virus isolates. Most isolates infecting potato belong either to the main three strain groups, PVY^{O}, PVY^{C} (minor pathotype) and PVY^{N} according to biological (symptomatology and resistance response) and/or serological properties, or to the variants PVY^{NTN} and $PVY^{N}W$ according to molecular analysis (Tribodet et al., 2005). The majority of theses variants are genomic recombinants between PVY^{O} and PVY^{N} (Glais et al., 1998; Glais et al., 2002; Nie and Singh, 2003).

Based on virus symptoms induced on tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) plants, isolates of the PVY^{O} group induce mottling and mosaic symptoms on tobacco and mild to severe mosaic and leaf drop on potato, while isolates of the PVY^{N} group induce veinal necrosis on tobacco and cause very mild mottling with occasional necrotic leaves on potato (for reviews, see Robert et al. (2000) and Ragsdale et al. (2001). Therefore, the ability of PVY potato isolates to cause necrosis is one of the most important factors involved in taxonomy and the principal cause of the agronomic impacts of PVY (Tribodet et al., 2005). Other economically important strain variants are derived from recombination events, including variants that cause tuber necrotic symptoms (PVY^{NTN}) and PVY^{O} serotypes causing tobacco veinal necrosis (PVY^{N}-W, $PVY^{N:O}$) (Lorenzen et al., 2006). PVY^{NTN} and PVY^{N}-W variants were first reported in Europe and most of these variants display a recombinant genome with exchanges of sequences in the HC-Pro (PVY^{NTN} and PVY^{N}-W), NIa and CP genes (PVY^{NTN}) between PVY^{N} and PVY^{O} sequence types (Glais et al., 1998; Glais et al., 2002 and Nie and Singh, 2003).

This paper provides updated information on incidence of the most common viruses (secondary infections) affecting late season potato crops in Central Tunisia. Biological, serological and molecular characterization of PVY, the most prevalent potato virus were then involved respectively by indexing on one indicator tobacco cultivar, serotyping using strain-specific antibodies and immunocapture RT-PCR test targeting polymorphism in two genomic regions.

MATERIALS AND METHODS

Surveys

Surveys were carried out from the late season crops (autumn 2009) in many fields in the center of Tunisia: Sidi bouzid (3 fields) and Gafsa (4 fields). A total of 201 leaf samples, 123 from Gafsa and 78 from Sidi Bouzid, showing typical secondary symptoms of viruses (mosaic, dwarfing, yellowing, leaf curl, necrotic spots or necrosis) were collected from Spunta, Nicola, Gredine and Belle de Fontenay potato varieties.

Viral isolates

5 standard isolates representing the main strains and variants of PVY were used in each experiment: PVY^{N} (B203), PVY^{NTN} (-H and NZ), PVY^{N}-W (-iP) and PVY^{O} (Irl) (Djilani-Khouaja et al., 2010).

Detection of potato virus infections

Potato samples were tested using enzyme-linked immunosorbent assay (ELISA) with the anti-PVY, -PLRV, -PVA, -PVX, -PVM and -PVS polyclonal antibodies (from Bioreba, Basel, Switzerland) according to a standard DAS-ELISA protocol (Clarks and Adams, 1977).

Biotyping and serotyping

PVY positive samples assessed by DAS-ELISA were confirmed by mechanical inoculation onto *N. tabacum* cv. Xanthi plantlets under insect-proof greenhouse and regulated conditions (20 to 23°C) (Le Romancer et al., 1994). Symptoms on tobacco plantlets were observed daily and recorded at 18-20 days post-inoculation. Tobacco samples were ELISA tested using an anti-PVY serum (from Bioreba, Basel, Switzerland), anti-PVY^{N} specific monoclonal antibodies (from Bioreba, Basel, Switzerland) and anti-PVY^{O-C} specific monoclonal antibodies (from Adgen, Ayr, UK).

Molecular typing

Immunocapture RT-PCR assays were performed as previously described (Glais et al., 2005; Djilani-Khouaja et al., 2010). Amplified products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and photographed under UV rays. Primer names, sequence, polarity, location in genome and expected PCR products are listed in Table 1.

Primer sequences, previously described by Glais et al. (1996; 1998 and 2002) were deduced by comparing nucleotide sequences of three PVY isolates: PVY^{NTN}-H (accession number M95491; Thole et al., 1993), PVY^{N}-605 (accession number X 97895; Jakab et al., 1997) and PVY^{O}-139 (accession number UO9509; Singh and Singh, 1996). Specific detection of both recombinant and non-recombinant PVY^{NTN} variants was conducted with the primer pair Fr_{2000}/F2-d, which has been based on nucleotide sequence

Table 1. Sequence and genomic location of primer pairs used in molecular typing.

	Primer name	Polarity	Sequence 5' – 3'	Location[a]	Expected products	References
RT	d	Antisense	TG(CT)GA(CTA)CCACGCACTATGAA	955-974		Glais et al., 1996
	$3'^{NTR}C$	Sense	GTCTCCTGATTGAAGTTTAC	9684-9703		Glais et al., 1998
PCR	FR_{2000}/F_2	Sense	TCAAACTCTCGTAAATTGCAGA	159-180	815 bp	Glais et al., 2001
	d	Antisense	TG(CT)GA(CTA)CCACGCACTATGAA	955-974		Glais et al., 1996
PCR	$_{9132/51}Y^NF$	Sense	TCGTAATCTGCGCGATGGAA	9132-9151	368 bp	Glais et al., 2001
	$_{9481/00}Y^OR$	Antisense	CCCTGCCACCTCTATCTATT	9481-9500		Glais et al., 2001

[a] :Numbered in PVY^{NTN}-H (Thole et al., 1993).

Table 2. Percentage of potato virus infections, assessed by DAS-ELISA test, in plants collected from late potato crops growing in central Tunisia.

Potato viruses	Percentage of virus infection	
PLRV	4/201	2
PVX	4/201	2
PVM	1/201	0.5
PVS	4/201	2
PVA	4/201	2
PVY	143/201	71

Percentages of virus infections: number of virus-infected plants/number of collected plants.

polymorphism observed in 5'NTR/P1 region (Glais et al., 1996). Specific detection of recombinant PVY^{NTN} isolates was conducted with the primer pair $_{9132/51}Y^NF/_{9481/00}Y^OR$ (Glais et al., 2002) targeting the recombination site within CP/3'NTR region. The primers $_{9132/51}Y^NF$ and $_{9481/00}Y^OR$ amplified PVY^N and PVY^O-type nucleotide sequences, respectively.

RESULTS

Incidence of potato viruses

ELISA tests performed on collected potato leaf samples, resulted in the following virus infection rates: 2% (4/201) for PLRV, PVS, PVX and PVA, 0.5% (1/201) for PVM and 71% (143/201) for PVY (Table 2). All viruses were detected in single or double infection (Table 3). No cases of single plant infection by more than 2 viruses were identified.

Characterization of PVY isolates

Serological typing

136 of the 143 PVY isolates were simply infected with PVY and were then tested for further characterization. One hundred twenty of the 136 simply infected with PVY (88.2%) reacted positively only with anti-PVY^N antibodies (with high ELISA signals for most of them). Only one of the 136 (0.7%) PVY isolates reacted positively only with anti- PVY^{O-C} antibodies. Fifteen samples (11%) reacted positively with both anti-PVY^{O-C} and anti-PVY^N antibodies (Table 3).

Biological typing

115 out of the 120 PVY^N serotype 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, that is, typical mottle without distortion of the tobacco leaves, were obtained after inoculation from one potato sample only.

Molecular typing

Immunocapture RT-PCR was used to determine the genotype of the 120 PVY isolates of the serotype N, first by amplifying the 5'NTR/P1 region. A PCR product of around 800 bp was amplified from 88 isolates of the PVY^N serotype (73.3%) Table 3. The same size product was amplified from the standards isolates PVY^{NTN}-H and PVY^{NTN}-NZ but not from the isolates PVY^OIrl, PVY^N B203 and PVY^NWi-P (Figure 1a).

RT-PCR were then used to test for the presence of recombinant junction in CP/3'NTR region in these 88 isolates. Electrophoresis patterns obtained from the standard PVY^{NTN}-H isolate displayed one band of approximately 360 bp. Similar pattern was obtained from 83 of 88 isolates (94.3%). The amplified product for five of 88 isolates, as well as for PVY^{NTN}-NZ, PVY^OIrl, PVY^N B203 and PVY^NWi-P did not correspond to any of these bands. The Figure 1b illustrates some examples of electrophoresis pattern.

PVY diversity pattern

From the 136 simply infected PVY isolates, only one was Y^{O-C} serotype (0.7%), 120 Y^N serotype (88.2%) and 15 Y^{N+O-C} (11%) (Table 3 and Figure 2). The molecular typing revealed 32 Y^N strains (23.5%) and 88 Y^{NTN} variants from which 83 were recombinants (61%) and 5

Table 3. Serological and molecular variability of PVY isolates.

Potato viruses	Percentage of virus infection assessed by ELISA	
PVY	136/201 [a]	67.6
PLRV	1/201 [a]	0.5
PVA	1/201 [a]	0.5
PVX	1/201 [a]	0.5
PVY+PLRV	2/201 [a]	1
PVY+PVA	1/201 [a]	0.5
PVY+PVS	3/201 [a]	1.5
PVY+PVX	2/201 [a]	1
PVA+PVM	1/201 [a]	0.5
PVA+PLRV	1/201 [a]	0.5
PVX+PVS	1/201 [a]	0.5
PVY^{N}	120/136 [b]	88.2
$PVY^{O/C}$	1/136 [b]	0.7
$PVY^{N+O/C}$	15/136 [b]	11

PVY diversity	Percentage of PVY^{NTN} variants assessed by immunocaptutre RT-PCR	
PVY^{NTN}	88/120 [c]	73.3
NTN recombinant	83/120 [c]	69.1
NTN non recombinant	5/120 [c]	4.2

[a]: Percentages of virus infections: number of virus-infected plants/number of collected plants; [b] : Percentages of PVY serotypes: number of PVY serotype-infected plants/number of PVY simply infected plants; [c] : Percentages of PVY variants: number of PVYvariants-infected plants/number of PVY^{N} infected plants.

non recombinant (3.6%) in the CP/3'NTR site (Figure 2).

DISCUSSION

This paper reports result of virus indexing of symptomatic potatoes harvested from the late season crops in Central Tunisia using serological methods. It also provides information on incidence and diversity of PVY using molecular markers. Thus, it consists on the complement characterization of PVY strains previously assessed in Northern (Djilani Khouadja et al., 2010) and Southern (Boukhris-Bouhachem et al., 2008) Tunisia.

Here, PVY was by far the most common of the six viruses investigated in central Tunisia. This data is consistent with previous studies on PVY incidence in Northern Tunisia (Djilani Khouadja et al., 2010) showing infection rates of 5.4, 7, 3.8, 4.3 and 4.8% for PLRV, PVS, PVA, PVX and PVM, respectively. Such limited spread of these viruses could be due to the variable relative efficiency of aphid clones for vectoring different viruses (Boukhris-Bouhachem et al., 2007). Moreover, PVY may just be more prevalent in seed stocks used in the regions surveyed due to the absence of well-established systems for multiplication and distribution of virus-free potato seed of high quality. Indeed, farmers in Tunisia often resort to local production of potato seeds which are selected as the smallest tubers or those having no value. Such practice would inevitably lead to the spread of viral infections in the same area of potato cultivation as well as virus spread from one region to another. Furthermore, alternate PVY hosts or the tendency of farmers to replant the smallest tubers will be the definite explanations. Our results were also consistent with the situation described in many potato growing areas in the world, especially in South America (Salazar et al., 2000) and in Western Europe (Valkonen, 2007). In addition, the low rates of the combined infections in the Tunisian potato crops showed the rarity of the presence of the PVY with another virus. This could be due to the fact that the infection of a plant with a virus could make it resistant to another infection and could be a complement evidence explaining the low incidence of the other weakly detected viruses. It is also noted that multiple infection of potato viruses were not detected contrary to Jendoubi (unpublished data, 2001) and Djilani-Khouaja et al. (2010).

Serological data clearly shows here the extent of necrotic PVY^{N} strains over the PVY^{O-C} ones (Table 3 and Figure 2). The same situation was previously confirmed in Poland (Chrzanowska, 1991), in Spain (Blanco-Urgoiti et al., 1998), in France (Kerlan et al., 1999), in USA (Crosslin et al., 2002), in Canada (Nie and Singh, 2002), in Germany (Lindner and Billenkamp, 2005) and in Tunisia (Boukhris-Bouhachem et al., 2008 and Djilani-Khouadja et al., 2010). Molecular characterization of PVY isolates based on the polymorphism of the 5'NTR/P1 region, showed an extremely high frequency of PVY^{NTN} variants

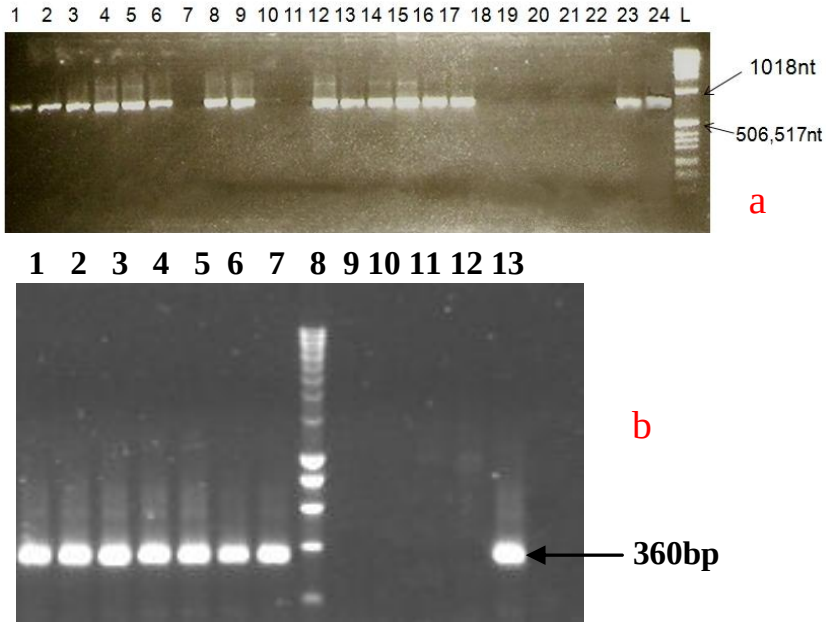

Figure 1. Electrophoresis analysis in two genomic regions amplified by immunocapture RT-PCR: (a): 5'NTR/P1 region amplified by « d » and « FR_{2000}-F_2 » primer pairs : 1-17: Tunisian PVY isolates, 18: negative control, 19: healthy tobacco sample, 20: PVY^{N}B203, 21: PVY^{O}Irl, 22: PVY^{N}Wi-P, 23: PVY^{NTN}-NZ, 24: PVY^{NTN}-H, L: smart ladder 1k. (b): CP/3' NTR recombination site amplified by « $^{9132}/_{51}$ $Y^{N}F/_{9481/00}Y^{O}R$» primer pairs: 1-7 : Tunisian PVY isolates; L : Smart Ladder (Eurogentec); 8 : healthy tobacco control; 9 : PVY^{O} Irl; 10 : PVY^{N}B203; 11 : PVY^{N}Wi-P; 12 : PVY^{NTN}-H; 13 : PVY^{NTN}-NZ.

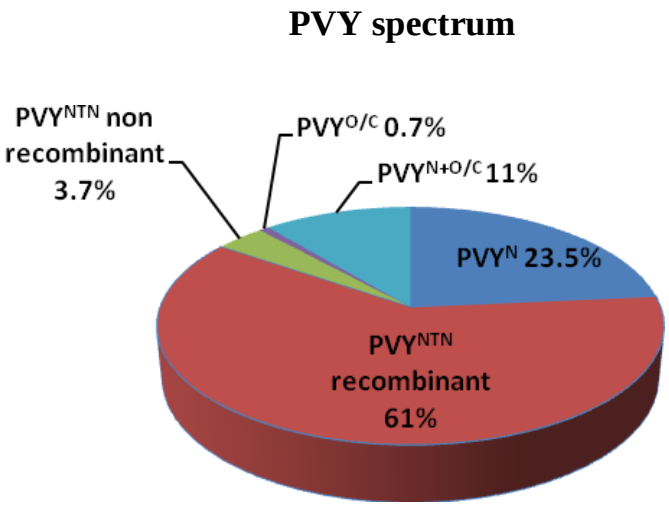

Figure 2. Spectrum of PVY diversity in a set of leaf potatoes in Central Tunisia. All infection percentages were assessed from the 136 simply infected PVY isolates (number of virus infected plants/136).

among PVY^{N} strains (Table 3 and Figure 2). This is in according to previous studies conducted in Southern (Boukhris-Bouhachem et al., 2008) and Northern (Djilani-Khouaja et al., 2010) Tunisia. This high frequency of the PVY^{NTN} variant, causal agent of PTNRD (Beczner et al., 1984) is not correlated with a significant presence of this disease in Tunisia. In fact, PTNRD was only reported once in two locations representing ware potato areas, much further south of seed potato growing areas (Boukhris-Bouhachem et al., 2008). This could be due to the widely cultivated Spunta in Tunisia, a tolerant potato variety to this disease, although susceptible to PVY, i.e., it does not express typical tuber necrosis symptoms under natural conditions of infection (Kerlan, unpublished). The high incidence of PVY^{NTN} will be a major risk for growers planting cultivars susceptible to PTNRD, such as Nicola and because warm climatic conditions in Tunisia are known to favor expression of this disease. This problem, leading significant losses, is known in most countries producing potato (Weidemann and Maiss, 1996). Recent outbreaks due to this disease have occurred in recent years in France (Crouau and Gokelaere, 1997), Italy (Tomassoli and Lumia, 1998), Greece (Bem and Varveri, 1999), Japan (Ohshima et al., 2000), Peru (Salazar et al., 2000) and the United States and Canada (Crosslin et al., 2002).

In addition, this study revealed that most the PVY^{NTN} variants are recombinants in the CP/ 3'NTR recombination regions and for all considered potato

varieties. The pattern of PVY diversity found in central Tunisia (Figure 2) resemble, but not mirror, to the diversity in Northern Tunisia in which no non-recombinant PVY^{NTN} variants were found (Djilani-Khouaja et al., 2010). Similarly, van de Haar and van den Bovenkamp (2007) showed a large fraction of PVY^{NTN} variants occurring in Netherlands.

In this study, diagnosis of PVY^{N}-W variant has not been undertaken since only one PVY^{O-C} serotype was found. The absence of this variant in Tunisia could be due to local climatic conditions and cultural practices unfavorable to its propagation, to the sanitary status of imported seed potatoes and to the specific resistance to $PVY^{N}W$ in cv. Spunta. This variant has become prevalent for over a decade in potato crops under continental climates, especially in Poland (Chrzanowska et al., 1998; Chrzanowska, 2001) and Germany (Linder and Billenkamp, 2005). However, despite its scarcity, PVY^{N}-W variant may be present in cases of co-infection making it a source of infection more or less asymptomatic so not easily detectable during field inspections.

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