

## **A *Bromoviridae* member associated with chlorotic leaf symptoms on sunflowers**

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### **ABSTRACT**

- A bromovirus isolated from sunflower (*Helianthus annuus* L.) was characterised and shown to be highly related to *Pelargonium zonate spot virus* (PZSV), a virus reported in Italy, Spain, France, USA and Israel. Sunflower plants showing chlorotic concentric ring and linear pattern symptoms were observed near Paraná city (Entre Ríos Province) in commercial sunflower crops. This was the first observation of this type of symptoms in sunflower in Argentina, but similar ones have been reported in Africa and Mexico. The aim of this study was to characterize this new sunflower disease.
- The characterization was based on virus transmission, host range, electron microscopy and comparison of its sequence with those of related viruses.
- Virus transmission efficiency by mechanical inoculation to sunflower plants at the V2 vegetative stage was nearly 100%. Seed transmission was negative as plants derived from seeds of systemically infected plants showed no symptoms. A host range including 27 species of the families *Amaranthaceae*, *Asteraceae*, *Chenopodiaceae*, *Cucurbitaceae*, *Dipsacaceae*, *Fabaceae* and *Scrophulariaceae* was tested, and 59.26% of the inoculated species showed positive plants to viral infection. Electron microscopy of rapid preparations from samples enriched with the virus revealed high concentration of quasi-spherical particles of ~33 nm diameter. Total RNA extracted from a viral enrichment sample was pyrosequenced, and 54,506 sequences were obtained, three of them with 3383, 2433 and 2655 pb showed 90.0%, 94.7% and 93.9% of identity with RNA1, RNA2 and RNA3, respectively, of PZSV isolated in Apulia, Italy.
- These results indicate that the virus associated with chlorotic concentric ring and linear patterns symptoms in sunflower is PZSV, a member of the *Bromoviridae* family, *Anulavirus* genus. This is the first mention of a PZSV infecting sunflower and is the first mention of this virus in Argentina.
- This study provides basic information on this new sunflower disease in Argentina. These results will allow further epidemiological and characterization studies of the disease.

Key words: concentric rings - linear patterns - characterization - pyrosequencing - Bromoviridae - PZSV

## INTRODUCTION

Several sunflower virus diseases have been reported worldwide, at least 36 viruses have been mentioned to affect naturally or artificially sunflower plants (Brunt et al. 1996). In general, these were reported as secondary diseases but in India the *Tobacco streak virus* -TSV (Bhat et al. 2002) and in Uganda the Sunflower yellow blotch virus-SuYBV (Aritua 2006) became major diseases. In Argentina two potyviruses have been detected in cultivated sunflower, the Sunflower chlorotic mottle virus (SuCMoV) and Sunflower mild mosaic virus (SuMMoV), both from Entre Ríos province (Lenardon 1994; Dujovny et al. 1998; Giolitti et al. 2010). In this province, in the 2003-2004 crop season, plants showing chlorotic concentric ring and linear pattern symptoms were observed in commercial sunflower crops (Figure 1). Symptoms like these had been reported in Uganda (Aritua 2006) and Mexico (Fucikovsky 1976), but these diseases were not fully characterized. The aim of this study was to characterize this new Argentinean sunflower disease as it affects one of the most important commercial crops in Argentina and could become a major problem for its production and export.

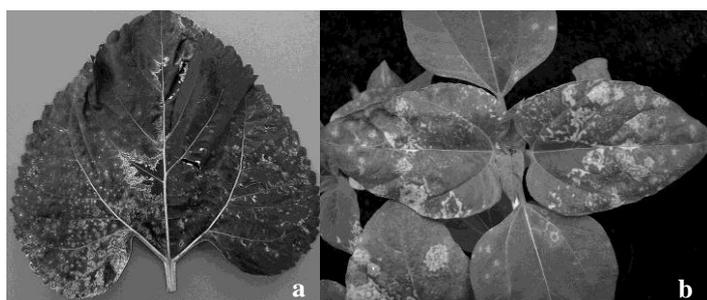


Figure 1: (a) leaf and (b) inoculated plant of sunflower showing chlorotic concentric ring and linear pattern symptoms.

## MATERIALS AND METHODS

**Plant sample:** Leaves of sunflower plants showing chlorotic concentric rings and linear pattern symptoms were collected near Paraná city in the 2003-2004 crop season. The inoculum was maintained under greenhouse conditions by mechanical inoculation on sunflower and *Nicotiana glutinosa* plants. Symptomatic leaves were ground 1:5 (w/v) using 0.01M phosphate buffer pH 7, 0.1% Na<sub>2</sub>SO<sub>3</sub>, plus silicon carbide (600 mesh) as abrasive, the resulting slurry was rubbed onto sunflower healthy plants.

**Virus transmission efficiency:** The percentage of mechanical transmission to sunflower plants was determined using 200 healthy sunflower plants (cv. Contiflor 17 DRM), that were mechanically inoculated at V2 (Schneider and Miller 1981). The experiment was divided into four replicates of 50 plants each and inoculated plants were observed for symptoms development.

**Seed transmission:** Seeds were obtained from sunflower plants (cv. Contiflor 17 DRM) systemically infected with the virus after mechanical inoculation. Four sowings of 250 seeds each were made, and the plants were observed for symptoms development.

**Host range:** 27 species from seven families (see results) were mechanically inoculated. Plants were obtained from seeds and at least 25 plants per species were inoculated. Noninoculated plants of each species were grown as negative controls and *N. glutinosa* plants were inoculated as positive controls each time. The inoculated plants were observed for symptoms development and plants with and without symptoms were used as inoculum sources for back inoculation of sunflower or *N. glutinosa* plants.

**Viral enrichment:** Leaves of systemically infected *N. glutinosa* plants were collected 30 days after inoculation and stored at -70°C. Twenty grams of infected plant tissue were ground 1/5 (w/v) in grinding buffer (Jones et al. 2006), plus 25% chloroform and subjected to one cycle of differential centrifugation. The quality of the enrichment was checked by transmission electron microscopy (TEM), for this purpose, rapid preparations (dips) were made by floating formvar-coated grids on drops of viral enrichment, washed with water and contrasted with 2% uranyl acetate solution (Kitajima 1997). Dips were inspected using a Jeol JEM EXII (Jeol, Tokio, Japan) and 100 viral particles were measured to calculate their diameter.

**Sequencing:** Total RNA was extracted from 200 µl of virus enriched preparation using the RNeasy Plant Mini kit (Qiagen, California, USA) and 200 µl (20 ng/µl) suspended in water, were submitted to INDEAR (Rosario, Santa Fe, Argentina) for its pyrosequencing using a 454 Genome Sequencer FLX Titanium System (Roche, Brandford, CT, USA). Contigs were assembled *de novo* from the dataset using Newbler v 2.6 software (Roche), edited manually and were subjected to both BLASTN and BLASTX analysis (NCBI 2011). Sequence and phylogenetic analysis were performed using the Lasergene 8.0.2 software package (DNASTAR, Inc., Madison, WI, USA). Sequences of 25 viruses belonging of

*Bromoviridae* family, used for comparative analyses, were retrieved from the GenBank. Multiple sequences alignments produced by ClustalW for the full length nt sequences of RNA1, RNA2 and RNA3 and protein sequences were used as input data for reconstructing phylogenetic trees by the Neighbor-Joining method. Statistical significance was estimated by performing 1000 replications of bootstrap resampling of the original alignment. The following RNA1, RNA2 and RNA3 sequences have been used in this study: **Genus Alfamovirus:** *Alfalfa mosaic virus*-AMV (HQ316635, HQ316636, HQ316637), **Genus Anulavirus:** *Pelargonium zonate spot virus*-PZSV (AJ272327, AJ272328, AJ272329), **Genus Bromovirus:** *Broad bean mottle virus*-BBMV (NC\_004008, NC\_004007, NC\_004006), *Brome mosaic virus*-BMV (GU584131, GU584130, GU584129), *Cassia yellow blotch virus*-CYBV (AB194806, AB194807, AB194808), *Cowpea chlorotic mottle virus*-CCMV (AF325739, AF325740, AF325741), *Melandrium yellow fleck virus*-MYFV (NC\_013266, NC\_013267, NC\_013268), *Spring beauty latent virus*-SBLV (NC\_004120, NC\_004121, NC\_004122), **Genus Cucumovirus:** *Cucumber mosaic virus*-CMV (AB188234, AB188235, AB188236), *Gayfeather mild mottle virus*-GfMMV (NC\_012134, NC\_012135, NC\_012136), *Peanut stunt virus*-PSV (AB360968, AB360969, AB360970), *Tomato aspermy virus*-TAV (NC\_003837, D10663, NC\_003836), **Genus Ilarvirus:** *American plum line pattern virus*-APLPV (NC\_003451, NC\_003452, NC\_003453), *Apple mosaic virus*-ApMV (NC\_003464, NC\_003465, NC\_003480), *Asparagus virus 2*-AV-2 (NC\_011808, NC\_011809, NC\_011807), *Citrus leaf rugose virus*-CiLRV (NC\_003548, NC\_003547, NC\_003546), *Elm mottle virus*-EMoV (NC\_003569, NC\_003568, NC\_003570), *Fragaria chiloensis latent virus*-FCILV (NC\_006566, NC\_006567, NC\_006568), *Humulus japonicus latent virus*-HJLV (NC\_006064, NC\_006065, NC\_006066), *Parietaria mottle virus*-PMoV (NC\_005848, NC\_005849, NC\_005849), *Prunus necrotic ringspot virus*-PNRSV (JN416771, JN416772, JN416773), *Spinach latent virus*-SPLV (NC\_003808, NC\_003809, NC\_003810), *Tobacco streak virus*-TSV (FJ403375, FJ403376, FJ403377), *Tulare apple mosaic virus*-TAMV (NC\_003833, NC\_003834, NC\_003835) and **Genus Oleavirus:** *Olive latent virus 2*-OLV2 (C\_003673, C\_003674, C\_003671)(ICTV 2011).

## RESULTS

**Virus transmission:** Sunflower plants mechanically inoculated at V2, developed chlorotic concentric ring and linear pattern symptoms 7 to 10 days after inoculation in 98.5% of cases. 978 plants were evaluated for seed transmission, none of them developed symptoms.

**Host range:** Of the 27 species in seven families of plants inoculated, 16 species (59.26%) showed positive plants. Back inoculated plants were positive when symptomatic plants were used as inoculum source and did not react when asymptomatic plants were used, except for *Petunia hybrida*. Inoculated species and their percentages of infection were: **Amaranthaceae:** *Amaranthus retroflexus* (0.00%), *Gomphrena globosa* (0.00%), **Asteraceae:** *Bidens pilosa* (0.00%), *B. subalternans* (65.00%), *Chrysanthemum coronarium* (66.10%), *Cichorium intybus* (100.00%), *Dipsacus fullonum* (19.23%), *Helianthus annuus* (98.50%), *H. petiolaris* (44.44%), *H. tuberosus* (0.00%), *Matricaria recutita* (62.16%), *Lactuca sativa* (0.00%), **Cucurbitaceae:** *Cucumis metuliferus* (0.00%), **Chenopodiaceae:** *Chenopodium amaranticolor* (100.00%), *C. quinoa* (100.00%), **Fabaceae:** *Arachis hypogea* (0.00%), *Desmodium tortuosum* (0.00%), *Glycine max* (8.33%), *Pisum sativum* (100.00%), **Scrophulariaceae:** *Antirrhinum majus* (0.00%), **Solanaceae:** *Capsicum annuum* (20.00%), *Nicotiana glutinosa* (100.00%), *N. occidentalis* (100.00%), *N. rustica* (75.00%), *Petunia hybrida* (7.14%), *Solanum lycopersicum* (0.00%) and *S. tuberosum* (0.00%). Systemic symptoms of chlorotic concentric ring and linear pattern were observed in positive plants; except for *Chenopodium* species that showed local lesions and for *P. hybrida* that were cryptic symptoms (data not shown).

**Viral enrichment:** Viral enrichment was successful and dips observed by TEM showed a high concentration of quasi-spherical viral particles of ~33 nm in diameter (Figure 2).

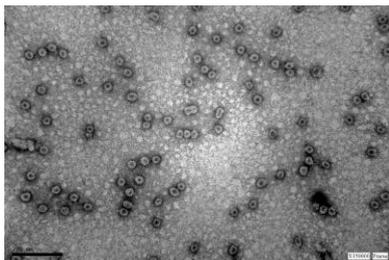


Figure 2: Electron micrograph of dip of viral enrichment showing quasi-spherical viral particles.

**Sequencing:** A dataset of 54,506 sequences were obtained. *De novo* assembly yielded 655 contigs including the 90.92% of readings, BLAST analysis indicated that 6, 11 and 176 of these contigs were highly related to RNA1, RNA2 and RNA3, respectively of members of *Bromoviridae* family. These fragments revealed:

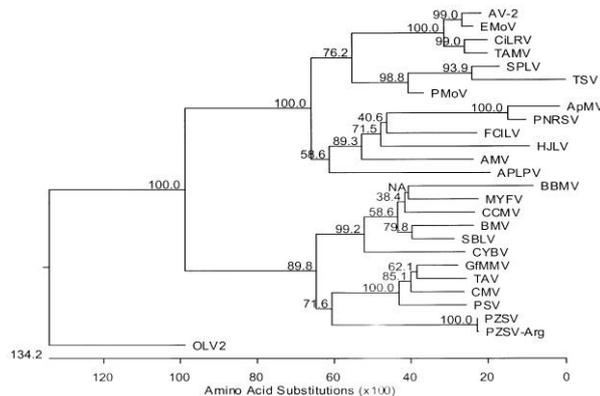
**RNA1:** It is 3383 nt long and contain a open reading frame (ORF1) of 2886 nt, beginning at ATG (nt 84-86) in the context (CATAATGGTCTGC) and ending at TGA (nt 2970-2972). ORF1 encodes a putative polypeptide of 962 amino acids (aa), named protein 1a, which include two putative proteins, the methyltransferases (277 aa long, from aa 80-356) and the helicases (249 aa long, from aa 693-941). The 5' and 3' non coding regions (NCR) have 81 and 414 nt long, respectively.

**RNA2:** It is 2433 nt long, it contains a ORF2 of 2061 nt, beginning at ATG (nt 82-84) in the context (ATAAATGGCTA) and ending at TAA (nt 2143-2145). ORF2 encodes a putative polypeptide of 687 aa (protein 2a), including a putative RNA dependent RNA polymerase (RdRp) of 425 aa long (from aa 236-660) and a GDD motif is observed from aa 525-527. The 5' and 3' NCR have 81 and 291 nt long, respectively.

**RNA3:** It is 2655 nt long, it contains two ORFs, the first of which (ORF3) begins at ATG (nt 335-337, in the context GAAAATGTCTC) and ends at TGA (nt 1262-1264). The polypeptide encoded by ORF3 (protein 3a) is a putative movement protein (MP) consisting in 309 aa. The second ORF (ORF4) starts at ATG (nt 1619-1621, in the context GCATAATGCCCCC) and ends at TAA (nt 2243-2245). ORF4 encodes a putative product of 208 aa, which presumably corresponds to putative capsid protein (CP). The 5' and 3' non coding regions (NCR) have 334 and 413 nt long, respectively. The ORFs of RNA3 are separated by an intergenic region of 357 nt.

In 5' NCRs of RNAs 1 and 2, 11 nt sequence (GGTTCAATTCC) resembling the internal control region (ICR-2) of eukaryotic tRNA gene promoters are present, in both cases from nt 25-35. The same sequence is present in intergenic region of RNA3 starting at nt 1406 and ending at nt 1416.

Comparisons of sequence similarities between Argentine sunflower virus and 25 other virus species of the *Bromoviridae* family were in the range: RNA1: 90.0, 50.6 - 37.5 (PZSV, MYFV - AV-2); RNA2: 94.7, 48.4 - 35.9 (PZSV, GfMMV - SPLV) and RNA3: 93.9, 37.7 - 27.5 (PZSV, BMV - OLV2) at the nt level, and for methyltransferase: 97.4, 51.9 - 27.5 (PZSV, CCMV - APLPV); helicase: 97.2, 52.8 - 31.0 (PZSV, GfMMV - FCILV); RdRp: 99.3, 53.3 - 26.8 (PZSV, GfMMV - FCIL); PM: 98.4, 29.4 - 8.6 (PZSV, CYBV - TSV) and CP: 95.2, 18.4 - 7.3 (PZSV, SBLV - HJL) at the aa level, (the remaining results are not showed). Phylogenetic trees derived from the comparison of nt sequences of RNA1, RNA2 and RNA3, and aa sequences of methyltransferases, helicases, RdRp, MP and CP grouped to Argentine sunflower virus with European isolate and were clearly separated from other genera members (*Alfamovirus*, *Bromovirus*, *Cucumovirus*, *Iilarvirus* and *Oleavirus*). Only is shown the phylogenetic tree derived from RdRp (Figure 4).



**Figure 4:** Phylogenetic trees derived from amino acid sequences of RdRp of Argentine sunflower virus and 25 other bromoviruses analyzed.

## DISCUSSION

The causal agent of chlorotic concentric ring and linear pattern symptoms in sunflower was identified as a new isolate of PZSV (PZSV-Arg). This virus was isolated originally from tomato (*Solanum lycopersicum*) in Italy and mentioned as *Tobacco streak virus* (Martelli and Cirulli 1969), it was later described to affect *Pelargonium zonale* plants showing concentric chlorotic rings in the leaves, from which it derived its name (Gallitelli 1982). It was also identified infecting tomato in Italy (Gallitelli 1982; Vovlas et al. 1989), Spain (Luis-Arteaga and Cambra 2000), France (Gebre-Selassie et al. 2002) and recently in USA (Gulati-Sakhuja et al. 2009).

PZSV is readily transmitted mechanically from the sap of infected plants (Lapidot et al. 2010) and has been effectively sap-transmitted to sunflower, its natural host in Argentina, and a high percentage of species tested as host range in this study were positive. PZSV-Arg induced symptoms on *Chenopodium quinoa*, *C. amaranticolor*, *Capsicum annuum*, *Nicotiana glutinosa* and *N. rustica* resembling those described previously for other isolates of this virus (Luis-Arteaga and Cambra 2000; Gebre-Selassie et al. 2002; Liu and Sears 2007; Lapidot et al. 2010). The Spanish isolate induced systemic symptoms on *Gomphrena globosa* (Luis-Arteaga and Cambra 2000) and the Israel isolate local symptoms on *Lactuca sativa* (Lapidot et al. 2010) species that not reacted with PZSV-Arg, whereas this isolate reacted with *Pisum sativum* and the French did not (Gebre-Selassie et al. 2002). *Petunia hybrida* positive plants showed no symptoms when were inoculated whit PZSV-Arg, but showed systemic symptoms when were inoculated with Spain or French isolates (Luis-Arteaga and Cambra 2000; Gebre-Selassie et al. 2002). The major difference between host ranges of PZSV was tomato, a frequent host reported for this virus (Vovlas et al. 1989; Luis-Arteaga and Cambra 2000; Gebre-Selassie et al. 2002; Liu and Sears 2007; Lapidot et al. 2010) it was not infected with PZSV-Arg. Perhaps the reaction of tomato plants to PZSV-Arg is cultivar-dependent as was found for cucumber plants (Lapidot et al. 2010), thus it would be important to test more tomato cultivars.

PZSV has been shown to be seed-transmitted in *N. glutinosa* and *Diploaxis erucooides*, both with an efficiency of ~5% (Vovlas et al. 1989) and in tomato with an efficiency of ~29% (Lapidot et al. 2010). PZSV-Arg cannot be transmitted when sunflower seeds harvested from infected plants were sown, probably because different host species, different cultivars of the same host species and different viral isolates vary in their ability to be seed transmitted (Lapidot et al. 2010).

Total RNA extracted from a virus enriched sample was used for pyrosequencing and satisfactory results were obtained (Roossinck et al. 2010). PZSV is a virus with quasi-spherical particles of 25-35 nm in diameter (Gallitelli et al. 2005), this range includes the ~33 nm in diameter determined for PZSV-Arg. BLAST analysis indicated that 6, 11 and 176 of the contigs obtained were related to RNA1, RNA2 and RNA3 respectively, of members of *Bromoviridae* family, this greater abundance of RNA3 is probably because it is the most abundant species encapsidated by PZSV particles (Finetti-Sialer and Gallitelli 2003).

The genome of PZSV-Arg is distributed in three RNA species encoding four proteins and is typical of members of the *Bromoviridae* family. The genomic structure described here for PZSV-Arg is highly similar to that reported for the Italian isolate (here PZSV-It) (Finetti-Sialer and Gallitelli 2003). The main differences between Italian and Argentinean isolates are: RNA1 of PZSV-It have a 5'NCR of 78 nt, instead PZSV-Arg have 81 nt, so the star and stop codons of ORF1 are moved five nt. ORF2 of PZSV-It, encoded by RNA2, ends in a TGA codon, followed by a 3'NCR of 289 nt, while ORF2 of PZSV-Arg ends in a TAA codon and its 3'NCR is two nt shorter than the Italian isolate. The RNA3 of PZSV-It is four nt longer than PZSV-Arg. The movement protein (310 aa) and the capsid protein (209 aa) of PZSV-Arg both have one aa less than PZSV-It. The ORF4 that encodes the putative CP of PZSV-It ends in a TGA codon but the ORF4 of PZSV-Arg ends in a TAA codon. ORF3 and ORF4 are separated by an intergenic region that in the PZSV-Arg is three nt longer than in PZSV-It. In PZSV-Arg we have the same: nt context regions for start codons of four ORFs, GDD conserved motif of RdRp and the 11 nt sequences of internal control regions of 5'NCRs of RNA1, RNA2 and in intergenic region of RNA3, than in PZSV-It (Finetti-Sialer and Gallitelli 2003; Gallitelli et al. 2005).

Comparisons of sequence similarities between PZSV-Arg and 25 virus species of the family *Bromoviridae* were carried out for nt sequences of RNA1, RNA2 and RNA3 and for aa sequences of methyltransferase, helicase, RdRp, MP and CP. In all cases the highest similarities were between PZSV-Arg and PZSV-It, confirming they are two isolates from the same virus and that they are different from others viruses analyzed. Tentative phylogenetic trees constructed by comparing the same eight sequences of PZSV-Arg with those of other 25 bromovirus, generated trees with clearly distinct clusters, one comprising the two isolates of PZSV in a subcluster, that is highly related with the cucumovirus subcluster and in less degree with the bromovirus subcluster. The second cluster compares the ilarvirus with the AMV (alfalmovirus), and OLV-2 (oleavirus) is in the third cluster. Similar results were obtained when PZSV-It was compared with 18 other bromovirus (Finetti-Sialer and Gallitelli 2003). Differences between PZSV-It and other bromovirus were enough to propose that this virus should belong to a new genus within *Bromoviridae* family (Gallitelli et al. 2005).

This study provides information on this new sunflower disease in Argentina. This knowledge is necessary to begin studies aimed at avoiding potential damage caused by this disease in sunflower or other economically important crops of our country. These results clearly support the idea that the virus isolated from sunflower in Argentina is the Pelargonium zonate spot virus, member of *Anulavirus* genus,

*Bromoviridae* family. This is the first mention of PZSV infecting sunflower and it is its first identification in Argentina and South America.

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