Wheat: A New Natural Host for the Mal De Río Cuarto Virus in the Endemic Disease Area, Río Cuarto, Córdoba Province, Argentina

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ABSTRACT

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The fijivirus known as "Mal de Río Cuarto" that affects corn is endemic to the area of Río Cuarto, Córdoba Province, Argentina. One of the preferred species for the development of its vector, the insect *Delphacodes kuscheli* Fennah, is wheat. In this area, wheat plants with deformed leaves, spikes and spikelets, shortened internodes, leaves with serrated borders, and sterile spikelets were detected, suggesting the possibility that Mal de Río Cuarto Virus could also be infecting this crop. Samples originating in Río Cuarto, Sampacho, and La Carlota (Córdoba Province) that showed symptoms, were analyzed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), polyacrylamide gel electrophoresis, and electron microscopy, confirming, for the first time, the occurrence of the disease in wheat. The frequency of the disease was assessed in random samples from 14 wheat plots located in the Department of Río Cuarto (Córdoba Province). The samples were analyzed using the DAS-ELISA immunoenzymatic technique, and the disease was detected in the majority of the fields assessed, with levels of incidence that ranged between 2.5 and 24%. We must be aware of the presence of this virus in the wheat crop, where it appears to play a double role in the epidemiology of the disease, acting both as a virus reservoir and as a preferred host for the development of populations of the vector virus, *D. kuscheli*.

Additional keywords: Mal de Río Cuarto virus, reservoir, wheat

"Mal de Río Cuarto" is the most important disease in maize in Argentina, and it can account for yield losses higher than 70% in the years of its greatest incidence (14). The disease is caused by a member of the subgroup II, genus *Fijivirus* Reoviridae family (12,30). It was initially thought to be a geographic strain of the maize rough dwarf virus (MRDV) detected in Italy (6), but molecular hybridization tests showed no complementation between them in at least 7 of the 10 genomic fragments (5).

Mal de Río Cuarto virus (MRCV) has 65- to 70-nm diameter icosahedric particles that contain 10 segments of double-stranded RNA (19). It is naturally transmitted by the insect *Delphacodes kuscheli* Fennah (*Homoptera: Delphacidae*) in a persistent, propagative way, and this form

of transmission constitutes the initial source of the inoculum (24,26). Transmission by seeds has not yet been reported. It has been detected in 12 species of weeds of the families *Poaceae* and *Cyperaceae* found in the endemic area of the disease, among them *Eleusine indica* (L.) Gaer, *Cynodon dactylon* (L.) Pers, *Sorghum halepense* (L.) Pers, *Cyperus cayennensis* (Lam) Britt, *Eragrostis viriscences* Pers, *Digitaria sanguinalis* (L.) Scop, *Cenchrus equinatus* L., *Cyperus rotundus* L., *Setaria geniculata* (L.) Beauv, *Arundo donax* L., *Echinochloa colonum* (L.) Link, and *Setaria verticillata* (L.) Beauvois (13,22,25, 27); and in sorghum *Sorghum bicolor* L. Moench (10,20), millet *Panicum milliaceum* L., foxtail millet *Setaria italica* (L.) Beauv. (16,17,25), and oat *Avena sativa* L. (9). Studies carried out in the disease endemic area (15,23,24), showed that the vector's populations develop mainly in oats and wheat, then migrate to maize plants in their first stages of growth. This suggested that wheat may participate in the virus epidemiology. This, and the detection of plants with uncertain symptomatology, prompted the present research, which aimed to detect and identify the Mal de Río Cuarto virus in wheat crops in the endemic

disease area (the Río Cuarto department in the Province of Córdoba, Argentina).

MATERIALS AND METHODS

Source of inoculum and symptoms.

The incidence of Mal de Río Cuarto was assessed in wheat plots located in Río Cuarto, La Carlota, Sampacho, Espinillo, and Las Vertientes, in the province of Córdoba. Virus identification was carried out in samples of plants exhibiting symptoms at the vegetative or reproductive stage. Wheat plants were found in fields in Río Cuarto, Sampacho, and La Carlota which, in the vegetative stage, showed a large number of tillers, with shortening of the internodes, which gave them a stunted appearance (Fig. 1A) and short, erect, leathery leaves with enlarged veins (Fig. 1B). At the reproductive stage, plants had short internodes and showed enlarged walls. The leaves had serrated borders and curled in on themselves, giving them a corkscrew appearance (Fig. 1C). The spikes were found to be partially or totally sterile, and had shortened, curved rachis (Fig. 1D). Plants with similar symptoms were found in Pergamino and Colón (Buenos Aires Province) and Diego de Alvear (Santa Fe Province). The identification tests began with this material. Samples were also taken to determine the incidence of the disease in wheat fields in the areas of Río Cuarto, La Carlota, Sampacho, Espinillo, and Las Vertientes (Province of Córdoba).

Serology. *Virus purification.* Roots of infected maize, collected from corn fields, when plants were flowering, were the source of tissue for virus extraction. One hundred grams of root were ground in a mortar with 4 vol of 0.2 M sodium-potassium phosphate buffer, pH 7.0, containing 5 mM EDTA and 10 mM sodium sulfite (extraction buffer), then squeezed through a cheesecloth. The homogenate was mixed with 1 vol (400 ml) of CCl_4 , stirred for 30 min, and clarified by a 10-min centrifugation at $10,000 \times g$. The supernatant was centrifuged for 2 h at $75,000 \times g$, and pellets were resuspended in 12 ml of extraction buffer and clarified at $10,000 \times g$ for 10 min. The supernatant obtained was ultracentrifuged for 1 h at $100,000 \times g$. Pellets were resuspended in 2 ml of a 0.1-M

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sodium-potassium phosphate buffer pH 7.0 containing 5 mM EDTA, and layered onto cesium sulfate gradients from 20 to 50% (from 5 in 5) for 2 h 30 min. at $90,000 \times g$. The light scattering bands observed at approximately 30% density were diluted in 10 vol of the same buffer, and centrifuged for 1 h at $100,000 \times g$. The pellet obtained was resuspended in a 0.1 M potassium phosphate buffer, pH 7.0. All the protocol was carried out at 4°C with sterilized materials. Virus concentrations were estimated

by spectrophotometer using an extinction coefficient of 5.

Antiserum production. Polyclonal antiserum was produced by injecting rabbits with 3 doses of 20 to 25 μg of viral protein at 18-day intervals. Each dose consisted of 2 intramuscular injections and various subcutaneous ones in the loin. The first immunization was made with Freund's complete adjuvant. For the other two, incomplete adjuvant was used. Bleeding was done 21 days after the last dose.

From the serum obtained, gammaglobulin was purified by ammonium sulfate precipitation and later DEAE sephacel (Sigma Chemical Co., St. Louis) chromatography in half-strength phosphate-buffered saline (PBS; 0.02 M phosphate plus 0.15 M K- NaCl , pH 7.4). It was adjusted at a final concentration of 1.0 mg/ml (optical density $[\text{OD}]_{280} = 1.4$). One ml of the above immunoglobulin G (IgG) was conjugated to alkaline phosphatase (type VII T, Sigma Chemical Co.) according to the original protocol by Clark and Adams (4). The antiserum reacted by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) in the presence of the MRCV maize antigen with high absorbency values (8), showed no reaction in the presence of maize dwarf mosaic potyvirus, (MDMV), but did react slightly with maize leaf infected with French origin MRDV. The immunosorbent electron microscopy (ISEM-D) of the antiserum reacted with roots of maize infected with Italian origin MRDV (M. P. Giménez Pecci and R. G. Milne, unpublished data).

Sample preparation. Leaf and root samples were ground in PBS + 0.05% Tween 20 + 2% polyvinyl pyrrolidone (PVP) at a 1:4 (wt/vol) dilution.

ELISA (4). ELISA plates (Nunc-Immunoplate MaxiSorp, 96 wells) were coated with 1 $\mu\text{g}/\text{ml}$ of IgG in 0.05 M sodium carbonate (pH = 9.6) and incubated for 4 h at 37°C ; the antigen was added to the wells and incubated for 18 h at 5°C . The specific gammaglobulin for MRCV conjugated with alkaline phosphatase was added at a dilution of 1/1000 in conjugate PBS + 0.05% Tween 20 + 2% PVP + 0.2% chicken egg albumin and was incubated for 4 h at 37°C for 60 min. After adding the substrate (0.75 mg/ml p-nitrophenyl phosphate [Sigma Chemical Co.] in 10% diethanolamine, pH 9.8), the reaction was quantified using a Dynatech MR4000 ELISA plate spectrophotometer. Samples were considered positive when OD_{450} values were higher than 0.100 or the mean of the healthy controls plus three times the standard deviation (six healthy controls were used per plate).

Three washings with PBS + 0.05% Tween 20 were performed between steps and 100 μl were added to each well in all steps.

PAGE. In order to confirm the presence of the virus, roots of DAS-ELISA positive plants were analyzed with polyacrylamide gel electrophoresis (PAGE) for the detection of RNA according to the Giordano et al. protocol (11) modified by Giménez Pecci (8). Positive and negative controls were DAS-ELISA-positive maize and DAS-ELISA-negative wheat plants, respectively.

Sample preparation. Samples were ground in a ratio of 1:4 (wt/vol) in PBS containing 2% PVP and 0.05% Tween 20. Two hundred μl of the aqueous phase were

Fig. 1. Wheat plants with (A) shortened internodes and stunted aspect; (B) enlarged veins; (C) curled wheat leaves, with corkscrew appearance; and (D) partial sterility, curved spikes, and shortening of rachis.

mixed with 200 μ l LiCl buffer + 1% sodium dodecyl sulfate (SDS) and 10 mM EDTA; 400 μ l of phenol chloroform 1:1 were added and samples were vortexed, heated at 56°C for 10 min, and centrifuged at 16,000 $\times$ g for 30 min. One ml ethanol was added to the resulting supernatant (+/- 200 μ l), mixed vigorously, and kept at -20°C for 18 h. It was centrifuged again at 16,000 $\times$ g for 30 min, and the pellet was kept and dried at 37°C. Finally, it was dissolved in 20 μ l sample buffer (Tris-HCl, 0.06 M, pH 6.8 + 5% 2-mercaptoethanol + 3% glycerol + 3% SDS + 0.0.1% bromophenol blue).

A polyacrylamide 1 gel, composed of a 5% stacking gel in 0.125 M Tris-HCl buffer, pH 6.8, + 0.1% SDS and a 10% running gel in 0.375 M Tris-HCl buffer, pH 8.7 + 0.1% SDS 10% (29:1) was run. in a Mini PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA) at

11 mA for 11 h in Tris-glycine buffer (0.025 M Tris, 0.2 M glycine, pH 8.9 + 0.1% SDS). Then the gel was intensely washed, fixed for 30 min with 0.5% acetic acid in 10% ethanol, stained with 0.011 M AgNO₃ for 30 min under gentle agitation, and developed with a solution composed of 0.75 M NaOH and 0.76% formaldehyde.

Electron microscopy. Inclusions of small pieces of leaves were prepared in Spurr resin for observation with the electron microscope, fixed with 2% paraformaldehyde/glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h, washed three times (15 min each) with 0.05 M cacodylate buffer, and postfixed with 1% OsO₄ (osmium tetroxide) in 0.05 M cacodylate buffer for 2 h. They were gradually dehydrated in a 30, 50, 70, and 90% acetone series from 15 to 20 min each, and in 100% acetone three times for 15 min each. Finally, they were infiltrated in acetone/Spurr (1:1) for 2 h, then in pure Spurr all night, to be later polymerized in pure Spurr at 70°C for 24 h (29). Sections were cut on a RMC MT6000-XL microtome with a diamond knife, mounted on copper grids, stained with 2% uranyl acetate and lead citrate, and observed in an electron microscope (Jeol JEM 1200 EX II).

Disease frequency studies. Frequency studies were carried out during two consecutive crops in 1993 and 1994. Ten plots were sampled in Río Cuarto, La Carlota, and Sampacho (Department of Río Cuarto) in the first year, and four plots in Espinillo, Sampacho, and Las Vertientes (Department of Río Cuarto) in the second year. Thirty random samples were collected along the diagonal in each plot, and each of them was analyzed with the DAS-ELISA test.

RESULTS AND DISCUSSION

Serological tests gave positive results in plants with symptoms in the presence of the antiserum used. Absorbency values for positive samples ranged from OD₄₁₀ 0.122 to 2.000 in the analysis of leaves, and between OD₄₁₀ 0.163 and 2.000 in that of roots. Healthy controls showed absorbency values between 0.023 and 0.050 in leaves

and between 0.031 and 0.056 for roots. The DAS-ELISA test was shown to be appropriate for the detection of Mal de Río Cuarto virus in wheat, as it could detect the virus both in leaf and in root tissues. No non-specific reactions were observed in healthy wheat plants.

In the PAGE analysis, the 10 characteristic double-stranded RNA segments were observed with a pattern which coincided with the one observed in the positive control and agreeing with the one described for MRCV in corn when it was first associated with maize rough dwarf fijivirus (19). Bands were absent in healthy controls (Fig. 2A). The electron microscope showed viroplasma containing virus-like particles in the parenchyma phloem cells. The particles were approximately 55 to 60 nm in diameter, some of them looking empty (incomplete particles) while others had an electron-dense core surrounded by an electron lucent layer (complete particles; Fig. 2B). The characteristics and size of the particles observed are similar to those reported for MRCV (21) and other fijiiviruses (7,12). Table 1 summarizes the results of the DAS-ELISA test for the evaluation of disease incidence. Mal de Río Cuarto was detected in the majority of the wheat plots assessed in the first year of the study, with frequency values between 2.5 and 17.5%, while in the 1994 season, frequency increased to 24%.

Previously, other fijiiviruses have been reported as affecting wheat, in natural infections. Among them are oat sterile dwarf virus, which belongs to the subgroup III of the fijiiviruses but is serologically unrelated to the rest of the group and has a quite different electrophoretic pattern from that of other known fijiiviruses. (2,7); and rice black streak dwarf virus which, according to Azuhata et al. (1), should be considered as a geographical variant of MRDV. Both these viruses induce milder symptoms in wheat than those described for the MRCV (3,28,31). MRDV has been cited only in artificial infections of wheat (18).

As a result of the positive reaction in the DAS-ELISA serological technique, the

Fig. 2. (A) dsRNA fragments of "Mal de Río Cuarto" virus in infected wheat, observed in polyacrylamide gel electrophoresis in polyacrylamide gels with silver staining. References R = roots of positive wheat, Te = positive control, and Ts = healthy wheat. (B) Viroplasma (Vir) and complete and incomplete virus particles (V) in phloem parenchyma cells.

Table 1. Mal de Río Cuarto incidence in wheat plots in the Río Cuarto Department (Córdoba Province, Argentina) in 1993 and 1994

Locality	Year of sampling	Plot number	% infection ^a
Río Cuarto	1993	1	2.4
Río Cuarto	1993	2	3.9
Río Cuarto	1993	3	16.6
Río Cuarto	1993	4	0.0
Río Cuarto	1993	5	0.0
La Carlota	1993	6	6.0
Sampacho	1993	7	4.5
Sampacho	1993	8	9.5
Sampacho	1993	9	17.4
Sampacho	1993	10	6.6
Sampacho	1994	11	3.5
Sampacho	1994	12	24.1
Espinillo	1994	13	0.0
Las Vertientes	1994	14	3.3

^a Determined by double-antibody sandwich enzyme-linked immunosorbent assay.

appearance of the 10 RNA fragments, and the virus particles observed in electron micrographs of ultra-thin sections, it may be concluded that the wheat plants with the described symptoms were infected with the Mal de Río Cuarto virus. This is the first time this fijivirus has been observed in wheat.

Symptom severity, as well as the infection levels registered in the fields, which reached values of 24%, indicate that this is an important disease in wheat. This crop would also appear to be acting as a winter reservoir for the virus. If we add to this its role as a preferred habitat for the development of *D. kuscheli* (the main vector of the virus), there is a clear need to look for strategies for disease control in wheat, in the endemic area of the Mal de Río Cuarto, in order to reduce its incidence.

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