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COMUNICACIÓN

Phylogenetic analysis of *Groundnut ringspot virus isolates* from peanut and identification of potential thrips vectors in peanut crop in Argentina

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SUMMARY

Groundnut ringspot virus (GRSV), genus *Tospovirus*, is a thrips-transmitted virus infecting peanuts (*Arachis hypogaea* L.) in Córdoba province, Argentina. Fourteen viral isolates were recovered from *Tospovirus*-like symptomatic plants from different peanut fields. Viral isolates as GRSV were identified by serological and molecular tests. Nucleotide and derived amino acid sequence analyses of the nucleocapsid (*N*) gene indicated a high degree of identity between the GRSV peanut isolates, indicating that there is no molecular variability in the *N* gene of the GRSV that infects peanuts in the cropping area of Córdoba. In this study, we determined the presence of thrips species in the crop, which can potentially transmit the virus. Thrips were observed in all the evaluated peanut fields. *Frankliniella schultzei* was the most frequently identified species followed by *Caliothrips phaseoli* and *Frankliniella occidentalis*. This work reports the presence of *F. schultzei* and *F. occidentalis* in peanuts in Argentina for the first time. These results along with the high degree of similarity between the GRSV peanut isolates suggest that the virus could be transmitted by *F. schultzei*, which has been cited as its most efficient vector.

Key words: *Arachis hypogaea*, *Tospovirus*, molecular characterization, *Frankliniella schultzei*

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RESUMEN

Groundnut ringspot virus (GRSV, género *Tospovirus*) es un virus que infecta naturalmente el cultivo de maní (*Arachis hypogaea* L.) en la región productora de Córdoba, Argentina. En distintas localidades de la provincia, se

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colectaron 14 aislamientos virales provenientes de maníes que manifestaban síntomas característicos de *Tospovirus*. Todos los aislamientos virales fueron identificados como GRSV mediante pruebas serológicas y moleculares. El análisis de las secuencias nucleotídicas y de amino ácidos deducidas del gen de la nucleoproteína (*N*) reveló un alto grado de identidad entre los 14 aislamientos, indicando que no existe variabilidad molecular en el gen *N* del GRSV que infecta maní en la provincia de Córdoba. En este estudio se determinó la presencia de trips en el cultivo que pueden potencialmente transmitir la enfermedad. Estos insectos fueron observados colonizando maní en todos los lotes evaluados. La especie identificada con mayor frecuencia fue *Frankliniella schultzei*, seguida de *Caliothrips phaseoli* y *Frankliniella occidentalis*. Este es el primer reporte de *F. schultzei* y *F. occidentalis* afectando maní en Argentina. Estos resultados, junto con el elevado grado de similitud encontrado entre los distintos aislamientos de GRSV, sugieren que el virus puede ser transmitido por *F. schultzei*, citado como el vector más eficiente del GRSV.

Palabras clave: *Arachis hypogaea*, *Tospovirus*, caracterización molecular, *Frankliniella schultzei*

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Argentina is one of the world's main peanut (*Arachis hypogaea* L.) exporting countries. The principal peanut growing area is located in the center-south of Córdoba province (central Argentina), accounting for approximately 91% of the total production (Source: MAGyP – SIIA).

Peanut is affected by several tospoviruses worldwide, including: *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV), *Peanut bud necrosis virus* (PBNV), *Peanut yellow spot virus* (PYSV) and the tentative species Peanut chlorotic fan-spot virus (PCFSV) (Reddy *et al*., 1992; De Avila *et al*., 1993; Satyanarayana *et al*., 1998; Chu *et al*., 2001). TSWV, the type member, is the most important species. It was first recorded in Texas and has become one of the most serious peanut diseases in the United States. To keep TSWV incidence at low levels, it is necessary to combine resistant cultivars with cultural measures such as planting date, plant population, row pattern, tillage, herbicide and insecticide application (Culbreath *et al*., 2003). In the peanut growing area of Córdoba, the crop is naturally affected by GRSV, an emerging *Tospovirus* whose infection causes plant stunting, and smaller and distorted leaves showing severe chlorosis and concentric ringspots (de Breuil *et al*., 2007; Pappu *et al*., 2009). GRSV was found to be distributed in the central-northern area of the peanut-growing region of Córdoba, including the departments of Río Segundo, Tercero Arriba, Gral. San Martín and Juárez Celman (de Breuil *et al*., 2008). The distribution is probably related to the presence of insect vectors, since tospoviruses are transmitted exclusively by thrips (*Thysanoptera*) in nature (Whitfield *et al*., 2005). GRSV is efficiently transmitted in a propagative manner by the thrips species *Frankliniella schultzei* (Tribom) and *Frankliniella occidentalis* (Pergande) (de Borbón & Gracia, 1996; Nagata *et al*., 2004) and by *Frankliniella gemina* under experimental conditions (de Borbón *et al*., 2006).

The aims of this work were to determine the phylogenetic relationship between GRSV isolates and to assess the presence of *Tospovirus* thrips vectors in peanuts in the main producing area of Argentina in order to generate epidemiological information about GRSV disease.

The survey was carried out in the province of Córdoba in February and March 2009 and 2010. During the study, 92 farm fields were arbitrarily selected and surveyed for virus infection. Young leaf samples were collected from plants showing *Tospovirus*-like symptoms and stored at 4°C until analyses. Samples were tested by double-antibody sandwich-enzyme-linked immunosorbent

assay (DAS-ELISA) using commercial ELISA kits with specific GRSV/TCSV antibodies with their respective positive and negative controls, following the manufacturer's protocol (Agdia Inc., Elkhart, IN). ELISA-positive samples were assayed by antigen-capture (AC) followed by reverse transcription-polymerase chain reaction (RT-PCR). For AC, PCR tubes were coated with 50 µl of a 1:200 (w/v) dilution of leaf tissue extract in carbonated coating buffer, and incubated overnight at 4ºC. RT-PCR was performed with two GRSV-specific primers for the nucleoprotein (*N*) gene (Boari *et al.*, 2002) using the Access RT-PCR System (Promega Corp., Madison, WI, USA). The following cycling parameters were used: 48ºC for 45 min, 94ºC for 4 min, (30 cycles of 94ºC for 1 min, 50ºC for 2 min, 68ºC for 1 min), and a final extension of 68ºC for 7 min. Electrophoresis of the PCR products was conducted on a 1.4% agarose gel and visualized with 0.01% ethidium bromide under UV light. The amplified fragments were purified using Wizard-PCR columns (Promega) and then ligated into pGEM-T plasmid (Promega) according to the manufacturer's instructions. Positive recombinant clones were sequenced in both directions at Macrogen Inc. (Korea Republic). The sequences were compared with each other and with other *Tospovirus* sequences available in the GenBank (NCBI, 2014). Multiple alignments of the nucleotide and deduced amino acid sequences were obtained using ClustalW and phylogenetic trees were generated with the MegAlign program (DNASTAR, Lasergene Software, 2001). Accordingly, the *N* gene nucleotide and protein sequence analysis have been selected as important criteria for *Tospovirus* species designation (King *et al.*, 2012).

In addition, all the surveyed fields were visually evaluated for presence or absence of thrips. Insect sampling was made in 19 fields planted in different locations and growing seasons. In each field, a sample included 20 peanut flowers collected randomly from the interior and margin of the plot, or 20 leaves when flowers were not present. At some sites, thrips were taken from weeds growing within the crop. Samples were stored separately in plastic bags; thrips were then transferred to vials containing 70% ethanol using a brush, and tagged with date and origin. All adult specimens collected were individually mounted on slides and examined under stereoscopic microscope with 60X magnification. Identifications were made by confrontation with previously identified material and keys for *Thysanoptera*.

Fourteen *Tospovirus*-symptomatic peanut leaves collected during 2009 from different locations across the center-south of Córdoba province were positive to GRSV/TCSV infection according ELISA test. During the 2010 cropping season, no *Tospovirus*-infected plants were detected. ELISA-positive samples were assayed by RT-PCR and yielded an expected 800-bp fragment corresponding to the *N* gene of GRSV. They were designated according to their collection site as GRSV-ARo, GRSV-Baig, GRSV-Beng, GRSV-Cab, GRSV-DVe, GRSV-Etr, GRSV-Euf, GRSV-MGa, GRSV-Mol, GRSV-Oli, GRSV-RCua, GRSV-RTer, GRSV-Tan and GRSV-TPu. Comparisons of nucleotide and deduced amino acid sequences of the *N* fragment confirmed their identities as GRSV. The 14 peanut isolates had a high nucleotide identity to other GRSV sequences (95.1-99.8%), showing the highest percentage values with the Argentine GRSV isolate (GRSV-AR) (Table 1). They also had identity values of 82.8-84.0% with TCSV and of 77.0-78.2% with TSWV. Comparisons of amino acid sequences of peanut isolates with other GRSV isolates revealed percent identities ranging from 95.0% to 99.5%, whereas with TCSV and TSWV, identities ranged from 86.9% to 88.0% and 79.2% to 80.3%, respectively (Table 1). When the GRSV peanut isolate sequences from Córdoba were compared with each other, the percentages of identities ranged between 97.2-100% for nucleotides and 98.1-100% for derived amino acid (Table 1), indicating a lack of molecular variability in the *N* gene of the GRSV infecting peanuts in the cropping area of Córdoba.

Phylogenetic analyses based on the nucleotide sequence of the GRSV *N* gene showed segregation into three clusters. The isolates from Córdoba clustered with GRSV-AR, whereas isolates from Brazil and South Africa clustered in separate clades (Figure 1). This result indicates that the *N* gene is highly conserved and that the degree of relatedness between GRSV isolates is correlated with their geographic origin, which agrees with previous studies of *Tospovirus* (Nischwitz *et al.*, 2007; Yeturu *et al.*, 2014). This finding may be related with the coordinated co-evolution between thrips, tospoviruses, and their plant hosts (Ullman *et al.*, 2005; Whitfield *et al.*, 2005; Ananthakrishnan & Annadurai, 2007; Pappu *et al.*, 2009; Sundaraj *et al.*, 2014).

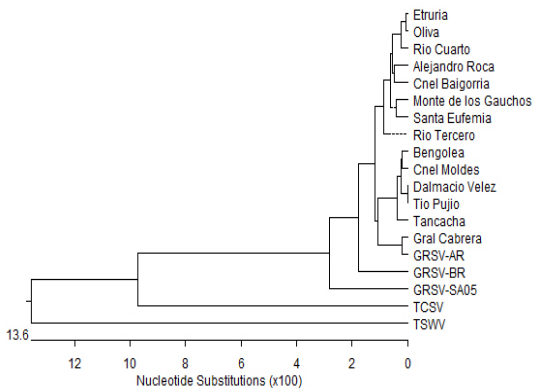
In all the evaluated fields (100%) many thrips were detected in flowers and leaves of peanut plants. They cause damage by inserting their needlelike mouthparts into the leaf surface and feeding, and according to their abundance, symptoms could be negligible or significant. In this survey, 19 thrips samples were taken from different locations and in two years. A total of 1025 individuals were analyzed (782 and 243 in the 2009 and 2010 seasons, respectively). The most frequently observed

Table 1. Percent nucleotide identities (above diagonal) and derived amino acid identities (below diagonal) of the *N* gene of GRSV peanut isolates with other reported tospoviruses.

Virus	GRSV -ARo	GRSV -Baig	GRSV -Beng	GRSV -Cab	GRSV -DVe	GRSV -Etr	GRSV -Euf	GRSV -MGa	GRSV -Mol	GRSV -Oli	GRSV -RCua	GRSV -RTer	GRSV -Tan	GRSV -TPu	GRSV -AR	GRSV -BR	GRSV -SA05	TCSV	TSWV
GRSV-ARo	-----	99.1	98.1	97.9	98.1	98.6	98.6	99.1	98.3	98.8	98.8	98.7	97.6	98.1	97.7	96.9	95.1	83.8	77.5
GRSV-Baig	99.2	-----	97.9	98.1	97.9	98.7	98.7	99.2	98.2	99.0	99.0	98.8	97.4	97.9	97.6	96.8	95.4	84.0	78.1
GRSV-Beng	99.6	99.2	-----	98.1	99.2	97.7	97.7	97.9	99.5	97.9	97.9	97.8	98.3	99.2	97.9	96.5	95.8	83.4	77.7
GRSV-Cab	99.6	99.2	99.6	-----	97.8	97.6	97.8	98.1	98.1	97.8	97.8	98.1	97.2	97.8	99.8	96.4	95.1	83.4	78.1
GRSV-DVe	98.8	98.5	98.8	98.8	-----	97.7	97.7	97.9	99.2	97.9	97.9	97.8	98.3	100	97.7	96.3	95.5	83.1	77.5
GRSV-Etr	98.8	98.5	98.8	98.8	98.1	-----	98.2	98.7	97.9	99.5	99.2	98.3	97.2	97.7	97.1	96.3	95.5	83.5	77.7
GRSV-Euf	98.8	98.5	98.8	98.8	98.1	98.1	-----	99.0	97.7	98.4	98.5	98.6	97.3	97.7	97.4	96.8	95.1	83.4	77.0
GRSV-MGa	99.6	99.2	99.6	99.6	98.8	98.8	98.8	-----	98.2	99.0	99.0	98.8	97.4	97.9	97.7	96.8	95.2	83.7	77.7
GRSV-Mol	99.6	99.2	99.6	99.6	98.8	98.8	98.8	99.6	-----	98.2	98.2	97.8	98.3	99.2	97.9	96.5	95.8	83.4	78.2
GRSV-Oli	100	99.6	100	100	99.2	99.2	99.2	100	100	-----	99.5	98.6	97.4	97.9	97.6	96.5	95.7	83.5	77.7
GRSV-RCua	99.6	99.2	99.6	99.6	98.8	98.8	98.8	99.6	99.6	100	-----	98.6	97.4	97.9	97.4	96.5	95.8	83.7	77.7
GRSV-RTer	98.8	98.5	98.8	98.8	98.1	98.1	98.1	98.8	98.8	99.2	98.8	-----	97.3	97.8	97.9	96.7	95.1	83.7	77.5
GRSV-Tan	99.2	98.8	99.2	99.2	98.5	98.5	98.5	99.2	99.2	99.6	99.2	98.5	-----	98.3	96.9	96.4	95.2	82.8	77.0
GRSV-TPu	98.8	98.5	98.8	98.8	99.6	98.1	98.1	98.8	98.8	99.2	98.8	98.1	98.5	-----	97.7	96.3	95.6	83.1	77.5
GRSV-AR	99.5	99.0	99.5	99.5	98.5	98.5	98.5	99.5	99.5	99.5	99.5	99.0	99.0	98.5	-----	96.4	94.5	82.4	78.2
GRSV-BR	98.8	98.5	98.8	98.8	98.1	98.1	98.1	98.8	98.8	99.2	98.8	98.1	98.5	98.1	99.5	-----	94.2	83.0	77.0
GRSV-SA05	95.7	95.3	95.7	95.7	95.0	95.0	95.0	95.7	95.7	95.7	95.7	95.0	95.3	95.0	95.1	95.0	-----	82.9	77.7
TCSV	87.6	88.0	87.6	87.6	86.9	86.9	86.9	87.6	87.6	87.9	87.6	86.9	87.6	86.9	89.2	86.9	84.9	-----	77.7
TSWV	79.9	80.3	79.9	79.9	79.2	79.2	79.2	79.9	79.9	80.2	79.9	79.2	79.9	79.2	82.4	79.2	78.7	79.9	-----

The origin of the Córdoba peanut isolates of GRSV and the origin and the accession numbers of the tospovirus sequences available in the GenBank are indicated in parentheses: GRSV-ARo (Alejandro Roca), GRSV-Baig (Cnel Baigorria), GRSV-Beng (Bengolea), GRSV-Cab (Gral Cabrera), GRSV-DVe (Dalmacio Vélez), GRSV-Etr (Etruria), GRSV-Euf (Sta Eufemia), GRSV-MGa (Monte de los Gauchos), GRSV-Mol (Cnel Moldes), GRSV-Oli (Oliva), GRSV-RCua (Río Cuarto), GRSV-RTer (Río Tercero), GRSV-Tan (Tancacha), GRSV-TPu (Tío Pujio), GRSV-AR (DQ973171; Argentina), GRSV-BR (AF251271; Brazil), GRSV-SA05 (S54327; South Africa), TCSV (AF282982; Brazil) and TSWV (AB175809; Korea).

Figure 1. Phylogenetic tree showing relationships of nucleotide sequences of the *N* gene between the GRSV peanut isolates from Córdoba and reported sequences of GRSV, TCSV and TSWV in the GenBank.



species was *F. schultzei* (tomato thrips) (442 and 231 in 2009 and 1010, respectively), followed by *Caliothrips phaseoli* Hood (bean thrips) (321 only in 2009) and *F. occidentalis* (western flower thrips), of which only 30 thrips were collected in both years (Table 2). Of these species, those belonging to the *Frankliniella* genus can transmit GRSV. *F. schultzei*, the most efficient vector of GRSV (Nagata *et al.*, 2004), was present in almost all samples studied both in the crop and in the associated weeds shortpod mustard (*Hirschfeldia incana* (L.) Lagr.-Fossat, family *Brassicaceae*), and tall morning-glory (*Ipomoea purpurea* (L.) Roth., family *Convolvulaceae*), (Table 2). *H. incana* is an annual to biennial plant species and has been reported as natural host of another *Tospovirus*, the TSWV (Lavina *et al.*, 1996), so it could be a GRSV alternative host and/or a reproductive host for thrips. *F. schultzei* is a poliphagous species that is widespread throughout Córdoba's peanut growing region and because of the long distance movements of winged thrips carried by winds it could be responsible for GRSV spread. Other thrips species present in peanut crops with high frequency was *C. phaseoli*. It was detected on peanut and other widely cultivated crops such as soybean (*Glycine max* (L.) Merr.) and lucerne (*Medicago sativa* L.) where it causes plant damage (Boito *et al.*, 2006). However, it has not been reported as a *Tospovirus* vector.

This work shows that peanut crops are usually attacked by thrips. Accordingly, our study reports

Table 2. Thrips species infecting peanuts and weeds sampled at different locations and on different dates in the surveyed growing area of Córdoba province.

Thrips samples			Presence of GRSV in peanut fields	Number of thrips identified		
Host plant	Location	Date		*Frankliniella schultzei*	*Frankliniella occidentalis*	*Caliothrips phaseoli*
Peanut flowers	Oliva	February 2009	Yes	45	---	---
Peanut flowers	Dalmacio Vélez	February 2009	Yes	51	---	---
Shortpod mustard flowers	Dalmacio Vélez	February 2009	Yes	46	10	---
Tall morning-glory flowers	Gral Fotheringham	February 2009	No	43	---	---
Peanut flowers	Bengolea	February 2009	Yes	51	---	---
Peanut flowers	Chazón	February 2009	No	30	---	6
Peanut flowers	Paso del Durazno	February 2009	No	44	---	---
Peanut flowers	Alejandro Roca	February 2009	Yes	38	---	---
Peanut flowers	Malena	March 2009	No	26	---	70
Peanut flowers	Cnel Moldes	March 2009	Yes	35	8	6
Peanut flowers	Monte de los Gauchos	March 2009	Yes	7	---	75
Peanut leaves	Ruiz Díaz de Guzmán	March 2009	No	11	---	84
Peanut flowers	Gral Lavalle	March 2009	No	---	---	70
Peanut flowers	Nicolás Bruzone	March 2009	No	16	---	10
Peanut flowers	Manfredi	March 2010	No	48	12	---
Peanut flowers	Gral Cabrera	March 2010	No	5	---	---
Peanut flowers	Carnerillo	March 2010	No	22	---	---
Peanut flowers	Charras	March 2010	No	124	---	---
Peanut flowers	Olaeta	March 2010	No	32	---	---

the presence of *F. schultzei* and *F. occidentalis* on peanuts in Argentina for the first time. They are important pests because besides causing leaf damage to crops, they could be acting as vectors of GRSV. It is likely that viruliferous *F. schultzei* thrips are responsible for the dispersion of the GRSV strain infecting the peanut crops. However, despite the presence of the competent thrips vectors, no plants infected with GRSV were detected in 2010, so further investigations are needed to elucidate the components involved in the viral disease.

Understanding the vector-virus relationship between thrips and *Tospovirus* is fundamental to develop disease management measures. Currently, ongoing research works are focused on the population dynamics of thrips within the peanut fields and the role of weed host reservoirs in viral infection cycle and virus spread.

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