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Plant Pathology

First report of sunflower chlorotic mottle virus infecting sunflower plants in Brazil

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ABSTRACT: Sunflower (*Helianthus annuus*) plants showing symptoms of chlorosis, mosaic, chlorotic ringspot, and necrosis on younger leaves were found in a small experimental plot in Piracicaba, in the state of São Paulo, Brazil. Preliminary examinations by transmission electron microscopy of symptomatic leaf tissue revealed flexuous filamentous particles 13-15 nm wide and 700-750 nm long, and cytoplasmatic cylindrical inclusions typical of those found in plant cells infected by members of the *Potyvirus* genus. Total RNA extracted from symptomatic leaves and subjected to RT-PCR followed by partial nucleotide sequencing confirmed the presence of a potyvirus in the affected plants, which was identified as sunflower chlorotic mottle virus (SuCMoV), a member of the *Sunflower chlorotic mottle virus* (genus *Potyvirus*, family *Potyviridae*) species. Mechanical transmission assays with extracts of symptomatic sunflower leaves reproduced the original symptoms in sunflowers, mosaic symptoms in *Zinnia elegans*, and chlorotic local lesions in *Chenopodium amaranticolor* and *C. quinoa*. Sunflower and zinnia plants became infected after aphid transmission experiments with *Myzus persicae*. RT-PCR tests using specific primers for SuCMoV confirmed the presence of this virus in experimentally infected plants, meeting the criteria of Koch's postulate. This is the first report of SuCMoV infecting sunflower plants in Brazil.

Keywords: *Helianthus annuus*, *Potyvirus*, aphid transmission, diagnose

Introduction

The sunflower (*Helianthus annuus* L.) is a herbaceous annual plant that belongs to the Asteraceae family and is native to North American regions (Blackman et al., 2011). This plant is important as its seeds are an accessible source of premium oil dedicated primarily to human consumption.

Aside from other factors, sunflower production can be affected by diseases caused by nematodes, bacteria, fungi, and viruses. Sastry et al. (2020) listed 19 distinct viruses which can infect the sunflower. Among them, the potyvirus sunflower chlorotic mottle virus (SuCMoV) is regarded as an emerging pathogen most frequently found in sunflower fields in Argentina (Lenardon et al., 2005; Mederos et al., 2020). In Brazil, three viruses have been described as natural sources of infection of sunflower: the potyvirus bidens mosaic virus, causing systemic necrosis (Costa and Kitajima, 1966); the ilarvirus tobacco streak virus, inducing stunting, tip blight, vein clearing and necrosis (Costa and Costa, 1972), and the alphanecrovirus tobacco necrosis virus, recovered from the root of asymptomatic plants kept in the greenhouse (Costa and Carvalho, 1960). All of them were first found in the state of São Paulo.

Potyviruses (genus *Potyvirus*, family *Potyviridae*) are one of the largest groups of plant RNA viruses that affect various crops worldwide. The particles are flexuous and filamentous, 680-900 nm long and 11-20 nm wide, and can be found in the cytoplasm and leaf parenchyma cells (Inoue-Nagata et al., 2022). They have a single-stranded, positive-sense RNA genome with approximately 10,000 nucleotides containing a long open reading frame (ORF) (Inoue-Nagata et al., 2022). Aphids transmit potyviruses in a non-persistent relationship, with *Myzus persicae* and *Aphis gossypii* being the most important vectors (Gadhave et al., 2020).

In June 2021, sunflower plants showing virus-like symptoms were observed in a small experimental plot in Piracicaba, in the state of São Paulo, Brazil. This work reports details in identifying the causal virus as an isolate of SuCMoV.

Materials and Methods

Plant samples

Five sunflower plants from cv. Zebulon Tall were collected from a small experimental plot (22°42′30.5″ S, 47°37′45.9″ W, altitude 500 m) in Piracicaba, in the state of São Paulo, Brazil. Infected plants (from the field and greenhouse) showed symptoms of chlorosis, mosaic, chlorotic ringspot, and systemic necrosis on young leaves (Figures 1A and B).

Transmission electron microscopy

Negatively stained extract from five (individually) symptomatic sunflower leaves was examined by transmission electron microscopy (TEM) to detect virus particles. Small pieces of sunflower leaves were ground in a 0.05 M phosphate buffer (pH 7.2), and one drop of the extract was transferred onto a formvar/carbon-coated 200-µm mesh coper grid and incubated for 5 min, washed with distilled water, and negatively contrasted with 1 % uranyl acetate.

Ultrathin sections were obtained by fixing 2-mm^2 pieces of symptomatic leaves in a modified Karnovsky

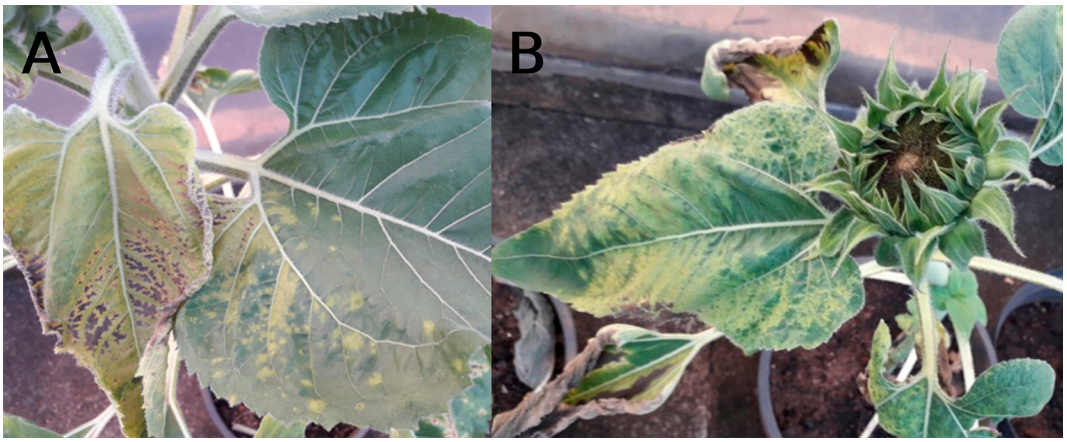

Figure 1 – A and B Symptoms of chlorosis, mosaic, chlorotic ringspot, and necrosis on young leaves of sunflower plants (*Helianthus annuus* cv. Zebulon Tall) caused by sunflower chlorotic mottle virus (SuCMoV)-infection.

solution and post-fixed in 1 % OsO_4 for one hour, followed by dehydration with acetone, infiltration, and embedding with Spurr's low viscosity epoxy resin (Kitajima and Nome, 1999). Finally, the blocks were sectioned with an ultramicrotome equipped with a Diatome diamond knife. Ultrathin sections were then collected on copper grids and stained with uranyl acetate and Reynold's lead citrate. All observations were made using a transmission electron microscope, and the images were recorded digitally with a camera.

Molecular identification

Total RNA was separately extracted from the symptomatic leaves of five field plants using the PureLink Viral RNA/DNA Kit following the manufacturer's instructions. A single-step reverse transcription polymerase chain reaction (RT-PCR) was carried out using the potyvirus universal primers WCIEN (5′-ATG GTT TGG TGY ATY GAR AAT-3′) and PV1 (5′-$T^{(17)}$ VGC-3′), which amplify 840 bp of part of the capsid protein gene and part of the 3′ UTR (Mota et al., 2004). The amplicons from two samples were Sanger sequenced using the WCIEN primer, and the sequences were compared with those deposited in GenBank using the Blastn algorithm (http://www. ncbi.nlm.nih.gov/Blast.cgi) to identify the potyvirus. Additionally, the same RNAs from the five infected plants were used in RT-PCRs using the specific primer for SuCMoV CP2R (5′-ACA TGT TAC GAA CCC CAA GC-3′) (Giolitti et al., 2009) combined with the WCIEN primer, which amplify a fragment of 522 bp corresponding to part of the capsid protein region. The amplicons from two samples previously sequenced using WCIEN primer were selected, and Sanger sequenced using the CP2R primer as described above.

The total RNAs from the five infected field plants were also used for RT-PCR using specific or universal primers to detect different viruses that induce similar symptoms to those observed in sunflower plants. The detections were undertaken using primer pairs for orthotospoviruses (BR60 and BR65) (Eiras et al., 2001), tobacco streak virus (TSV) (3TbS3 and TbCP5U) (Almeida et al., 2005), and bidens mosaic virus (BiMV) (8331 and 9046) (Suzuki et al., 2009).

Host range

Leaf extract from the sunflower plant was ground in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.1 % sodium sulfite at a ratio of 1:10 (w/v). The extract was mechanically inoculated onto leaves of different plant species: *Bidens pilosa*, *Chenopodium amaranticolor*, *C. quinoa*, *Cichorium intybus*, *Datura stramonium*, *Gomphrena globosa*, *Helianthus annuus* cv. Amarelo Alto, *Lactuca sativa* cvs. Americana and Lisa, *Nicotiana tabacum* cv. TNN, and *Zinnia elegans*. The leaves had been previously dusted with 600-mesh carborundum and sap-inoculated with the leaf extract. Groups of five plants of each species were inoculated and kept under greenhouse conditions to observe symptoms for up to 30 days. RT-PCR was carried out using the WCIEN/CP2R primer pair to confirm virus infection.

Aphid transmission

Vector transmission of the potyvirus was investigated using a *Myzus persicae* (Hemiptera: Aphididae) colony obtained from the Instituto Agronômico de Campinas (IAC, Campinas, in the state of São Paulo, Brazil) and maintained on healthy *D. stramonium* plants in insect-proof cages under controlled conditions [26 °C, 12L: 12D (6h00-18h00)]. Initially, the aphids were placed into empty Petri dishes for a starvation period of 30 min. Starved aphids were transferred onto sunflower-infected leaves for a 10 min acquisition access period (AAP). Subsequently, the aphids (ten insects per plant) were transferred to healthy seedlings with three true leaves for an inoculation access period (IAP) of 10 min. Ten

sunflower plants (cv. Amarelo Alto), *B. pilosa*, *C. intybus*, *L. sativa* (cvs. Americana and Lisa), and *Z. elegans* were individually inoculated. After IAP, the aphids were removed manually with a fine brush, and all plants were maintained inside insect-proof cages under greenhouse conditions. Virus infection was evaluated by symptoms and confirmed 30 days later by RT-PCR using the WCIEN/CP2R primer pair.

Results and Discussion

TEM examination of extracts from five symptomatic sunflower plants, collected from among 60 plants in the experimental plot, revealed the consistent presence of flexuous filamentous particles ca. 13 nm in diameter and 700-750 nm in length (Figure 2A), typical of *Potyviridae* members (Inoue-Nagata et al., 2022). Fixed ultrathin section analysis revealed the presence of cylindrical inclusions, producing a pinwheel configuration in the cytoplasm of leaf cells of symptomatic sunflower plants (Figure 2B), characteristic of infection by viruses belonging to the *Potyviridae* family (Inoue-Nagata et al., 2022).

The 840 bp amplicon obtained by RT-PCR with the universal primer pair WCIEN/PV1 confirmed the presence of a potyvirus in the five samples taken from the field. Comparison of the two obtained nucleotide sequences (OK539551 and OK539553) with those sequences available from GenBank showed 99.77 % and 98.25 % identity, respectively, with the corresponding nucleotide sequence of sunflower chlorotic mottle virus (SuCMoV) found infecting *Z. elegans* plants in Brazil (AY344048) (Maritan et al., 2004). The same sequences share 90.2 % and 89.65 % identity, respectively, with sequences of SuCMoV found to infect sunflower plants in Argentina (MG885786 and MG885787) (Mederos et al., 2020). The 522 bp amplicon obtained by RT-PCR with the specific CP2R primer for SuCMoV, in combination with the WCIEN primer, confirmed the presence of SuCMoV in the five samples taken from the field. The nucleotide sequences (OK539552 and OK539554) obtained also share 98.77 % and 97.65 % identity, respectively, with the corresponding sequence of SuCMoV (AY344048) found to be infecting *Z. elegans* plants in Brazil. In contrast, the sequences share 90.02 % and 89.21 % identity, respectively, with a sequence of SuCMoV found to be infecting sunflower plants in Argentina (MG885787) (Mederos et al., 2020). Orthotospoviruses, TSV and BiMV were not detected in the same five sunflower plants in which SuCMoV was detected, demonstrating that SuCMoV causes the observed symptoms in sunflower.

Based on the species demarcation threshold for *Potyviridae* members, potyviruses of the same species share more than 76 % nucleotide and 82 % amino acid identities of the complete ORF or similar criteria can be used for the CP coding region if the complete ORF sequence is not available (Inoue-Nagata et al., 2022). The nucleotide sequences obtained in the present work confirmed the presence of SuCMoV in field symptomatic sunflower plants. However, the possibility of recombination with other potyviruses (e.g., BiMV) cannot be completely discarded. To clarify, it is essential to sequence the entire genome and verifies the occurrence of recombination.

The virus was mechanically transmitted to all sunflower, *C. amaranticolor*, *C. quinoa*, and *Z. elegans* plants. *Myzus persicae* also transmitted it to all sunflower and *Z. elegans* plants tested. However, all the inoculated plants (mechanically or by aphid) of *B.*

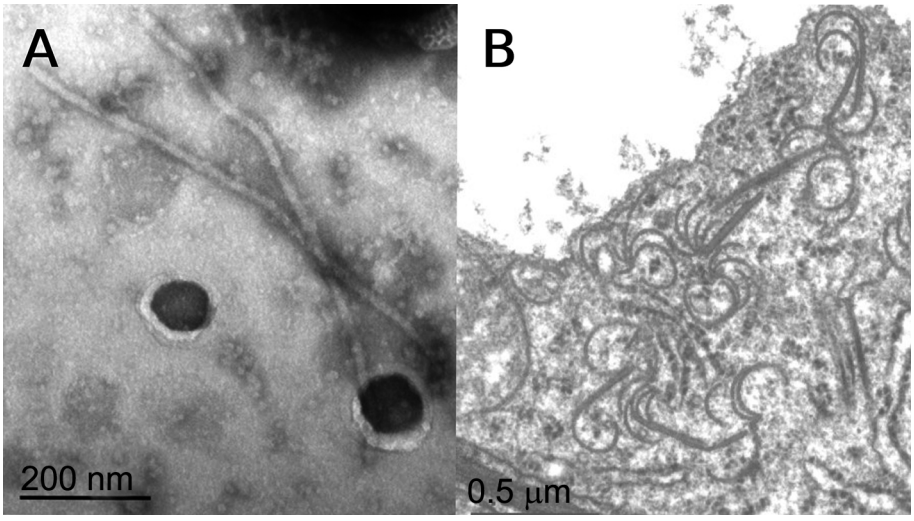

Figure 2 – Transmission electron micrographs of sunflower chlorotic mottle virus (SuCMoV) - infected sunflower (*Helianthus annuus* cv. Zebulon Tall) leaf sample. (A) Flexuous filamentous particles present in a negatively stained extract from infected sunflower leaf. (B) Typical potyvirus cylindrical inclusions forming pinwheel profiles in a section of leaf parenchyma cell of infected sunflower.

pilosa, *G. globosa*, *C. intybus*, *D. stramonium*, *L. sativa* (cvs. Lisa and Americana), and *N. tabacum* cv. TNN were asymptomatic and tested negative for viral infection by RT-PCR (Table 1). Symptoms of chlorosis, mosaic, chlorotic ringspot, and necrosis on the younger leaves of inoculated sunflower plants appeared 10-15 days post-inoculation. Symptoms were like those observed in field symptomatic plants. The symptoms in zinnia plants appeared ten days after inoculation and consisted of mosaic, like that described by Maritan et al. (2004). The partial host range result of the sunflower isolate of SuCMoV was like that reported in the virus found in *Z. elegans* that caused chlorotic local lesions in *C. quinoa* and *C. amaranticolor*, mosaic in *Z. elegans*, and systemic infection in *H. annuus* (Maritan et al., 2004). All symptomatic plants tested positive in RT-PCR assays using the specific CP2R primer in combination with the WCIEN primer for SuCMoV (Table 1).

Infection of sunflowers by SuCMoV was first described in Argentina (Dujovny et al., 1998), and it is considered the most important and widely distributed virus in Argentina in sunflowers. Moreover, weeds such as *Dipsacus fullonum*, *Ibicella lutea*, *Helianthus petiolaris*, and *Eryngium* sp. were reported to be naturally infected by SuCMoV in Argentina and can serve as reservoir hosts for the virus in the field (Bejerman et al., 2013; Giolitti et al., 2009; Mederos et al., 2020). Outside Argentina, SuCMoV has only been reported in Brazil, infecting the ornamental plant zinnia in São José do Rio Preto, in the state of São Paulo (Maritan et al., 2004).

Table 1 – The reaction of plants of different species to sunflower chlorotic mottle virus mechanically and aphid (*Myzus persicae*) inoculated.

Plant species	Mechanical transmission	
	Symptoms[a]	RT-PCR[b]
Helianthus annuus cv. Amarelo Alto	Ch, M, CRS, N	+
Bidens pilosa	ns	-
Cichorium intybus	ns	-
Chenopodium amaranticolor	CLL	+
C. quinoa	CLL	+
Datura stramonium	ns	-
Gomphrena globosa	ns	-
Lactuca sativa cv. Americana	ns	-
Lactuca sativa cv. Lisa	ns	-
Nicotiana tabacum cv. TNN	ns	-
Zinnia elegans	M	+
	Aphid transmission	
Helianthus annuus cv. Amarelo Alto	Ch, M, CRS, N	+
B. pilosa	ns	-
C. intybus	ns	-
L. sativa cv. Americana	ns	-
L. sativa cv. Lisa	ns	-
Z. elegans	M	+

[a]Symptom abbreviation: Ch = chlorosis; M = mosaic; CRS = chlorotic ringspot; N = necrosis; CLL = chlorotic local lesions; ns = no symptom. [b]Infection confirmed by RT-PCR using the primers WCIEN and CP2R; – negative reaction; + positive reaction.

This is the first report of SuCMoV infecting sunflower plants in Brazil. As SuCMoV causes severe symptoms in sunflower plants, it is essential to assess its occurrence in other producing areas of Brazil and estimate losses. Furthermore, sunflower genotypes must be tested for resistance to virus infection and/or tolerance to the disease.

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Authors' Contributions

Conceptualization: Bello, V.H.; Kitajima, E.W. **Data curation**: Bello, V.H.; Kitajima, E.W. **Formal analysis:** Bello, V.H. **Funding acquisition:** Rezende, J.AM.; Kitajima, E.W. **Investigation:** Bello, V.H.; Rezende, J.AM.; Favara, G.M.; Benardi, G.V.; Kitajima, E.W. **Methodology:** Bello, V.H.; Rezende, J.AM.; Favara, G.M.; Salaroli, R.B.; Kitajima, E.W. **Project administration:** Bello, V.H. **Resources:** Rezende, J.AM.; Kitajima, E.W. **Software:** Bello, V.H.; Favara, G.M. **Supervision:** Bello, V.H.; Rezende, J.AM.; Kitajima, E.W. **Validation:** Bello, V.H.; Favara, G.M. **Visualization:** Bello, V.H. **Writing – original draft:** Bello, V.H. **Writing – review & editing**: Bello, V.H.; Rezende, J.AM.; Favara, G.M.; Kitajima, E.W.

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