eISSN 1981-1829

Ciência e Agrotecnologia
Science and Agrotechnology

Quantification of two *Potato virus Y* strains in single and mixed infections by RT–qPCR highlights its epidemiological landscape in Brazil

Quantificação de duas estirpes do *Potato* vírus Y em infecções simples e mistas por RT-qPCR realça o seu panorama epidemiológico no Brasil

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ABSTRACT

Potato virus Y (PVY) is recognized as one of the most common and destructive pathogens seriously affecting potato producing areas worldwide. More recently PVY^{NTN} and PVY$^{N:O/N-Wi}$, have emerged as the main strains present in the PVY infected plants detected in Brazilian potato fields. In this study, samples of potato collected in south part of Minas Gerais – Brazil were first tested by DAS-ELISA and then by RT-PCR multiplex in order to discriminate the PVY strains. Afterward, part of them was tested by RT–qPCR to confirm and quantify the viruses in infected tissues. The sensitivity of the techniques for detecting PVY isolates present in the sampled locations was investigated, as well as the occurrence of mixed infections, aiming to understand the general epidemiological picture of this pathogen in potato producing fields. In the multiplex RT-PCR test, the samples with O and N serotypes were identified as infected with PVY^{NTN} and PVY$^{N:O/N-Wi}$ strains. When tested by RT-PCR for amplification of PVY^{E}, 41 samples (67,2%) were positive, having a characteristic electrophoretic profile for this recombinant strain, and 9 isolates were also observed with atypical patterns for recombinant PVYE. The best technique to detect mixed infection was RT-qPCR, with the concentration of PVYNTN being much higher than that of PVYN:O/N-Wi. These results show the importance of using the most suitable method for the diagnosis and surveying of PVY strains in crop fields and reveal, for the first time, the dissemination of PVYE recombinants to several Brazilian potato fields.

Index terms: TAS-ELISA; Potyvirus; PVY; PVY^{E}.

RESUMO

O *Potato* vírus Y (PVY) é reconhecido como um dos patógenos mais comuns e destrutivos que ocorre nas áreas produtoras de batata em todo o mundo. Recentemente a PVY^{NTN} e a PVY$^{N:O/N-Wi}$ emergiram como as principais estirpes presentes nos campos de batata brasileiros. Neste estudo, amostras de batata provenientes do Sul de Minas Gerais foram testadas inicialmente por DAS-ELISA e RT-PCR multiplex para discriminar as estirpes de PVY. Posteriormente, parte deste material foi testada por RT-qPCR para confirmar e quantificar os vírus nos tecidos infectados. Foi investigada a sensibilidade das técnicas para detecção de isolados de PVY presentes nos locais amostrados, bem como a ocorrência de infecções mistas, visando entender o quadro epidemiológico geral deste patógeno nos campos produtores de batata. No teste RT-PCR multiplex, as amostras com sorotipos O e N foram identificadas como infectadas pelas estirpes PVY^{NTN} e PVY$^{N:O/N-Wi}$. Quando testadas por RT-PCR para amplificação de PVY^{E}, 41 amostras (67,2%) foram positivas, mostrando o perfil eletroforético característico para esta estirpe recombinante, e 9 amostras mostraram padrões atípicos para PVY^{E} recombinante, mostrando a variabilidade dos isolados de PVY que ocorrem no campo. A melhor técnica para detectar infecção mista foi RT-qPCR, sendo que a concentração de PVY^{NTN} foi muito maior que a de PVY$^{N:O/N-Wi}$. Esses resultados mostram a importância da utilização de método mais adequado para o diagnóstico e levantamento de estirpes de PVY em lavouras de batata e revelam, pela primeira vez, a disseminação do recombinante de PVYE para diversos campos de batata brasileiros.

Termos para indexação: TAS-ELISA; Potyvirus; PVY; PVY^{E}.

Introduction

Potato virus Y (PVY) is currently considered the main virus present in potato crops worldwide (Chikh-Ali et al., 2020; Green et al., 2020; Kreuze et al., 2020; Torrance & Taliansky, 2020; Rashid et al., 2021). Great diversity in PVY has been observed and results mainly from genomic recombination among virus strains, which may be related to the occurrence of mixed infections with two or more strains. During the viral replication process, new recombinant events may occur and generate virus isolates carrying adaptive advantages in their survival and ease of spread by vectors in the field (Galvino-Costa et al., 2012a; Bai et al., 2019; Mao et al., 2019; Green et al., 2020; Gao et al., 2020, Bhoi et al., 2022).

PVY presents a complex of strains in practically all potato-producing regions worldwide, and based on its genomic structure,

Agricultural Sciences

Ciênc. Agrotec., 48:e006024, 2024
http://dx.doi.org/10.1590/1413-7054202448006024

Editor: Renato Paiva

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Received in March 26, 2024 and approved in June 25, 2024

the virus can be classified into nonrecombinant (PVY^{O}, PVYC, and PVY^{N}) and recombinant (PVY^{NTN}, PVY^{N-Wi}, $PVY^{N:O}$, PVY^{E}, PVY^{NE-11}, PVY^{NA}, $PVY^{NA-N/NTN}$, PVY^{ZNTN} and PVY^{NTN-NW}) strain groups (Nie et al., 2012; Galvino Costa et al., 2012a; Karasev & Gray, 2013; Green et al., 2020; Kuzmitskaya et al., 2021, Dupuis et al., 2024). Nucleotide sequence and genomic recombination analyses can distinguish these different recombinants by comparing their sequences with the parental PVY^{O} and PVY^{N} sequence types. In Brazil, the main tool used in routine tests to detect the different recombinants is still conventional RT–PCR using specific primers in a multiplex reaction (Nie & Singh; 2002; Lorenzen et al., 2006a; Galvino-Costa et al., 2012b). Thus, the diversity of Brazilian PVY has not yet been completely described, considering that only a few PVY isolates have been fully sequenced. Therefore, additional evaluation of the types of recombinants present in this country is necessary.

In temperate countries, cold seasons limit the vector population and interrupt its cycle, thus allowing the reduction of PVY inoculum potential available in the field for the next annual potato crop. In tropical countries, such as Brazil, the reproductive cycles of aphids are not broken, and potatoes can be grown three times a year. In addition, there are a great number of alternative hosts that can act as sources of PVY strains in the field; these strains can be rapidly and efficiently spread in a nonpersistent manner by several aphid species between consecutive crops (Syller & Grupa, 2014; Bhoi et al., 2022). Taken together, these phenomena have led to an increase in the incidence of single and mixed infections, which likely contributes to the emergence of new recombinants (Syller & Grupa, 2014, Onditi et al., 2022, Tran et al., 2022).

As there is no possibility of recover a PVY-infected plant, control of this virus is a very difficult task, and preventive measures must be taken. Reliable detection methods with high sensitivity are required to detect the different PVY strains present in both single and mixed infections (Polder et al., 2019, Rubio et al., 2020; Ji et al., 2020). These methods should support producers in deciding when to eliminate potato seeds carrying high infection levels, thus avoiding greater losses in the next generation. Some misidentifications of PVY isolates and errors in serological and molecular tests have resulted in reduced disposal of contaminated potato seeds by potato growers. PVY-infected Agata, the most commonly planted potato cultivar in Brazil, does not show significant leaf and tuber symptoms, acting as a silent source of inoculum in the field. This lack of symptoms increases the importance of diagnostic tests (Galvino-Costa et al., 2012b).

Many RT–PCR tests specific for PVY strains reported in other countries commonly require constant updating due to the growing number of new variant strains. The frequent occurrence of recombination events in the PVY population creates many challenges for plant pathologists, both in the accuracy of the identification of these strains and in forecasting their impact on plant yield. Because of the large variation in the symptoms caused by the different recognized PVY strains, the use of specific diagnostic techniques that can be applied to rapid diagnostic tests on a large scale is crucial to determine the effect of the virus in the field.

In Brazil, a few studies have been conducted to identify the predominant PVY strain in potato fields. Therefore, the sensitivity of DAS- and TAS-ELISA, RT–PCR, and RT–qPCR methodologies was compared in this work. The goals were to check the accuracy of these tests to detect the two main PVY strains present in Brazilian fields and to determine the proportion of single and mixed infections in different potato crops.

Material and Methods

Viral isolates: Origin and maintenance

The potato samples were collected from lots of seed potatoes initially collected in a random manner by seed potato growers who submitted tubers and leaves for postharvest testing to a certificate laboratory at the Federal University of Lavras– UFLA (Virus Indexing Center - CIV/MG), and these were considered random samples. The category of seed potatoes samples were basic G3 and first generation certified C1, according to Brazilian IN Nº 32, from November, 2022.

The seed potato lots came from fields located in southern and western (also called Alto Paranaiba region) areas of Minas Gerais state, which is the largest Brazilian potato-producing region. As part of the certification process, the lots were first submitted to DAS-ELISA (double sandwich antibody – enzyme-linked immunosorbent assay) using polyclonal antisera from Agdia to detect the presence of the viruses PVY, PVX, PVS and PLRV according to the manufacturer's instructions.

The tubers and leaves that tested positive for PVY and negative for PVX, PVS and PLRV were selected to be employed in this study: 39 leaves of potato plants collected at a near-senescence stage from São Gotardo (western region); 53 tubers from Ouro Fino; 52 tubers from Maria da Fé; 10 tubers from Cristina (from the southern region) and 11 tubers from Lavras marketing (unknown origin), resulting in a total of 165 samples. The isolates used as positive controls in all tests performed were LUI-AGA and PED-AX, which belong to the $PVY^{N:O/N-Wi}$ and PVY^{NTN} strains, (Galvino-Costa et al., 2012a). Both standard isolates are part of the UFLA Plant Virology Laboratory Collection; they were maintained lyophilized and recovered in tobacco plants by mechanical inoculation.

To standardize the positive control for mixed infection, the two strains were mechanically inoculated together in tobacco plants cv. Turkish NN using equal proportions of each isolate (LUI-AGA (PVY^{NTN}) + PED-AX ($PVY^{N:O/N-Wi}$). For this purpose, the leaf extract of infected tobacco plants cv. Turkish NN, obtained by maceration in a mortar in the presence of 0.01M phosphate buffer containing sodium sulfite at the same molarity,

was rubbed on the recipient test plant previously sprayed with carborundum. After washing the leaves, all plants were grown in an insect-proof greenhouse before and after inoculation under natural light throughout the experiment. The infected plants were analyzed by DAS- and TAS-ELISA, multiplex RT–PCR and RT–qPCR approximately 3-4 weeks after inoculation.

Evaluation of symptoms in plants

Potato tubers infected with PVY were established in pots with a capacity of 4 kg containing sterilized substrate in an insect-proof greenhouse. After the emergence of leaves, the plants were evaluated for the presence of symptoms. The type and intensity of predominant symptoms observed in the plants grown from the infected tubers was recorded 6-8 weeks after planting. Each plant was individually evaluated by comparing the severity of the viral symptoms and classifying the intensity as light (+), mild (++) and severe (+++). The tubers obtained from these original infected potato plants were kept and periodically multiplied to maintain the original inoculum.

ELISA tests

The DAS-ELISA (Clark & Adams, 1977) to determine the sanity for seed potato samples were applied to tuber sprouts that were 1 cm to 2 cm long. The TAS-ELISA (triple antibody sandwich – enzyme-linked immunosorbent assay) tests were performed in the potato leaves originated from tubers analyzed by DAS-ELISA, planted in pots at the greenhouse conditions. They were performed using antibodies from different origins to ensure the detection of strains. In one test, antisera from Agdia were employed: the polyclonal antibody UID8 and two monoclonal antibodies, MAb2, which detects PVY^{O}, $PVY^{N:O}$, $PVY^{N:O/N-Wi}$ and PVY^{C} (Mcdonald & Kristjansson, 1993), and IF5, which detects PVY^{N}, PVY^{O-O5}, PVY^{NTN} and PVY^{E} (Karasev et al., 2010). In the other test, the polyclonal antibody G500 and the monoclonal antibodies SASA-O, which is specific for PVY^{O}, $PVY^{N:O}$, $PVY^{N:O/N-Wi}$ and PVY^{C}, and SASA-N, which is specific for PVY^{N} and PVY^{NTN} were employed. In both tests, the methodology described by the manufacturer was used with some modifications, as reported by Karasev et al. (2010). The ELISAs were always conducted in duplicate.

Total RNA extraction

RNA was extracted from each of the 165 collected samples: 39 leaves from São Gotardo and also leaves of the remaining 126 plants originated from tubers of other municipalities. The RNA extraction followed the Trizol method, described by Shi & Bressan (2006). The total RNA extracted was analyzed on a 0.7% agarose gel and contrasted with Red Nucleic Acid Gel Stain (Biotium) before being used in the subsequent experiments to evaluate the particle integrity. The assessment of RNA purity was performed by analyzing the 260/280 and 260/230 ratios obtained by NanoVue Plus™ (GE healthcare) equipment. Total RNAs showing good integrity and purity were aliquoted and stored at -80 °C for subsequent use in RT–PCR and RT–qPCR tests.

Singleplex and multiplex RT–PCR

The multiplex RT–PCR described by Lorenzen et al. (2006a) was used to classify all 165 PVY isolates and to check for possible mixed infections. The technique employed a set of eight primers combined with a single multiplex RT–PCR test to differentiate PVY^{O}, PVYN, $PVY^{N:O/N-Wi}$, $PVYNT^{N}$, and PVY^{NA-N}, or mixtures of these strains. An additional single-plex RT–PCR using a pair of specific primers that differentiate PVYE from PVY^{NE-11} and ordinary PVY^{NTN} isolates was used, as described by Galvino-Costa et al. (2012a). Reverse transcription was performed as described by Galvino-Costa et al. (2012b), using the Superscript II Reverse Transcriptase (Invitrogen) and the Oligo DT primer (Promega). The DNA amplification was done using the enzyme Taq High Fidelity Pol (Cellco Biotec do Brasil Ltda, São Carlos, SP, BRA).

Taqman® probe and primer design

Perform the RT–qPCRs, specific probes were designed to identify PVY^{NTN} and $PVY^{N:O/N-Wi}$ based on the regions of the genome where the unique recombination junctions (RJs) for each strain cited above occur. The choice of probe location was done using the genomic positions of the specific RJ provided for each strain by the RDP4 program (Martin et al., 2010). Probe design was performed using Primer Quest (Integrated DNA Technologies, IDT®).

The primers developed for RT–qPCR were also designed considering regions with specific recombination points in each strain. For $PVY^{N:O/N-Wi}$, the primer pairs were located in the P1 gene region, with the forward primer (qWi-F: 5-ACGCGCATCCAGAAGAAA-3') located at nucleotides 402-420 and the reverse primer (qWi-R: 5'-TCTCCACCAGCAATAGTGATCTTTTGAC-3') located at nucleotides 506-527. The specific $PVY^{N:O/N-Wi}$ probe (qWi-P: 5'Cy5-TCACTTCCAGATGGCAGCTCCTAGTA-3') was located at nucleotides 431-441 and carried the Cy5 fluorophore.

For PVY^{NTN}, the forward primer (qNTN-F: 5'-GGGCTGGCTTTGAAATTGAC-3') was designed between nucleotides 5767-5787 within the VPg protein gene, and for the reverse primer, the A6032m primer was used (H6032m: 5'-CTTGCGGACATCACTAAAGCG-3') (Nie & Singh 2003). Between these two primers, a PVY^{NTN}-specific probe labeled with the HEX fluorophore was built (qNTN-P:5'HEX-TGGATCTGCATACAGGAAGAAGGGA-3') and located at nucleotides 5824-5849.

RT–qPCR assay

RT–qPCR was conducted in two steps, and cDNA synthesis was carried out using Superscript II Reverse Transcriptase

(Invitrogen) and the Oligo DT primer (Promega) according to the manufacturer's instructions. TaqMan® RT-PCRs (20 µl) were set up in 48-well reaction plates using the TaqMan® core reagent kit (Applied Biosystems). qPCR was carried out using 10 µl of TaqMan Universal Master Mix II (Applied Biosystems), 0.9 µM of each primer, 0.25 µM of the specific probe for each strain and 2 µl of cDNA. Real-time PCR was performed using an Eco Real-Time PCR machine (Illumina), and thermocycling was conducted at 50 °C for 2 min of incubation, 95 °C for 10 min, and 40 cycles of 95 °C for 30 and 60 °C for 1 min (for $PVY^{N:O/N-Wi}$) or 2 min (for PVY^{NTN}). The processed samples were analyzed in triplicate using EcoStudy v5 software.

Standard curve

The RT–PCR products obtained for each standard strain (PVY^{NTN} and $PVY^{N:O/N-Wi}$) were analyzed in two steps: the first involved using Superscript II Reverse Transcriptase to perform cDNA synthesis, and the second included Taq DNA polymerase to amplify the specific fragments in individual PCR assays. In both reactions, the manufacturer's instructions were followed (Invitrogen®). The original RT–qPCR primers described (qWi-F/qWi-R) for $PVY^{N:O/N-Wi}$ and (qNTN-F/A6032m) PVY^{NTN} were used in these amplification reactions under the following thermocycle profile: 94 °C for 2 min; 30 cycles of 94 °C/1 min, 60 °C/1 min and 72 °C/2 min; and a final extension of 72 °C for 5 min. Amplicons of 265 bp and 125 bp for PVY^{NTN} and PVY, respectively, were purified from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega). These purified fragments were ligated into the pGEM®-T Easy vector (Promega) and cloned into Escherichia coli αDH6 according to the manufacturer›s instructions.

Plasmids were extracted using the alkaline lysis method (Miniprep) and quantified by measuring the OD260 with a NanoVue Plus™ (GE Healthcare) spectrophotometer to calculate the number of viral copies/µl and to evaluate the purity of the DNA. After quantification, the number of viral copies was calculated using the formula described by Dai et al. (2013). Plasmids with higher levels of purity (A260/280 and A260/230 ratios) were selected for linearization and enhancing the standard curve. The process of plasmid linearization was performed using the restriction enzyme SalI (Fermentas) according to the manufacturer's instructions. The linearized plasmids were purified using a phenol:chloroform:isoamilic alcohol extraction, followed by ethanol precipitation, as recommended by the manufacturer. The linearized and purified plasmids were used to construct the qPCR standard curve. Eight serial dilutions ranging from 101 to 109 viral copies/µl were prepared and used in the qPCR assays in triplicate. Before being used to detect the PVY strains in the samples, the probes were tested with the isolates PVY^{NTN}: PVY-FORTE, YN-IBIA and YN-VELOX and $PVY^{N:O/N-Wi}$: MAF-VIV, PED-CA and Y-BR2 belonging to DFP collection, earlier identified by Galvino-Costa et al. (2012b).

Results and Discussion

Evaluation of symptoms in potatoes

The 39 samples from São Gotardo, which contained leaves collected at the end of the crop cycle in the near-senescence stage, did not show visible symptoms, likely because they came from potato seed crops. Considering that the potato plants sampled in the field were generated by healthy tubers, all infections detected were probably of the primary type; therefore, the plants could have been infected at any stage of their life cycle. Consequently, plants infected at the end of the cycle would certainly have very low concentrations of PVY and would show no symptoms. The remaining 126 samples consisting of tubers with a positive reaction for PVY by DAS-ELISA planted, and symptoms appeared in the emerging plants after 6-8 weeks. The classifications in light mosaic (Mo+), mild mosaic (Mo++), severe mosaic with no leaf deformation (Mo+++) and severe mosaic with leaf deformation (Mo+++/LD) are shown in Figure 1.

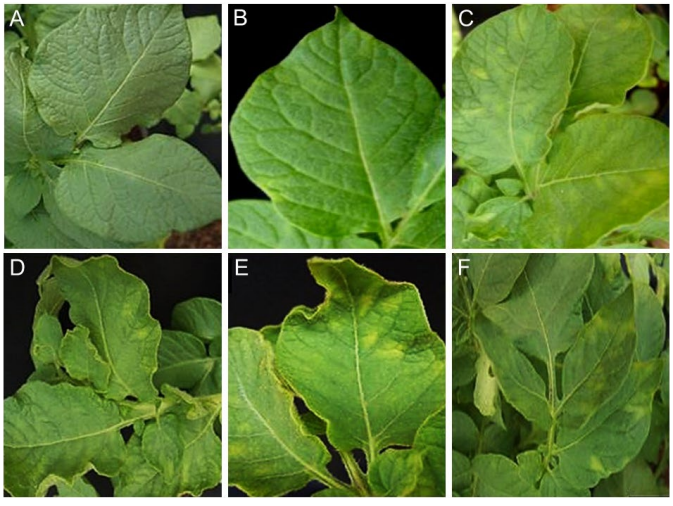

Figure 1: The main symptoms induced by Potato virus Y in potato plants from the tubers planted. A: healthy plant; B: light mosaic (MO+); C: mild mosaic (MO++); D and E: severe mosaic with leaf deformation (Mo+++/LD); F: severe mosaic with no leaf deformation (MO+++).

Of a total of 53 potato plants from tubers collected in Ouro Fino fields, 4 (7.6%) showed no apparent symptoms; 27 (50.9%) presented light mosaics, which was almost always imperceptible to potato farmers; 13 (24.5%) showed mild mosaics; 5 (9.4%) showed severe mosaics with no leaf deformation and 4 (7.6%) showed severe mosaics and leaf deformation.

Of the 52 plants originating from the tubers coming from Maria da Fé, 13 (25%) had light mosaic; 10 (19,2%) presented severe mosaics and 29 (55.8%) of the plants presented mild

mosaics. Among the ten samples evaluated from the Cristina field, 2 (20%) presented light mosaics 9 (70%) presented mild mosaics and 1 (10%) presented severe mosaic. Finally, of the eleven plants from tubers purchased at local markets and fairs, 3 (27.3%) showed light mosaic, and 8 (72.7%) had mild mosaics. Most samples with positive serology for PVY^{N} showed light to severe mosaic, and in mixed infections (N:O/N-Wi +NTN), the symptoms tended to be severe and/or with a reduction in leaf area.

In the last two decades, the emergence of recombinant strains of PVY worldwide has increased considerably (Galvino Costa et al., 2012a; Karasev & Gray, 2013; Green et al., 2020, Rodriguez-Rodriguez et al.,2020, Glasa et al., 2021, Kuzmitskaya et al., 2021, Tran et al., 2022). Although the main reasons for this epidemiological phenomenon are not yet clear, recombinant strains tend to produce milder foliar symptoms than parental strains (Nie et al., 2012). These milder symptoms have prevented the removal of infected potato plants from production fields, contributing to the selection of recombinant types of PVY (Karasev & Gray, 2013). Another important issue is that plants with late infections usually do not show significant yield losses, although the viruses can translocate to the tubers, causing seed degeneration in successive plantings.

The intensity and appearance of symptoms observed in potato plants vary considerably between cultivars and are generally associated with the stage at which these plants were infected and with the PVY strain present in the infection, as well as the environmental conditions in which these plants encountered in the field (Karasev & Gray 2013). In this study, symptom evaluations showed only variations in the intensity of the mosaics presented by the potato plants, and the absence of symptoms may be related to the fact that most of the collected tubers belong to the Ágata cultivar (Table1). Apparently, Ágata is different from other cultivars because it does not show tuber rings when infected with PVY and rarely shows visible leaf symptoms, because of its genetic resistance (European Cultivated Potato Database - ECPD, 2016).

Experiments carried out by Ramalho (personal communication) showed that the PVYN isolates O/N-Wi and NTN did not induce symptoms in Ágata during primary infections when the plant was infected in the current season. Symptoms only appeared with different intensities during secondary infections in the second and third generations when the plant was infected with PVYNTN, and in some cases, the PVYN:O/N-Wi isolates did not induce apparent symptoms until the third generation. Therefore, potato plants that show stronger symptoms may be the product of successive plantations of infected tubers or even the synergistic effect of mixed infections (Pacheco et al., 2012).

Although many symptoms other than mosaics are attributed to infections caused by PVY, such as necrotic lesions, systemic yellowing and tissue necrosis (Karasev & Gray, 2013) and necrotic rings in the potato tuber (Le Romancer & Nedellec, 1997), none of these symptoms were observed in this work, except for leaf reduction associated with strong mosaics. Although Cultivar Caesar is considered to have high resistance to PVY (EPCD, 2011), this characteristic has not been confirmed in the field or in experiments such as the one carried out in this work. This is probably due to new strains of PVY that have emerged over time, as symptoms are known to vary with the strain.

Mixed infections with two or more different PVY strains occur naturally in the environment, and the symptoms caused by one strain may occasionally prevail or mask the symptoms of another strain, making the diagnosis process difficult (Nie & Singh, 2002). Syller and Grupa (2014) studied the effect of coinfection and superinfection of several PVY strains in potato and tobacco plants experimentally inoculated with *Myzus persicae*. They observed that the severity of symptoms in doubly infected plants was similar to that shown by plants infected with a single virus, regardless of the virulence of the isolate, indicating the occurrence of competitive interactions between isolates within the plant. The authors also observed that the reaction of plants infected with different virus strains depended on both the host and the virus.

As mixed infection can result from coinfection, when viruses infect the plant simultaneously, or superinfection, when each virus infects the plant at different times (Saldaña, Elena, & Solé, 2003), there is no way to know which process occurred in the mixed infection samples detected in this work. Syller and Grupa (2014) observed that aphids can simultaneously transmit two strains, albeit with low efficiency. Therefore, the mixed infections detected here can be either coinfection or superinfection.

Serology and molecular pattern of the Brazilian PVY isolates

When analyzed by TAS-ELISA, the control tobacco plants inoculated with the two know strains, (LUI-AGA (PVY^{NTN}) + PED-AX ($PVY^{N:O/N-Wi}$) reacted with both monoclonal antisera for N and O serotypes, showing the presence of mixed infection.

Among the potato samples, 78 (47.3 %) showed serological behavior compatible with strains belonging to the O serotype group, 54 (32.7%) reacted with antisera against the N group, and 19 (11.5%) reacted with both sets of antisera for the O and N groups, indicating mixed infections (Table 1). The remaining 14 (8.5%) samples, 2 from Ouro Fino, 7 from São Gotardo and 5 from the Lavras market, with unknown origin, did not react with any antiserum, indicating either a lower virus concentration than the test sensitivity or diverse serological patterns.

Although one of the limitations of serological methods is distinguishing recombinant strains from their parental types, these methods have been considered one of the main detection tools due to their practicality and low cost. However, the sensitivity of serological tests is not suitable for detecting low virus concentrations, which can be quite common in infections that occur at the end of the plant's life cycle.

Table 1: Number of single and mixed infections detected by TAS-ELISA and RT-PCR multiplex in potato samples from different Brazilian fields.

Number of Samples	Cultivar	Local of sampling	Number and percentile of PVY strains detected by each diagnostic test											
			TAS-ELISA						RT-PCR					
			N strain		O-Wi strain		N + O:Wi strains		N strain		O:Wi strain		N + O:Wi strains	
			Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%
39[1]	Ágata	São Gotardo	8	20.5	11	28.2	13	33.3	14	35.9	8	20.5	17	43.6
53[2]	Ágata	Ouro Fino	33	62.3	15	28.3	3	5.60	31	58.5	13	24.5	9	17.0
52	Caesar	Maria da Fé	7	13.5	42	80.8	3	5.70	7	13.5	42	80.8	3	5.7
10	Caesar	Cristina	2	20.0	8	80.0	0	0	2	20.0	8	80.0	0	0
11[3]	Ágata	Lavras market	4	36.4	2	18.2	0	0	7	63.6	2	18.2	2	18.2
Total Nº 165			54	32.7	78	47.3	19	11.5	61	37.0	73	44.2	31	18.8

[1] Seven samples were seronegative for the N and O-Wi strains; [2] Two samples were seronegative for the N and O-Wi strains; [3] Five samples were seronegative for the N and O-Wi strains.

In our initial tests using DAS-ELISA, 165 samples composed of potato leaves and tubers were considered PVY positive. However, when submitted to TAS-ELISA with strain-specific monoclonal antibodies, 14 (8.5%) samples tested negative. This result could have happened for two reasons: first, a low virus concentration below the detection sensitivity of the technique, and second, the diversity of viral strains, which could avoid recognition by monoclonal antibodies (Nikolaeva et al., 2012). Galvino-Costa et al. (2012b), working with Brazilian PVY isolates, concluded that some recombinant isolates had anomalous serological behavior, which could impede their correct detection and identification by serological tests.

In the samples from São Gotardo, nine isolates showed positive results for monoclonal antibody MAb2 but negative results for SASA-O. In addition, three isolates showed positive results for monoclonal antibodies that detect serotype N (1F5 and SASA-N) and serotype O (SASA-O) but showed negative results for the MAb2 antibody. This pattern of showing differences between detected isolates, such as those positive for monoclonal antibody SASA-O and negative for MAb2, has not been reported in the literature.

Among the samples from Ouro Fino, the monoclonal antibody SASA-N failed to detect one isolate, but this isolate reacted positively with antibody 1F5. This result was also observed among the PVY-AGA and PVY-AST isolates that similarly reacted only with the 1F5 antibody but not with SASA-N (Galvino Costa et al., 2012b).

It is known that the serological profile of viruses is determined by specific regions that make up the coat protein, known as the epitope. Mutations in the epitope are common in recombinant strains; they prevent the recognition of antibodies and thus reduce the detection efficiency of serological tests (Inoue-Nagata et al., 2022,). Nikolaeva et al. (2012) identified some distinct epitopes in the PVY coat protein that allow them to be recognized by monoclonal antibodies specific for the N serotype, and the authors found that the single substitution of an amino acid in the region of these epitopes in the viral coat protein can lead to the complete loss of reactivity with commercially available monoclonal antibodies for the TAS-ELISA test. Furthermore, the occurrence of errors in serological tests is also considered common in some cases, which results in serious problems, since the tubers can also act as a source of viral inoculum for the aphid vectors in subsequent generations, facilitating the spread of the virus to neighboring areas and introducing new recombinants (Singh et al., 2003, da Silva et al., 2020).

Multiplex RT–PCR (Lorenzen et al., 2006a) was able to distinguish between the strains present in control tobacco plants with mixed infection as well as in all the PVY-infected samples studied in this work (Table 1). Positive serological tests were confirmed by RT–PCR results, but among the 14 seronegative samples, 9 showed a PVY^{NTN} molecular pattern (181 and 452 bp) and 5 showed amplified bands for both strains, indicating a mixed infection with PVY^{NTN} + $PVY^{N:O/N-Wi}$ strains (181, 452 and 689 bp) (Figure 2A). In addition, 2 samples with the serological pattern of PVY^{NTN} (Figure 2A) and 2 samples with that of $PVY^{N:O/N-Wi}$ (181 and 689 bp) from Ouro Fino, as well as 2 samples with the $PVY^{N:O/N-Wi}$ pattern from São Gotardo, showed a mixed infection by RT–PCR. Therefore, in the analysis with RT–PCR, 73 samples were positive for $PVY^{N:O/N-Wi}$, which corresponds to 44.2% of the samples characterized, and 61 samples showed amplification of the characteristic bands of the PVY^{NTN} pattern, corresponding to 37% of the total samples. Finally, the remaining 31 samples were classified as a mixed infection with PVY^{NTN} + $PVY^{N:O/N-Wi}$ strains, representing 18.8% of samples.

Among the 61 PVY^{NTN} samples detected by multiplex RT–PCR and subsequently analyzed by singleplex RT–PCR using

a specific primer pair to differentiate PVY^{E} from PVY^{NE-11}, 42 samples showed amplification of a 995 bp band (Figure 2B) characteristic of the recombinant Brazilian isolate PVY-AGA. It is the first time that this isolate was found in Brazilian potato crops after being described by Galvino-Costa et al. (2012a). The extent of the dissemination of PVY-AGA in Brazil is still unknown, mainly because its detection is more difficult than the other strains, since PVY^{E} is easily misidentified as PVY^{NTN} through the commonly used multiplex RT–PCR method (Lorenzen et al., 2006b, 2008; Galvino-Costa et al., 2012b). Other seven samples from Maria da Fé and two from Cristina, tested with the same primers pair, showed an odd amplification of only one band much larger with about 1500 bp (Figure 2B).

In general, the samples that reacted only with the MAb2 and SASA-O antibodies, which detect the common and Wilga strains, respectively, were identified as having a $PVY^{N:O/Wi}$ molecular pattern in multiplex RT–PCR, with rare exceptions. Of the 39 samples from São Gotardo, 10 reacted with one or both of these antibodies. When submitted to multiplex RT–PCR, eight of these 10 samples showed amplification of bands characteristic of $PVY^{N:O/N-Wi}$, while two showed mixed infection, with amplification of the specific bands of the Wilga and NTN strains.

Among the 53 samples from Ouro Fino, 15 showed an O serotype: 13 of them were identified as $PVY^{N:O/Wi}$, and 2 revealed mixed infections in the multiplex RT–PCR. Of the samples collected in Maria da Fé, 42 showed the $PVY^{N:O/N-Wi}$ serotype in the ELISA test, and all of them were later identified as the $PVY^{N:O/N-Wi}$ strain. Among the 10 samples from the Cristina district, eight of them showed the O serotype and were subsequently classified as the $PVY^{N:O/N-Wi}$ strain by RT–PCR. On the other hand, of the 11 samples collected in the markets, only two samples with the O serotype were identified as $PVY^{N:O/N-Wi}$ based on molecular tests.

Similarly, the majority of the samples that presented the N serotype in the ELISA test were subsequently classified as belonging to the PVY^{NTN} strain based on multiplex RT–PCR. Eight samples from São Gotardo showed the N serotype and were identified as PVY^{NTN}. Among the 33 samples originating from Ouro Fino with the N serotype, 31 had the PVY^{NTN} molecular pattern, and nine showed the molecular pattern reported for mixed infection with PVY^{NTN} and $PVY^{N:O/N-Wi}$. The seven samples from Maria da Fé and two samples from Cristina, all with N serotypes, were classified as PVY^{NTN}. Similar results were found for four samples collected in the markets' samples.

Comparing the results of serological and molecular techniques, TAS-ELISA failed to detect virus in 14 (8.5%) samples that tested negative, as well as mixed infections in 12 (7.3%) samples, showing the higher sensitivity of RT–PCR.

Detection of PVY strains in single and mixed infections by RT–qPCR

After the TAS-ELISA and RT–PCR multiplex tests, 151 samples were selected for RT–qPCR to quantify the strain concentrations and confirm the previous results. The criterion used to make this selection was to analyze all 134 samples classified as single infections, 61 infected only by PVY^{NTN} and 73 infected only with $PVY^{N:O/N-Wi}$, as well 17 samples classified as mixed infections (9 from São Gotardo, 5 from Ouro Fino and 3 from Maria da Fé), which were randomly selected among the 31 samples initially detected in the multiplex RT–PCR. the tobacco control plants with mixed infections were also included for comparison.

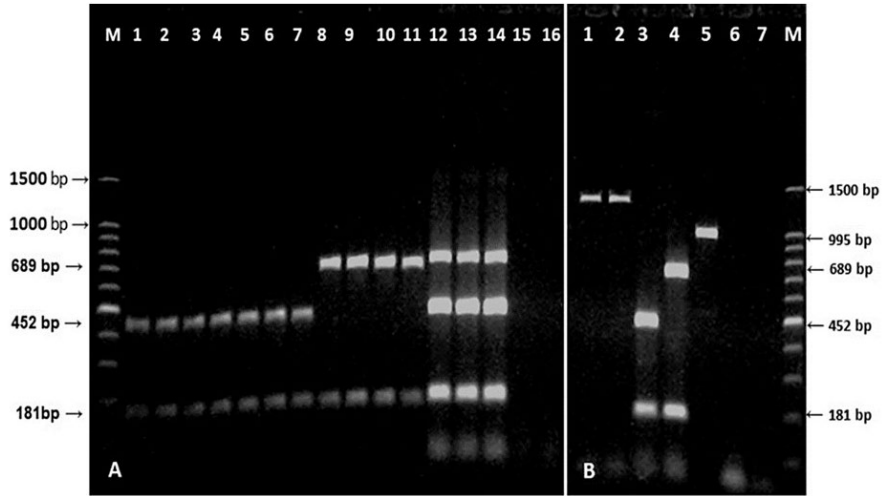

Figure 2: Electrophoretic profile of the strains detected by RT-PCR multiplex (A) and by PCR multiplex and singleplex (B). A) M: 100 bp ladder; 1 to 7: PVY^{NTN}; 8 to 11: PV $PVY^{N:O/N-Wi}$; 12 10 14: PVY^{NTN} + $PVY^{N:O/N-Wi}$; 15 and 16: negative controls. B) 1 and 2: atypical bands amplified by singleplex RT-PCR using specific primers for PVY^{E}; 3 and 4: controls amplified by multiplex RT-PCR with primers for PVY^{NTN} and $PVY^{N:O/N-Wi}$, respectively; 5: band with 995 bp specific of PVY^{E}, similar to isolates –PVY-AGA; 6 and 7: negative controls; M= 100 bp ladder.

When tested with the isolates used as control: PVY-FORTE, YN-IBIA, YN-VELOX, MAF-VIV, PED-CA and Y-BR2 belonging to DFP collection (Galvino-Costa et al., 2012b), and the control tobacco plants with mixed infections, the set of primers and probes for real-time RT-PCRs was efficient for the detection of PVY^{NTN} and $PVY^{N:O/N-Wi}$ strains, in single and mixed infections. The regression analysis of the standard curve showed high linearity, with y = -4.48 and -3.06 for PVY^{NTN} and $PVY^{N:O/N-Wi}$, respectively, and a coefficient of variation (r2 = 0.997 for PVY^{NTN} and r2 = 0.993 for $PVY^{N:O/N-Wi}$) suggesting an efficiency close to 1 (Figure 3 A→D), indicating that our methodology can be used to measure the concentrations of these two PVY strains in infected samples.

All 17 samples that tested positive for mixed infection by RT–PCR had this result confirmed by RT–qPCR. Based on the slope and R2 values obtained in the quantification curves, the methodology developed here was efficient and reliable for detecting mixed infections in analyzed samples with high accuracy.

Among the 61 samples infected with PVY^{NTN}, according to RT-PCR, only 11 (18.0%) samples showed single infection, while 24 (77.4%) showed mixed infection when analyzed by RT-qPCR (Table 2). Therefore, the number of mixed infections detected by RT–qPCR was substantially higher than that of the other two techniques, suggesting that this method was better for the detection of $PVY^{N:O/N-Wi}$ in samples supposed to be infected only with the PVY^{NTN} strain.

Similar results were obtained with the 73 samples characterized as $PVY^{N:O/N-Wi}$ in the multiplex RT–PCR: 52.1% were confirmed as single infections with this strain, but 47.9% showed mixed infection when submitted to RT–qPCR. Considering the incidence of both strains in the 134 samples that were supposed to have a single infection when analyzed by RT–PCR, 85 (63.4%) were revealed to have mixed infection, showing more RT–PCR detection errors for PVY^{NTN} in samples with mixed infections.

More errors in the detection of mixed infection were seen in the samples from Ouro Fino, where the RT–PCR test failed to detect 36 samples (81.8 %) of mixed infections, followed by the 15 samples of São Gotardo (68.2 %) and Cristina 6 (60%), when both strains were evaluated together. In the samples from Maria da Fé, which showed a higher incidence of $PVY^{N:O/N-Wi}$, the errors were also significant but were found at a comparatively lower level of 23 (46.9%). Finally, among the 9 samples collected at fairs and markets, 5 of them were detected as mixed infections by real-time analysis, totaling about 55,6% of the total analyzed (Table 2). The amount of mixed infections detected in RT-qPCR was about four times bigger when compared with the results obtained in TAS-ELISA and three times bigger than the numbers detected in multiplex RT-PCR.

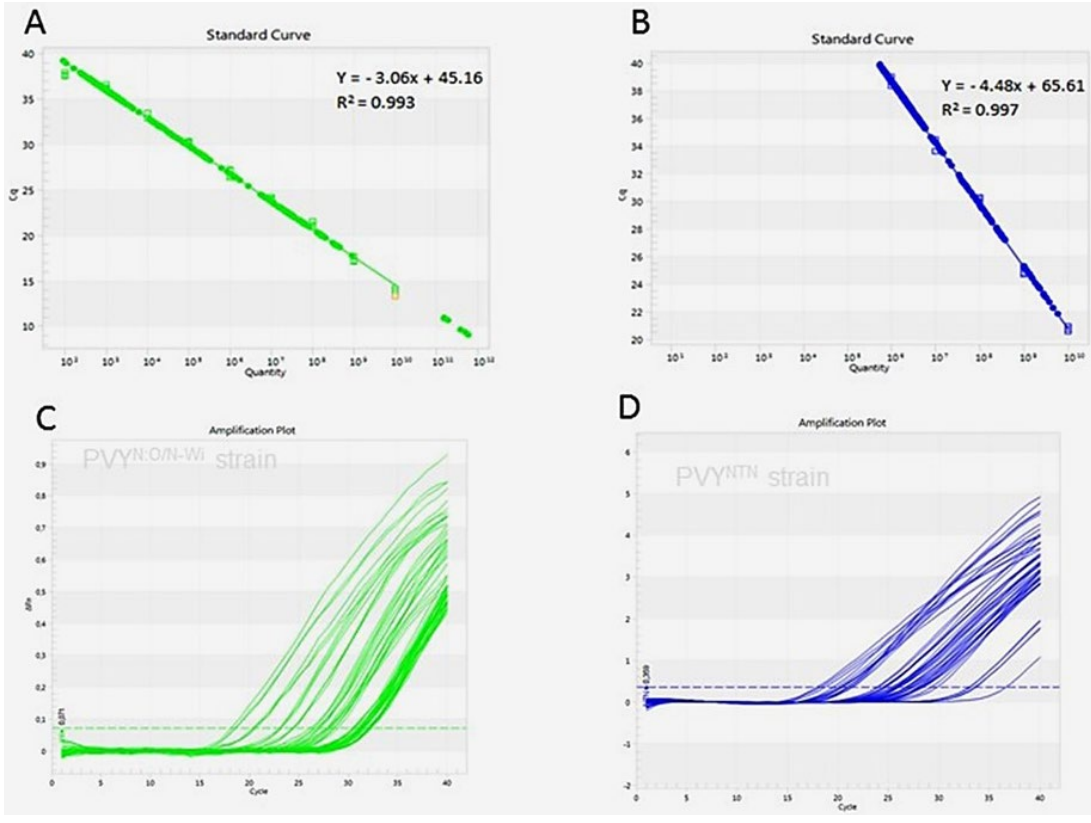

Figure 3: Quantification and Ct for the standard curves of RT-qPCR for each reaction strain-specific. A) PVYN:O/N-Wi Ct; B) PVYNTN Ct; C and D: amplification plots for PVYNTN and PVYN:O/N-Wi strains, respectively.

Table 2: Comparison among the numbers of single and mixed infections with PVY strains detected by RT-PCR multiplex and RT-qPCR in potato samples collected in Brazil.

Origin of samples	Number of Single infection detected by RT-PCR multiplex			Number and percentile of single and mixed infection Detected by RT-qPCR*					
	N strain	O-Wi strain	Total	N-Strain		O-Wi strain		N + O Wi	
				Nº	%	Nº	%	Nº	%
São Gotardo	14	8	22	1	4.5	6	27.3	15	68.2
Ouro Fino	31	13	44	4	9.1	4	9.1	36	81.8
Maria da Fé	7	42	49	3	6.2	23	46.9	23	46.9
Cristina	2	8	10	0	0	4	40.0	6	60.0
Lavras Market	7	2	9	3	33.3	1	11.1	5	55.6
Total Numbers	61	73	134	11	8.2	38	28.4	85	63.4

* All the 17 samples detected by RT-PCR with mixed infections multiplex were confirmed by RT-qPCR.

These results allow us to infer that the NTN strain apparently had some advantage in the viral interaction with the Wilga strain, since, in all the mixed samples mentioned above, the concentration of NTN particles was on average, 4,000 times higher than that of Wilga particles. Much higher viral concentrations were found, comparing the absolute values observed for each strain, in one sample from São Gotardo, which presented a concentration of PVY^{NTN} particles $2.87x10^5$ times higher than that of PVY^{N-Wi}.

The 17 samples with mixed infection detected by RT–PCR showed high concentrations of both strains in the three repetitions, and all were on the order of 10^4-10^9 particles/µL, which explains why most samples were also detected by TAS-ELISA tests.

Comparing all the results obtained it can be noted that, depending on the sensitivity of the test used, the detection of strains can lead to misrepresentative results, especially in the case of mixed infections. Sensitivity was clearly observed when the 14 TAS-ELISA-negative samples were detected and identified by multiplex RT–PCR, and the mixed infection rate increased from 11.5% to 18.8%. These data confirm that errors can occur in serological tests and have great impacts on future seed potato fields due to the misidentification of tubers as virus-free when they are actually infected with one or more PVY strains (Hühnlein et al., 2013, Khelifa, 2019).

The initial tests identified only 11.5% of the samples with mixed infection, which means that 7.3% of the mixed infections were missed when compared with the results of the RT–PCR and 40% were missed when compared with RT–qPCR. On the other hand, the TAS-ELISA results did not allow the identification of all strains and genetic variants present in the samples, exposing the need to use more sensitive and reliable detection methods, mainly due to the great variability that can be observed in the seed potato fields from Minas Gerais and probably from Brazil as a whole.

The same increase in sensitivity was seen in the real-time RT–qPCR results when compared with those of RT–PCR. There was a large difference in the detection of mixed strains, which increased from 18.8% to 63.4%. The data showing that the PVY^{NTN} strain was more concentrated than the $PVY^{N:O/N-Wi}$ strain could lead to mistakes in field surveys when using multiplex RT–PCR. Ávila et al. (2009) carried out a survey of potato viruses in seven Brazilian states using RT–PCR and serological techniques, and concluded that most PVY-infected samples presented the PVY^{NTN} strain. It is possible that the scenario could change if the survey was carried out using the RT–qPCR technique.

Balme-Sinibaldi et al. (2006) also developed a real-time RT–qPCR assay for the detection of two specific PVY groups (PVY^O and PVY^N) with high specificity in both assays. Their assays showed simultaneous detection and quantification of each of the strains even in samples containing mixed infections. These procedures allowed the detection and quantification of samples containing known fractions of 103 to 108 RNA transcripts of PVY^O and PVY^N. In their study, potatoes grown in fields under an uncontrolled environment were analyzed to assess the natural conditions that occur in seed potato fields.

It is not very clear if the difference in strain concentrations in mixed infections is related to competitive advantage or the timeline of plant inoculation with each strain by the aphid vector. These possibilities could result in a difference in the final concentrations of each strain in plants with systemic coinfection (Syller, 2012; Tran et al., 2022, Xu et al., 2022). Experiments under controlled conditions should be carried out to solve this problem. Furthermore, a larger survey using RT–qPCR should confirm the incidence and prevalence of PVY strains in Brazilian fields.

The divergence in fragment sizes shown by the virus isolates from Maria da Fé and Cristina, which showed an atypical band size of almost 1500 bp, could be attributed to a potential recombination or even some mutation event that occurred in the genomes of these isolates. However, this result must be verified in future studies involving genomic sequencing and analysis. These results highlight the need for more specific field surveys in Brazil using a more reliable technique to accurately identify the types of PVY

isolates that are prevalent in potato fields, especially in mixed infections, to assess the impacts on each planted potato cultivar.

Conclusions

The best technique to detect single and mixed infections with PVY strains was RT-qPCR, while DA-ELISA showed the lowest sensitivity. Two strains were detected: PVY^{NTN} and $PVY^{N:O/N-Wi}$ in single and mixed infections, inducing mild to severe mosaic. The concentration of PVY^{NTN} was, in average, about 4,000 times higher than the concentration of $PVY^{N:O/N-Wi}$. Most samples infected with the N strain were characterized as PVY^{E}, showing for the first time its spread and prevalence in Brazilian potato fields.

Author Contribution

Conceptual idea: Alves, M.C.; Methodology design: Alves, M.C.; Galvino-Costa, S.B.F.; Data collection: Alves, M.C.; Galvino-Costa, S.B.F.; Geraldino-Duarte, P.S.; Data analysis and interpretation: Alves, M.C.; Galvino-Costa, S.B.F.; Geraldino-Duarte, P.S.; and Writing and editing: Alves, M.C.; Figueira, A.R.; Silveira, A.T.L; Carvalho, C.M.

Acknowledgements

The authors would like to thank Brazilian Institutions: Conselho Nacional de Pesquisa e Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de aperfeiçoamento de Pessoal de Nível Superior (Capes) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the resource funds to support the current work.

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