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Short communication

First report of Rice stripe necrosis virus in Argentina

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| ARTICLE INFO | A B S T R A C T |
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| Keywords: Oryza sativa Benyvirus RSNV Plant virus | Since 2008/2009, we have seen symptoms of viral infection in rice plants from crop regions in Argentina. The main objective of this work was to identify the causal agent of the rice plants' crinkling. We therefore analyzed samples of symptomatic rice plants from Berón de Astrada (Corrientes province) and San Javier (Santa Fe province) by serological, electron microscopy and RT-PCR methods, and determined the presence of <i>Rice stripe necrosis virus</i> (RSNV). Helically constructed rod-shaped virus particles, with an axial canal with an estimated modal length of 228 nm, 370 nm and a last interval of 87–181 nm and a diameter of 20 nm, were observed in ultrathin sections and ISEM-D from rice leaf samples. Transmission of RSNV by viruliferous soil was achieved. Phylogenetic analysis, based on a partial sequence (828 nt) of the helicase gene of 10 RSNV isolates, grouped the |

1. Introduction

Rice (Oryza sativa L.) is fifth in the ranking of Argentina's cereal production (https://datos.agroindustria.gob.ar/dataset/estimacionesagricolas). It is grown mainly in the provinces of Corrientes (45%), Entre Ríos (37%) and Santa Fe (14%) (Buryaile et al., 2016). Diseases are one of the main limitations to rice productivity. Several species of fungi infecting rice have been reported In Argentina, but there were no reports of viruses infecting rice in the country (http://www.sinavimo. gov.ar/plagas?populate=arroz). Since 2008/2009, we have seen symptoms of viral infection in rice plants like those described for "Rice Crinkle Disease" (Louvel and Bidaux, 1977; Fauquet and Thouvenel, 1983) from several locations of the provinces of Corrientes, Santa Fe and Tucumán. In Berón de Astrada, around 28% of rice crops (3270 of 11700 has) presented symptoms during 2017/2018, with up to 65 symptomatic plants per m2 (Carbajal, L., ADECOAGRO, personal communication). There are approximately 30 diseases caused by viruses in rice plants (Abo and Fadhila, 1998; Yang et al., 2017). Rice stripe necrosis virus (RSNV) is the causal agent of "Rice Crinkle Disease", one of the most severe viral infections in rice (Fauquet et al., 1988; Morales et al., 1999). Rice plants infected with RSNV present conspicuous chlorotic stripes and systemic leaf necrosis, growth retardation, panicle malformation and low seed production (Fauquet and Thouvenel, 1983).

Argentinian sequences in the same clade as the African, rather than with the sequence from Colombia. This is the first report not only of the presence of RSNV in Argentina, but of a viral infection in rice plants in the country.

RSNV is a virus of *Benyvirus* genus, transmitted by *Polymyxa graminis* (Fauquet et al., 1988; Kanyuka et al., 2003), an obligate parasite of plant roots, protist of the order *Plasmodiophorales* (Dick, 2001). This parasite has recently been reported in Argentina in rice plants from Berón de Astrada (Cundom et al., 2018). Up to five genomic RNAs have been reported for *Benyvirus*, though only two have been found for RSNV: RNA1 with 6614 nucleotides (nt) in length and RNA2 with 4631 nt in length (Gilmer and Ratti, 2011; Lozano and Morales, 2009).

Several papers and technical reports relate the presence of symptoms of RSNV infections and yield losses in rice fields. The effect of RSNV on production depends on the developmental stage in which the plant was infected, the number of infected tillers on each plant, the severity of the symptoms and the rice variety. Among 73 rice varieties tested in Côte d'Ivoire between 1981 and 1983, the percentage of infected plants ranged from 0 to 100%, depending on the variety (Fauquet et al., 1988). In Colombia and Ecuador, infected rice fields showed between 20% and 40% of yield losses (Pardo and Muñoz, 1994; Paz et al., 2009). The presence of RSNV was reported in Colombia

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Fig. 1. Rice plants (*Oryza sativa* L.) showing symptoms of a viral infection. A) Leaf crinkling and necrosis; B and C) Panicle deformation; D) Leaf crinkling, chlorotic striping and necrosis; E) Leaf striping; F) Striping and crinkling in seedlings.

(Morales et al., 1999), Ecuador (Paz et al., 2009), Côte d'Ivoire (Louvel and Bidaux, 1977), Liberia, Nigeria and Sierra Leone (Fauquet et al., 1988), Panama (Lozano and Morales, 2009), Brazil (Maciel et al., 2006), Burkina Faso (Sereme et al., 2014), Benin (Oludare et al., 2015) and Mali (Decroës et al., 2017).

The main objective of this work was to confirm the identity of the causal agent of rice plants crinkling in crop regions in Argentina.

2. Material and methods

2.1. Rice sample collection

In February 2017, five symptomatic rice plants were collected from San Javier (Santa Fe Province) showing leaf veins thickening, leaf blades crinkling and young leaves unfolding from the spindle, dwarfism, lax and compact panicles, and leaf blades with a darker color or with chlorotic stripes that eventually turn necrotic (Fig. 1). These plants were used for the serological, molecular and microscopic identification of the causative agent of the disease. Subsequently, 58 symptomatic rice plants were collected from affected rice crops from Berón de Astrada (Corrientes Province).

Additionally, six plants were collected of different weed species that grow in rice crops: three plants of *Ludwigia* sp., one *Panicum* sp., one *Eleusine* sp. and one *Echinochloa* sp., from the surroundings of symptomatic rice plants in San Javier and Berón de Astrada. These also presented symptoms of leaf mosaic and twisting. The plant material collected was maintained under fresh conditions until arrival at the laboratory, where it was stored at 6 °C until analysis.

Rice plants obtained from seeds sown in sterile soil, maintained under controlled conditions in the greenhouse and without symptoms, were used as a negative control.

2.2. Serological detection

Seven rice plants, the five symptomatic plants from San Javier and two negative controls, were analyzed by Plate-trapped antibody enzyme-linked immunosorbent assay (PTA-ELISA) according to Lommel et al. (1982), using RSNV antiserum at a final dilution of 1:250 (v/v) (Fauquet and Thouvenel, 1983). The antiserum to an isolate of RSNV from Côte d'Ivoire (Fauquet and Thouvenel, 1983) was kindly provided by Dr. Eugénie Hébrard, Institut de Recherche pour le Développement (IRD), Montpellier, France.

2.3. Electron microscopy

Leaf samples of the five symptomatic rice plants from San Javier were fixed in a modified (2% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M pH7 phosphate buffer) Karnovsky solution (Karnovsky, 1965) for at least 24 h. After being rinsed in a 0.05 M pH 7 phosphate buffer, the samples were post-fixed for 2 h in 1% osmium solution, contrasted in 0.5% uranyl acetate for 24 h, dehydrated in serial dilutions of acetone and included in Spurr resin (Spurr low viscosity embedding media kit, Polysciences, INC.). Ultrathin sections were contrasted in 2% uranyl acetate and examined under a Jeol JEM EXII (Jeol, Tokio, Japan) transmission electron microscope (Kitajima and Nome, 1999).

Additionally, the plants were analyzed by leaf dip and immunosorbent electron microscopy plus decoration (ISEM-D) methods, according to Milne and Luisoni (1977). Briefly, specific RSNV antiserum (Fauquet and Thouvenel, 1983) was used to coat grids (diluted 1:500, v/v) and to decorate the virus particles (diluted 1:20, v/v). The viral decorated particles were observed with a JEOL 1200 transmission electron microscope. From a sample with decorated particles (SF10), measurements and virion modal lengths were estimated using InfoStat software (version 2013, Universidad Nacional de Córdoba, Córdoba, Argentina) by comparing 300 viral particles with scales of known size.

2.4. Detection of RSNV by RT-PCR

Total nucleic acids were extracted from leaf tissue of all the plants, the five symptomatic plants from San Javier (Santa Fe), 58 rice plants collected from Berón de Astrada (Corrientes) and six weed plants, using the CTAB method (Hoisington et al., 1994). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using specific primers, RSNV1-3827R/and RSNV1-3827R, targeting the helicase gene of RSNV with expected amplicons of 927 base pairs (bp) (Oludare et al., 2015).

The amplicons obtained from three symptomatic rice plants (isolate "Cor4" from Corrientes and isolates "SF5" and SF10″ from Santa Fe Province) were cloned into pGEm-T Easy Vector System (Promega, Madison, WI, USA), following the manufacturer's instructions. High efficiency cells were transformed using the recombinant plasmids, and the colonies containing the insert were then selected and multiplied. Plasmid DNA was purified from the clones of interest using Wizard Plus SV Minipreps DNA Purification System (Promega). One clone of each isolate was sequenced in both senses using universal primers M13 F/R and Sanger technology (Instituto de Biotecnología, CICVYA-INTA, Buenos Aires).

2.5. Soil transmission test

One soil sample was collected from the surroundings of the RSNV symptomatic rice plants from Berón de Astrada. Sixty rice seeds (20 of each cultivar) of three cultivars widely sown in recent years in the country (121 CL, IC 107 and Taim), treated with Vitavax and Zinc, were sown in viruliferous soil. Plants were maintained in a greenhouse under controlled conditions at 20–27 °C and checked daily for virus symptoms. Plant infection was confirmed by RT-PCR.

2.6. Sequence identity and phylogenetic analysis

The three RNSV ORF1 nt sequences obtained in this work were compared to all available sequences of RSNV from GenBank (six sequences from Africa with 828 nt length, and one from Colombia with 927 nt length), using BLASTn facility at NCBI (http://www.ncbi.nlm. nih.gov/). The nt identity was analyzed using Sequence Demarcation Tool (SDTv1.2; Muhire et al., 2014).

A phylogenetic analysis of nt sequences of the ten RSNV, 828 nt corresponding to the fragment of ORF 1, was performed using the maximum likelihood method, with a general time-reversible model (GTR + G + I) and Mega 7.0.18 software (Kumar et al., 2016). The bootstrap consensus tree was inferred from 2000 replicates.

3. Results and discussion

3.1. Serological detection

Three of five symptomatic plants analyzed by PTA-ELISA showed strong positive reaction for RSNV. The other two symptomatic plants and the negative controls showed no reaction at all.

3.2. Electron microscopy

Disperse (Fig. 2A) and aggregates (Fig. 2B) of oblique and crosssectioned virus particles were observed in ultrathin sections of symptomatic rice leaves.

Helically constructed rod-shaped particles with an axial canal were observed by the leaf dip method (Fig. 2C) and ISEM-D with specific RSNV antiserum.

Measurements of 300 particles decorated with RSNV antiserum showed a higher relative frequency of particles with an estimated modal length of 228 nm, followed by particles of 370 nm and an interval of 87–181 nm (Fig. 3). The particles' diameter measured 20 nm.

3.3. Detection of RSNV by RT-PCR

RT-PCR products of the expected size (927 bp) were obtained from 25 of the 63 analyzed rice plants (three from San Javier and 22 from Berón de Astrada). All weed plants (6) resulted negative to RSNV infection by RT-PCR analysis using specific primers.

3.4. Soil transmission test

Only 1.6% of the rice seedlings (1/60, cv IC107) developed the characteristic symptoms (Fig. 1-F), 60 days after sowing in viruliferous (RSNV) soil. The transmission was confirmed by RT-PCR.

3.5. Sequence identity and phylogenetic analysis

The percentage identity for three sequences obtained in this work, using BLASTn and the GenBank data, was 96% with an isolate from Colombia (EU099844, 927 nt, 100% coverage) and 97–99% with six shorter fragments of RSNV from Africa (Benin, Burkina Faso and Mali, 828 nt, 89% coverage).

Using SDTv1.2, the nt identities of the three Argentinian sequences



Fig. 2. Electron micrographs of *Rise stripe necrotic virus*. (A) Disperse and (B) aggregates of oblique and cross-sectioned virus particles were observed in ultrathin sections of symptomatic rice leaves. (C) Helically constructed rod-shaped particles with an axial canal observed by the leaf dip method. (Courtesy of C.F.D. Nome).



Fig. 3. Histogram of relative frequencies of the number of RSNV viral particles between 75 and 500 nm.

among them (GenBank access n° MG792544, MG792545, MG792546) ranged from 99.4 to 99.9%. When comparing a shorter fragment (828 bp), Argentinian isolates still presented the highest identity levels with





Fig. 4. Consensus phylogenetic tree constructed using maximum likelihood method and Mega software (v 7.0. 18, Kumar et al., 2016), based on the alignment of nt sequences (828 nt) of the helicase gene. Values in the nodes indicate bootstrap values (%) with 2000 replicates.

the isolates reported from Africa: 97.9–98.6% with an isolate from Benin (KP099623), 97.3–98.1% with two isolates from Burkina Faso (MF115600 and MF115599), 97.6–98.3% with three isolates from Mali (MF115603, MF115602 and MF115601) and 95.4–96% with the Colombian isolate (EU099844).

Phylogenetic analysis based on a partial sequence (828 nt) of the helicase gene of the ten RSNV isolates displayed two defined clusters: one included the RSNV isolates from Argentina with others from Benin (1), Burkina Faso (2) and Mali (2), whereas the Colombian isolate was separate (Fig. 4).

The presence of RSNV in Argentina was confirmed by different methods. The rice plants collected from Argentina presented the typical symptoms of RSNV infection: conspicuous chlorotic stripes, systemic leaf necrosis, growth retardation, panicle malformation and low seed production of mature plants, as described by several authors (Fauquet and Thouvenel, 1983; Fauquet et al., 1988; Lozano and Morales, 2009; Oludare et al., 2015).

Virus particles decorated with RSNV antiserum were observed by electron microscopy, with lengths of 87–181 nm, 228 nm and 370 nm. The length of RSNV particles appeared to be very variable. Estimates made by some authors have found lengths of 110–160 nm, 270 nm and 380 nm (Fauquet and Thouvenel, 1983), 260 nm and 360 nm (Morales et al., 1999), or 120 nm and 200–325 nm (Paz et al., 2009).

We managed to amplify a genomic fragment of the expected size using specific RNA 1 RSNV primers (Oludare et al., 2015), and the nt identity of three Argentinian sequences ranged from 99.4 to 99.9% among themselves, and from 95.4 to 98.3% when compared to other available RSNV isolates, thus confirming the presence of RNSV in Argentina. Decroës et al. (2017) determined that isolates from Burkina Faso, Mali and Benin presented a higher identity among themselves than to the only American sequence previously available (Colombia). The detection and sequencing of new American RSNV isolates made in this work showed that the African isolates present higher nt identity with the Argentinian than with the Colombian isolate.

The phylogenetic analysis of RNA1 showed the closeness of Argentinian to African isolates, rather than to the isolate reported from Colombia. This would suggest that the RSNV from Argentina probably has its origin in Africa. On the other hand, as Brazil reported the presence of the virus and its vector in 2006 (Maciel et al.), and considering that the trade in Argentinian rice seeds is mostly from bordering countries, Brazil seems to be a more probable source of the entry of this disease to the country. However, a comparison of the Argentinian isolates with the Brazilian ones could not be performed in the present work, as there are no RSNV genomic sequences available from Brazil. According to Lozano and Morales (2009), the international trade in rice

seeds produced in fields contaminated with *P. graminis* infected with the virus, is the main form of propagation of this disease.

RSNV has caused severe disease outbreaks in the eastern Colombian plains (Morales et al., 1999) and only a few genotypes show some resistance to infection by *P. graminis* and/or RSNV (Fauquet et al., 1988; Lozano and Morales, 2009; Gutiérrez et al., 2010). The first report of the presence of RSNV in rice fields in Argentina requires new monitoring and management action from the farming sector.

Further studies about the epidemiology of the RSNV (distribution and incidence) and its vector in Argentina are suggested in addition to screening rice germplasm for resistance.

The presence of RSNV in Argentina was confirmed by serological, electron microscopy, and RT-PCR methods in rice plants from Corrientes and Santa Fe. The transmission of RSNV by viruliferous soil was achieved. This is the first report not only of the presence of RSNV in Argentina, but of a viral infection in rice plants in the country.

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