Association of Spiral Filamentous Viruslike Particles with Rice Hoja Blanca

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ABSTRACT

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Fine, spiral, filamentous particles ~3 nm in diameter were consistently associated with hoja blanca-affected rice plants. The filamentous particles were isolated from infected plants following organic solvent clarification, concentration with polyethylene glycol, and equilibrium centrifugation in Cs_2SO_4 or CsCl gradients. Purified preparations exhibited typical nucleoprotein ultraviolet absorption spectra with a maximum at $A_{260\ nm}$ and an $A_{260/280\ nm}$ ratio of 1.4. The preparations contained one major and one minor protein of molecular weights 34 and 17.5×10^3 daltons, respectively, as determined by SDS-PAGE. In double immunodiffusion tests, an antiserum (prepared by injecting rabbits with purified preparations) produced specific precipitin reactions with leaf extracts of hoja blanca-affected rice plants, but not with leaf extracts of healthy rice plants. No serological reaction was observed in similar tests of leaf extracts from hoja blanca-infected *Echinochloa colonum*.

Additional key words: Sogatodes orizicola.

The hoja blanca (white leaf) disease of rice was first observed in Colombia in 1935 (5). This disease is now known to occur in almost all rice-growing countries of Latin America, and in the United States (3,5). Yield losses due to hoja blanca have been estimated to range from 25 to 100% (10).

The vector of the disease was shown to be the planthopper *Sogatodes orizicola* (Muir) in 1957 (15). However, the causal agent has not yet been unequivocally characterized. Spherical particles, approximately 42 nm in diameter, were first observed in dip and purified preparations obtained from hoja blanca-infected rice leaves (8). Later, flexuous threadlike particles, 8–10 nm in diameter, were demonstrated by transmission electron microscopy of thin sections of cells of both hoja blanca-infected rice leaves and viruliferous insect vectors (17). The authors concluded that the flexuous particles were similar to those of the closterovirus group. These filamentous particles were also observed in leaf cells of *Echinochloa colonum* showing hoja blanca symptoms (11). Recent outbreaks of rice hoja blanca have occurred in Colombia.

The purpose of this work was to isolate the causal agent, and contribute towards a better understanding of its pathogenic variability and epidemiological potential in rice and other hosts such as *E. colonum*.

MATERIALS AND METHODS

Pathogen source. Rice (*Oryza sativa* L. 'CICA 8' and 'IR 22') plants, showing hoja blanca symptoms, were collected 60–70 days after emergence in a rice field located in Espinal (Tolima), Colombia. The plants were transported in an ice-box to CIAT, Palmira, where they were processed within 48 hr after collection. Hoja blanca-affected rice Bluebonnet 50 plants, inoculated with *S. orizicola* vectors under glasshouse conditions, were also used to isolate the pathogen. Symptomless CICA 8 rice plants collected in the field and healthy Bluebonnet 50 plants grown in a glasshouse were included in this study as controls.

Purification. Either the roots or symptomatic leaves of hoja blanca-affected plants were selected for processing. Plant tissue was homogenized in a blender with three volumes of a cold mixture of 0.2 M potassium phosphate buffer (pH 7.6) containing 0.1% thioglycollic acid, 1 mM sodium diethyldithiocarbamate, and one volume of a chloroform:carbon tetrachloride mixture (1:1, v/v). The homogenized emulsion was centrifuged at 8,288 *g* for 5 min. The supernatant was filtered through glasswool and treated with 10% (w/v) polyethylene glycol (MW 6,000). After being stirred for 2 hr at 5 C, the treated supernatant was centrifuged at 9,514 *g* for 20 min. The pellet was resuspended overnight at 5 C in 0.1 M potassium phosphate buffer, pH 7.6. The suspension was clarified by centrifugation at 12,000 *g* for 10 min and concentrated by ultracentrifugation at 102,900 *g* for 2 hr. The pellet was resuspended overnight in 0.01 M potassium phosphate buffer, pH 7.6. The suspension was clarified by centrifugation at 12,000 *g* for 10 min and further subjected to equilibrium centrifugation (90,000 *g* for 17 hr) in 30% (w/w) cesium sulphate (Cs_2SO_4) or cesium chloride (CsCl) suspensions prepared either in 0.01 M sodium phosphate buffer (pH 7.0) or 0.05 M potassium phosphate buffer (pH 7.6), respectively. Some Cs_2SO_4 gradients were also preformed (20–30%, w/w) to shorten centrifugation time to 5 hr at 100,000 *g*.

Visible bands observed in gradients were collected in a dropwise manner through a needle hole punched in the bottom of the tubes after centrifugation, and diluted twofold with 0.01 M potassium phosphate, pH 7.6. The preparations were clarified by centrifugation at 12,100 *g* for 10 min, and concentrated by ultracentrifugation at 102,900 *g* for 120 min. The pellets were finally resuspended overnight at 5 C, in 0.01 M potassium phosphate buffer, pH 7.6. These preparations were used for spectrophotometry, electrophoresis, and as an immunogen.

Spectrophotometry. The absorption of the purified preparations was determined with a Beckman DB spectrophotometer. The extinction coefficient, E (mg/ml)/ 1 cm (260 nm) of 2.3, estimated for a similar virus, maize stripe virus (6), was used to estimate the concentration of the isolated nucleoprotein.

Electron microscopy. Preparations at various stages of purification were negatively stained with 2% phosphotungstic acid, pH 6.5, and examined with a JEOL-7A electron microscope.

Polyacrylamide gel electrophoresis. The electrophoretic analysis of the purified preparations in polyacrylamide gels, containing SDS, was performed according to the techniques of Weber and Osborn (19) as modified by Hiebert and McDonald (9). Electrophoresis was carried out in 7.5% acrylamide gels using a vertical apparatus. The samples for electrophoresis were treated with a dissociation solution containing 0.01 ml of the sodium

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phosphate buffer used for electrophoresis, 0.25 ml 10% SDS, 0.025 ml 2-mercaptoethanol, and 0.25 ml 60% sucrose. One volume of the sample was added to two volumes of the dissociation solution, and the mixture was boiled for 1 min. Serum albumin (67×10^3 daltons), ovalbumin (45×10^3 daltons), carbonic anhydrase (29×10^3 daltons), β-lactoglobulin (18×10^3 daltons), tobacco mosaic virus (17.5×10^3 daltons), bean common mosaic virus ($29–32 \times 10^3$ daltons), and bean southern mosaic virus (30×10^3 daltons) coat proteins were used as markers for molecular weight determinations.

Serology. An antiserum was prepared by injecting a New Zealand white rabbit with Cs_2SO_4-purified, pooled preparations obtained from hoja blanca-infected rice CICA 8 and IR 22 plants, standardized to a concentration of approximately 1 mg/ml. A series of four injections were given at weekly intervals, using the foot pad technique of immunization (20). Each injection consisted of 0.15 ml of the purified preparation emulsified with an equal volume of Freund's complete (first injection) or incomplete (subsequent injections) adjuvant. Bleedings were made at weekly intervals, after the last injection, for 2 mo.

Double immunodiffusion (Ouchterlony) tests were performed in a medium containing 0.8% Noble agar, 0.5% sodium dodecyl sulfate (SDS), and 1% sodium azide, all in water (7). The agar medium was poured into 9-cm-diameter plastic petri dishes, and the wells were punched in a hexagonal arrangement with the center well spaced 4–5 mm from the peripheral wells. The leaf extracts of hoja blanca-infected plants were prepared by homogenizing 1 g of tissue in 1 ml of deionized water. Plates were incubated in a moist chamber at 24 C, and reactions were recorded 24 hr after preparation of the plates.

Membrane acquisition test. Artificial acquisition tests were conducted by caging third- or fourth-instar, virus-free, nymphs of *S. orizicola* in cellulose butyrate tubes, 2.6 cm in diameter, with ventilation holes covered with a nylon net. The tubes were maintained vertically inside a pot with moist sterile soil, and the top end was closed with two parafilm membranes containing drops of purified preparations of hoja blanca-affected leaves, and 5% sucrose, at a concentration of 0.1 mg/ml of nucleoprotein.

The nymphs were allowed a 24-hr access period to the purified preparation and, subsequently, transferred to 7- and 15-day-old Bluebonnet 50 rice seedlings, in varying numbers (2, 4, 5, 7, and 10 insects) per plant. A total of 72 nymphs were used in this test. Purified preparations obtained from healthy rice CICA 8 plants were also included as controls. The insects remained on the test plants (for a month) until results were recorded.

RESULTS

Two to three closely spaced light-scattering bands were commonly observed in Cs_2SO_4 (Fig. 1) and CsCl gradients of preparations obtained from hoja blanca-affected plants grown under field or glasshouse conditions. The bands were located 30–32.5 mm (in 30% Cs_2SO_4); 25–27 mm (in 20–30% Cs_2SO_4); or 17–19 mm (in CsCl) from the bottom of 13×51 mm tubes. These bands were not observed in gradients containing preparations of symptomless rice plants included as controls. An examination of fractions of these bands collected in a dropwise manner, with the aid of the electron microscope, revealed the presence of varying amounts of fine, filamentous particles, approximately 3 nm in diameter and of variable length (Fig. 2). At high magnification, some particles were shown to possess a tightly spiraled configuration (Fig. 3). No qualitative differences were observed in the fractions collected. Isolated phytoferritin and other small isometric particles, probably fraction 1 protein (8,14) of similar diameter (11 nm), were frequently observed in partially purified preparations (Fig. 2). Isometric viruslike particles, such as those demonstrated by Herold et al (8) in rice plants with hoja blanca symptoms, were not observed in any of the preparations examined by electron microscopy in this study.

The purified preparations, obtained by clarification and ultracentrifugation of pooled bands recovered from gradients, exhibited an ultraviolet absorption spectrum typical of a nucleoprotein, with an $A_{260/280\ nm}$ ratio of 1.4 ± 0.02. The concentration of the nucleoprotein was 1.73 mg/ml (uncorrected for light scattering), and the average yield 10 mg per kilogram of leaf tissue. The nucleoprotein was not recovered when plant tissue was kept frozen prior to processing. Comparative yield assays of purified leaf and root preparations, obtained from hoja blanca-

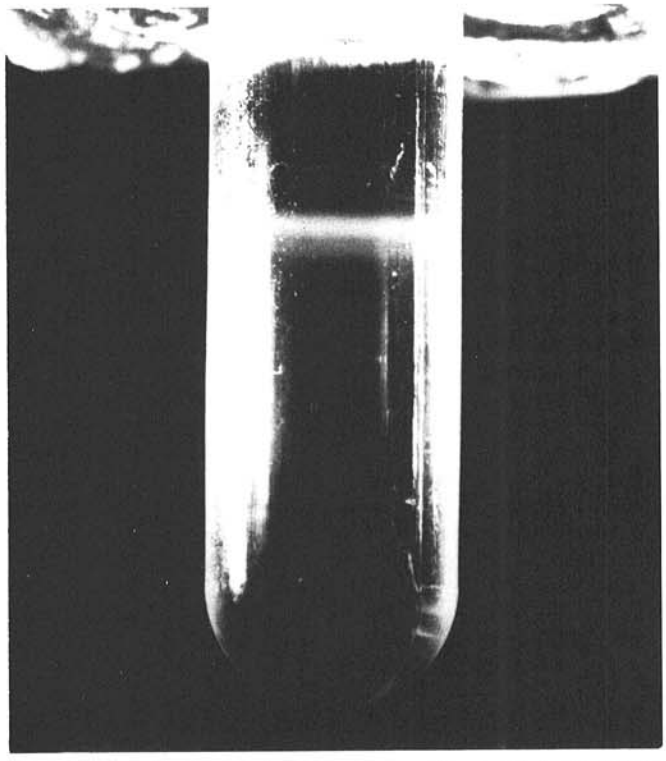

Fig. 1. Light-scattering bands in a cesium sulfate gradient obtained by equilibrium centrifugation of partially purified extracts of hoja blanca-affected rice plants.

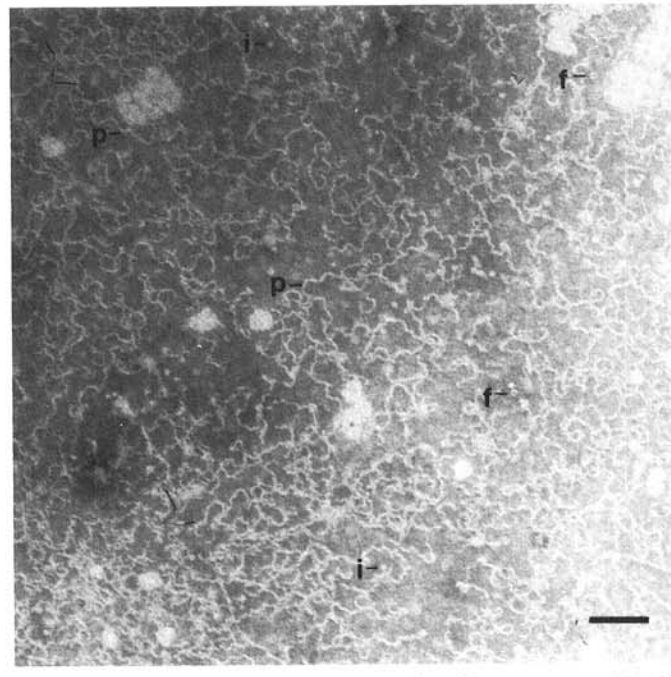

Fig. 2. Electron micrograph of negatively stained filamentous particles (p) isolated from hoja blanca-affected rice plants. Phytoferritin particles (f) and some isometric particles (i) of similar size (~11 nm) can also be observed. Scale bar represents 100 nm.

infected rice plants, indicated that leaves contained, on average, 30% more of the nucleoprotein that was isolated.

Purified preparations from hoja blanca-affected leaves, assayed by polyacrylamide gel electrophoresis in the presence of SDS, contained a predominant protein species of molecular weight 34 $\times 10^3$ daltons (Fig. 4j). In most preparations, a second molecular species of approximately 17.5×10^3 daltons was also present in a comparatively low concentration (Fig. 4j). Only traces of the heavier protein species were observed in samples prepared from symptomless CICA 8 rice plants collected in hoja blanca-affected fields. Neither one of these two proteins was observed in samples obtained from symptomless Bluebonnet 50 plants grown under glasshouse conditions. Another common protein species found in partially purified leaf preparations (Fig. 4e and g), with a molecular weight of approximately 56×10^3 daltons, probably represents a subunit of fraction-1 protein (14). This protein was present in leaf preparations of symptomless rice plants, but not in root preparations (Fig. 4f), as expected for fraction-1 protein.

The antiserum prepared by injecting a rabbit with the purified hoja blanca preparations assayed by SDS-PAGE (Fig. 4j) gave positive precipitin reactions in Ouchterlony tests with leaf extracts of hoja blanca-affected rice plants grown under field or glasshouse conditions. Leaf extracts prepared from symptomless rice plants or *E. colonum* plants, with or without hoja blanca symptoms, did not react with the antiserum in tests carried out with either field- or glasshouse-collected plants (Fig. 5). No serological relationship, either, could be demonstrated in this study between a virus of similar characteristics (6), maize stripe virus (MStpV) and the rice hoja blanca nucleoprotein isolated here, using both an antiserum to MStpV (kindly provided by R. E. Gingery, Department of Plant Pathology, Ohio Agricultural Research and Development Center, Wooster 44691), and the rice hoja blanca antiserum. Homologous reactions with their respective antigens, however, were observed in both cases.

None of the *S. orizicola* individuals allowed access to purified preparations containing the filamentous particles transmitted hoja blanca to any of the test plants.

DISCUSSION

The isolation of viruslike, filamentous particles from hoja blanca-affected rice plants in this investigation is in agreement with the findings of the cytological study carried out by Shikata and Gálvez (17). The particles isolated here, however, do not resemble those of closteroviruses as suggested by the above authors but, rather, are similar to those reported as the causal agents of rice stripe (12,13) and maize stripe (6). As pointed out for these diseases,

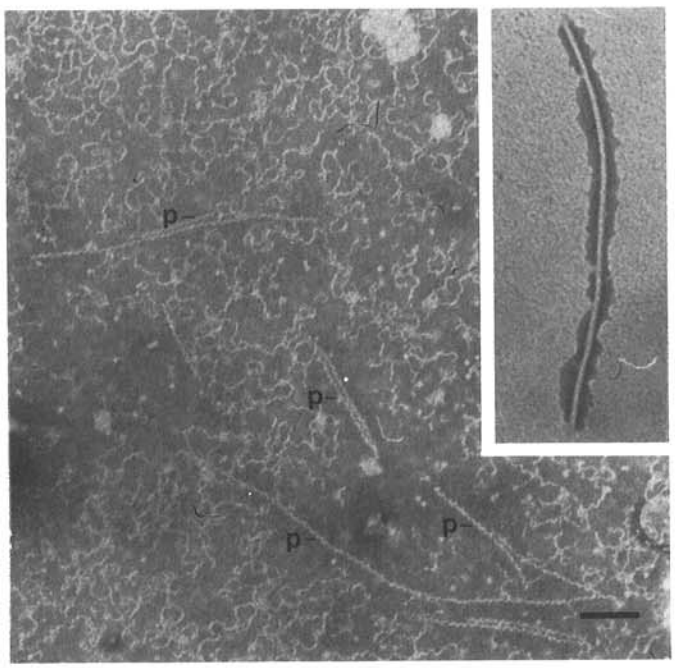

Fig. 3. Electron micrograph of magnified fine, filamentous particles (p), isolated from hoja blanca-affected rice plants, showing spiraled configuration. Inset shows filamentous particle of bean common mosaic virus at the same magnification. Scale bar represents 100 nm.

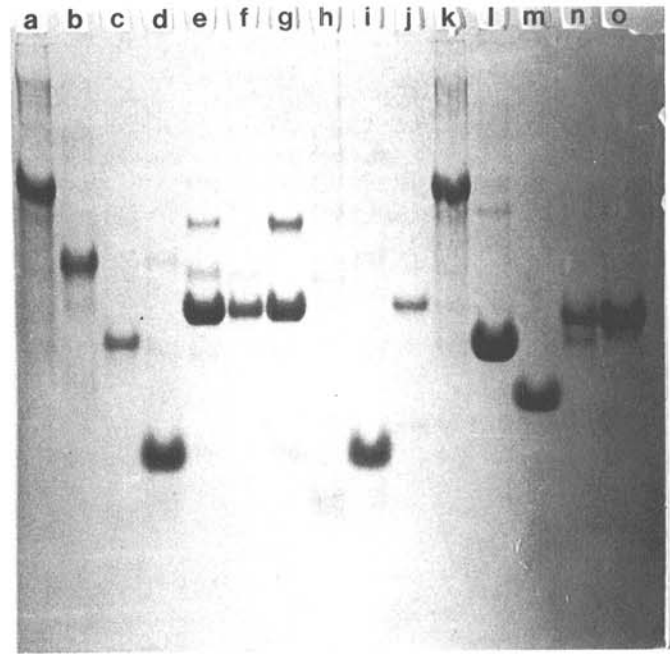

Fig. 4. SDS-polyacrylamide gel electrophoresis of proteins prepared from purified preparations obtained from hoja blanca-affected rice plants, and protein markers. Lanes: a, serum albumin; b, ovalbumin; c, carbonic anhydrase; d, β-lactoglobulin; e, fresh leaf; f, root; g, fresh leaf; and h, frozen leaf (partially purified) preparations of hoja blanca-affected plants; i, β-lactoglobulin; j, purified preparation of hoja blanca-affected rice plants; k,serum albumin; l, bean southern mosaic virus; m, tobacco mosaic virus; and n–o, bean common mosaic virus, coat proteins.

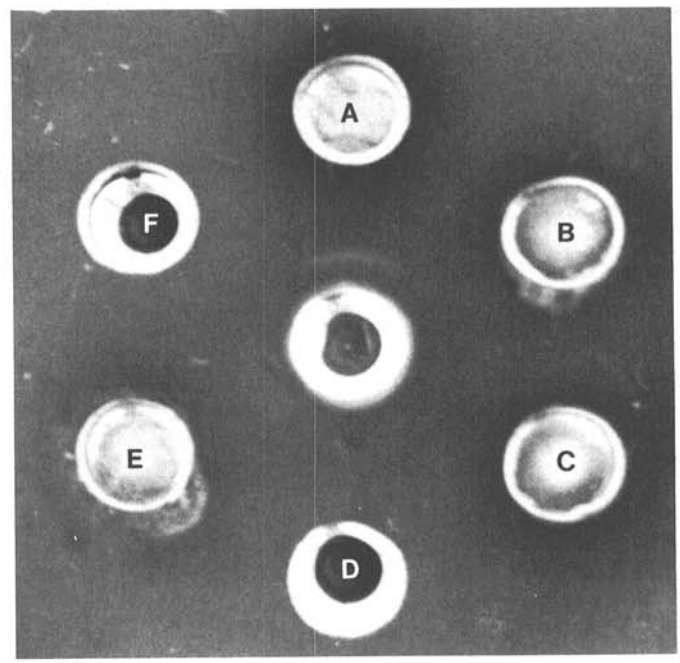

Fig. 5. Double immunodiffusion test. Center well contains antiserum prepared to a filamentous nucleoprotein isolated from hoja blanca-affected rice plants. Peripheral wells contain leaf extracts of: A, hoja blanca-affected rice plant; B, hoja blanca-affected *Echinochloa colonum* plant; C, healthy *E. colonum*; D, empty well to prevent lateral diffusion of precipitin reactions; E, healthy rice; and F, empty well.

these fine threadlike particles seem to constitute a new group of viruses.

The similarities between the filamentous nucleoprotein isolated in this study and both rice stripe virus (RSV) and MStpV, include: morphology; planthopper vectors; transovarial transmission in the vector (3); $A_{260/280\,nm}$ ratio of, approximately, 1.4; the existence of a predominant protein species of molecular weight $32–34 \times 10^3$ daltons; and the presence of a second protein of molecular weight $16.5–21 \times 10^3$ daltons in infected plants.

The threadlike particles associated with hoja blanca were not branched like RSV but some particles of both nucleoproteins have a tightly spiraled helical structure. Similarly, MStpV has not been visualized in a branched configuration. Unlike the case of RSV, the infectivity of purified preparations of both the nucleoprotein isolated in this investigation and MStpV (6) could not be demonstrated. It has been suggested that the branched configuration of RSV (6) or special components (18) could be required to retain infectivity. We also have to consider the possibility that the insects selected for the transmission tests were not vectors. In the case of hoja blanca, several attempts at selecting a colony of virus-free, potential vectors by other workers (2) and at CIAT have been unsuccessful.

The presence of multiple, closely spaced bands in cesium gradients of hoja blanca preparations could be due to contamination of the virus with the second protein of molecular weight $16.5–21 \times 10^3$ daltons. For maize stripe, this protein exhibited a density of 1.28 g/ml compared to 1.27 g/ml for the virus, following isopycnic centrifugation in CsCl (6). Moreover, a recent investigation on rice stripe virus suggests that this new group of plant viruses might be multicomponent (18). The protein contaminant, as determined by SDS-PAGE, is greatly reduced during concentration of the recovered gradient fractions by ultracentrifugation, due to its low sedimentation coefficient (12).

Despite the lack of evidence to demonstrate the infectivity of the purified preparations obtained, the similarities of the hoja blanca-associated nucleoprotein with RSV and MStpV, together with the homologous serological reactions shown in this paper, leads us to propose that the filamentous nucleoprotein isolated is the rice hoja blanca virus (RHBV).

Although there are obvious similarities between rice stripe and hoja blanca, there are also marked differences in varietal reaction to the respective causal viruses (1). Moreover, the serological relationship reported to exist between RSV and MStpV (6), as well as the broad host range of MStpV, RHBV, and RSV (6,16), within the Gramineae, suggest that pathogenic variability could also be consequence of vector preference. This hypothesis is supported by the propagative nature of these viruses in their vectors, and by the observation that *Sogatodes cubanus*, vector of the hoja blanca disease of *E. colonum*, can also transmit the rice hoja blanca disease by forced feeding to rice plants (4). This vector, however, prefers *E. colonum* as a host. Our serological results suggest that the causal agents of rice and *E. colonum* hoja blanca are not identical. It must be considered, however, that, first, the immunization route used here produces highly specific antisera and, secondly, that extracts of *E. colonum* could contain inhibitors or a low concentration of the virus.

In conclusion, RHBV seems to be another member of a new class of viruses transmitted, predominantly, to species in the Gramineae by planthoppers. As pointed out by other authors (12,18), it is possible that other virus members, such as the causal agent of European wheat striate mosaic, might exist.

LITERATURE CITED

1. Atkins, J. G., Goto, K, and Yasuo, S. 1961. Comparative reactions of rice varieties to the stripe and hoja blanca virus diseases. Int. Rice Commission Newsl. 10:5-8.
2. Everett, T. R. 1967. Vectors of hoja blanca virus. Pages 111-121 in: IRRI Proc. The Virus Diseases of the Rice Plant. Johns Hopkins Press, Baltimore, MD. 354 pp.
3. Everett, T. R., and Lamey, H. A. 1969. Hoja blanca. Pages 361–377 in: Viruses, Vectors, and Vegetation. K. Maramorosch, ed. Interscience, New York. 66 pp.
4. Gálvez, G. E. 1968. Transmission studies of the hoja blanca virus with highly active virus-free colonies of *Sogatodes oryzicola*. Phytopathology 58:818-821.
5. Garcés-Orejuela, C., Jennings, P. R., and Skiles, R. L. 1958. Hoja blanca of rice and the history of the disease in Colombia. Plant Dis. Rep. 42:750-751.
6. Gingery, R. E., Nault, L. R., and Bradfute, O. E. 1981. Maize stripe virus: characteristics of a member of a new virus class. Virology 112:99-108.
7. Gooding, G. V., Jr., and Bing, W. W. 1970. Serological identification of potato virus Y and tobacco etch virus using immunodiffusion plates containing sodium dodecyl sulfate. Phytopathology 60:1293.
8. Herold, F., Trujillo, G., and Munz, K. 1968. Viruslike particles related to hoja blanca disease of rice. Phytopathology 58:546-547.
9. Hiebert, E., and McDonald, J. G. 1973. Characterization of some proteins associated with viruses in the potato Y group. Virology 56:349-361.
10. Jennings, P. R. 1963. Estimating yield loss in rice caused by hoja blanca. Phytopathology 53:492.
11. Kitajima, E., and Gálvez, G. E. 1973. Flexuous, threadlike particles in leaf cells of *Echinochloa colonum* infected with rice hoja blanca virus. Cienc. Cult. 25:979-982.
12. Koganezawa, H. 1977. Purification and properties of rice stripe virus. Pages 151–154 in: Symposium on Virus Diseases of Tropical Crops. Trop. Agric. Res., Ser. No. 10., Ministry. Agric. Forestry, Jpn. 222 pp.
13. Koganezawa, H., Doi, Y., and Yora, K. 1975. Purification of rice stripe virus. Ann. Phytopathol. Soc. Jpn. 41:148-154.
14. King, S. 1976. Tobacco fraction-1 protein: a unique genetic marker. Science 191:429-434.
15. Malaguti, G., Diaz, H., and Angeles, N. 1957. La virosis "hoja blanca" del arroz. Agron. Trop. 6:157-163.
16. Ou, S. H. 1972. Rice diseases. Commonw. Mycol. Inst., Kew, Surrey, England. 368 pp.
17. Shikata, E., and Gálvez, G. E. 1969. Fine flexuous threadlike particles in cells of plants and insect hosts infected with rice hoja blanca virus. Virology 39:635-641.
18. Toriyama, S. 1982. Characterization of rice stripe virus: a heavy component carrying infectivity. J. Gen. Virol. 61:187-195.
19. Weber, K., and Osborn, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.
20. Ziemiecki, A., and Wood, K. R. 1975. Serological demonstration of virus-specific proteins associated with cucumber mosaic virus infection of cucumber cotyledons. Physiol. Plant Pathol. 7:171-177.