Scientific Note

Partial sequencing of a putative *Alstroemeria necrotic streak orthotospovirus* isolate detected on lettuce in Colombia[1]

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ABSTRACT

Lettuce is the most cultivated leafy salad vegetable in Colombia, being the municipality of Madrid, in the Department of Cundinamarca, the second largest producer. In this region, lettuce plants with foliar symptoms characterized by brown necrotic spots forming an extended necrotic area, chlorosis, leaf distortion and plant stunting have been detected, possibly caused by a viral infection associated with the *Orthotospovirus* genus. This study aimed to identify the orthotospovirus species associated with those symptoms, contributing to updating the lettuce phytosanitary status in this region. The presence of orthotospovirus was confirmed by the enzyme-linked immunosorbent assay (DAS-ELISA), although the sequence of the nucleocapsid (N) gene confirmed the presence of *Alstroemeria necrotic streak orthotospovirus*, disregarding the *Tomato spotted wilt orthotospovirus* and *Impatiens necrotic spot orthotospovirus* previously reported for this crop, being this its first report in lettuce crops in Colombia.

KEYWORDS: *Lactuca sativa* L., *Tomato spotted wilt orthotospovirus*, *Impatiens necrotic spot orthotospovirus*, nucleocapsid protein.

RESUMO

Sequenciamento parcial de isolado putativo de *Alstroemeria necrotic streak orthotospovirus* detectado em alface na Colômbia

A alface é o vegetal folhoso para salada mais cultivado na Colômbia, sendo o município de Madrid, no Departamento de Cundinamarca, o segundo maior produtor. Nessa região, foram detectadas plantas com sintomas foliares caracterizados por manchas necróticas de coloração castanha formando uma extensa área necrótica, clorose, distorção foliar e atrofia das plantas, causados por infecção viral possivelmente associada ao gênero *Orthotospovirus*. Objetivou-se identificar as espécies de orthotospovirus associadas aos sintomas, contribuindo para a atualização do estatuto fitossanitário da alface nessa região. Foi constatada a presença de orthotospovirus pelo ensaio de imunoabsorção enzimática (DAS-ELISA), embora a sequência do gene nucleocapsid (N) tenha confirmado a presença de *Alstroemeria necrotic streak orthotospovirus*, desconsiderando-se o *Tomato spotted wilt orthotospovirus* e *Impatiens necrotic spot orthotospovirus* previamente relatados para essa cultura, sendo este o seu primeiro relato para o cultivo de alface na Colômbia.

PALAVRAS-CHAVE: *Lactuca sativa* L., *Tomato spotted wilt orthotospovirus*, *Impatiens necrotic spot orthotospovirus*, proteína nucleocapsid.

Lettuce is one of the most cultivated leafy salad vegetables worldwide. In Colombia, the principal producing region is the Department of Cundinamarca, with Madrid being the second largest producer, with a yield of 13,514 ton ha^{-1}, in 2019 (Agronet 2021).

This crop is affected by several pathogens, being viruses, after fungi, the most limiting biotic agents, causing devastating losses (Raid 2004, Lebeda et al. 2014). Among the main viruses affecting lettuce crops so far reported are the *Lettuce mosaic virus*, *Mirafiori lettuce virus*/*Lettuce big vein virus*, *Beet western yellows virus* and *Cucumber mosaic virus*, as well as *Lettuce necrotic stunt virus*, *Tomato spotted wilt orthotospovirus* - TSWV (Lebeda et al. 2014) and *Impatiens necrotic spot orthotospovirus* - INSV (Koike et al. 2008, Beris et al. 2020), the latter two being species of the *Orthotospovirus* genus (ICTV 2019).

Currently, there are 26 plant pathogenic species of orthotospovirus (ICTV 2019) with high economic impact due to their broad host range and

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geographical distribution (Kamberoglu & Alan 2011), notably *Tomato spotted wilt orthotospovirus* (Margaria & Rosa 2015), *Impatiens necrotic spot orthotospovirus* (Nekoduka et al. 2015), *Groundnut ringspot orthotospovirus* (Hallwass et al. 2012), *Tomato chlorotic spot orthotospovirus* (Gonzalez-Arias et al. 2010) and *Alstroemeria necrotic streak orthotospovirus* (Olaya et al. 2017).

The symptoms associated with TSWV and INSV in lettuce crops are indistinguishable from one another, consisting of brown necrotic spots forming an extended necrotic area, leaf chlorosis, leaf distortion and plant stunting (Koike et al. 2008, Moreno & Fereres 2012, Beris et al. 2020).

All types of lettuce (iceberg, romaine, greenleaf, redleaf, butter) are susceptible to TSWV and INSV, and both viruses are not seedborne, i.e., they infect several crops, weed species and are spread by thrips.

Alstroemeria necrotic streak orthotospovirus (ANSV) is endemic in Colombia, being found naturally infecting ornamental alstroemeria (*Alstroemeria* sp.) crops (Hassani-Mehraban et al. 2010). Furthermore, other surveys have identified natural infections in solanaceous crops, including tomato (*Solanum lycopersicum*), bell pepper (*Capsicum annuum*) and lulo (*Solanum quitoense*), in Colombia (Olaya et al. 2017, Gallo et al. 2018, Gallo et al. 2019). Recently, symptoms similar to those of orthotospovirus infection have been reported in commercial lettuce crops in the municipality of Madrid, Cundinamarca. Thus, to establish the causal agent thereof, the present study aimed to identify the viral species associated with those symptoms, in order to update the lettuce phytosanitary status in this region.

Random sampling was used to evaluate seven romaine lettuce commercial crops, with samples collected from 30 points in each crop, of which 6 plants per point were evaluated, during the first crop cycle, in 2017. The disease incidence was 30 % in the municipality of Madrid, Department of Cundinamarca, Colombia, with foliar symptoms characterized by brown necrotic spots forming an extended necrotic area, chlorosis, leaf distortion and plant stunting (Figure 1).

The collected plant material underwent serological and molecular testing. The serological tests were performed according to the decision scheme for some of the orthotospoviruses reported in diagnosis protocols for regulated pests (EPPO 2004), avoiding indicator plants (Figure 2). Enzyme-linked immunosorbent assays (DAS-ELISA) for *Orthotospovirus* (Agdia Inc., Elkhart, IN) and lateral flow ImmunoStrip™ assays (Agdia Inc.) were carried out for TSWV and INSV using sap obtained directly from collected symptomatic lettuce plants.


Photo: Diego Alejandro Jurado-Rincón

Figure 1. Symptoms in lettuce plants (brown necrotic spots forming an extended necrotic area, chlorosis, leaf distortion and plant stunting), in the municipality of Madrid, Cundinamarca, Colombia.

RNA extraction was performed from symptomatic lettuce plants using the Thermo Scientific GeneJET™ kit, according to the manufacturing instructions. The first-strand cDNA synthesis was done using a universal degenerate primer for the coding region of the orthotospovirus nucleocapsid protein (N) gene (Uga & Tsuda 2005), in a volume of 20 µL. The reaction mixture was composed of 0.1 µM final concentration of universal primer

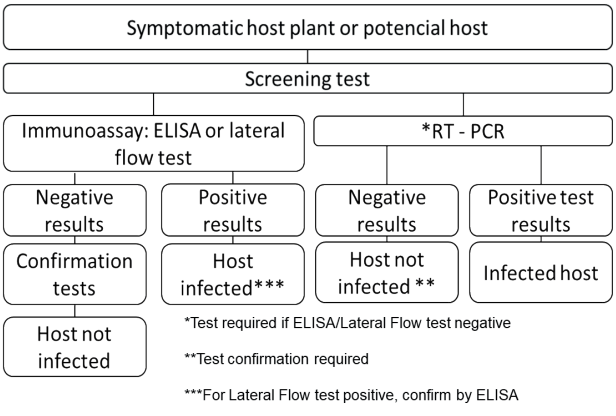

Figure 2. Decision scheme for orthotospovirus detection and identification, modified from EPPO (2004).

TOS-R15 (Table 1), 0.5 mM dNTPs (Thermo Fisher Scientific™), 1X buffer (Invitrogen™), 0.01 M DTT (Invitrogen™), 40 U RNasa-out (Invitrogen™), 200 U M-MLV (Invitrogen™) and 9 µL ARN, incubating the mixture at 25 ºC for 15 min, 37 ºC for 50 min and 70 ºC for 15 min.

The PCR was carried out in a volume of 20 µL, containing 0.1 µM of the specific primers reported by Liu-Yuan et al. (2017) and Uga & Tsuda (2005) (Table 1), with 2 µL of cDNA, 1X buffer (Invitrogen™), 1.5 mM of $MgCl_2$ (Invitrogen™), 0.2 mM dNTPs (Thermo Fisher Scientific™), 0.2 µM of each primer and 1.25 U Taq Pol (Invitrogen™). It was carried out following this thermal profile: 95 ºC for 5 min, followed by 35 cycles at 95 ºC for 30 s, 55 ºC for 40 s and 72 ºC for 30 s, and a final step of 6 min at 72 ºC. Alstroemeria lyophilized material infected with TSWV was used as a positive control. The PCR products were analyzed in 1.5 % agarose gel electrophoresis and sequenced, and the BioEdit software (version 7.1.9) was used for the analysis, whose results were compared with the sequences available in the GenBank, in which the consensus sequences generated in this study were deposited.

The visualized symptoms (Figure 1) corresponded to those associated with both the TSWV and INSV species (Kuo et al. 2014), which were reported in Europe (Moreno & Fereres 2012), Asia (Al-Saleh et al. 2014), Brazil (Pavan et al. 2008) and the United States (Abad et al. 2005). However, the differentiation between both viruses in the field is difficult, due to the ressemblance of symptoms produced by these two species (Kuo et al. 2014). In this way, the identification of such viral species in this study was carried out following the decision scheme for orthotospovirus detection (EPPO 2004), which was modified, avoiding indicator plants (Figure 2). The symptomatic samples were positive for the *Orthotospovirus* genus by the DAS-ELISA test and showed a positive reaction to TSWV and negative to INSV by the ImmunoStrip™ test, thus suggesting that TSWV is the causal agent of this disease. A cross reaction of TSWV antiserum with other orthotospovirus species, such as *Groundnut ringspot orthotospovirus*, *Tomato chlorotic spot orthotospovirus* and ANSV, was reported by Margaria & Rosa 2015, Hassani-Mehraban et al. 2016, Agdia 2017 and Olaya et al. 2017, based on the highly conserved capsid protein used for these tests (Gallitelli 2004). For this reason, we used molecular tests to verify the viral species.

The RT-PCR for nucleocapsid N gene detection was performed using the primers TSWV-709/TOS-R15 (Uga & Tsuda 2005) and Pr-dTS-f/Pr-dTS-r (Liu-Yuan et al. 2017), and the sequence analysis confirmed that the disease is caused by orthotospoviruses. To determine the viral species, a product of 709 pb was obtained from the symptomatic lettuce samples (accession number MK085115) and the positive control (Alstroemeria infected with TSWV, accession number MK08511), using the primer combination TSWV-709 (specific for TSWV)/TOS-R15 (universal for *Orthotospovirus*) (Figure 3; Table 1). The sequence analysis of the lettuce samples showed nucleotide identity higher than 99 % for the ANSV genome segment S and the N gene (MK275264.1, MK275263.1, GQ478668.1, KX833218.1), and 78.44 % of nucleotide identity for the TSWV nucleocapsid protein N gene (AJ296600.1). Meanwhile, the Alstroemeria control infected with TSWV (MK085117) showed nucleotide identity higher than 99 % for the TSWV genome segment S and the N gene (KC261967.1, HQ830187.1). Additionally, there was not an amplification product using the primers INSV-589 (specific for INSV)/TOS-R15 (universal for *Orthotospovirus*), confirming the absence of INSV in the samples.

Table 1. PCR with universal and specific primers to detect the nucleocapsid (N) gene of *Orhtotospovirus*.

Primer	Sequence 5'-3'	Specificity	Product (bp)	Temperature (ºC)	Author
TSWV-709/ TOSR15	GTGTCATACTTCTTTGGGTC GGGAGAG-CAATYGWGKYR	TSWV *Orthotospovirus*	709	53	Uga & Tsuda (2005)
INSV-589/ TOSR15	CCCAAGACACAGGATTTCA GGGAGAGCAATYGWGKYR	INSV *Orthotospovirus*	589	53	Uga & Tsuda (2005)
Pr-dTS-f/ Pr-dTS-r	ATGTMTAAGGYHAAGHTYAC GAAGCWATVAGAGGNADRCTWCCTCC	*Orthotospovirus*	460	50	Liu-Yuan et al. (2017)

According to these results, in order to confirm the presence of ANSV, the pair of primers Pr-dTS-f/ Pr-dTS-r (Table 1) was used, which identified six orthotospovirus species. As a result, a 460 bp product (accession number MK085116.1) (Figure 4) was obtained, with nucleotide identity of more than 99 % for the ANSV genome segment S and the N gene (MK275264.1, GQ478668.1, KX833218.1).

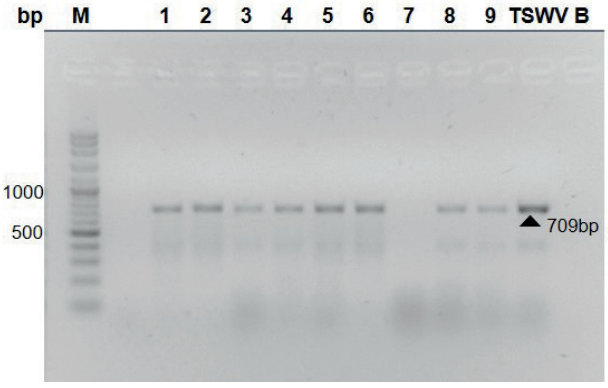

Figure 3. RT-PCR for the *Tomato spotted wilt orthotospovirus* (TSWV) gene N detection using primers reported by Uga & Tsuda (2005). M: molecular marker GeneRuler 100 bp Plus (Thermo Fisher Scientific™); 1-6 and 8-9: symptomatic plants; 7: healthy plant, Alstroemeria positive control infected with TSWV; B: blank. The head arrow indicates amplification product corresponding to 709 bp. Electrophoresis in agarose gel: 1.5 %.

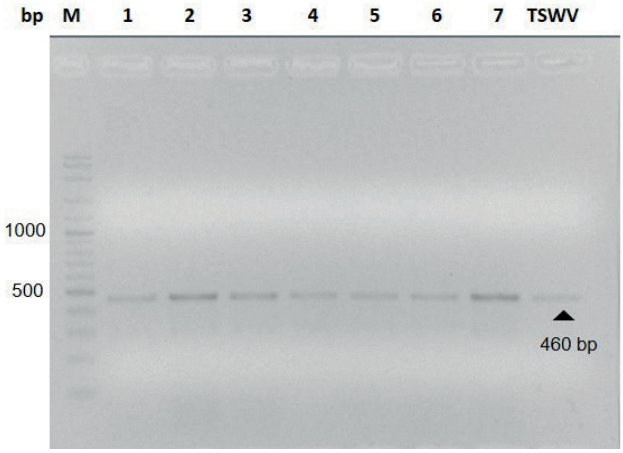

Figure 4. RT-PCR for the orthotospovirus N gene detection using primers reported by Liu-Yuan et al. (2017). M: molecular marker GeneRuler 100 bp Plus (Thermo Fisher Scientific™); 1-7: symptomatic plants; TSWV: Alstroemeria positive control infected with *Tomato spotted wilt orthotospovirus*. The head arrow indicates amplification product corresponding to 460 bp. Electrophoresis in agarose gel: 1.5 %.

Meanwhile, the positive control (Alstroemeria infected with TSWV, accession number MK085117.1) showed nucleotide identity of 99 % for the TSWV genome segment S and the N gene (KC261967.1, HQ830187.1, AJ297611.1).

According to the molecular analysis, all sequences showed nucleotide identity higher than 99 % for ANSV, discounting the presence of the TSWV identified by the ImmunoStrip™ test. Therefore, ANSV is another orthotospovirus present in lettuce which had not yet been reported for this crop in Colombia, as this species had been previously detected only for solanaceous like tomato, lulo and pepper (Olaya et al. 2017, Gallo et al. 2018, Gallo et al. 2019), what supports the extensive host range previously proposed for this virus (Hassani-Mehraban et al. 2016). It would be interesting to sequence the complete genome of ANSV infecting lettuce to establish whether it belongs to the American clade, as the ANSV infecting solanaceous plants, and evaluate in different regions such viral presence in lettuce crops and thrips vectors. However, specific primers have been recently designed from the genome sequencing of ANSV from Colombian solanaceous samples, which could as well be used to confirm the presence of ANSV in other vegetables by RT-PCR and RT-qPCR (Gallo et al. 2018, Gallo et al. 2019).

In conclusion, an accurate orthotospovirus diagnosis between *Tomato spotted wilt orthotospovirus* and *Alstroemeria necrotic streak orthotospovirus* requires the use of molecular RT-PCR test with different sets of specific primers followed by sequence analysis, because of cross reactions when immunological tests are used. Specific molecular tests detected the presence of *Alstroemeria necrotic streak orthotospovirus* in lettuce, and could be used in other crops and regions to update the phytosanitary status of this viral species in Colombia.

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