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=== REVIEW ===

Challenges in the detection and identification of potato virus Y, an important pathogen of potato

Adriana Aurori[1,✉], Carmen Liliana Bădărău[2,3] and Elena Rakosy-Tican[1]

SUMMARY. Potato virus Y (PVY) has become one of the most important pathogens of potato. Being a RNA virus, one of the main characteristic is it's great genetic variability. Several well characterized strains du exist but the continuous emergence of new forms, the fast spreading of the existent ones and the shifting toward the increased prevalence of the recombinant necrotic strains raised a huge interest in finding ways for reducing the propagation of the disease. Two important strategies were adopted aiming for the same outcome, the reduction of PVY incidence. The first one, consists in improving the genetic background of potato and creating cultivars resistnt to PVY. This objective may be achieved by the integration of PVY resistance genes into potato gene pool. The second one, and this will be the subject of this review, relies on the finding of suitable methods for PVY detection which has to be fast and economical competitive for being applied to a large scale. The seed certification represents the most important step for multiannual and/ or interregional or international PVY spread prevention. There is no tolerance for necrotic PVY strains in seed, especially in the case of those batches that are obtained by biotechnology, and therefore, the screening for PVY became part of the protocol for potato seed certification.

Keywords: bioassay, ELISA, molecular detection, PVY^{NTN}, PVY^{N-Wi}

[1] *Babeş-Bolyai University, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 5-7 Clinicilor Str., Cluj-Napoca, Romania*

[2] *National Institute of Research and Development for Potato and Sugar Beet Braşov, 2 Fundăturii Str., Braşov, Romania*

[3] *Transilvania University, Faculty of Food and Tourism, 2 Castelului, Braşov, Romania*

✉ ***Corresponding author: Adriana Aurori***, *Babeş-Bolyai University, Faculty of Biology and Geology, 5-7 Clinicilor Str., Cluj Napoca, Romania*
E-mail: aaurori2002@gmail.com

Introduction

Potato is one of the most important crops ranking on the fifth place in the world as human food source after wheat, rice, corn and barley (Fageria *et al*., 1997). It is considered one of the key elements in nutrition having in the history an important contribution to the population growth and prosperity (Nunn and Qian, 2011). In the current context, in which the population is growing continuously, the need for food leads to a higher pressure for increasing the productivity in agriculture but without the possibility of expanding the cultivated areas. In addition, we assist to climate changes that endanger the crop production, due to both, abiotic and biotic stress, the last one being caused in particular by modification of plant-pathogen relationship (Halterman *et al*., 2012). The potato virus Y (PVY) has become lately one of the most threatening pathogens of potato that can lead to significant financial losses (Karasev and Gray, 2013).

PVY is transmitted mechanically but its most significant propagation is made by aphids in a non-persistent manner. An exception to this way of transmission was encountered in the case of a substrain of PVY^C (Blanco-Urgoiti *et al*., 1998 a). This mode of transmission is the main limiting factor in stopping the spread of infection by applying insecticides, a very short probing of the aphids being enough for transmitting the virus before insecticide to become effective (Hühnlein *et al*., 2013). Early PVY detection and prevention of the cultivation of contaminated potato seed represents the main strategies for limiting the PVY spread besides the utilization of PVY resistant cultivars, the last one not being the subject of this review. The PVY detection starts in the field and immediately after harvest, based on the overt symptoms (Nolte *et al*., 2004). But the detection based on phenotype manifestations is not always easy due to the variety of strains, the emergence of new recombinant PVY forms who have new symptoms, sometimes not obvious, depending on the cultivars and climatic factors (Ellis *et al*., 1996; Rykbost *et al*., 1999; James *et al*., 2003; Nolte *et al*., 2004; Schubert *et al*., 2007; Hühnlein *et al*., 2013). For an effective management of PVY control it is mandatory that precise biochemical and molecular methods of detection and identification are established, regardless on the fact that they are addressed to: the preventive screening of the potato seed (Halterman *et al*., 2012), the rapid identification during epidemics, determination of the aphid vectors in the field (Singh, 1998) or to test for the contamination of the water in the case of hydroponic cultures (Mehl *et al*., 2014).

The analysis of PVY in potato follows two close directions. The first one, based on PVY sequencing is necessary for full characterization of strains (Robaglia *et al*., 1989; Singh and Singh, 1996 b) and it's imposed each time when strains with atypical biological, serological or molecular features are found (Thole *et al*., 1993; Nie and Singh, 2003 a; Chikh Ali *et al*., 2007; Lorenzen *et al*., 2008; Hu *et al*., 2009 b; Chikh Ali *et al*., 2013 a) or when PVY incidence in a new geographical area is explored (Ohshima *et al*., 2000; Chikh Ali *et al*., 2007; 2013 a; Ogawa *et al*., 2008; Schubert *et al*., 2014).

Such studies are meant to reveal the degree of PVY variability due to mutations/ recombination (Ogawa *et al*., 2008) or the origin of certain strains (Lorenzen *et al*., 2006 a; Nie and Singh, 2002 a, 2003 a). Eventually, the findings of such studies can lead to the understanding of the molecular evolution of PVY offering the opportunity to find the important features of the virus biology, especially of the virulence determinant factors (Tribodet *et al*., 2005; Hu *et al*., 2009 b). The second direction, rely on the previous one findings, aims to establish methods for rapid identification of the virus during the routine purposes such are PVY detection and indexing in the potato seed certification protocol (Halterman *et al*., 2012). In addition, these studies follows to determine the geographical distribution of different strains aiming the localization of virus outbreaks source (McDonald and Kristjansson, 1993; Piche *et al*., 2004; Ogawa *et al*., 2008) which is an essential information for developing strategies to control the spread of infection.

In this review we will make a quick scanning of the methods addressed in time for the identification and characterization of PVY and we will highlight in particular those assays that offer the greatest advantages for the routine screening in the seed certification programs and fast PVY indexing.

The molecular structure, genetic variability and nomenclature of PVY

PVY belongs to the genus *Potyvirus*, *Potyviridae* family. All the members of this family include cylindrical, flexible viruses (Glais *et al*., 1996). PVY genome consists in a single strand of positive sense RNA molecule of about 10 kb with a VPg protein attached to the 5' end and a poly A tail to the 3' end. Viral RNA encodes a single large polypeptide molecule which is subsequently cleaved by three proteases of viral origin in ten proteins (review of Quenouille *et al*., 2013). The viral proteins (Fig. 1) are represented by P1 which is among the most variable proteins within the PVY strains (Marie-Jeanne Tordo *et al*., 1995), protease helper component (HC-Pro), protein P3, inclusion cellular protein (CI) flanked by 6K2 and 6K1 proteins, genome-linked viral protein (VPg), first nuclear inclusion protein (NIa), second nuclear inclusion protein (NIb) and coat protein (CP) (Hühnlein *et al*., 2013).

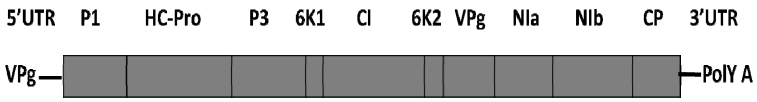

Figure 1. PVY genome structure (after Hühlein *et al*., 2013); VPg - viral genome linked protein, Poly A - poly A tail, P1- P1 protein, HCPro - helper component protease, P3 - P3 protein, Ci - cellular inclusion protein, NIa - nuclear inclusion protein a, NIb - nuclear inclusion protein b, CP - coat protein

Table 1.

The main PVY strains (after Singh *et al*., 2008, with modifications)

Strain name	Hypersensitive reaction in potato Yes (gene)/ No	Serotype	Bioassay in tobacco	PTNRD	Synonymous codes
PVY^{O}	Yes (*Ny*)	O	M, VCL	No	PVY^{O5}
PVY^{N}	No	N	VN	No	PVY^{EU-N}, PVY^{NA-N}, $NA\text{-}PVY^{N}$, PVY^{R}, PVY^{-TVN}
PVY^{C}	Yes (*Nc*)	O	M, VCL	No	PVY^{C1}, PVY^{C2}
PVY^{Z}*	Yes (*Nz*)	O or N	M, VCL	Yes, occasionally	$PVY^{Z\text{-}NTN}$
PVY^{E}**	No	N	M, VCL	Yes	PVY^{ZE}
PVY^{D}***	Yes (putative *Nd*)	NA	M, VCL	NA	
PVY^{NTN}	No	N	VN	Yes	$EU\text{-}PVY^{NTN}$, $Eu\text{-}PVY^{NTN}$, $PVY^{EU\text{-}NTN}$, PVY^{NN} $PVY^{NA\text{-}NTN}$, $NA\text{-}PVY^{NTN}$
$PVY^{N\text{-}Wi}$	No	O	VN	No	$PVY^{N\text{-}Wilga}$, $PVY^{N\text{-}W}$, PVYN-Wi-P, $PVY^{N:O}$

M – mosaic, NA – not assessed, PTNRD - potato tubers necrotic ring spot disease,
VCL – vein clearing, VN – vein necrosis
* PVY^{Z} described after Jones, 1990; Blanco-Urgoiti *et al*., 1998 b; Kerlan *et al*., 1999; 2011
** PVY^{E} described after Ggalvino-Costa *et al*., 2012
*** PVY^{D} described after Kehoe and Jones, 2015

Their role in establishing the infection, polyprotein processing, replication, translation, virion assembly, cell-to-cell and systemic movements and aphid transmission is partially known (reviewed by Dougherty and Carrington, 1988; Quenouille *et al*., 2013).

PVY was initially classified, based on the pathotype and the hypersensitivity reactions (HR) resulted by elicitation of the resistance genes present in certain cultivars of potato, in several strains named O, N, C, Z, E (review of Singh *et al*., 2008) (Table 1). PVY^{O}, the common form of PVY (O from "ordinary") is characterized by the induction of HR in cultivars carrying Ny gene, produce mosaics in tobacco plants and various degrees of mosaic in the most varieties of potato. Necrotic PVY (PVY^{N})

overcomes all known *N* genes, causes tobacco systemic vein necrosis (TVN) but in potato the symptoms can be invisible or may manifest as mild mosaic. PVY^{C} leads to HR in the cultivars having *Nc* gene and systemic mosaic in the rest of potato varieties (Karasev *et al*., 2011).

The PVY^{Z} strain was accepted based on the induction of HR in cultivars carrying the putative gene *Nz* (Jones, 1990; Blanco-Urgoiti *et al*., 1998 b; Kerlan *et al*., 1999; 2011) and now proved to be a true resistance gene (Chikh Ali *et al*., 2014). It seems to be a recombinant form between PVY^{O} and PVY^{N} having a serotype O, the restrictotype of coat protein like PVY^{O}, but a RFLP pattern at the 5' end similar with PVY^{N} (Blanco-Urgoiti *et al*., 1998 b). Another recombinant form of PVY assigned to the PVY^{E} strain overcomes all the resistance genes (*Ny*, *Nc* and putative *Nz*) in potato cultivars but in the same time it does not induce TVN (Galvino-Costa *et al*., 2012). Recently, a new isolate of Australian origin was found, manifesting new biological features. It is eliciting a putative *Nd* resistance gene apparently present in the cultivars King Edward, Russet Burbank and White Rose inducing the HR (Kehoe and Jones, 2015). Although it has a genome similar with the PVY^{C} strain based on the HR it can be classified in a new strain, PVY^{D} (Kehoe and Jones, 2015) (Table 1).

In the '80s, new PVY^{N} isolates were reported in Europe and later in North America. One of them, named PVY^{NTN}, induce tuber necrosis causing potato tubers necrotic ring spot disease (PTNRD) (Beczner *et al*., 1984). A few years later, another PVY variant was discovered, named PVY^{N-Wi} (noted after Wilga, the potato cultivar from which was isolated) which induce only mild phenotype in potato but it is responsible for braking the resistance in some potato cultivars (Chrzanowska, 1991). The new PVY strains, PVY^{N-Wi} and PVY^{NTN}, have a great variability. They are supposed to be derived by recombination between PVY^{O} and PVY^{N} strains having one or two recombination junctions in the case of PVY^{N-Wi} (Glais *et al*., 2002; Lorenzen *et al*., 2008) and 3 - 4 in the case of PVY^{NTN} giving at least nine recombination patterns (Hu *et al*., 2009a). PVY^{N-Wi} has also a great variability at the molecular level, especially in the 5' UTR (untranslated region) - P1 region resulting three lines (Chachulska *et al*., 1997; Glais *et al*., 2002; 2005). The first contains isolates that are similar to PVY^{N} at the 5' UTR - P1 end, the second includes isolates that are similar to PVY^{O} in the sequence of P1 and the third contain one isolate having a sequence PVY^{O}-like in the 5' UTR - P1 region. Despite of this heterogeneity in the region P1 of PVY^{N-Wi} the rest of the genome is similar, with that of PVY^{O} serotype. This heterogeneity resulted from the recombination at the C-terminus of HC-Pro region. To some extent there are similarities between the sequences of PVY^{N-Wi} and PVY^{NTN}, but unlike to the first one, in the case of PVY^{NTN} the recombination occurred at several points in the genome (Boonham *et al*., 2002; Glais *et al*., 2002). Almost similar with PVY^{N-Wi} (of European origin) is the American form noted $PVY^{N:O}$ (Singh *et al*., 2003; Nie and Singh, 2003 b) but this one is supposed to be derived by recombination with a different line of PVY^{O} compared with the European form (Karasev *et al*., 2011).

A different PVY^{NTN} isolate, Tu 660, originating from North America called NA-PVY^{NTN} is believed to have resulted by mutagenesis and not by recombination (Nie and Singh, 2003 a). Isolates with similar pathotype were reported in Japan also (Ohshima *et al*., 2000) but a later analysis revealed that those were non-recombinant PVY^{NTN} variants as well (Ogawa *et al*., 2008). An isolate of PVY having similarities with the European PVY^{NTN} recombinant form, induce HR in Maris Bard cultivar carrying the putative *Nz* gene, thus being classified in the PVY^{Z} group being called $PVY^{Z\text{-}NTN}$. Although it is capable of inducing PTNRD similarly to PVY^{NTN}, the TVN was not associated with it (Kerlan *et al*., 2011). It still have the molecular determinants in the HC-Pro (K-400 and E-419) that have been considered responsible for inducing of the TVN phenotype (Tribodet *et al*. 2005), but the substitution of a single amino acid (D-205 to G-205) was correlated with loss of this phenotype, so the strain causes only mosaic symptoms and mild vein clearing in tobacco rather than TVN characteristic to the PVY^{NTN} strain (Hu *et al*., 2009 b). In Syria, there were detected recombinant forms of PVY which show both genomic and biological features intermediate between PVY^{NTN} and $PVY^{N\text{-}Wi}$ (Chikh Ali *et al*., 2007 a) named PVY-SYRIII and $PVY^{NTN\text{-}NW}$ (Chikh Ali *et al*., 2010 a). These strains present three recombinant junctions HC-Pro/ P3, 6K2/ VPG, NIb/ CP, but at the 3' end the recombination junction occurs at different nucleotides resulting at least three different viral subtypes. All variants, excepting one who wasn't tested on potato yet, produce PTNRD and TVN but have O serotype (Chikh Ali *et al*., 2010 a).

The emergence of the new PVY forms, some of them presenting several recombination patters between the parental lines and inducing new phenotypes in the host plants impose an improved PVY classification which has to include serological and molecular criteria along to the first classification criteria which are based mainly on the presence or not of the HR and TVN response in the indicator plants (review of Singh *et al*., 2008; Karasev and Gray, 2013; Kehoe and Jones, 2015).

Biological assay

The main indicator plants for the presence of PVY are *Nicotiana tabacum* (Samsun varieties, NC95, NC in 2326, Burley 21), *Lycopersicon esculentum* (cv. Sheyenne), *Capsicum frutescens* (cv. Calwonder), *Solanum tuberosum*, *S. demissum*, *Physalis floridana*, *Physalis angulata*, *Chenopodium amaranticolor*, *C. quinoa* (McDonald and Kristjansson, 1993). The general symptoms induced in the indicator plants are vein clearing, vein necrosis, mottle, mosaic, interveinal necrosis, leaf drop, systemic necrosis or cupping of uninoculated leaves (McDonald and Kristjansson, 1993).

The symptoms induced in various indicator plants may give a clue about the viral strain involved. For instance, PVY^{O} and PVY^{C} induce local lesions in *C. amaranticolor* but this remains asymptomatic to the infection with PVY^{N} (Yin *et al*., 2012). For the detection of PVY necrotic forms (PVY^{N}) different species and cultivars belonging to

Nicotiana genus are used as indicators. *N. tabacum* cv. White Burley shows symptoms after 10-14 days, in the *Nicotiana affinis* cv. Lime Green the first symptoms appear after 5-10 days (Rose et al., 1987) but also *N. tabacum* cv. Samsun (10-21 days symptoms appearance) or Xanthi, can be used (Singh and Singh, 1994). *S. brachycarpum* is also a good differentiator between different PVY strains, being affected by necrosis when inoculated with PVY^{N} and mosaic in the case of infection with PVY^{O}, giving a relatively fast reaction (7 - 10 days) and offering the advantage of a lack of interference with PVX if a mixed infection do exist (Singh and Singh, 1994).

Of a particular importance for PVY indexing are the potato varieties that possess known resistance genes such are Désirée, Pentland Crown, Delikat, Maris Bard, Pentland Ivory, Allegany having *Ny* gene, Pentland Ivory or Maris Bard with *Nc*, *Ny* and *Nz* genes or King Edward, Maris Bard, Pentland Ivory, Eersteling having *Nc* gene (review by Singh *et al*., 2008; Blanco-Urgoiti, 1998 b; Kerlan *et al*., 1999; Baldauf *et al*., 2006; Chikh Ali *et al*., 2014). Depending on the resistant gene the HR appears at the interaction with specific PVY strains (Table 1). The cultivar Eva has $Ry_{adg,}$ an extreme resistance gene (derived from *S. tuberosum* subsp. andigena) that confer resistance to all the PVY strains (Baldauf *et al*., 2006) and also the Sante cultivar having the resistance gene Ry_{sto} form *S. stoloniferum* (Mehle *et al*., 2004). On the contrary, Atlantic and NY115 cultivars have no resistance to any form of PVY and may serve as susceptible controls (Baldauf *et al*., 2006).

Although important for detection and PVY indexing the bioassay tests are not infallible. Very often different strains can induce atypical phenotypes in the indicator plants. The PVY^{N} usually does not induce local lesions in *C. amaranticolor* but there are isolates that can produce these lesions (Yin *et al*., 2012). Also, the North American correspondent for PVY^{N-Wi}, $PVY^{N:O}$, usually do not cause tuber necrosis but this affection was observed under the form of an atypical PTNRD (Piche *et al*., 2004) and the uncommon symptoms were spotted later by Baldauf *et al*. (2006). Another situation is of PVY^{NTN} isolate L26, which despite of the fact that manifest an N positive serotype cannot induce TVN (Hu *et al*., 2009 b).

The biological assay can thus be inconclusive due several reasons such are the conditions in the greenhouse (Baldauf *et al*., 2006), the existence of mixed natural infections (Damirdagh and Ross, 1967), the possible contamination during analysis (Rose *et al*., 1987) or to the occurrence of new isolates that can give atypical reactions in the indicator plants (Baldauf *et al*., 2006).

Detection by immunological methods (ELISA)

As a routine, the first indicators of PVY infection are the symptoms usually present in the host. Clark and Adams (1977) established the microplate method of enzyme-linked immunosorbent assay (ELISA) for virus detection proving its versatility for a series of viruses. The method was adapted for PVY detection in potato as well.

First, there were established polyclonal antibodies PVY specific but very often the ELISA contradicted the bioassay results giving false positive or negative results and raising questions about its reliability (Rose *et al*., 1987). Subsequently, the production of a large number of more and more improved sets of monoclonal antibodies (Rose and Hubbard, 1986; Sanz *et al*., 1990; Ellis *et al*., 1996; 1997; Cerovska, 1998) and the application of different ELISA variants (triple antibody sandwich-ELISA, direct and indirect double antibody sandwich enzyme-linked immunosorbent assay, nitrocellulose membrane - ELISA) (Gugerli and Fries, 1983; Rose and Hubbard, 1986; Ohshima *et al*., 1990; Sanz *et al*., 1990; Ellis *et al*., 1996) lead to a significant increase in sensitivity and specificity being possible to distinguish between different groups of strains (Rose *et al*., 1987; Ellis *et al*., 1997; Blanco-Urgoti *et al*., 1998; Lizarraga and Fernandez-Northcote, 1989; Lorenzen *et al*., 2006). The monoclonal antibodies that give the best results in the tests were included in commercially available kits (Bioreba, Agdia, SASA, Neogen). ELISA has become a routine method for PVY detection (Nolte *et al*., 2008), now being available even immuno-strips that simplifies the test, enabling their execution in field (Halterman *et al*., 2012). By using monoclonal antibodies the serotype of PVY isolate can be determined. Accordingly with Ellis *et al*., (1997) a serotype represents a "subclass of the viral strain group with distinguishable antigenic specificities" and although the classification in strains and serotypes is arbitrary they contribute to a better characterization of the PVY isolates.

ELISA can be performed directly at the tuber level, a positive reaction being obtained after breaking the dormancy of the tubers while in the dormant state the PVY titer can be too low, sometimes under the limit of detection and leading to false negative results (Gugerli and Gehringer, 1980; De Boks and Cuperus, 1987; Barker *et al*., 1993). As a consequence, the North American Plant Protection Organization (NAPPO) and European and Mediterranean Plant Protection Organization (EPPO) which implemented ELISA tests as a mandatory step in the seed certification protocol, strongly recommend the utilization in the assays of sprouts and leaves from plants grown from sample tubers instead of using directly the sap from tubers in order to ensure a high concentration of virus and to prevent the false negative results.

However, the use of ELISA has certain limitations, by this method is not possible, for example, the differentiation of PVY^{O} from the necrotic one PVY^{N-Wi}/ $PVY^{N:O}$ (Crosslin *et al*., 2005; Lorenzen *et al*., 2006). Also, PVY^{N} and PVY^{NTN} cannot be distinguished as separate strains be ELISA (Rigotti and Gugerli, 2007; Karasev *et al*., 2010). The emergency of new recombinant isolates, other than PVY^{N-Wi} that induce TVN but not react with expected N specific monoclonal antibodies (Galvino-Costa *et al*., 2012) raises serious concerns for the efficiency of immunological analysis. A reverse situation was encountered in the case of PVY^{O5} which although have a typical PVY^{O} genotype and pathotype reacts with 1F5, a PVY^{N} specific monoclonal antibody, having apparently N serotype (Karasev *et al*., 2010). This confusion is due probably to an amino acid substitution in the CP that results in an

epitope recognized by 1F5. Luckily another N specific monoclonal antibody, SASA-N, gives the expected reaction assigning the strain to the adequate serotype, which is O (Karasev *et al*., 2010).

PVY detection/ indexing by molecular methods

The prerequisite for molecular detection of PVY is the unrestricted access to a database meant to provide the informative support. This starting point has become more and more solid, complete or partial genome sequences being available for several PVY isolates in the National Center for Biological Information (NCBI) GenBank repository. New powerful methods may be used in perspective for PVY sequencing. The next generation sequencing (NGS) which allows the whole genome sequencing became sensitive enough to identify different sequences of substrains of the same virus from mixed infections (Kehoe and Jones, 2015). Now, that it becomes more and more financially accessible it is predictable that it will be extensively used for finding the sequence of new isolates, the only limitation of the method being the huge amount of data resulted after analysis that requires a high amount of time for processing (Kehoe *et al*., 2014).

The molecular detection was a continuous process that was mainly based on PCR (Polymerase Chain Reaction) method which has to be constantly adapted to the detection of more and more PVY isolates and to assign them to the proper strain group. For the pre-PCR preparations, obtaining of a high quality RNA in regard to integrity, quantity and purity was a struggle but step by step all the drawbacks were addressed and overcome. Nowadays, this is no longer a problem regardless of the starting plant tissue (leaves or tubers), an accurate identification being possible using sap of dormant potato tuber (Agindotan *et al*., 2007), RNA extraction kits or ready to use reagents being now available (Chikh Ali *et al*., 2007 a; Kogovsek *et al*., 2008; Gawande *et al*., 2011; MacKenzie *et al*., 2015). The purification of the virus is no longer a requirement for an accurate indexing (Lorenzen *et al*., 2006 a). Of equal importance, the reverse transcription (RT) step was optimized in regard to the chosen PCR protocol, by using PVY specific primers (Glais *et al*., 1996: Singh *et al*., 1998 b; Weilguny and Singh, 1998; Moravec *et al*., 2003; Lorenzen *et al*., 2006 b) or random hexamers (Nie and Singh, 2001; 2003 b) and/ or oligo(dT) (Nie and Singh, 2000; 2003 b; Chikh Ali et al., 2010 b) in one step (Rigotti and Gugerli, 2007; Crosslin *et al*., 2005; Kogovsek *et al*., 2008) or two steps RT-PCR approaches (MacKenzie *et al*., 2015).

The first concern was to distinguish PVY among several other viruses infecting potato (Potato virus Y, Potato leafroll virus, Potato virus A, Potato virus X, Potato mop-top pomovirus, Tobacco rattle tobravirus, etc), but this wasn't such a heavy task due to the high degree of genetic variability among the species, several methods being established (Crosslin and Hamlin, 2011) including multiplex qPCR (quantitative PCR)

based ones (Boonham *et al*., 2000; Agindotan *et al*., 2007). The greatest fight was made for an accurate PVY detection/ indexing within the PVY clades and in this review the most significant molecular methods will be highlighted. For the purpose of developing methods that in perspective may be applied at an industrial level some criteria have to be accomplished, they have to be efficient in terms of time and costs requirements but without compromising the high sensitivity and specificity.

In the first approaches for PVY detection/ indexing the PCR was coupled with RFLP (Restriction Fragment Length Polymorphism) technique. Using the 5' end of PVY genome sequence, including the UTR and the P1 protein and several restriction enzymes (Taq I, Ava H and Hinc H) the assignation of o series of PVY isolates to the common PVY^{N} or PVY^{NTN} groups was accomplished (Glais *et al*., 1996). Later, more specific enzymes were found for the differentiation between common necrotic PVY forms and PVY^{NTN} (Rosner şi Maslenin, 1999) or between North American and European PVY^{NTN}, including the non-recombinant PVY isolate (Nie şi Singh, 2002 a). Blanco-Urgoiti *et al*., (1996), using available sequences of CP gene, pointed out the equal importance of RFLP in genotyping of the PVY strains. The term "restrictotype" was proposed, suggesting that the pattern resulted by RFLP can be a used for the calculation of genetic distance as a viable alternative to the classification of PVY based on sequence comparison (Blanco-Urgoiti *et al*., 1996). Although precise and bringing significant steps forward in PVY indexing the RFLP technique is considered expensive and time-consuming imposing the need to find more simple and fast ways for PVY identification (Rigotti and Gugerli, 2007).

Some studies focused on finding a specific way of identification of a particular strain of PVY while others, using combinations of primers in individual or in multiplex reactions offered solutions for indexing of a series of PVY strains or substrains. The most challenging but extremely important is the detections of the recombinant substrains or pathotypes of PVY, especially of the PVY^{NTN} which affects the tubers quality and is lately arising with an increased frequency worldwide (Weidemann and Maiss, 1996; Karasev and Gray, 2013).

Several methods were established for PVY^{NTN} indexing by RT-PCR. Targeting P1 protein using three-primer combination suitable methods were developed to differentiate detection of PVY^{NTN} of European origin and PVY^{N} (Weidemann and Maiss, 1996; Weilguny and Singh, 1998; Singh *et al*., 1998 b). Nie and Singh (2002 a) have shown, by sequencing the 5' region (comprising 5'- UTR and P1 cistron) of PVY^{N} and PVY^{NTN} from Europe and North American that the PVY^{N} and PVY^{NTN} clustered better together if they originate from the same region. A three-primer combination was established for specific differentiation of NA-PVY^{NTN} from EU-PVY^{NTN} (Nie and Singh, 2002 a). This primer set was adopted by North American Plant Protection Organization (NAPPO, 2011) for differentiation of PVY^{NTN} by other forms of necrotic PVY, EU-PVY^{NTN} including. Later, the method was improved by optimization of a multiplex PCR allowing simultaneous detection of strains or substrains of PVY

from any combination of PVY^{O}, EU-$PVY^{N/NTN}$, NA-PVY^{N} and NA-PVY^{NTN} (Nie and Singh, 2002 b). The recombination points within the CP region were also used as a marker for PVY^{NTN} indexing. By performing 4 sets of PCR primer mixes in a competitive PCR combined with mutagenically separated PCR the synthesis of enhanced specific bands was promoted leading to the discrimination between PVY^{NTN}, PVY^{O}, PVY^{N}, and PVY^{C} (Boonham *et al*., 2002). Moravec *et al*. (2003) adopting a three-primer strategy were able to differentiate recombinant PVY^{NTN} among the other PVY variants, targeting the CP region as well.

Exploiting the recombinant point within the HC-Pro/P of the PVY genome a RT-PCR reaction was designed for specific detection of PVY^{N-Wi} strain, from mixed PVY^{NTN} / PVY^{N-Wi} infections (Glais *et al*., 2005). This method is fast and can be successfully used for potato seed certification being officially applied for this purpose in France (Glais *et al*., 2005). Nevertheless, the protocol requires care, a 4114 pb product being obtained during amplification, and therefore, for preventing the RNA degradation the RT-PCR must be performed immediately after RNA isolation (Glais *et al*., 2005). For the differentiation of $PVY^{N:O}$ and PVY^{NTN} a triplex PCR was established, in which, based on the recombination sites within the PVY genome, one fragment is obtained for $PVY^{N:O}$ and three bands were amplified for PVY^{NTN} (Nie and Singh, 2003 b). The functionality of this method was validated in assays on isolates of different geographic origin and allowed the discovery of new type of isolates with an intermediate number of recombination points (Chikh Ali *et al*., 2007 a).

The most promising PCR based methods are those who facilitate the detection/ indexing of several strains or substrains in one multiplex reaction. Although multiplex methods for discriminating between PVY isolates were developed (Nie and Singh, 2002 b; 2003 b) some weaknesses were observed in identification from strain mixes from within the necrotic group (PVY^{N}, PVY^{NTN}, $PVY^{N:O}$, NA-PVY^{N} or NA-PVY^{NTN}) (Lorenzen *et al*., 2006 b). A touchdown multiplex PCR method in which eight different primers (Table 2) can be combined in different ways allows the detection of the main PVY strains (Lorenzen *et al*., 2006 b). Further, for the specific indexing of $PVY^{N:O}$ type A and B and for NA- and EU-$PVY^{N/NTN}$ discrimination specific sets of primers were in addition designed (Lorenzen *et al*., 2006 b). This became one of the methods extensively use for PVY indexing worldwide (Lorenzen *et al*., 2008; Chikh Ali *et al*., 2010 b; Karasev *et al*., 2010; Crosslin and Hamlin, 2011). In Europe, another method is that of Rigotti and Gugerli (2007) who made possible the detection of PVY^{N}, PVY^{O}, PVY^{NTN} (recombinant types), PVY^{N-Wi} and PVY^{C} in one-step triplex PCR (Table 2). One of the most comprehensive multiplex RT-PCR based detection (Table 2) developed by Chikh Ali *et al*., (2010 b) and further improved by coupling it with immunocapture instead of RNA extraction (Chikh Ali *et al*., 2013 b) allows the accurate detection of the main strains and substrains of PVY [PVY^{O} (both PVY^{O} and $PVYO^{O5}$), PVY^{N}, NA-PVY^{N}, PVY^{NTN}, PVY^{Z}, PVY^{E}, PVY-NE11, PVY^{N-Wi} and $PVY^{N:O}$].

Table 2.

Primers (after Lorenzen *et al*., 2006 b; Rigotti and Gugerli, 2007; Chikh-Ali *et al*., 2010 b) that can work in multiplex PCR for detection/ indexing of the main strains and substrains of PVY

Primer name	Sequence (5' - 3')	PCR results, amplicon/ strain
o2172	CAACTATGATGGATTTGGCGACC	181/ NTN, N:O
n2258	GTCGATCACGAAACGCAGACAT	267/ O
o2439c	CCCAAGTTCAGGGCATGCAT	398/ N
n2650c	TGATCCACAACTTCACCGCTAACT	328/ N,NA-N/NTN
n5707	GTGTCTCACCAGGGCAAGAAC	452/ NTN
o6266c	CTCCTGTGCTGGTATGTCCT	689/ N:O,O
S5585m	GGATCTCAAGTTGAAGGGGAC	
A6032m	CTTGCGGACATCACTAAAGCG	
PVYc3	CAACGCAAAAACACTCA(CT)AAA(AC)GC	440+1110/ N
PVYf	TAAGTG(AG)ACAGACCCTCT(CT)TTCTC	440+1110/ NTN(nR)
PVY3+	TGTAACGAAAGGGACTAGTGCAAAG	440/ NTN (R)
PVY3−	CCGCTATGAGTAAGTCCTGCACA	660+530/ O
CP2+	CCAGTCAAACCCGAACAAAGG	530/ Wi (Wi-P)
CP1−	GGCATAGCGTGCTAAACCCA	440+530/ Wi (N242) 660/ C
n156	GGGCAAACTCTCGTAAATTGCAG	853+532/ O
o514	GATCCTCCATCAAAGTCTGAGC	1307/ NA-N
n787	GTCCACTCTCTTTCGTAAACCTC	853+441/ NW(B)
n2258[a]	GTCGATCACGAAACGCAGACAT	1307+633+441/
o2172[a]	CAACTATGATGGATTTGGCGACC	NTN(A)
n2650c[a]	TGATCCACAACTTCACCGCTAACT	1307+441/ NTN (B)
o2700	CGTAGGGCTAAAGCTGATAGTAG	1307+633+441/
S5585m[a]	GGATCTCAAGTTGAAGGGGAC	NTN(A)
o6400	GTAACTCCTAAACAAATGGTGGTTCG	1076+633+441/ NTN-
n7577	ACTGCTGCACCTTTAGATACTCTA	NW ($^{SYR-I}$)
YO3-	CTTTTCCTTTGTTCGGGTTTGAC	1076 + 441/ NTN-NW
8648[b]	GTTTCTCCTATGTCGTATGCAAGTT	($^{SYR-II}$)
SeroN[c]		1076+441+278/ SYRIII

The need of developing detection protocols fast and safe, suitable for high throughput applications, lead to establishment of a series of methods by incorporating fluorescent dyes into the PCR assays. One of the first such methods was carried out by Walsh *et al*., (2001) who used competitive fluorescent RT-PCR assay for the differentiation of PVY^O and PVY^N. The great advantage of using fluorescent dyes lies in the possibility of performing so called "one-tube reactions" in which the detection can be made directly, no electrophoresis being required, advantage that was not provided by the pioneering method (Walsh *et al*., 2001). Later, by applying

real time PCR methods (qPCR) this disadvantage was overcome. The SNaPshot assay combined with TaqMan technology was used for discrimination between PVY^{O} and PVY^{N} (Jacquot *et al*., 2005) or PVY^{N}, PVY^{O}, PVY^{N-Wi} and PVY^{NTN} (Rolland *et al*., 2008). The high sensitivity of the methods based on fluorescent quantitative assays, regardless of the fact that TaqMan (Balme-Sinibaldi *et al*., 2006; Kogovsek *et al*., 2008), SYBR green (Hühnlein *et al*., 2013; Zhang *et al*., 2015) or multiplex qPCR (quantitative real time PCR) based on EvaGreen dye (Cheng *et al*., 2012) are involved, was pointed out during the assays, these being considered powerful tools for future PVY detection.

From the same category of the high throughput methods of detection highlighted above is the microarray test which is based on the hybridization of a probe attached to a solid surface with a target DNA (cDNA, in the case of PVY detection) which is labeled with a fluorescent dye. Its functionality was proven for the detection of several strains of PVY (Boonham *et al*., 2003). Although extremely powerful, the use of this method to a higher scale is restricted by the high costs associated to the fixation of the probe on the substrate, the purchasing of reading instruments and reagents and the maintenance procedures (Agindotan and Perry, 2007). The development of a more simple method, named macroarray (Agindotan and Perry, 2007) combined with the possibility of using synthetic probes (Bystricka *et al*., 2005; Sip *et al*., 2010) opened the way for using this procedure, in perspective, for the routine PVY detection/ indexing.

Another direction in the developing pathogen detections ways goes toward finding of easy to perform methods that can be applied directly in the field. The loop-mediated isothermal amplification (LAMP) assay, developed by Notomi *et al*., (2000), apparently qualifies for on-the-spot tests for pathogen detection (De Boer and Lopez, 2012). The method can be a substitute for PCR presenting the huge advantage of being an isothermal reaction that don't requires a termocycler and can be performed in one-tube, the versatility of the method depending only on the finding of the suitable primers. Such a method was settled for the detection of one PVY strain but although it meets all the requirements for sensibility and sensitivity the possibility of being performed in the field remains a problem to be solved (Przewodowska *et al*., 2015).

Conclusions

There has to be a continuous research activity, meant to keep up with the PVY evolution, which has to provide for the Agricultural Scientific Services reliable upgraded methods, including biochemical and molecular materials (monoclonal antibodies and specific primer sequences) for an accurate virus detection/ indexing in the continuous process of seed potato certification and quarantine. Although each assay, biological, immunological and molecular, independently offers valuable data

for PVY isolate characterization, the complete picture of a new emerged isolate that often results in a new classified strain, relies on an integrative view of the results of the combined analysis. If we refer to the main issue of the whole PVY related problem, which is the rapid detection/ indexing, the molecular methods approaches have become increasingly important and in the near future the implementation of their application in the routine screening will be mandatory due to the high variability of PVY for which accurate detection by ELISA simply will be not enough.

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