Natural co-infection of *Solanum tuberosum* crops by the *Potato yellow vein virus* and potyvirus in Colombia

Co-infección natural de *Potato yellow vein* virus y potivirus en cultivos de *Solanum tuberosum* en Colombia

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ABSTRACT

The *Potato yellow vein virus* (PYVV), a *Crinivirus* with an RNA tripartite genome, is the causal agent of the potato yellow vein disease, reported in Colombian since 1950, with yield reductions of up to 50%. Co-infection of two or more viruses is common in nature and can be associated with differences in virus accumulation and symptom expression. No evidence of mixed infection between PYVV and other viruses has been reported. In this study, eight plants showing yellowing PYVV symptoms: four *Solanum tuberosum* Group Phureja (P) and four Group Andigena (A), were collected in Cundinamarca, Colombia to detect mixed infection in the isolates using next generation sequencing (NGS). The *Potato virus Y* (PVY) complete genome (similar to N strain) and the *Potato virus V* (PVV) partial genomes were detected using NGS and re-confirmed by RT-PCR. Preliminary field screening in a large sample showed that PYVV and PVY co-infect potato plants with a prevalence of 21% within the P group and 23% within the A group. This is the first report of co-infection of PYVV and potyvirus in Colombia and with the use of NGS. Considering that potyviruses enhance symptom severity and/or yield reductions in mixed infections, our results may be relevant for disease diagnosis, breeding programs and tuber certification.

Key words: viral diseases, *Potato virus V*, prevalence, NGS, potato, root vegetables.

RESUMEN

El *Potato yellow vein virus* (PYVV) es un *Crinivirus* de genoma RNA tripartita, agente causal de la enfermedad del amarillamiento de las nervaduras de la papa reportada desde 1950 en Colombia con reducción de producción hasta 50%. La coinfección entre virus en un mismo hospedero es común en la naturaleza y se asocia con cambios en acumulación viral y en la expresión de síntomas. Hasta el momento no se ha reportado coinfección entre PYVV y otros virus en papa. En este estudio se colectaron en Cundinamarca-Colombia ocho plantas con síntomas de PYVV: cuatro *Solanum tuberosum* Grupo Phureja (P) y cuatro del Grupo Andigena (A). Utilizando secuenciamiento de alto rendimiento (NGS) en los aislamientos se detectó infección mixta de PYVV con potyvirus que se reconfirmó con RT-PCR. Estudios preliminares en una muestra mayor de campo confirmaron que PYVV y PVY coinfectan papa con 21% de prevalencia para el grupo P y 23% para el grupo A. Este es el primer reporte de coinfección de PYVV y potyvirus en Colombia. Teniendo en cuenta que los potyvirus pueden incrementar la severidad de síntomas y/o reducir el rendimiento en infecciones mixtas, los resultados presentados pueden ser relevantes para el diagnóstico de enfermedades virales, programas de propagación y de certificación de semillas.

Palabras clave: enfermedades virales, *Potato virus V*, prevalencia, NGS, papa, hortalizas de raíz.

Introduction

Co-infection is a natural event involving different viruses or strains from the same virus being present in a host at the same time (Bennett, 1953). Such viruses interact in some cases, thereby increasing (synergism) or decreasing (antagonism) the accumulation and expression of the symptoms of one or more viruses, thus affecting crop yield (Goodman and Ross, 1974; Vance, 1991; Pruss *et al.*, 1997). The world's potato production is affected by at least 40 different viruses (Vreugdenhil, 2007); potato-infecting viruses are spreading fast worldwide; mostly because of infected tubers being used as seed-tubers for propagating crops, thereby leading to the possibility of interaction (Davey, 2013); and also by dispersion caused by natural vectors (Salazar *et al.*, 2000).

Mixed *Potato virus X* (PVX) and *Potato virus Y* (PVY) infection in *Solanaceae* sp. has been one of the most studied viral synergisms; it causes increased PVX accumulation without interfering with PVY accumulation, but can reduce yield by around 80% (Vance, 1991; Anjos *et al.*, 1992; Vance *et al.*, 1995). PVY (family *Potyviridae,* genus *Potyvirus*) is one of the most important potato pathogens worldwide. Its

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genome consists of a single-stranded, positive-sense RNA molecule of about 10 kb in length (Dougherty and Carrington, 1998; Hall *et al.*, 1998). Gene interactions involving different strains have been characterized based on host response and resistance, including the ordinary PVY (PVY-O), Tobacco venial necrosis (PVY-N) and stipple streak (PVY-C) strain groups of PVY (Schubert *et al.*, 2007; Singh *et al.*, 2008; Moury, 2010). The North American (NA) isolate of the potato tuber necrotic strain of PVY (PVY-NTN) is serologically related to PVY-N; however, PVY-NTN has been found to be a recombinant of PVY-O and PVY-N in the CP gene at the molecular level (Boonham *et al.*, 2002). Phylogenetic analysis of CP sequences from Colombian and Chilean isolates, which had previously been characterized as PVY-NTN, has clustered them together in a new group which is closely related to PVY-N and PVY-NTN (Moury, 2010; Gil *et al.*, 2011).

Reports of co-infection involving potyviruses and different viral groups are common. Co-infection with *Comovirus* in the soybean (Anjos *et al.*, 1992), with *Crinivirus* in the sweet potato (Untiveros *et al.*, 2007) and with *Potexvirus* in potato plants (Vance, 1991) has been reported. Previous studies have indicated that this is most likely due to P1/HC-Pro post-transcriptional gene silencing (PTGS) suppressor expression which allows non-potyvirus accumulation levels to increase without being repressed by plant PTGS defences (Anjos *et al.*, 1992; Vance *et al.*, 1995; Pruss *et al.*, 1997; Anandalakshmi *et al.*, 1998).

Crinivirus co-infection with other viral groups has been reported, *e.g. Potyvirus, Carlavirus, Cucumovirus, Ipomovirus* and *Cavemovirus* (Untiveros *et al.*, 2007; Cuellar *et al.*, 2011). Regarding *Crinivirus*, *Sweet potato chlorotic stunt virus* (SPCSV) co-infection with the *Sweet potato feathery mottle virus* (SPFMV) results in increased symptom severity and yield loss in the sweet potato, which is believed to be mediated by SPCSV endoribonuclease III (RNAse3) acting as a silencing suppressor and, thus, allowing synergism with SPFMV (Untiveros *et al.*, 2007; Kreuze *et al.*, 2008; Cuellar *et al.*, 2009).

PYVV is a re-emergent and quarentenary *Crinivirus* known as the causal agent of the Potato yellow vein disease (PYVD), affecting production in Colombia by 25 to 50% (Salazar *et al.*, 2000; Guzmán-Barney *et al.*, 2012), and has a prevalence of 5.6 to 11.0% in potato crops of Group P reported in three Colombian states (Franco-Lara *et al.*, 2012). The viral genome consists of three single-stranded, positive-sense RNAs and at least two defective RNAs (Livieratos *et al.*, 2004; Eliasco *et al.*, 2006). Major coat

protein (CP) gene studies have indicated low variability (Offei *et al.*, 2004; Guzmán *et al.*, 2006; Rodríguez-Burgos *et al.*, 2009; Chaves-Bedoya *et al.*, 2013) compared to high variability regarding the minor coat protein (mCP) gene and the homologue heat shock protein (HSP70h) gene (Chavez-Bedoya *et al.*, 2014).

Considering the importance of PYVV in Colombia and other Andean countries and the widespread occurrence of potyviruses, such as PVY, this study aimed to detect PYVV co-infection of field potato plants with other viruses which might partly explain the leaf symptom expression variability and yield loss which have been observed by our research group in previous studies. Next-generation sequencing (NGS) and RT-PCR were used to detect the viruses and establish co-infection. Phylogenetic analysis of the sequenced amplicons was used to identify and cluster the detected viruses. An NGS approach has thus been used for the first time to demonstrate that PYVV can co-infect potato plants along with PYV and PVV.

Materials and methods

Plant material

Eight *Solanum tuberosum* Group Phureja (P) (4) and Group Andigena (A) (4) plants expressing leaf yellowing symptoms were collected near Chipaque, Cundinamarca (Colombia), for RT-PCR diagnosis and NGS analysis. Leaf samples were collected at different times of the year from 61 *S. tuberosum* Group P (51 symptomatic and 10 symptomless) and 39 of Group A (28 symptomatic and 11 symptomless) potato crops grown in Cundinamarca, using random sampling to establish virus prevalence.

RNA and siRNA extraction

For siRNA extraction, an initial total RNA extraction was performed from 4 g symptomatic leaves from each of the eight plants using Trizol reagent (Invitrogen™, Thermo Fisher Scientific, Waltham, MA); 50 ug were separated on 4% agarose gel with microRNA markers (New England Biolabs®, Ipswich, MA) and visualised using SYBRSafe (Invitrogen™, Thermo Fisher Scientific, Waltham, MA). Bands located between 21 and 30 bp were excised and purified using a gel extraction spin column kit (Bio-Rad Laboratories, Hercules, CA). The pellet was washed with 75% ethanol and dried at room temperature, according to the procedure described by Kreuze *et al.* (2009). All small interference RNAs (siRNA) were sent to Fasteris Life Science (Fasteris, Plan-les-Ouates, Switzerland) for processing and sequencing on an Illumina Genome Analyzer II (Illumina®, San Diego, CA). Total RNA for further

analysis was obtained from 1 g leaves using Trizol reagent (Invitrogen™, Thermo Fisher Scientific, Waltham, MA). The RNA was purified using chloroform, precipitated with isopropanol and washed with 70% ethanol.

NGS analysis

Reads obtained by Illumina sequencing were assembled using Velvet (Zerbino, 2008) and Assembly-Assembler script software. The PVY genome sequence (NC_001616, AY884984) was initially used as a template for aligning siRNA reads using MAQ software (Redmond, WA). Different contigs were produced depending on the software used and different parameters, as published elsewhere (Flores *et al.*, 2011). Larger contigs were assembled using SeqMan (DNASTAR software, Madison, WI), combining the consensus contigs and sequences produced using MAQ and Velvet. The assembled contigs were compared with the NCBI nucleotide and protein databases using the NCBI BLAST® (Bethesdam, MD) database search tool (Altschul *et al.*, 1997); their translated peptides were matched to the corresponding viruses in each plant. Virus-specific contig coverage and distribution by siRNA was determined using MAQ (default parameters) and the results were exported to R statistical software (version 2.13.0) and Microsoft Excel® for further analysis.

PYVV and PYV coat protein gene amplification

Two hundred ng total RNA were denatured at 72°C for 10 min and chilled on ice for 2 min to confirm the presence of PYVV and the potyviruses. RNA was reverse-transcribed for 1 h at 42°C in the presence of 0.4 µM of the corresponding reverse primer (Tab. 1), 1X reaction buffer (Epicentre, Illumina®, San Diego, CA), 1 mM dNTPs (Bioline, London), 10 mM DTT (Epicentre, Illumina®, San Diego, CA), 1.6U RNAse inhibitor (Fermentas, Thermo Fisher Scientific, Waltham, MA), and 8U MMLV HP (Epicentre, Illumina®, San Diego, CA) to 10 µL final volume. Final denaturing took place at 72°C for 10 min.

PCR was carried out using 1.6 µL of the RT-PCR product diluted five times in 1X buffer NH_4 (Bioline, London), 2.0 mM $MgCl_2$ (Bioline, London), 0.4 µM dNTPs (Bioline, London), 0.4 µM of each primer (Tab. 1) and 1U of Biolase (Bioline, London) to 10 µL final volume. Samples were initially denatured at 94°C for 4 min and 35 cycles of PCR with 30 s denaturing at 94°C, 30 s of annealing at 60°C and 30 s of extension at 72°C. The series of cycles was followed by a final extension step for 10 min at 72°C.

Seven µL of each PCR product were analyzed on a 2% agarose gel in TAE buffer, stained with SYBRSafe (Invitrogen™, Thermo Fisher Scientific, Waltham, MA), ran for 45 min at 70 V and photographed with a gel digitalizer (BioRad). Amplicons obtained with the potyvirus degenerate primers were cloned in PCR 4-TOPO (Invitrogen™, Thermo Fisher Scientific, Waltham, MA) and recombinant plasmids were purified from *E. coli* using a Quicklyse miniprep kit (Quiagen, Hilden, Germany). Plasmid preparations were sequenced using both forward and reverse primers. Macrogen, Korea, did the sequencing.

Sequence alignment and phylogenies

To confirm the presence of PVY and PVV in the NGS analyzed samples, isolates of PYV and PVV were amplified with the degenerated primers POT1 and POT2 (Tab. 1). Amplicons were cloned and sent to Macrogen (Seoul, Korea) for Sanger sequencing. Molecular Evolutionary Genetics Analysis (MEGA) software (version 4.0) (Tamura et al., 2007) was used for bioinformatics analysis and Clustal W for aligning sequences (Thompson *et al.*, 1994). The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004), units being the number of base substitutions per site. Evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). Statistical confidence was evaluated using a bootstrap test with 1,000 replicates (Felsenstein, 1985). Pea seed-borne mosaic virus (PSbMV) was used as the outgroup.

TABLE 1. Primers and probes used to detect PYVV, PVY and *Potyvirus*.

Target	Primer	Sequence (5'→3')	Expected amplicon size	Fragment amplified	Reference
PYVV	Forward: 3'	AAGCTTCTACTCAATAGATCCTGCTA	769 pb	CP	Rodríguez *et al.* (2009)
	Reverse: F2	CTCGAGGATCCTCATGGAAATCCGAT			
PVY	Forward:	ACGTCCAAAATGAGAATGCC	480 pb	CP	Nie and Singh (2001)
	Reverse:	TGGTGTTCGTGATGTGACCT			
*Potyvirus**	Forward: POT1	GACTGGATCCATTBTCDATRCACCA	~ 1 kb	NIb/CP	Colinet *et al.* (1994)
	Reverse: POT2	GACGAATTCTGYGAYGCBGATGGYTC			

* Degenerated primers.

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Results

NGS analysis

Eight leaf samples of PYVD symptomatic *Solanum tuberosum* Groups P and A plants were collected from crops in Cundinamarca, Colombia. siRNAs were purified from total RNA extracts and sent for NGS using the Illumina platform. Reads between 21 to 24 nt were used to assemble contigs; 0.2 to 1.2 million reads were obtained, having a higher proportion of host and unspecific siRNAs. Potyviral sequences accounted for 63% of the reads for virus-specific siRNAs, particularly represented by 21 and 22 nt siRNAs. While PYVV sequences accounted for 8% of the 21 and 22 nt siRNAs, the remaining contigs corresponded to sequences with similarity to sequences for other viral families, host sequences or unknown sequences.

The reads were assembled into contigs; Tab. 2 shows those having over 70% similarity with other viruses. Several contigs were found having similarity with virus sequences from the families *Betaflexiviridae*, *Caulimoviridae*, *Closteroviridae* and *Potyviridae*. However, most contigs were discarded due to the contig length (less than 100 nt) or the contig amount (one to three contigs) (Tab. 2), leading to not taking into account the family *Caulimoviridae* and some members of the family *Potyviridae* as candidate viruses.

Regarding *Cavemovirus*, *Turnip vein clearing virus* (TVCV) was also discarded as a candidate due to *Cavemovirus* genome similarity to sequences integrated in the host genome (Lockhart *et al*., 2000) and all the contigs found only matched open reading frames (ORF) 3 and 4.

The remaining candidate viruses were *Potyvirus* PVV and PVY. Most of the larger contigs had high similarity with sequences reported for PVY which lead to the complete assembly of sequences corresponding to the virus genome (Fig. 1A).

MAQ software was used for determining read frequency and distribution in the genome for both potyviruses and PYVV. Even when several contigs having high identity were obtained for PYVV and PVV, siRNA frequency was less than 50 reads for each position in most of the genome, so complete sequences could not be assembled (Fig. 1B to 1D). The results gave less than 50 reads for each point in the PVV and PYVV genome; whereas, frequency at each point was around 1,500 reads for PVY (Fig. 1).

The reads led to assembling 46% of the first PYVV RNA and 76% of the second and third RNAs; potyvirus coverage was 99% for PVY and 50 for PVV; coverage and frequency were lower at the 5' end than the 3' end for PVV.

The amount of each virus-specific siRNA (Fig. 1) showed that PVY siRNAs were 17 times more abundant than those for PYVV in dual-infected plants; PVV-specific reads were four times less abundant than PYVV-specific siRNAs in dual-infected plants while PYVV siRNAs were 1.6 times more abundant in single-infected plants than those for PVV. The amount of PYVV-specific siRNAs was almost twice as high when co-infecting with PVY and four times higher when co-infecting with PVY and PVV simultaneously (Fig. 2). This contrasted with the RT-PCR and sequencing results which confirmed the presence of PVV and PYVV (Fig. 3) even though the number of reads for these viruses was under-represented in the NGS data set as compared to PVY (Tab. 2, Fig. 4).

TABLE 2. Number of contigs assembled by Velvet and similarity to reported virus sequences. siRNAs obtained by NGS were assembled into larger contigs and evaluated by BLASTx, the number of contigs with a similarity over 70% and an e-value of 0.0000001 for each virus in each sample are shown.

Family	Genus	Species	A1	P1	P2	P3	A2	A3	P4	A4
Betaflexiviridae	*Carlavirus*	PVS	-	22	-	-	-	-	-	-
	Badnavirus	SPBV-A	-	-	3	-	-	-	-	-
	Cavemovirus	SPVCV	-	-	-	-	-	1	1	2
Caulimoviridae		TVCV	6	4	6	1	3	5	7	17
	Petuvirus	PVCV	-	-	-	-	-	-	-	1
		RuFDV	-	-	-	-	-	-	1	-
Closteroviridae	*Crinivirus*	PYVV	3	-	10	-	48	46	-	51
		PVV	-	56	-	14	-	-	-	-
		PVY	12	1	-	-	1	7	-	2
Potyviridae	*Potyvirus*	TNSV	1	-	-	-	-	-	-	-
		TuMMoV	-	-	-	-	-	-	-	1
		WPMV	-	8	-	1	-	-	-	-

the infection is responsible for 25 to 50% yield reduction (Salazar *et al.*, 2000; Franco-Lara *et al.*, 2009; Guzmán-Barney *et al.*, 2012).

Host co-infection by different viruses may cause increase in symptom severity and yield loss (Goodman and Ross,

1974; Vance, 1991; Pruss *et al.*, 1997). Dual PVY and PVX infection is responsible for large potato losses around the world, accounting for a 3- to 10-fold increase in symptom severity according to tobacco plant observations (Vance, 1991); nevertheless, co-infection does not always result in increased symptoms and/or yield loss. Some viruses
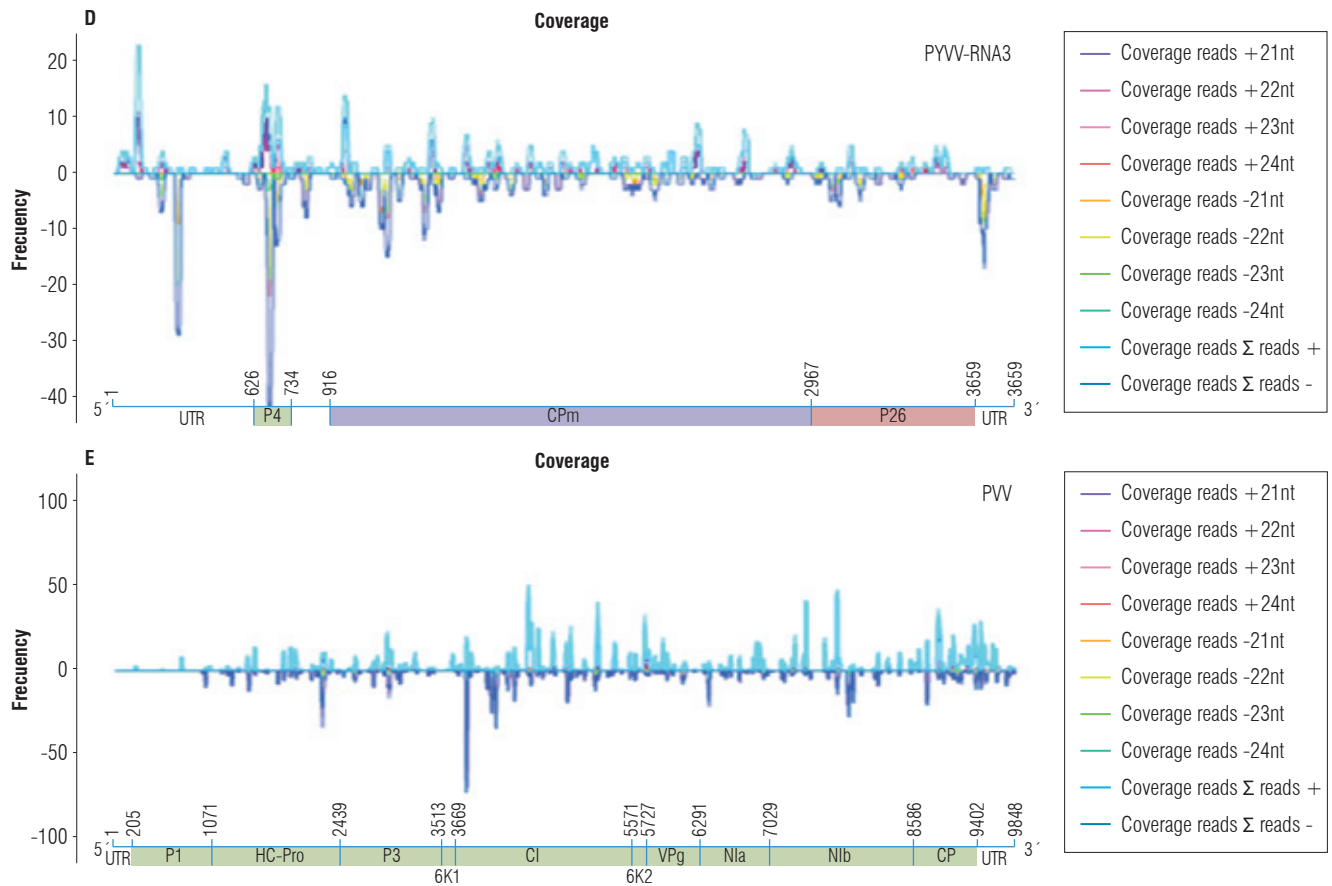

FIGURE 1. Coverage and distribution of specific PYVV, PVY and PVV siRNAs Upper Y axis shows positive sense siRNAs and lower Y axis shows negative sense siRNAs and the frequency and each color line represent a siRNA size (21 to 24 nt) light blue and dark blue lines represent total coverage of siRNAs, X axis represents the position of each virus genome. A, genome of PVY; B, PYVV RNA1; C, PYVV RNA2; D, PYVV RNA3; E, PVV.
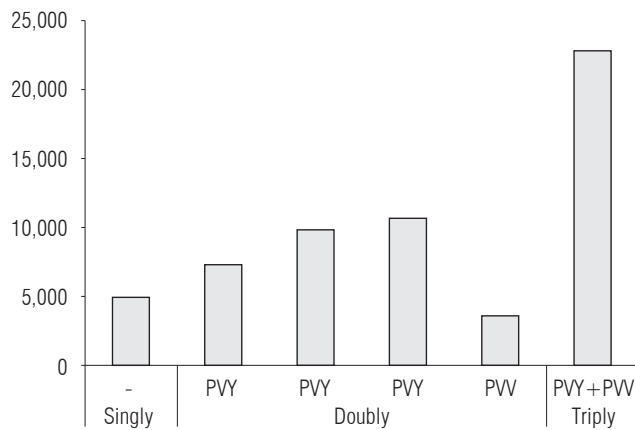
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FIGURE 1. Coverage and distribution of specific PYVV, PVY and PVV siRNAs Upper Y axis shows positive sense siRNAs and lower Y axis shows negative sense siRNAs and the frequency and each color line represent a siRNA size (21 to 24 nt) light blue and dark blue lines represent total coverage of siRNAs, X axis represents the position of each virus genome. A, genome of PVY; B, PYVV RNA1; C, PYVV RNA2; D, PYVV RNA3; E, PVV.

FIGURE 2. Amount of specific PYVV siRNAs in single infected plants, double infected with the potyvirus PVY or PVV and triple infected with both potyvirus. The amount of siRNAs was determined using MAQ results.

Sequence analysis and phylogenies

RT-PCR was performed with *Potyvirus* degenerate primers to amplify a segment of the NIb/CP gene. Amplicons were cloned and sent to sequencing to confirm the presence of PVV in the NGS samples. The sequence fragments were aligned using MEGA 4 along with fifteen full-length genomic sequences for non-recombinant PVY isolates, two for PVV and two for *Wild potato mosaic virus* (WPMV) and PSbMV as out-group (obtained from NCBI GenBank). The dendrogram (Fig. 3) was constructed using the Neighbor-Joining method (giving a total of 749 positions in the final dataset).

Sequence analysis of samples A1, P1 and P3 (Fig. 4) revealed similarity to PVV, thereby corroborating the results shown in Tab. 2. It also showed that the *Potyvirus* degenerate primers allowed PVY to be detected in just one of the tested samples.

PYVV and PVY Prevalence evaluation

The aforementioned results showed PYVV and PVY simultaneously infecting the same plant sample. A preliminary prevalence assay was made to corroborate co-infection in the field. Symptomatic and symptomless Group P (61
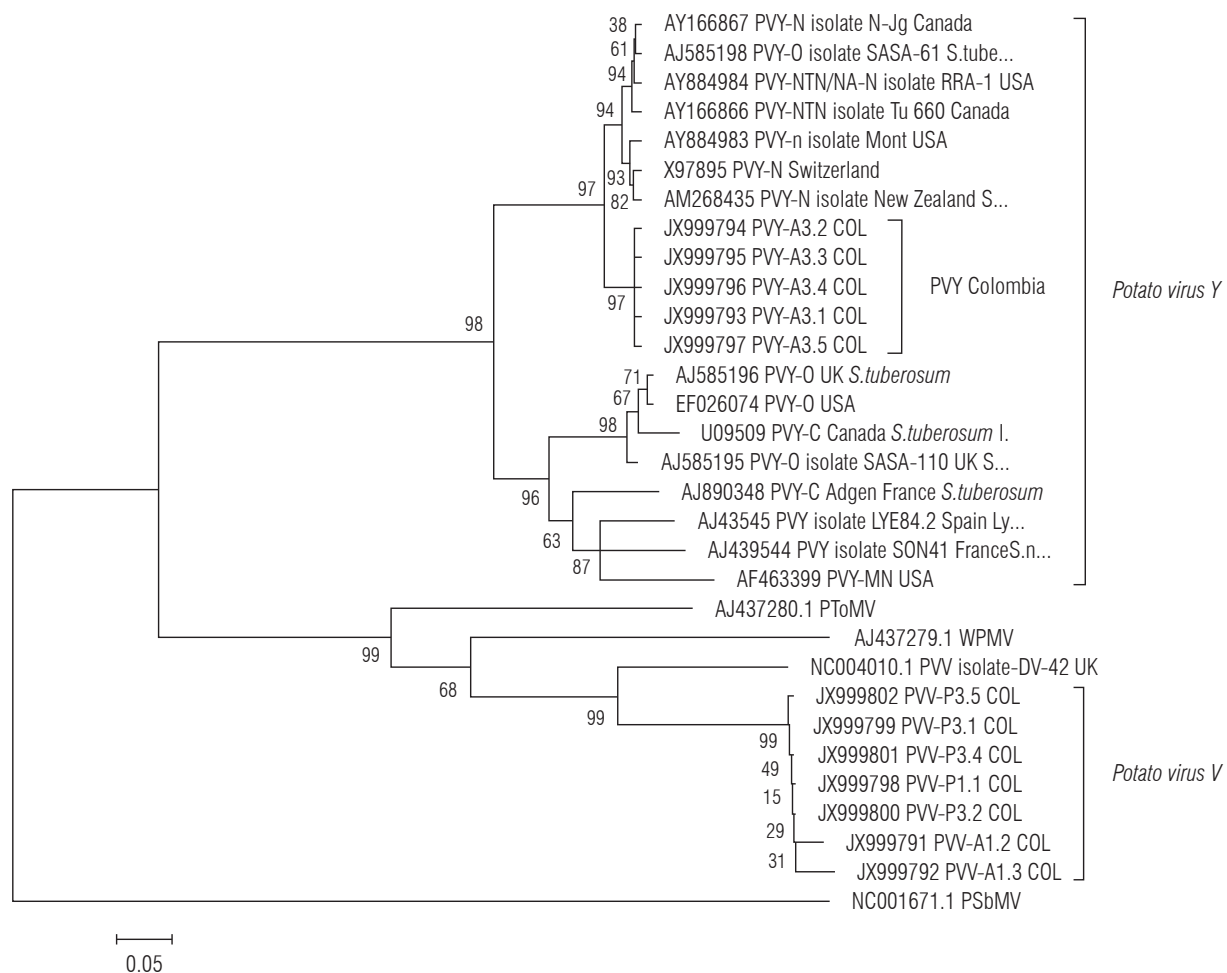
FIGURE 3. Dendrogram of PVY and PVV Colombian isolates. Neighbor-Joining tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. GenBank accession numbers are indicated at the end of each branch.

plants) and A (9 plants) samples were collected in Chipaque, Cundinamarca, and tested with specific primers. RT-PCR was used for estimating the number of singly- and dually-infected plants (Fig. 5). Results revealed that 21% of Group P and 23% of Group A samples were dual-infected and that just 15% of A samples and 16% of P samples were not infected by any of the viruses being tested.

Eleven symptomless plants were collected in the Group A and the percentages of singly- and dually-infected plants were calculated. The results showed that most PYVV singly-infected plants were symptomatic (54%) whereas most PVY singly-infected plants were symptomless (45%); all dually-infected plants evaluated here exhibited symptoms (Fig. 5).

Ten symptomless plants were collected in the Group P. The results showed that all symptomless plants were either PYVV singly-infected (50%) or were not infected by any of the viruses being evaluated (50%); whereas 25% of the symptomatic plants were dually-infected and similar amounts of singly-infected PYVV and PVY were found (Fig. 6).

All dually-infected plants evaluated here exhibited symptoms (Fig. 5) and their expression was documented; singly-infected plants exhibited typical symptoms of PYVV infection, meaning yellow leaves having green veins (Salazar *et al*., 2000). Dually-infected plants showed mild and severe mosaic with inter-venial yellowing in some cases; this differed greatly from previously-described symptoms caused by PYVV (Fig. 6).

Discussion

PYVV is a re-emergent Crinivirus becoming one of the most important potato viruses in Andean countries where
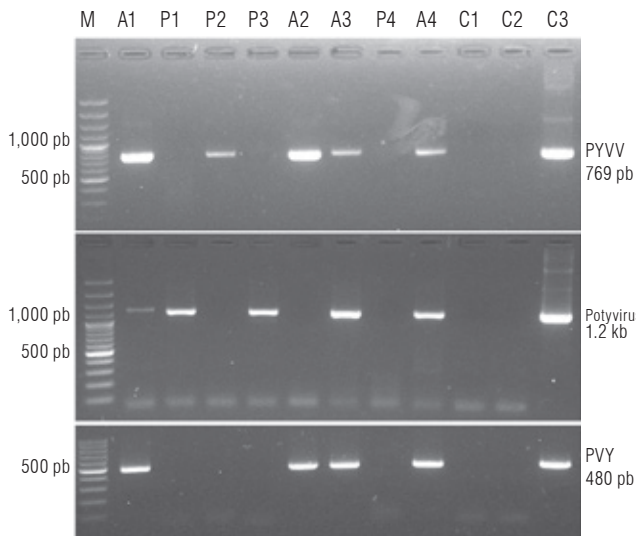
FIGURE 4. RT-PCR amplification of PYVV and PVY coat proteins and potyvirus NIb/CP fragment of samples used for NGS samples. M, generuler (Thermo Fisher Scientific, Waltham, MA) 100 bp and 1 kb. Lanes 2 to 9 samples; C1, RNA total from a *Citrus madurencisis* plant infected with CTV. C2, total RNA from a *S. tuberosum* Group Phureja plant maintained *in vitro*; C3, positive controls. For PYVV and *Potyvirus* plasmids and for PVY total RNA from an infected plant. P, Phureja Group plants; A; Andigena Group plants.
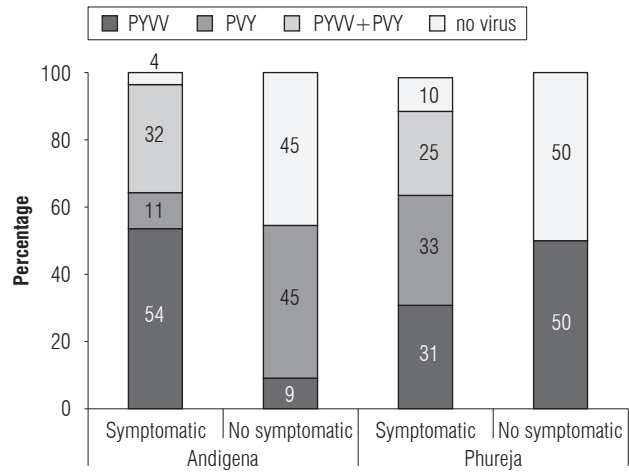

FIGURE 5. Prevalence of PYVV+PVY in Cundinamarca, Colombia. Thirty nine samples of *S. tuberosum* Groups Andigena and 61 of Phureja were evaluated by RT-PCR for the presence of PYVV, PVY and both viruses also considering symptomatic and asymptomatic plants.
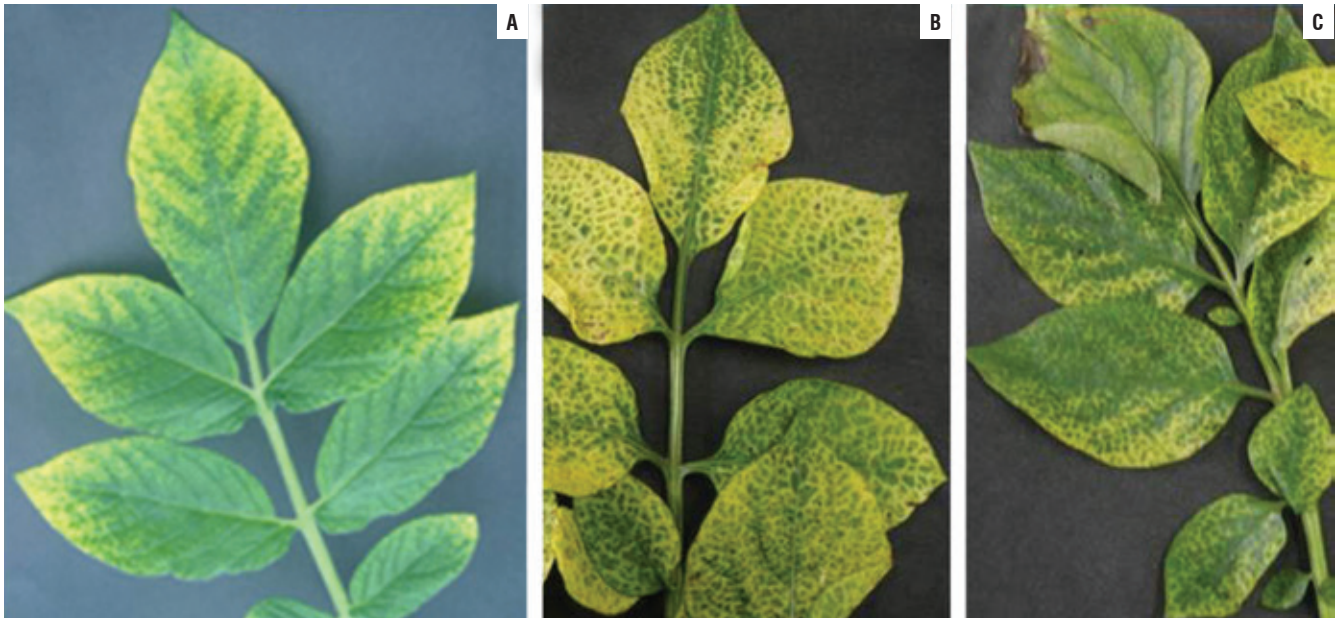

FIGURE 6. Symptoms of PYVV single infected and double infected (PYVV+PVY) under field conditions. A, symptoms characteristic of PYVV infection; B and C, symptoms observed in PYVV and PVY infected plants.

interact without affecting each another (*i.e.* neutralism) (Bennett, 1953). It is well known that virus interactions could affect virus accumulation, symptom expression and crop yield; for that reason, in the present study, next generation sequencing approach (NGS) was used to detect different viruses that could co-exist with PYVV in field potato plants and that may be responsible for some of the symptom expression differences observed in field-collected plants (Franco-Lara *et al.*, 2009).

Between 0.2 and 1.2 million 21 to 24 nt RNA reads were obtained and used to construct larger contigs which were

compared to other GenBank sequences using BLASTX. Several contigs having identity with several plant virus families were found; however, closer analysis using different parameters (similarity, e-value, contigs length, etc.) suggested that a large number of contigs had been misidentified (*e.g.* viruses in the families *Luteroviridae*, *Begomoviridae*, *Masteroviridae*, *Tymoviridae* and *Virgaviridae*).

The larger contigs matched PVY sequences, and the length and amount led to the assembly of full-length sequences that were used to perform phylogenetic analysis (Fig. 3). Those showed that the Colombian samples' PVY sequences grouped together with European and North-American NTN strain isolates, although they were not identical to those from previous reports (Gil *et al.*, 2011). Interestingly, *Potato virus S* (PVS) was detected in a P group sample. PVS has already been reported in Colombia in single (Sánchez de Luque *et al.*, 1991; Gil *et al.*, 2011) and mixed infections with PLRV, PVX, PVS and PVY (Guzmán *et al.*, 2010).

Preliminary studies for PYVV and PVV co-infection have been reported previously (Rodríguez *et al.*, 2011). Full-length genome sequences could not be formed for PYVV and PVV (<76% and 51% coverage, respectively); Fig. 1 shows that the number of PYVV siRNAs obtained was 17-fold lower than that obtained for PVY and only 1.6 higher than that obtained for PVV. PYVV-specific siRNAs were twice as high when co-infecting with PVY and four times higher when co-infecting with both PVY and PVV (Fig. 2). Larger contigs were obtained for PVY and up to 99% of the genome was assembled.

Contigs similar to *Cavemovirus* TVCV were found in all the analyzed samples (Tab. 2); nevertheless, this could have been due to the presence of pararetrovirus sequences integrated in the host genome, which has been already been described in other Solanaceae (Lockhart *et al.*, 2000).

A triple co-infection was noticed in one of the Group P samples analyzed here (this study's scope precluded further investigation). Triple infection including *Crinivirus* has already been reported as causing greater symptom severity than that in dual-infection (Untiveros *et al.*, 2007); the effects and prevalence of PVY, PVV and PYVV triple infection should thus be assessed.

A group of 100 field potato plants was used for preliminary estimation of dual-infection (PYVV-PVY) prevalence; up to 23% of the samples analysed here were dually-infected. In a screening done in Group P crops in three Colombian states during 2008, PYVV was reported as being present in 5.6 to 11% of the symptomatic samples and in 25% of the symptomless plants (Franco-Lara *et al.*, 2012); PVY prevalence was estimated at around 72% (Gil *et al.*, 2011). Prevalence analysis revealed that all dually-infected plants exhibited symptoms; in contrast, singly-infected plants in the Group A only had symptoms in 54% of the cases for PYVV and 45% for PVY and the Group P had 56% PYVV and 33% PVY (Fig. 5).

PYVV singly-infected plants showed typical symptoms: leaflets having clearing of the secondary and tertiary veins and also intense yellow leaflets having green central veins (Salazar *et al.*, 2000); whilst symptoms for the dually-infected plants (PVY-PYVV) included mild and severe mosaic, with yellow central veins in some cases (Fig. 6). Changes in symptom expression have been observed in virus interactions, namely *Lettuce infectious yellows virus* (LIYV, *Crinivirus*) and *Turnip mosaic virus* (TuMV, *Potyvirus*) or *Tomato chlorosis virus* (ToCV, *Crinivirus*) (Wang *et al.*, 2009) and *Tomato spotted wilt virus* (TSWV, *Tospovirus, Bunyaviridae*) (García-Cano *et al.*, 2006), whose interactions cause increased symptom severity leading to host death in some cases.

Potyvirus co-infection with non-related viruses can result in synergism, usually over-accumulation of non-*Potyvirus*, without affecting *Potyvirus* levels (Vance *et al.*, 1995). It was established that PYVV co-infects with PVY and also possibly with PVV. Helper component proteinase (HC-Pro) is known to act as a silencing suppressor in *Potyvirus*, thereby affecting siRNA accumulation (Mallory *et al.*, 2001) and explaining how PVY could allow other viruses to increase their effect in a particular host. By promoting higher virus accumulation, synergism could indirectly affect symptom severity and the efficiency of transmission by insect vectors. Types of *Crinivirus* have also been found to have a synergistic effect on unrelated viruses; when SPSCV (*Crinivirus*) interacts with SPFMV (*Potyvirus*) accumulation levels have not differed from single infected plants but SPFMV has increased its levels up to 600-fold and this has correlated with intensified symptom severity (Karyeija *et al.*, 2000). Such synergism is believed to be mediated by the SPCSV RNase3 protein which acts as a silencing suppressor (Kreuze *et al.*, 2008; Cuellar *et al.*, 2009). It would be interesting to identify the silencing suppressor protein in PYVV and ascertain its role in PYVV co-infection.

Preliminary assays were conducted using qPCR and ELISA tests; however, no PYVV and PVY synergistic interaction was detected; results that may be due to the reduced number of samples used and the lack of a follow-up study, which

led us to suggest that PYVV may have been interacting with PVY. Further studies are required to ascertain this.

The results are very important and should be taken into account by phyto-sanitary institutions. The prevalence study and symptom detection represent preliminary steps and should be enforced by research on a larger scale.

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