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Emergence and partial characterization of rice stripe necrosis virus and its fungus vector in South America

Francisco J. Morales[1], Elaine Ward[2], Mauricio Castaño[1], José A. Arroyave[1], Iván Lozano[1] and Michael J. Adams[2]
[1]*Virology Research Unit, International Center for Tropical Agriculture (CIAT), AA 6713, Cali, Colombia (E-mail: f.morales@cgiar.org);* [2]*Crop and Disease Management Department, IACR – Rothamsted, Harpenden, Herts AL5 2JQ, UK (E-mail: elaine.ward@bbsrc.ac.uk)*

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Abstract

A new disease of rice, known as 'entorchamiento' (crinkling), was first noticed in the Department of Meta, Colombia, in 1991. Symptoms include seedling death, foliar striping and severe plant malformation. Tissue extracts and purified preparations from diseased rice plants, contained virus-like particles ca. 20 nm in diameter, with a bimodal length of 260 and 360 nm. Particle aggregates were also observed in the cytoplasm of infected rice leaf cells. Electrophoretic analyses of purified preparations and dsRNA extracts, revealed a single protein species of M_r 22,500, and four dsRNA bands ca. 6300, 4600, 2700 and 1800 bp in size. Cystosori, characteristic of plasmodiophorid fungal vectors of plant viruses, were consistently observed in the roots of diseased rice plants. PCR and sequence analyses of amplified fungal DNA products from infected rice roots, revealed that the putative fungus vector was *Polymyxa graminis*. A Western blot of tissue extracts obtained from 'crinkled' rice plants from Colombia, using antiserum against a West African isolate of rice stripe necrosis furovirus (RSNV), resulted in the detection of a protein band of approximately M_r 22,000. The RSNV antiserum recognized the Colombian virus isolate in serologically specific electron microscopy tests. These results confirm the presence of RSNV in the Americas.

Abbreviations: BSBMV – beet soilborne mosaic virus; BNYVV – beet necrotic yellow vein virus; RSNV – rice stripe necrosis virus.

Introduction

In 1991, a new disease of rice (*Oryza sativa* L.), characterized by seedling death, severe plant malformation (Figure 1A) and foliar striping (Figure 1B) was noticed in the Department of Meta, in the Eastern Plains of Colombia. By 1994, the disease, referred to as 'entorchamiento' (crinkling), had spread to most of the rice-producing municipalities in the region, causing yield losses of over 20% (Pardo and Muñoz, 1994). Intensive pesticide applications followed reports from two independent investigations citing aphids (Tapiero, 1994) and nematodes (Pardo and Muñoz, 1994) as the causal agents of the 'crinkling' problem. Despite the use of highly toxic insecticides and nematocides applied at rates over five times the recommended dose, the disease continued to spread in the Eastern Plains and other rice-producing departments of Colombia. Subsequent investigations (Morales et al., 1995a,b) suggested that the 'crinkling' disease of rice in Colombia is a soil-borne viral disease similar to rice stripe necrosis, described in West Africa as a disease of upland rice caused by a furovirus transmitted by the fungus *Polymyxa graminis* (Fauquet and Thouvenel, 1983; Fauquet et al., 1988).

Given the importance of rice as a staple food in tropical America, this investigation was conducted to confirm the identity of the causal agent and its fungus vector.

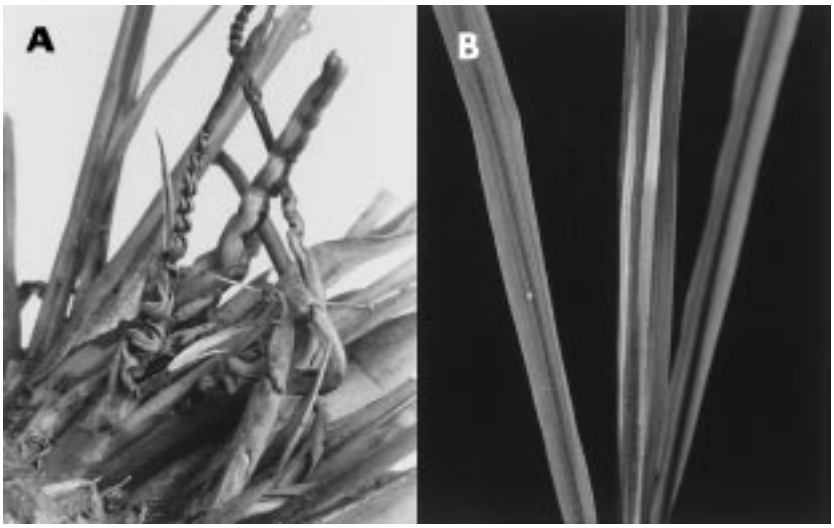

Figure 1. Severe plant malformation (A) and foliar striping (B) characteristic of the (crinkling) disease of rice in Colombia.

Materials and methods

Isolation and maintenance of the pathogen and its vector

Rice plants showing foliar malformation symptoms characteristic of the 'crinkling' problem, were originally collected near the city of Villavicencio, Meta, in the Eastern Plains of Colombia. The roots were washed, dried under vacuum, and subsequently ground to a powder, to be used as inoculum of rice seeds planted in a mixture of sand and soil under glasshouse conditions. Infected root samples were also collected in the department of Tolima, Colombia, for molecular characterization of the putative fungus vector.

Virus purification

Due to low virus recovery using the purification procedure described for the West African isolate of RSNV by Fauquet and Thouvenel (1983), the following procedure was finally adopted to purify the Colombian virus isolate. Infected rice tissue was homogenized for 3 min in a blender, in three volumes of 0.5 M KPO_4 buffer, pH 7.2, containing 0.75% sodium sulphite. The mixture was filtered through cheesecloth, and the filtrate centrifuged for 5 min at 4100*g*. The supernatant was treated with 2.5% Triton X-100 and 1 M urea, and then stirred for 1 h at 4 °C, prior to a low speed centrifugation for 5 min at 4100*g*. The resulting supernatant was subjected to high speed centrifugation for 3 h at 104,000*g*, over a 20% sucrose cushion. The pellet obtained was resuspended in 0.5 M KPO_4 buffer, pH 7.2, using 0.1 ml of buffer/g of infected tissue. Following a 5 min clarification, the suspension was centrifuged in a 10–40% sucrose gradient for 2 h at 65,000*g*. The virus band was recovered with a syringe, and finally diluted two-fold with 0.05 M KPO_4 buffer, pH 7.2.

Pathogenicity and seed transmission tests

Nicotiana benthamiana, Chenopodium album, C. amaranticolor, C. murale, C. quinoa, Datura stramonium, Oryza sativa 'Orizica 3', *Physalis floridana, P. peruviana, Sorghum bicolor* 'Rio' and *Zea mays* 'Sikuani', were mechanically inoculated with leaf tissue extracts from rice plants affected by the 'crinkling' disease, using 5 ml of KPO_4 buffer, pH 7.5, per g of infected rice tissue. For the seed transmission tests, 1500 seeds harvested from 'crinkled' Orizica 1 rice plants, were planted in a glasshouse in trays containing sterilized soil. These plants were maintained under observation in a glasshouse (average temperature of 27 °C and relative humidity of 75%) for two months.

Electron microscopy

Leaf extracts and partially purified preparations from infected rice plants were negatively stained in 2%

uranyl acetate, pH 3.7, and examined with a transmission electron microscope. The frequency distribution of the lengths was recorded for a total of 158 particles. Leaf tissue of symptomatic rice plants was prepared for cytology as described earlier (Morales et al., 1990). Scanning electron microscopy of fungal structures was performed according to the method of Wynn (1976).

Light microscopy and spectrophotometry

Roots of symptomatic rice plants were washed with sterile water and then stained for 15 min in 0.05% cotton blue in lactophenol. Absorption spectra of partially purified virus preparations were obtained with a spectrophotometer in the range of 360–240 nm.

Electrophoresis

For coat protein analysis, partially purified virus preparations were analyzed in 10% polyacrylamide gels containing sodium dodecyl sulphate (SDS) as described by Weber and Osborn (1969). Bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), and carbonic anhydrase (M_r 31,000) were used as markers for molecular weight determinations.

For the analysis of dsRNA, healthy and symptomatic rice plants were used as control and source of viral RNA, respectively, according to the procedure of Dodds and Bar-Joseph (1993). Approximate dsRNA weights were estimated using a 1-kb ladder (Bethesda Research Laboratory, Bethesda, Maryland, USA).

Serology

An antiserum to the West African isolate of rice stripe necrosis virus (Fauquet and Thouvenel, 1983) was kindly provided by Dr. Jean-Loup Notteghem, Plant Pathology Laboratory, CIRAD, Montpellier, France. Serologically specific electron microscopy (SSEM) tests were conducted as described by Derrick (1973). For the SSEM tests, the average number of particles in 20 different fields (mesh) of observation, were recorded for each treatment (with and without antiserum). The RSNV antiserum was also used in Western blotting. Samples were run in 10% polyacrylamide gels containing SDS at 100 V for 1 h, and the protein bands were transferred to a nitrocellulose membrane, using the trans-blot kit produced by Bio-Rad (Hertfordshire, England HP2 7TD). They were then analyzed using the ECL Western Blotting Analysis System (Amersham Life Science Ltd., Buckinghamshire, England HP7 9NA). This is a light emitting non-radioactive method for detection of antigens by the use of antibodies conjugated with horseradish peroxidase. The RSNV antiserum was used at a final dilution of 1 : 4000 (v/v).

Characterization of the fungal vector DNA

Two root samples obtained from 'crinkled' rice plants collected in the departments of Meta (sample 1) and Tolima (sample 2), were analyzed by molecular techniques. For sample 2, DNA was prepared from infected roots (freeze dried and ground) using the method of Lee and Taylor (1990), but including an additional RNase digestion. For sample 1 root extracts were used; DNA was released from a 1 cm long infected root by crushing it in an Eppendorf tube, adding 100 µl of 10 mM Tris (pH 8) and boiling for 10 min. Molecular characterization of the isolates involved analysis of their ribosomal DNA after amplification using fungal consensus primers (White et al., 1990). The region between primers NS5 (5′ AACTTAAAGGAATTGACGGAAG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) was amplified by PCR and the region between NS7 (5′ GAGGCAATAACAGGTCTGTGATGC 3′) and ITS4 was sequenced. The methods for PCR amplification, cloning of PCR products into pbluescript KS+, sequencing and sequence analysis were as described in Ward and Adams (1998).

Results

All rice plants showing foliar stripes and severe malformation symptoms characteristic of the 'crinkling' disease, were shown by electron microscopy to contain rod-shaped particles ca. 20 nm in diameter (Figure 2A), with a bimodal length distribution of 260 and 360 nm. Similar particles of shorter (90–250 nm) and longer (370–400 nm) lengths were also observed at a very low frequency, and were considered as fragments or end-to-end particle aggregates. The rod-shaped particles were not observed in symptomless rice plants. To date, we have processed hundreds of plant samples and have detected the virus in all the major rice-growing regions of Colombia. Purified preparations (Figure 2B) exhibited a typical nucleoprotein spectrum with a maximum ultraviolet absorbance peak at 260 nm and an $A_{260/280}$ ratio of 1.5. The approximate virus yield was estimated at 32 mg/kg of infected

rice tissue (uncorrected for light scattering), using an extinction coefficient of 3.0 (Fauquet and Thouvenel, 1983). In the mechanical inoculation tests, only the three *Chenopodium* species tested developed local (chlorotic) lesions. None of the remaining test plants inoculated, showed either local or systemic symptoms. None of the 1500 rice seedlings grown from seed harvested from 'crinkled' mother plants, developed symptoms. In transmission electron microscopy examinations, the cytoplasm of infected rice cells contained particle aggregates observed in longitudinal or cross section (Figure 3A). The disease also induced noticeable cytopathic changes in infected cells, particularly affecting mitochondria (Figure 3A) and the endoplasmic reticulum (Figure 3B), as observed for soil-borne wheat mosaic furovirus (Hibino et al., 1974).

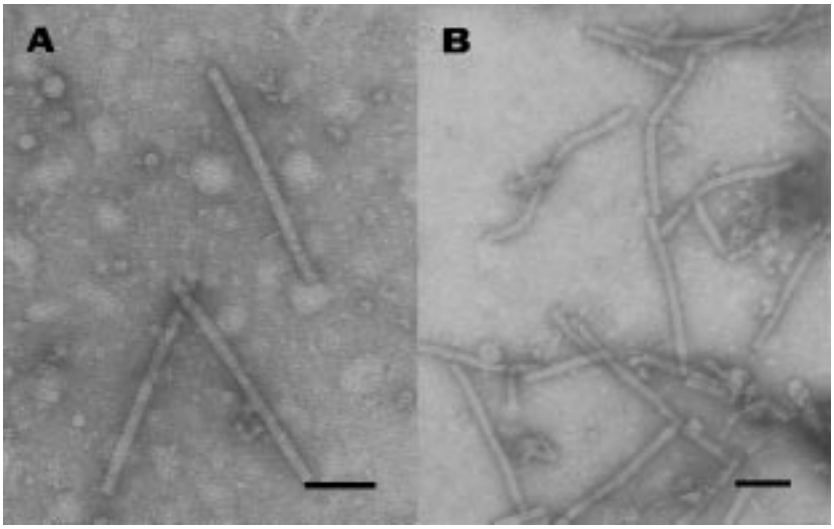

Figure 2. Virus particles observed in leaf extracts (A) and partially purified preparations (B) obtained from rice plants affected by the 'entorchamiento' disease of rice in Colombia. Bar = 100 nm.

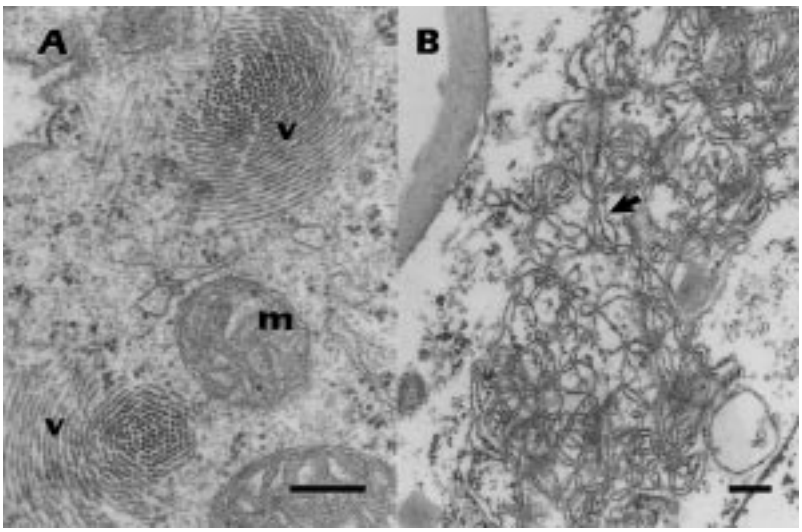

Figure 3. (A) Aggregates of virus rods (v), and abnormal mitochondria (m) in the cytoplasm of rice cells infected by rice stripe necrosis virus. (B) Inclusions consisting of convoluted endoplasmic reticulum (arrow) observed in the cytoplasm of virus infected rice cells. Bar = 200 nm.

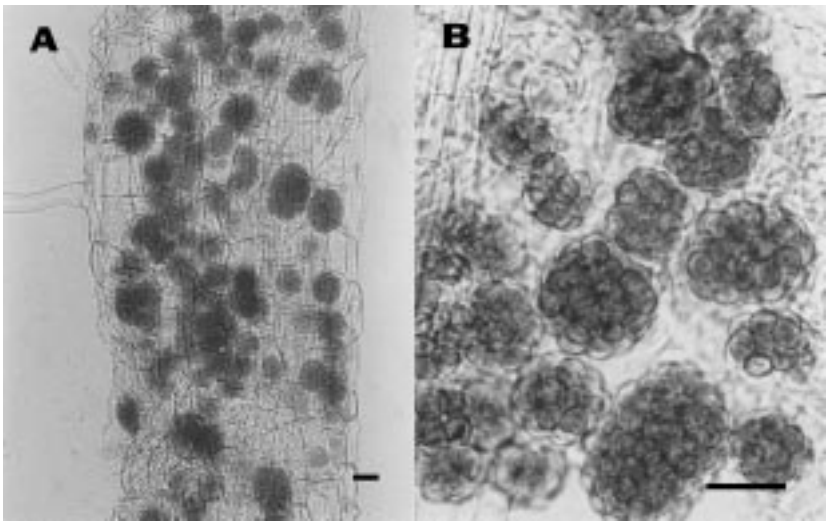

Figure 4. (A) Resting spore clusters (cystosori) and (B) close-up of mature cystosori in the rootlets of rice plants affected by rice stripe necrosis. Bar = 100 μm.

The examination of the root system by light microscopy revealed the presence of abundant cystosori (Figure 4A) containing irregular aggregates of spores (Figure 4B) characteristic of plasmodiophorid fungal vectors of plant viruses (Barr, 1979; Adams, 1991). SDS-PAGE analysis of partially purified virus preparations revealed the presence of a single protein species of ca. M_r 22,500. Four distinct bands of approximate size 6300, 4600, 2700 and 1800 bp, were observed in 5% acrylamide gels of dsRNA extracts from diseased rice plants (Figure 5).

In SSEM tests, the use of the RSNV antiserum resulted in a 16.5-fold increase in the average number (313) of virus particles trapped, with respect to the average number (19) of virus particles observed per mesh without treating the grid with antiserum. The Western blotting analysis of 'crinkled' rice plant extracts, using antibodies prepared to the capsid protein of a West African isolate of RSNV, resulted in the detection of a similar protein molecule of M_r 22,000, corresponding to the capsid protein of the Colombian virus isolate.

For root sample 1 from Meta, RFLP analysis showed that there were two types of fungally-derived clones and, initially, one representative clone of each type was sequenced using primer NS7 only. One clone showed a high homology to the plasmodiophorids, with the highest homology (99.5% in the NS7–NS8 region) being to *Polymyxa graminis* Type II isolates

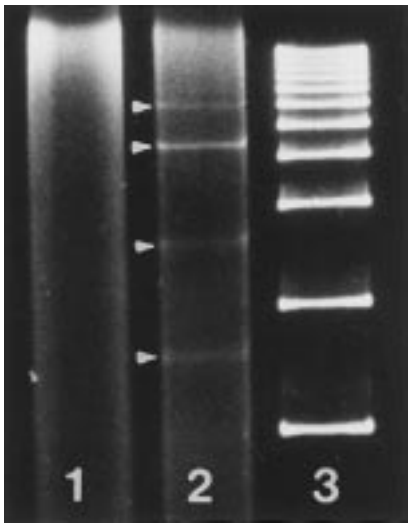

Figure 5. Analysis of dsRNA from rice plants affected by 'entorchamiento'. Lane 1: dsRNA extraction from healthy rice tissue; lane 2: dsRNA bands (marked) extracted from diseased rice plants; lane 3: 1-kb ladder.

(Ward et al., 1994; Ward and Adams, 1998). This clone and a second clone of this type (from an independent transformation) were then sequenced on both strands between the NS7 and ITS4 regions and found to be identical. The sequence has been deposited in

the EMBL database with accesssion no. AJ010424. The second type of clone proved not to be a plasmodiophorid sequence; it had no significant homology to *Polymyxa*, but rather showed high homology (92%, within the NS7–NS8 region) to the fungi *Cucurbidothis pityophila, Setosphaeria rostrata* and *Cochliobolus sativus*.

For root sample 2 from Tolima, almost all of the clones had an RFLP type identical to the *Polymyxa*-type clones from sample 1. Sequencing of three of these clones (from two independent transformations) gave identical results to those from sample 1.

The sequence of a *Ligniera* isolate was also completed [we previously reported the sequence between ITS4 and ITS5 (Ward and Adams, 1998)] to use in the phylogenetic analyses. The EMBL accession numbers allocated to these new sequences and those of other sequences used for comparisons are as follows: *Ligniera* sp. AJ010425, *Olpidium brassicae* Y12830, *Plasmodiophora brassicae* Y12831 and U18981, *Polymyxa* (Colombian isolate) AJ010424, *Polymyxa graminis* F1 Y12824, *Polymyxa graminis* F51 Y12826, *Polymyxa graminis* I1-229 Y12825, *Polymyxa betae* F67 Y12827, *Spongospora subterranea* Y12829.

The NS7–NS8 and ITS5–ITS4 regions were used separately in the phylogenetic analysis since complete NS7–ITS4 sequences are not available for some of the organisms with which the Colombian *Polymyxa* isolate was compared. The sequences were aligned with PILEUP (Genetics Computer Group, 1994) and then analyzed using programs in PHYLIP (Felsenstein, 1993). Figure 6 shows the phylogenetic tree obtained after NEIGHBOR analysis using all the known plasmodiophorid ITS4–ITS5 sequences. *Olpidium brassicae*, a zoosporic chytrid, was used as the outgroup. The phylogenetic tree obtained strongly supports the identity of the Colombian RSNV-associated fungus as *Polymyxa graminis*. It groups with Type II *Polymyxa graminis* isolates (Ward et al., 1994; Ward and Adams, 1998) with a high bootstrap value (96%).

For the NS7–NS8 sequence analysis a much wider range of eukaryotic taxa were used including fungi, protozoa, plants and stramenopiles. Details of these organisms and their Genbank/EMBL accession numbers are given in Ward and Adams (1998). The results of the new analyses (including the Colombian *Polymyxa* isolate) using NEIGHBOR, was essentially the same as that reported previously (Ward and Adams, 1998). The groupings within the plasmodiophorids were identical to those in Figure 6 although *Spongospora* was not included as its sequence in this region is not available.

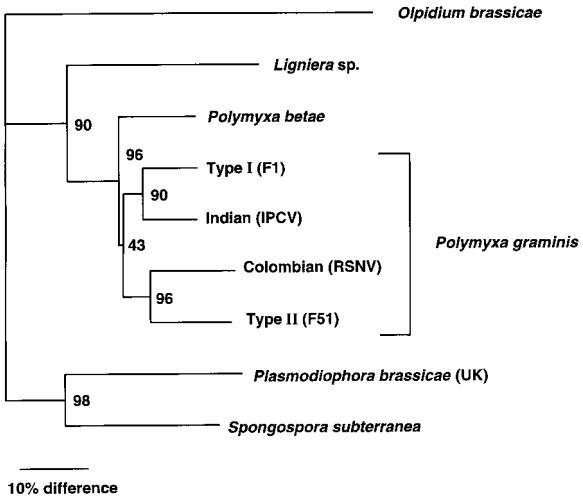

Figure 6. Phylogenetic tree of the ITS5–ITS4 regions obtained using NEIGHBOR analysis and displayed as a phylogram from VIEWTREE. The values at the forks indicate the number of times out of 100 trees that this grouping occurred after bootstrapping the data. The horizontal lines are to the scale shown at the bottom left of the picture. The EMBL/Genbank accession numbers for the sequences used are given in the results section.

Discussion

The characteristic symptoms of the 'entorchamiento' disease of rice in Colombia, are identical to those previously described for rice stripe necrosis in West Africa (Louvel and Bidaux, 1977). The results obtained in this investigation, show that the 'crinkling' disease of rice is associated with RSNV and the fungus vector *Polymyxa graminis*, as described by Fauquet and Thouvenel (1983) and Fauquet et al. (1988). The morphological characteristics of the Colombian isolate of RSNV, are also similar to those of the African isolate of RSNV, particularly their longer particle lengths (260–270 and 360–380 nm). These particle lengths differ from those reported (150–170 and 240–300 nm) for most furoviruses (Brunt and Richards, 1989; Putz, 1977). It is possible that RSNV may have more than two types of particles, but the shorter particles observed for the African (110–160 nm) and Colombian (140–160 and 195–215 nm) isolates of RSNV, may represent fragments of the predominant particles.

Within the original, predominantly bipartite furovirus group, beet necrotic yellow vein virus

(BNYVV) and beet soilborne mosaic virus (BSBMV) have been shown to have a multipartite genome consisting of four or five RNA species (Bouzoubaa et al., 1985, 1986, 1987; Heidel et al., 1997; Putz, 1977). BYNV and the Colombian isolate of RSNV also differ from other furoviruses (Brunt, 1988) in having longer particle lengths (260–265 and 360–390 nm). In this investigation, the two larger RNA species of the Colombian RSNV isolate, were similar in size to the corresponding RNA species of BNYVV and BSBMV (Bouzoubaa et al., 1985, 1986; Heidel et al., 1997; Putz, 1977; Putz et al., 1983), but some differences are apparent for the two smaller RNA species (Putz, 1977; Koenig et al., 1986; Kuszala et al., 1986; Heidel et al., 1997). However, similar differences in the size of RNAs 3 and 4, have been reported for different BNYVV isolates and hosts (Bouzoubaa et al., 1985; Burgermeister et al., 1986; Heidel et al., 1997; Koenig et al., 1986; Kuszala et al., 1986). These results suggest that RSNV, BNYVV and BSBMV may have a similar genomic organization and, thus, RSNV might be a species of the new *Benyvirus* genus. Benyviruses have other characteristic features, which differentiate them from other viruses previously assigned to the *Furovirus* genus. One of these characteristics is that the 3′ end of their RNAs is polyadenylated (Pringle, 1998). In preliminary work with the Colombian isolate of RSNV, the viral RNA was extracted from purified virus preparations using the Dynabeads Oligo $(dT)_{25}$ System (Dynal, Inc., Lake Success, NY, USA). The extracted viral RNA was used to produce cDNA for cloning and sequencing, which confirmed the presence of a poly-A tail in RSNV.

From the sequence studies, homology data and phylogenetic analyses, it is clear that RSNV-infected rice plants from Colombia contain the fungus *Polymyxa graminis*. This was also confirmed by using tests which allow rapid detection of *Polymyxa* species by PCR (Ward and Adams, 1998). The Colombian *Polymyxa* isolates were detected both in a *Polymyxa*-specific assay (primers Pxfwd1 and Pxrev7) and in a *P. graminis*-specific assay (primers Pxfwd2 and Pxrev7) (results not shown). These isolates form a distinct subgroup of *P. graminis*, which is most closely related to the Type II isolates originating from wheat, barley and oats (Ward and Adams, 1998). The two isolates studied (one from Meta and one from Tolima) have identical NS7–ITS4 rDNA sequences. Identification of *Polymyxa* spp. by classical means is a skilled, time-consuming and difficult task. Resting spores cannot always be distinguished from those of the genus *Ligniera*, and identification of the fungus to species level (*P. betae* or *P. graminis*) by conventional methods involves testing the host range (because there are no morphological differences), which can be inconclusive. The molecular techniques used here, discriminated the fungal species easily and are therefore an additional means of identification. Furthermore, these techniques assist in intra-specific differentiation and determination of taxonomic relationships.

Following the identification of RSNV in the Eastern Plains of Colombia, the virus and associated plasmodiophorid fungus have been shown to be widely distributed in the main rice growing departments of Colombia (Huila, Tolima, Meta, Casanare, Antioquia, Cordoba and Cundinamarca). Thus, it is possible that the distribution of RSNV in Latin America is broader than presently recognized. The main factor responsible for the relatively rapid spread of the ‘crinkling’ disease in Colombia, seems to be the use of contaminated agricultural machinery shared by different rice growers. The dissemination of RSNV in West Africa has been comparatively slower, probably due to the lower degree of mechanization of rice production in Africa.

The emergence of rice stripe necrosis in Colombia, constitutes a serious threat to most of the rice-producing countries in the Americas. Once viruliferous plasmodiophorid fungi invade a cultivated soil, it is nearly impossible to eradicate RSNV. The experience gained from the implementation of different measures to control furoviruses, suggests that the development of resistant cultivars is the most sustainable disease control strategy (Brunt and Richards, 1989). Fortunately, the original report of RSNV in West Africa, suggests that there are RSNV-resistant rice genotypes (Louvel and Bidaux, 1977). Moreover, the correct identification of the virus and vector of the ‘crinkling’ disease of rice in Colombia should now make feasible the implementation of complementary disease control measures.

Acknowledgements

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