Diagnosis of Rice Ragged Stunt Virus by Enzyme-Linked Immunosorbent Assay and Immunosorbent Electron Microscopy

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ABSTRACT

Luisoni, E., Milne, R. G., and Roggero, P. 1982. Diagnosis of rice ragged stunt virus by enzyme-linked immunosorbent assay and immunosorbent electron microscopy. Plant Disease 66:929-932.

Rice ragged stunt virus was purified and specific antisera were prepared that reacted only with the virus capsid and not with double-stranded ribonucleic acid. Enzyme-linked immunosorbent assay and immunosorbent electron microscopy detected the virus in infected rice plants. Immunosorbent electron microscopy was from 10 to more than 100 times more sensitive, but enzyme-linked immunosorbent assay is likely to find wider local application because it requires simpler, less costly equipment.

Rice ragged stunt virus (RRSV), the cause of a severe rice disease in Southeast Asia (4,6,16), has affinities with members of the fijivirus subgroup of the reovirus group but is not serologically related to them (13). Particles that may be either complete virions or subviral particles (10) have been partially purified, and low-titered antisera have been obtained that were used in immunodiffusion and immunoelectron microscopic "decoration" tests (13).

In this paper we report an improved method of purifying RRSV for production of antisera suitable for enzyme-linked immunosorbent assay (ELISA), and we compare ELISA with immunosorbent electron microscopy (ISEM) for diagnosis of the virus. Some preliminary results have been published (14).

MATERIALS AND METHODS

RRSV-infected rice plants were collected by K. C. Ling at the International Rice Research Institute, Philippines, brought to Turin, and fumigated (13). Some material was processed at once or after storage at 0 C. The rest was grown in submerged pots in the glasshouse at about 30 C with extra lighting and harvested periodically by pruning the roots and shoots without destruction of the plants, which remained vegetative but did not multiply.

The virus was purified from roots and stem bases essentially as described for oat sterile dwarf virus subviral particles (8).

Virus preparations were emulsified with Freund's adjuvant (using complete adjuvant for the first injection and incomplete adjuvant for the rest) and injected five times intramuscularly and twice into the footpads of a rabbit over a 6-wk period. Four bleedings were made between 6 and 12 wk after the first injection. Antiserum titers were determined by agar gel diffusion using partially purified virus or concentrated healthy plant material as antigens.

ELISA tests were conducted according to the procedures of Clark and Adams (2) with slight modifications, using M129A micro-ELISA plates (Dynatech Laboratories Inc., 900 Slaters Lane, Alexandria, VA 22314). The effects of several variables are given in the results.

ISEM was done as previously described (12) at about 23 C, using freshly carboned grids incubated with antiserum for 10 min and subsequently with virus extract for 30 min. In addition to crude antiserum, the same purified antibody preparation as used for ELISA was employed. As controls, grids were incubated either with 0.1 M phosphate buffer, pH 7, (PB) or with diluted normal serum before incubation with the virus extract. All preparations, including those of simple electron microscopy, were negatively stained in 2% aqueous uranyl acetate and examined in a Philips EM 300 electron microscope at 60 kV at a magnification of ×43,000.

For comparing ELISA and ISEM, sap was expressed from leaves of infected rice plants and diluted in phosphate-buffered saline plus Tween (PBS-Tween) for ELISA or in PB for ISEM, or in healthy sap for both tests, to simulate a sequence of undiluted samples containing decreasing amounts of RRSV. For ELISA, an absorbance value of more than twice the healthy background was taken as positive, as was the detection of an average of at least one virus particle per 400-mesh grid square for ISEM, with observation of at least two grid squares on each of two grids.

RESULTS

Virus purification and antiserum production. The best virus preparations (Fig. 1) were obtained after the cesium sulfate density gradient centrifugation step (8). Electron microscopy indicated that the virus particles were well purified except for traces of membrane material and occasional bacteriophages and bacterial flagellar fragments.

Titers of antisera ranged from 1:256 to 1:512. The antiserum used in the ELISA

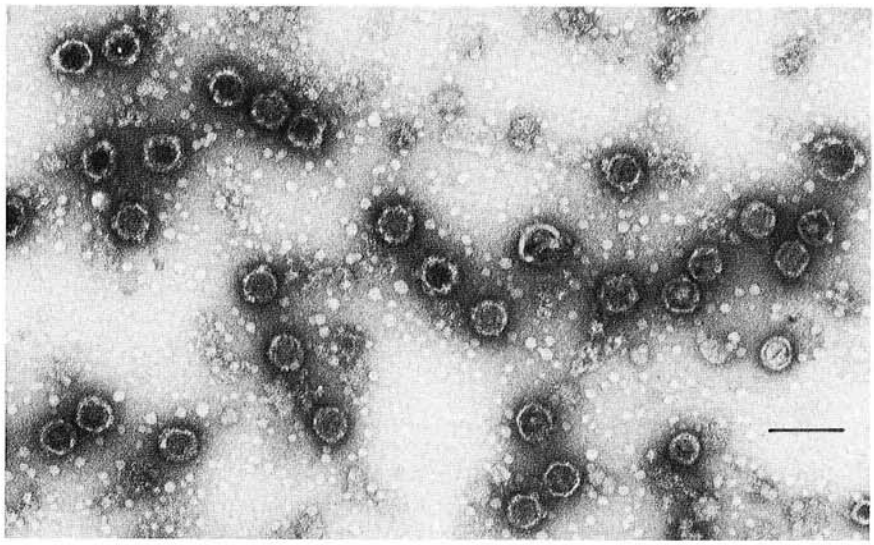

Fig. 1. Purified rice ragged stunt virus as used for obtaining the antisera, negatively stained in uranyl acetate. Bar = 100 nm.

Accepted for publication 11 February 1982.

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0191-2917/82/10092904/$03.00/0

and ISEM tests was obtained 8 wk after the first injection; had a titer of 1:512 against the virus; and gave no reaction with healthy rice preparations, the artificial double-stranded ribonucleic acid poly(I):poly(C), or the double-stranded ribonucleic acids of maize rough dwarf virus (9) and RRSV (1).

Table 1. Enzyme-linked immunosorbent assay activity of nine conjugates with different ratios of enzyme to antibody

Conjugate	Enzyme/antibody	Activity[a]	Protein[b] (mg/ml)
I	1:1	+	2.5
II	1:1	+++++	1
III	1:1	+++++	2
IV	1:1	++	2.2
V	1:5	+++	1.6
VI	1:5	++++	1.2
VII	1:5	+++++	1.2
VIII	1:5	++++++	1.2
IX	1:15	−	1.1

[a] The number of + signs indicates activity per milligram of conjugated protein, measured by the ratio I.D. infected/O.D. healthy; − = no appreciable activity.
[b] Enzyme plus antibody in the conjugate.

ELISA and ISEM. For ELISA, the optimum concentration of the coating antibody was 12 μg/ml. Ratios of enzyme to antibody in the conjugation mixture from 1:1 to 1:5 were used successfully, although there was much variability from one conjugate to another (Table 1). The best working concentration of protein (enzyme + antibody) in the conjugate was 2–6 μg/ml (ratio 1:5) and 7–10 μg/ml (1:1). In some cases, the conjugate was recycled (7). Generally, there was no "plate effect" (2), although corner wells occasionally showed higher absorbances.

ELISA tests indicated that green tissues contained more RRSV antigen then did roots; therefore, further experiments were conducted with green tissues for both ELISA and ISEM. Storing the sap from green tissues at 4 C for 1 wk did not alter ELISA values. Dilution of infected sap in PBS-Tween or in healthy sap did not significantly affect the sensitivity of the ELISA test (Fig. 2), which always gave positive results with sap diluted 1:25 and generally with sap diluted 1:100.

For ISEM, the optimal dilutions were 1:1,000 for crude antiserum and 1:100 (corresponding to protein at 60 μg/ml) for the purified antibody preparation. Crude serum and purified antibody worked about equally well; for comparison with ELISA, we used purified antibody (Fig. 3).

As with ELISA tests, dilution of infected sap in PB or in healthy sap did not significantly affect the sensitivity of ISEM (Table 2), although virus particles were more difficult to detect against the heavier background because of dilution in healthy sap. RRSV particles were consistently detected in saps diluted to 1:1,024 (Table 2) and sometimes at dilutions up to 1:8,192. Thus, the technique was generally about 10 times but occasionally more than 100 times more sensitive than ELISA.

On grids that were not pretreated or were preincubated with PB for 10 min, RRSV could only be detected in saps diluted to 1:10; the background of cell debris was heavy and made identification of the particles difficult (Fig. 3). Incubation of grids for 10 min with a normal serum diluted 1:1,000 gave relatively clean backgrounds, as did the homologous antiserum, but no virus particles were found beyond sap dilutions of 1:2.

Table 2. Detection of rice ragged stunt virus by immunosorbent electron microscopy in samples diluted in neutral 0.1 M phosphate buffer or healthy sap

	Virus particles per 400-mesh grid square in samples[a] diluted in:					
	Phosphate buffer			Healthy sap		
Sample dilution	Mean	SD[b]	n[c]	Mean	SD	n
1:32	234	71.5	4	233	13.4	2[d]
1:64	111	14.9	4	194	82.4	4
1:128	51	10.3	4	53	9.0	10
1:256	17	6.6	8	23	5.0	4
1:512	9	3.40	4	12	3.41	10
1:1024	2	0.75	6	3	0.82	4

[a] Same samples as used in Figure 2.
[b] Standard deviation.
[c] Number of observations.
[d] Only one grid examined.

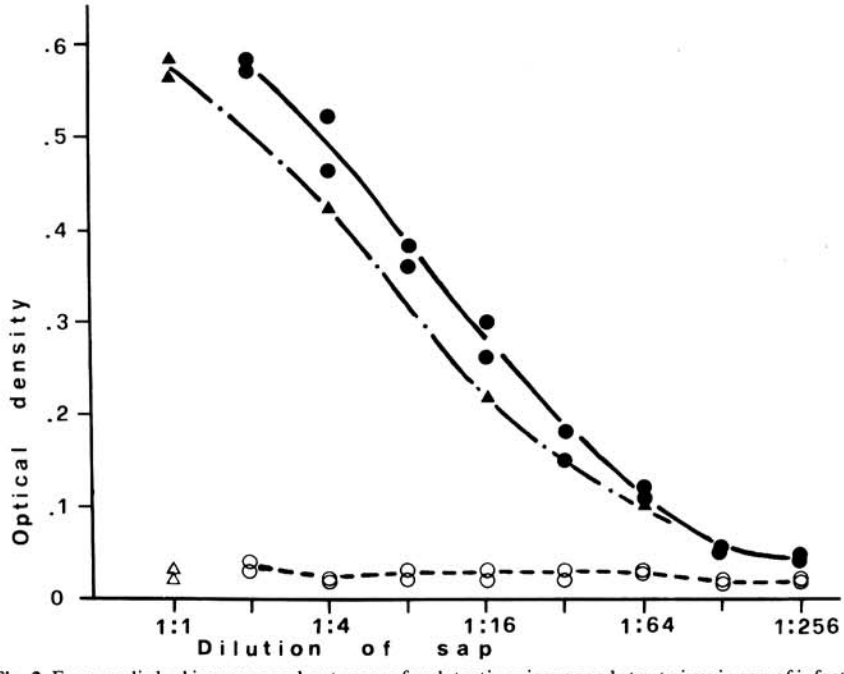

Fig. 2. Enzyme-linked immunosorbent assays for detecting rice ragged stunt virus in sap of infected rice leaves; same samples as used in Table 2. Hydrolyzed substrate solutions were diluted 1:5 and read at 405 nm with a 1-cm path length. • = infected sap diluted in PBS-Tween, ▲ = infected sap diluted in healthy sap, o = healthy sap diluted in PBS-Tween, and Δ = undiluted healthy sap.

DISCUSSION

With access to a supply of fresh plants, purification of RRSV should be easy, although extreme care should be taken that the plants are not coinfected with other viruses such as those of the tungro complex. Purification was more difficult for us because we avoided propagating the virus using its vector and therefore relied on small amounts of vegetatively propagated, infected rice or on packets of infected plants sent from Southeast Asia. Nevertheless, we were able to obtain an antiserum specific for RRSV and suitable for ELISA.

Diagnosis of RRSV by ELISA is important because it is the only reliable and sensitive method available that does not require expensive equipment and thus could be used in many regions where rice is grown. ISEM is more sensitive and, if combined with "decoration" (13), is extremely specific but would not always be feasible. Diagnosis based on symptomatology alone, although generally possible, might lead to confusion with rice black streaked dwarf virus (16) and might be less reliable in some stages of plant development (4), in mixed infections, or in some rice varieties.

RRSV in samples of rice sent to us by K. C. Ling from the People's Republic of China (14) was detected by ELISA and

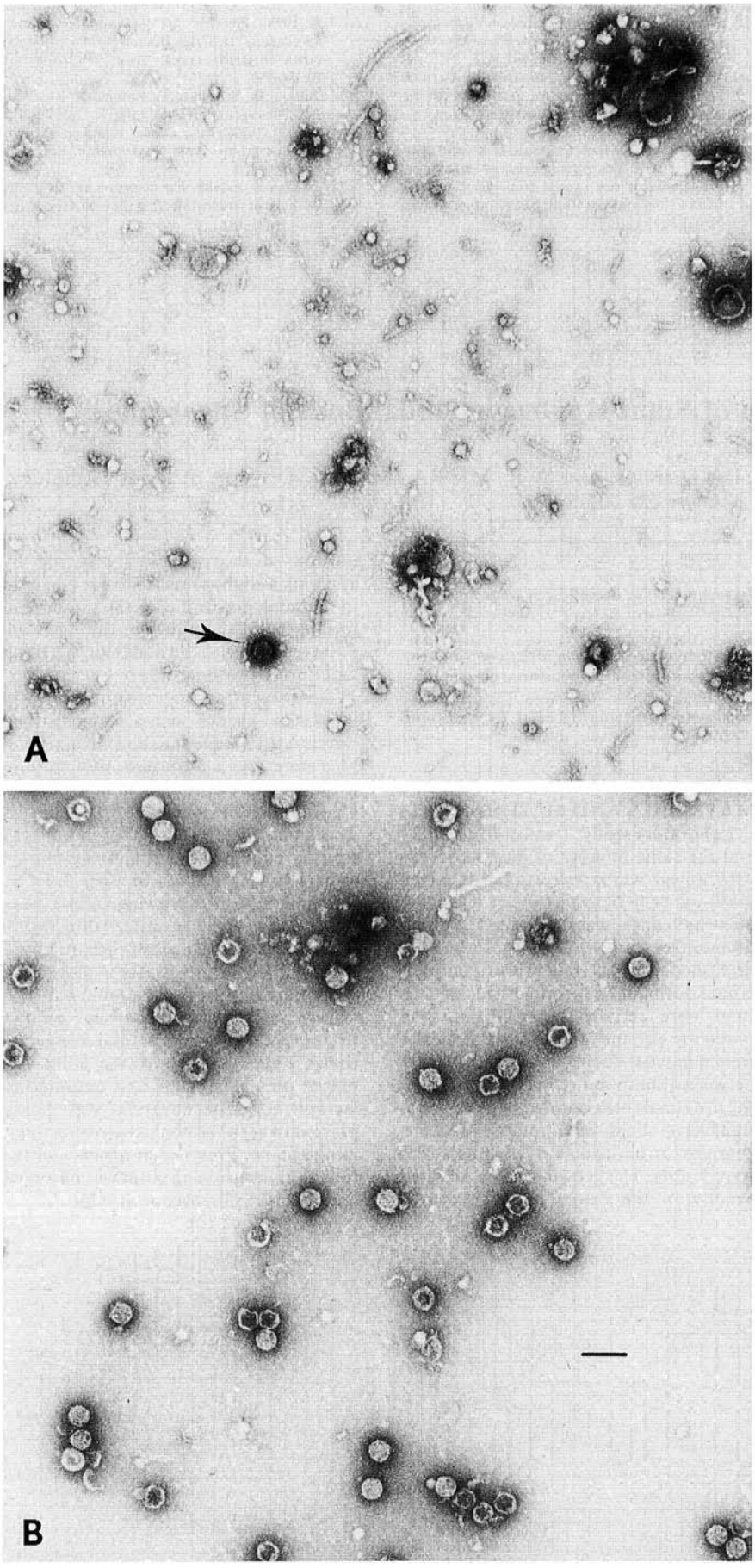

Fig. 3. Crude sap from leaves of plants infected with rice ragged stunt virus: **(A)** Incubated on a grid for 30 min, then negatively stained in uranyl acetate. Arrow indicates the single visible virus particle. **(B)** Incubated on a grid previously coated with rice ragged stunt virus antibody. Bar = 100 nm.

ISEM with the same sensitivity as the homologous virus from the Philippines; this indicates the possibility of applying these diagnostic tests far from the source of the disease.

The use of sap from leaves and stems instead of roots is suggested, particularly for ELISA, because it provides more virus antigen and fewer contaminating bacterial and bacteriophage antigens, with which in one case our antiserum reacted slightly. Roots remain the material of choice for virus purification because the sap is easier to clarify without great virus losses.

ISEM was highly sensitive because the virus particle is easily recognizable (10) and trapping efficiency is much higher than with some other viruses (11). ISEM is more sensitive than ELISA in detecting pea seedborne mosaic virus (3), arabis mosaic, prunus necrotic ringspot, and strawberry latent ringspot viruses (17), whereas the techniques are equally sensitive in detecting plum pox virus (15).

For comparative tests, we used sap from infected leaves. Enations or galls contain much higher concentrations of virus, which can be exploited by ISEM but not routinely by ELISA because of the small amounts of tissue available. We found that ELISA and ISEM can be used on dried material sent by mail.

Our results agree with those of Hibino and Kimura (5), who have developed ELISA mainly for detection of RRSV in the insect vector. When applied to infected rice leaves, their test was about three times more sensitive than ours. Our tests were made on plants kept for more than a year in the glasshouse, by which time symptoms were very weak and virus concentration was probably lower.

ACKNOWLEDGMENTS

We thank H. Hibino, Ibaraki, Japan, for sending us his manuscript before publication; G. Gaviani for printing Figure 2; and K. C. Ling, Manila, Philippines, whose cooperation in providing infected and healthy rice material made this work possible.

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