Infectivity Neutralization of Rice Tungro-Associated Viruses Acquired by Vector Leafhoppers

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ABSTRACT

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Rice tungro bacilliform virus (RTBV) depends on rice tungro spherical virus (RTSV) for its transmission by the vector leafhopper *Nephotettix virescens*. Adult leafhoppers transmit RTBV and RTSV in a semipersistent manner. Leafhoppers that had access to plants infected with both RTBV and RTSV were fed through membranes on anti-RTSV or -RTBV immunoglobulin (IgG). When the leafhoppers fed for 16 hr on anti-RTBV IgG diluted 25 times, RTBV infectivity was neutralized and mostly RTSV was transmitted. When the leafhoppers fed for 16 hr on anti-RTSV IgG diluted 25 times, RTSV transmission was markedly reduced and mostly RTBV was transmitted. In two of six trials, however, RTSV infectivity was completely neutralized by the anti-RTSV IgG treatment. When the leafhoppers that had access to RTSV-infected plants were fed on anti-RTSV IgG, they lost most of their ability to transmit RTSV but retained the ability to acquire and transmit RTBV. When the leafhoppers that had access to plants infected with both viruses were fed on a mixture of the anti-viral IgGs and then transferred to plants infected with both viruses for reacquisition access, they reacquired both viruses. These results indicate that RTSV virions may not bear the helper function for RTBV transmission by leafhoppers.

Rice tungro is a composite disease caused by rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (10,15). The green leafhopper, *Nephotettix virescens* Distant, transmits both RTBV and RTSV in a semipersistent manner (7,11,13). Leafhoppers transmit these viruses either together or singly from source plants infected with both viruses. RTSV can be transmitted independently once isolated, while RTBV depends on RTSV for its transmission by leafhoppers (1,6,7,10,11). RTBV transmission occurs only when leafhoppers are allowed to feed on RTSV-infected plants first and then on RTBV-infected plants. The reverse feeding sequence results in transmission of RTSV alone (1,6,7,10). Leafhoppers retained RTSV for 3 to 4 days but retained the ability to acquire and transmit RTBV for 7 days (1,7), which indicated that RTSV infectivity and the 'helper' activity for RTBV transmission by leafhoppers are differentiable. Viruliferous leafhoppers lost RTBV, RTSV, and the ability to acquire RTBV after molting (1,7).

To further characterize helper activity, biological assay techniques are essential. Infectivity of persistent viruses that have not been transmitted mechanically can be assessed by feeding the vector insects through membranes on viruses and subsequently testing the insects for infectivity (17). The technique has been used for infectivity neutralization by feeding the insects on viruses previously incubated with antiserum (2,18). Although the membrane feeding technique has been attempted for RTBV and RTSV, so far leafhoppers that have fed on virus extracts have not been infective (Hibino, *unpublished data*).

Here, we use the membrane feeding technique to neutralize infectivity of RTBV and RTSV in vivo and apply the technique to differentiate RTSV infectivity and helper activity. A preliminary report was made (9).

MATERIALS AND METHODS

Viruses, insects, and plants. A tungro isolate with which RTBV and RTSV were associated and virus-free *N. virescens* colonies that have been maintained in a greenhouse for several years at the International Rice Research Institute, Philippines, were used. Adult leafhoppers were given a 2- to 3-day acquisition access period on the rice cultivar Taichung Native 1 (TN1) infected with RTBV and RTSV and a 1-day inoculation access period on TN1 seedlings. One month after inoculation, inoculated plants were tested for the presence of RTBV and RTSV by latex serology. Seedlings infected with RTBV or RTSV alone, and seedlings infected with both RTBV and RTSV were selected and used as virus sources. Adult leafhoppers were given a 3-day acquisition access to one of these source plants as specified.

Antisera and IgG. Antiserum titers, determined by ring interface tests, were 1/1,028 for RTBV antiserum (15) and 1/640 for RTSV antiserum (8). Immunoglobulin (IgG) was precipitated in half-saturated ammonium sulfate (pH 6.5) and centrifuged at 10,000 rpm (Beckman type 20 rotor) for 10 min. Pellets were suspended in 2 ml of 0.01 M, pH 7.4, phosphate buffer containing 0.075 M NaCl and 0.01% NaN_3 (half-strength phosphate-buffered saline [PBS]), and dialyzed against PBS. Preimmune IgG was prepared similarly. Partially purified IgG solutions were diluted to the original volume with PBS and stored at 4 C.

Membrane feeding. Feeding solutions were prepared by diluting the IgG stock solution with 2% sucrose in 0.01 M phosphate buffer (pH 7.4). A specially designed feeding cage was used (Fig. 1). Approximately 0.5 ml of diluted IgG solution was sandwiched between Parafilm membranes, which covered one end of a glass tube, and the glass tube was covered with a glass cage. Twenty to thirty adult leafhoppers were introduced into each cage and the feeding cage was placed in a glass-bottomed dark chamber with illumination from the underside to attract the leafhoppers to the solution sandwiched between membranes.

Infectivity assays. Leafhoppers were individually confined with a 7-day-old TN1 seedling in a test tube for 1 day. Seedlings were transplanted in clay pots and grown in the greenhouse. Symptoms appeared 7–10 days after inoculation. One month after inoculation, the second or third youngest leaf was collected from each inoculated seedling and tested for presence of RTBV and RTSV by the latex test.

Infectivity of adult leafhoppers that had fed on the virus sources or virus-free plants was also tested from time to time. None transmitted either virus from source plants with RTBV alone or from virus-free plants. About 70% of the leafhoppers transmitted RTSV from RTSV-infected plants. About 70–90% of the leafhoppers that had fed on plants infected with both RTBV and

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RTSV transmitted the viruses with 60, 39, and 1% transmitting RTBV and RTSV together, RTBV alone, and RTSV alone, respectively.

Latex test. Latex particles (Difco Bacto-latex 0.81, Difco Laboratories, Detroit, MI) were sensitized with either anti-RTSV or -RTBV IgG by the procedure of Omura et al (14). Leaf samples about 10 cm long were homogenized in 1 ml of 0.05 M Tris-Cl buffer (pH 7.2) using a combined leaf and bud press (Erich Pollahne, Wennigsen, West Germany). Approximately 25 μl of sap and 25 μl of sensitized latex suspension were placed in each well of enzyme-linked immunosorbent assay plates (Dynatech, Alexandria, VA) and plates were shaken vigorously (160 oscillations per minute) for at least 30 min. Mixtures were observed under a light microscope (100). Clumping of latex particles indicated presence of the virus antigen. RTBV and RTSV were detected in extracts of infected TN1 plants up to dilution of 1/160 and 1/80, respectively. Extracts of virus-free TN1 plants reacted slightly when dilutions of 1/2 or less were used but no reactions occurred with either latex preparations when more dilute preparations of virus-free plants were used.

TABLE 1. Effect of immunoglobulin (IgG) concentration on neutralization of rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) by feeding *Nephotettix virescens*, which had fed on plants infected with RTBV and RTSV, through membranes on anti-RTBV, anti-RTSV, or preimmune IgG for 16 hr

IgG	Dilution[a] (times)	Seedlings (no.) infected with[b] RTBV+RTSV	RTBV	RTSV	None
RTBV	50	2	0	15	72
	500	10	4	13	74
	1,000	10	4	9	78
	5,000	22	21	1	56
RTSV	50	2	47	0	76
	500	4	39	1	30
	1,000	4	39	1	35
	5,000	9	31	0	26
Preimmune	50	124	81	12	35

[a] Diluted with 2% sucrose in 0.01 M phosphate buffer (pH 7.4).
[b] Rice seedlings were exposed to adult leafhoppers at one per seedling for 1 day and were tested for presence of RTBV and RTSV by latex serology 1 mo after inoculation.

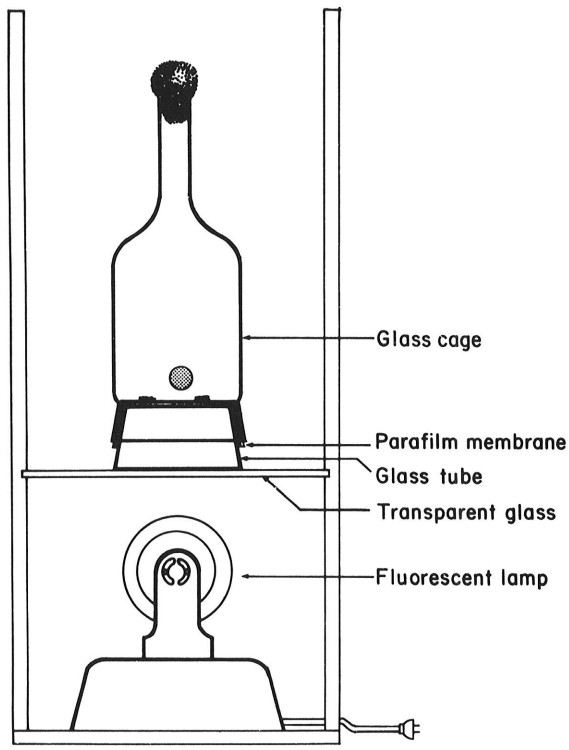

Fig. 1. Diagram of membrane feeding apparatus and accessories.

RESULTS

Effect of IgG concentration on infectivity neutralization. IgG to either RTBV or RTSV was diluted 50–5,000 times with a 2% sucrose solution. Leafhoppers that had fed on plants infected with RTBV and RTSV were allowed to feed on diluted IgG solutions for 16 hr and then tested for infectivity. RTBV infectivity was neutralized by treatment with anti-RTBV IgG diluted 50 times and leafhoppers transmitted mostly RTSV alone (Table 1). At higher IgG dilutions, leafhoppers that fed on anti-RTBV IgG occasionally transmitted RTBV, either singly or together with RTSV. On the other hand, leafhoppers that fed on anti-RTSV IgG diluted 50 times mostly transmitted RTBV alone, only two of 125 transmitted RTSV together with RTBV. The number of leafhoppers that transmitted RTBV and RTSV together increased at higher IgG dilutions.

Effect of feeding duration. Leafhoppers that fed on plants infected with RTBV and RTSV were allowed to feed on anti-RTSV, anti-RTBV, or preimmune IgG diluted 25 or 50 times for 8 or 16 hr. Their infectivity was then tested.

Infectivity of RTBV was completely neutralized when leafhoppers fed for 16 hr on anti-RTBV IgG diluted 25 times (Table 2). But 8-hr feeding was not sufficient to completely block RTBV transmission by leafhoppers. The anti-RTBV IgG treatment lowered RTSV transmission by leafhoppers, i.e., in 16-hr feeding on a 25-times dilution, about 23% of anti-RTBV IgG treated leafhoppers transmitted RTSV, while about 62% of preimmune IgG-treated leafhoppers transmitted RTSV singly or together with RTBV.

On the other hand, RTSV transmission by leafhoppers was markedly reduced when they were fed on anti-RTSV IgG (Table 2). Most of the treated leafhoppers transmitted RTBV alone, only a few transmitted RTBV and RTSV together or RTSV alone. Sixteen-hour feeding was more efficient than 8-hr feeding in blocking RTSV transmission. In a 16-hr feeding, treatment with 25- and 50-times dilutions gave similar results. In two trials, leafhoppers that fed for 16 hr at 25-times dilution transmitted RTBV alone. Treatment with anti-RTSV IgG also lowered RTBV transmission by leafhoppers, i.e., in a 16-hr feeding on a 25-times dilution, about 47% of the leafhoppers that were treated with anti-RTSV IgG transmitted RTBV singly or together with RTSV, whereas about 85% of preimmune IgG treated leafhoppers transmitted RTBV. Leafhoppers fed on preimmune IgG transmitted both viruses either together or singly at high rates.

TABLE 2. Effect of feeding duration on neutralization of rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) by feeding *Nephotettix virescens*, which had fed on plants infected with RTBV and RTSV, through a membrane on anti-RTBV, anti-RTSV, or preimmune IgG

IgG	Dilution[a] (times)	Feeding duration (hr)	Seedlings (no.) infected with[b] RTBV+RTSV	RTBV	RTSV	None
RTBV	25	8	1	1	10	59
	25	16	0	0	60	205
	50	8	8	2	24	45
	50	16	1	1	31	79
RTSV	25	8	2	18	1	53
	25	16	6	101	0	119
	50	8	9	32	0	46
	50	16	4	76	0	76
Preimmune	25	8	15	29	1	13
	25	16	62	26	1	13
	50	16	30	48	3	35

[a] Diluted with 2% sucrose in 0.01 M phosphate buffer (pH 7.4).
[b] Rice seedlings were exposed to adult leafhoppers at one per seedling and were tested for the presence of RTBV and RTSV with latex test 1 mo after inoculation.

Symptoms on seedlings inoculated by leafhoppers treated with anti-RTBV, anti-RTSV, or preimmune IgG were different depending on the viruses transmitted as reported previously (1,6,7,10).

Effect of RTSV neutralization on RTBV acquisition. Leafhoppers that had fed on plants infected with RTSV alone were allowed to feed again for 16 hr on anti-RTSV IgG diluted 25 or 50 times. After feeding, half of them were immediately tested for infectivity. The other half were allowed an 8-hr access to RTBV source plants and then tested for infectivity.

Leafhoppers that fed on anti-RTSV IgG diluted 25 times transmitted RTSV inefficiently but acquired and transmitted RTBV from RTBV-infected plants at a high rate (Table 3). Treatment at 50 times dilution had similar results. Apparently, the anti-RTSV IgG treatment greatly reduced RTSV infectivity but did not reduce the leafhopper's ability to acquire and transmit RTBV.

Effect of infectivity neutralization on reacquisition. Leafhoppers that had fed on plants infected with RTBV and RTSV were allowed to feed on anti-RTSV IgG, anti-RTBV IgG, or a mixture of both (1:1 in volume) for 16 hr. IgG was diluted 25 times. Then half of the leafhoppers from each treatment were immediately tested for infectivity. The remaining leafhoppers were given an 8-hr reacquisition access to source plants with either RTSV (for the leafhoppers fed on anti-RTSV IgG), RTBV (for the leafhoppers fed on anti-RTBV IgG), or both (for the leafhoppers fed on a mixture of anti-RTBV and RTSV IgG), and then tested for infectivity.

The leafhoppers that fed on anti-RTSV IgG, anti-RTBV IgG, and mixture of both IgG transmitted RTSV, RTBV, and both viruses, respectively, with low efficiencies or not at all (Table 4). Leafhoppers treated with IgGs readily reacquired and transmitted the respective viruses.

DISCUSSION

Infectivity neutralization, as it is commonly practiced, is based on a serological reaction that occurs when virus is incubated with antiserum in vitro. Infectivity assay of antiserum-treated virus can be by mechanical transmission, or by direct injection or membrane feeding of the vector insects. In these experiments, infectivity neutralization in vivo was done by feeding viruliferous vector leafhoppers on antisera through membranes. Viruliferous leafhoppers lost the ability to transmit RTBV following the anti-RTBV IgG treatment, whereas RTSV infectivity was markedly reduced by the anti-RTSV IgG treatment. This technique would be useful for other insect-transmitted viruses in which infectivity assay is difficult.

RTBV and RTSV have been purified separately or together (8,15,19) but infectivity of the purified virus suspensions has not been confirmed, thus the etiology of RTBV and RTSV has not been fully established to satisfy Koch's postulates. However, the neutralization of infectivity of RTBV and RTSV by their respective anti-viral IgGs confirmed that RTBV and RTSV were the virus agents that caused rice tungro disease in combination.

Several plant viruses are dependent on other viruses for their transmission by vector insects (16). Some of the dependent viruses are known to require a helper component produced in plants as a result of infection with the helper viruses (3,4,16). In the RTBV-RTSV complex, leafhoppers lost RTSV in 4 days after acquisition access, but retained the ability to acquire RTBV until the seventh day (1,7). In these experiments, anti-RTSV IgG treatment markedly diminished RTSV infectivity but did not reduce the leafhopper's ability to acquire RTBV. Thus, RTSV infectivity and the helper activity were differentiated. These results indicate that RTSV virions may not be the bearer of helper activity. Rice plants may produce a component similar to the helper component for other virus transmission systems when infected with RTSV to serve as a helper for RTBV transmission. In these experiments, it was not confirmed if the hypothetical helper component for RTBV transmission also functions in the transmission of RTSV by leafhoppers.

Viruliferous leafhoppers lose their ability to transmit tungro after molting (13). Retention of rice waika virus, which is closely related or identical to RTSV, also ends after molting (12). Recent investigations also indicated that retention of RTBV, RTSV, and the helper component ended after molting (1,7). The loss of RTBV, RTSV, and the helper component after molting and in vivo

TABLE 3. Transmission of rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV) by *Nephotettix virescens* that were given sequential feedings on RTSV-infected plants for 3 days; anti-RTSV immunoglobulin (SIgG) or preimmune IgG (PIgG) through a membrane; on RTBV-infected plants; and on Taichung Native 1 seedling for inoculation access

IgG dilution[a]	Feeding sequence[b] 1st (16 hr)	2nd (8 hr)	inoculation (24 hr)	Leafhoppers (no. that transmitted)[c] RTBV+RTSV	RTBV	RTSV	None
25×	SIgG	RTBV	TN1	0	18	0	2
50×	SIgG	RTBV	TN1	0	11	6	3
25×	SIgG	...[d]	TN1	0	0	2	18
50×	SIgG	...	TN1	0	0	5	15
25×	PIgG	RTBV	TN1	13	2	0	5
50×	PIgG	RTBV	TN1	15	4	1	0
25×	PIgG	...	TN1	0	0	36	4

[a] Diluted with 2% sucrose prepared in 0.01 M phosphate buffer (pH 7.4).
[b] Leafhoppers were first given a 3-day access to RTSV infected plants.
[c] Inoculated seedlings were tested by latex test for the presence of viruses 1 mo after inoculation.
[d] Adult leafhoppers directly tested for their infectivity on TN1 seedlings.

TABLE 4. Transmission of rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV) by *Nephotettix virescens* that were given sequential feedings on RTBV and RTSV-infected plants for 3 days; on anti-RTSV immunoglobulin (SIgG), anti-RTBV IgG (BIgG), mixture of SIgG and BIgG (B-SIgG), or preimmune IgG (PIgG) through a membrane for 16 hr; on plants infected with RTSV, RTBV, or both for 8 hr; and on Taichung Native 1 (TN1) seedling for inoculation access

Feeding sequence[a] 1st (16 hr)	2nd (8 hr)	Inoculation (24 hr)	Leafhoppers (no.) that transmitted[b] RTSV+RTBV	RTBV	RTSV	None	Virus Transmitter (%)
B-SIgG[c]	RTBV+RTSV	TN1	43	27	1	7	91.0
B-SIgG	...[d]	TN1	0	1	2	36	7.7
SIgG[c]	RTSV	TN1	9	1	20	9	76.9
SIgG	...	TN1	0	10	0	28	26.3
BIgG[c]	RTBV	TN1	8	11	5	11	68.5
BIgG	...	TN1	0	0	5	33	13.1
PIgG[c]	RTBV+RTSV	TN1	29	6	1	3	92.3
PIgG	...	TN1	14	19	0	6	84.6

[a] Leafhoppers were first given a 3-day access to plants infected with RTBV and RTSV.
[b] Inoculated seedlings were tested by latex test for the presence of viruses 1 mo after inoculation.
[c] Immunoglobulin stock solution was diluted 25 times with 2% sucrose in 0.01 M phosphate buffer (pH 7.4).
[d] Adult leafhopper directly transferred to TN1 seedling for inoculation access.

neutralization of virus infectivity are indirect evidences that RTBV and RTSV are adsorbed in the mouth or fore alimentary canal of the leafhopper.

Harris (5) showed by electron microscopy that the semipersistent maize chlorotic dwarf virus accumulates in the intima lining of the foregut of the leafhopper, *Dalbulus maidis*, and suggested that the leafhopper transmits the virus via an ingestion-egestion mechanism. Results of these experiments indicate that *N. virescens* may transmit RTBV and RTSV via a similar mechanism. The specific adsorption of RTBV in the mouth or fore alimentary canal may be completed under the presence of the helper component. The fact that neutralization of either RTBV or RTSV lowered the transmission of the other virus indicates that the RTBV and RTSV adsorption may take place at the same or nearby sites.

In these experiments, the leafhopper could reacquire RTBV and RTSV immediately after the infectivity of previously acquired RTBV and RTSV was neutralized. Leafhoppers fed on a mixture of IgG's (B-SIgG) had less than 8% transmitters but when transferred to a disease source for reacquisition access, percentage transmitters rose to 91%. In contrast, leafhoppers fed on preimmune IgG (PIgG) had 84% transmitters (Table 4) and leafhoppers that were transferred to a virus source for reacquisition access after PIgG treatment had only a slight increase (7.7%) in percentage transmitters. The low increase in percentage transmitters in PIgG-treated leafhoppers may suggest that the adsorption site was almost near saturation and that the virions were retained in the insect mouth parts despite PIgG treatment. The significant increase in percentage transmitters in B-SIgG treated leafhoppers could be explained by the desorption from the adsorption site of the virions after neutralization.

LITERATURE CITED

1. Cabauatan, P. Q., and Hibino, H. 1985. Transmission of rice tungro bacilliform and spherical viruses by *Nephotettix virescens* Distant. Philipp. Phytopathol. 21:103-109.
2. Gold, J. E., and Duffus, J. E. 1967. Infectivity neutralization–A serological method as applied to persistent viruses of beets. Virology 31:308-313.
3. Govier, D. A., and Kassanis, B. 1974. A virus-induced component of plant sap needed when aphids acquire potato virus Y from purified preparations. Virology 61:420-426.
4. Govier, D. A., Kassanis, B., and Pirone, T. P. 1977. Partial purification and characterization of the potato virus Y helper component. Virology 78:306-314.
5. Harris, K. F. 1981. Arthropod and nematode vectors of plant viruses. Ann. Rev. Phytopathol. 19:391-426.
6. Hibino, H. 1983. Transmission of two rice tungro-associated viruses and rice waika virus from doubly or singly infected source plants by leafhopper vectors. Plant Dis. 67:774-777.
7. Hibino, H. 1983. Relations of rice tungro bacilliform and rice tungro spherical virus with their vector *Nephotettix virescens*. Ann. Phytopath. Soc. Jpn. 49:545-553.
8. Hibino, H., and Cabauatan, P. Q. 1985. Purification and serology of rice tungro spherical virus. Int. Rice Res. Newsl. 10(4):10-11.
9. Hibino, H., and Cabauatan, P. Q. 1985. Serological blockage of rice tungro virus complex transmission by *Nephotettix virescens*. (Abstr.) Philipp. Phytopathol. 21:10-11.
10. Hibino, H., Roechan, M., and Sudarisman, S. 1978. Association of two types of virus particles with penyakit habang (tungro disease) of rice in Indonesia. Phytopathology 68:1412-1416.
11. Hibino, H., Saleh, N., and Roechan, M. 1979. Transmission of two kinds of rice tungro-associated viruses by insect vectors. Phytopathology 69:1266-1268.
12. Hirao, J., and Inoue, H. 1979. Transmission characteristics of rice waika virus by the green rice leafhopper, *Nephotettix cincticeps* Uhler (Hemiptera:Cicadellidae). Appl. Entomol. Zool. 14:44-50.
13. Ling, K. C. 1966. Nonpersistence of the tungro virus of rice in its leafhopper vector, *Nephotettix impicticeps*. Phytopathology 56:1252-1256.
14. Omura, T., Hibino, H., Usugi, T., Inoue, H., Morinaka, T., Tsurumachi, S., Ong, C. A., Putta, M., Tsuchizaki, T., and Saito, Y. 1984. Detection of rice viruses in plants and individual insect vectors by latex flocculation test. Plant Dis. 68:374-378.
15. Omura, T., Saito, Y., Usugi, T., and Hibino, H. 1983. Purification and serology of rice tungro spherical and rice tungro bacilliform viruses. Ann. Phytopathol. Soc. Jpn. 49:73-76.
16. Pirone, T. P. 1977. Accessory factors in nonpersistent virus transmission. Pages 221-225 in: Aphids as Virus Vectors. K. F. Harris and K. Maramorosch eds. Academic Press, New York.
17. Rochow, W. F. 1960. Transmission of barley yellow dwarf virus acquired from liquid extracts by aphids feeding through membranes. Virology 12:223-232.
18. Rochow, W. F. and Ball, E. M. 1967. Serological blocking of aphid transmission of barley yellow dwarf virus. Virology 33:359-362.
19. Singh, S. K., Anjaneyulu, A., and Lapierre, H. 1984. Use of pectino-cellulolytic enzymes for improving extraction of phloem-limited plant viruses as exemplified by the rice tungro virus complex. Agronomie 4:479-484.