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PRODUCTION OF ANTISERUM AND INITIAL HYBRIDOMAS AGAINST PAPAYA RING SPOT VIRUS

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ABSTRACT

Papaya ring spot virus (PRSV) was isolated and purified from infected papaya leaves. The particles were shown to be threadlike and filamentous measuring about 750 nm in length and 12 nm in diameter. The average yield of PRSV per 100 g samples was 443 ug.

The antiserum against PRSV was used for rapid screening of infected papaya plants from the provinces of Cavite, Batangas and Laguna using the enzyme linked immunosorbent assay (ELISA) double antibody sandwich technique. Four hybridomas which gave consistent positive reaction when assayed by the ELISA technique and fast grower were obtained from the 2 fusion experiments. Positive PRSV hybridomas were stored in liquid nitrogen for the cloning experiments and eventually for mass production of monoclonal antibodies.

Introduction

Papaya (*Carica papaya* L.) is one of the most important crops in the Philippines because of its several uses as food, medicine and in industry. Papaya fruits are available throughout the year. It ranked 6th in the area planted and in quality and value of production among fruits grown in 1975 as reported by BAECON (Philippine Recommends for Papaya, 1977). Both fruits and the enzyme papain from papaya show great economic potential for local consumption and for export.

Papaya is not free from disease attacks. Some diseases include papaya mosaic, a minor disease which is transmitted mechanically and by a vector, *Myzus persicae.* The other disease, leaf curl is presumed to be caused by tobacco leaf curl virus and transmitted by whitefly (Philippine Recommends for Papaya, 1977). Both diseases do not cause serious damage to the papaya trees grown locally.

Recently, a new destructive disease identified as papaya ring spot virus (PRSV) disease was reported in epidemic proportion in Silang, Cavite affecting about 200 hectares of papaya farms. The disease is reported to have spread in the papaya plantations in the Southern Tagalog region especially in Cavite, Batangas

and Laguna. The disease causes severe mottling, leaf malformation, reduction of laminae, streaks and ringspot symptoms especially in fruits. Initial studies were initiated at UP Los Baños and DAF Regional Crop Protection Center in Region IV and confirmed the viral nature of the disease. The virus is known to be transmitted by mechanical inoculation with 60-70% efficiency, by granting and by four species of aphids.

Papaya ring spot virus is made up of filamentous particles about 800 nm in length and 12 nm in diameter (Herold and Weibel, 1962; Purcifull, 1972). It is stylet-borne by aphids, mechanically transmissible and belongs to the potyvirus group (de Bokx, 1965; Harrison *et al.*, 1971; Brandes and Berks, 1965). The virus is serologically related to watermelon mosaic virus (Webb and Scott, 1965; Milne and Grogan, 1969; Purcifull and Hiebart, 1979; Gonsalves and Ishii, 1980).

A number of techniques to prevent the spread of plant viral diseases are available such as the use of tolerant or resistant varieties, vector control, use of virus – free planting materials, sanitation and cultural control (Bar-Joseph and Garnsey, 1981). There are no resistant papaya cultivars and attempts to develop effective control measures for PRSV were unsuccessful. Although roguing offers a feasible solution to the prevention of the disease, the method is not a permanent solution for other areas that do not have a geographical isolation and where the disease has become endemic (Yeh and Gonsalves, 1984).

In the Philippines, the diagnosis of plant viral diseases is done via conventional methods which are less rapid and less sensitive. Studies on the production of specific antisera and monoclonal antibodies against plant diseases are very limited. Hybridoma technology involves the use of monoclonal antibodies to sensitively detect the proteins of plant viral genes. This technology has recently been applied to plant virus research in several laboratories (Briand *et al.*, 1982; Diaco *et al.*, 1983; Halk *et al.*, 1982; Hsu *et al.*, 1983; Tremaine and Ronald, 1983). The use of of hybridomas offers many important benefits such as production of a highly purified antibody and the certainty that each hybridoma line is a clone producing antibodies to a single antigenic site. Monoclonal antibodies have also several advantages over polyclonal antibodies which include the availability of an unlimited supply of antibody, uniform antibody preparation and production of antibodies of predetermined specificity.

The main objective of this work is to produce antiserum and monoclonal antibodies against PRSV which can be used in the effective and rapid diagnosis of PRSV-infected papaya plants all over the Philippines.

Materials and Methods

Isolation and purification of the antigen (PRSV)

Papaya ring spot virus was isolated from infected papaya leaves collected from the nurseries of the Dept. of Horticulture and Institute of Plant Breeding, UPLB and from surrounding areas in Laguna where papayas were planted. The

purification procedure described by Gonsalves and Ishii (1980) was used in the isolation of PRSV. Minor modifications were made in the purification of PRSV. They included the following: (1) In the pelleting of the virus particles after PEG precipitation, centrifugation was done at 14,500 g instead of 10,000 g resulting in increased recovery of the virus precipitated; (2) In the final purification of PRSV using density gradient centrifugation, a 10-40% sucrose gradient was substituted for 10-40% cesium sulfate gradient due to the high cost and unavailability of cesium sulfate. Continuous isolation and purification of PRSV are done to have enough supply of antigen for immunization and for ELISA of antiserum and initial hybridomas.

Isolation and purification of host proteins from healthy papaya leaves were also done. Healthy papaya leaves were subjected through the same purification scheme as the infected papaya leaves except the sucrose density gradient centrifugation to obtain host proteins for cross absorption with antibodies. The concentration of the PRSV preparation was determined by assuming $A_{260} = 2.40 = 1$ mg/ml. The average value was obtained from the different absorbance values at 260 nm for reference viruses of the potato virus Y group to which PRSV belongs (Table 1).

Table 1. Viruses of the potato virus Y group with $A_{260} = 1$ mg/ml as reference values for determining PRSV concentration

Virus	*A_{260nm}*	*Reference*
Carnation vein mottle	2.10	Hollings and Stone, 1971
Dasheen mosaic	2.38	Zetter *et al.*, 1978
Pea seed borne mosaic	2.50	Hampton and Mink, 1975
Peanut mottle	2.60	Bock and Kuhn, 1975
Average	2.40	

Electron microscopy

Purified fractions from healthy and infected papaya leaves were negatively stained with 2% phosphotungstic acid (PTA), pH 7.2 and examined under the transmission electron microscope.

Production of antiserum and initial hybridomas

Culture of myeloma cells for cell fusion. The non-secreting mouse myeloma cell line used was P3-x63 – Ag8-U1. The myeloma cells were cultured in RPMI 1640 + HT + 12% FCS (Gibco) in an atmosphere of 5% Co_2 at 37°C.

Immunization of test animals

Each mouse (BALB/c AnNCrj, male, 4-11 weeks old) was injected intraperitoneally with an emulsion consisting of 0.1 ml purified antigen (75-100 ug) and 0.1 ml Freund's complete adjuvant. After four and three weeks interval, a second and third injections respectively of 0.1 ml antigen was given to each mouse. The mice were killed 3 days after the last booster injection.

Cell fusion

The immunized mouse was killed by neck bone dislocation. The blood was pumped from the heart and kept at 4°C overnight as a source of antiserum. The immunized spleen was removed under a sterile hood by spraying the mouse with 70% ETOH. Other tissues connected with the immunized spleen were removed to prevent contamination. The spleen was placed in about 40 to 50 ml DMEM and macerated until almost all spleen cells were in suspension. The methods described by Kohler and Milstein (1975), Miura (1980) and Ito *et al,* (1985) were followed for the fusion experiments.

After two or three weeks, hybrids growing in 96-well plates were screened for antibody activity using the enzyme linked immunosorbent assay (ELISA) double antibody sandwich technique. The procedure was adapted from the method described in Hybridoma Techniques (EMBO, SKMB Course 1980, Basel). Positive hybridomas were transferred in 2-cm dishes, cultured in RPMI 1640 + HT + 12% FCS, incubated at 37°C in a 5% CO_2 incubator, allowed to multiply and reassayed by the ELISA technique. Positive hybridomas were then kept in the above culture medium containing 10% DMSO and frozen in liquid nitrogen for cloning experiments using the limiting dilution technique.

For the assay of hybridomas by the ELISA technique, the antiserum against PRSV was used as the positive control whereas PBS buffer, mouse normal serum and purified extracts of healthy papaya leaves were used as negative control.

Results and Discussion

Isolation and purification of PRSV from infected papaya leaves

Infected papaya leaves (Fig. 1) were used to isolate PRSV. Fig. 2 shows the scanning patterns of the purified preparation of infected papaya leaves after density gradient centrifugation in 10-40% sucrose. A ten to 40% sucrose gradient with 0.1 M phosphate buffer containing 0.01 M EDTA, pH 7.0 was used as blank.

The purified preparation of PRSV gave a single peak which was located near the meniscus indicating its slow sedimenting property. The homogeneous preparation of PRSV showed the presence of threadlike, filamentous particles measuring about 750 nm in length and 12 nm in diameter (Fig. 3) when examined under the transmission electron microscope. The result conformed with the PRSV prepa-

ration obtained by Herold and Weibel (1962) and Purciful (1972). No virus particles were seen under the transmission electron microscope when the purified preparation of healthy papaya leaves was examined.

The average yield of 443 ug was obtained per 100 g samples. The concentration of the virus preparation was 419 ug/ml by assuming A_{260} = 2.40 = 1 mg/ml as indicated in the materials and methods. The $A_{260/280}$ value of 1.19 for PRSV was obtained and was comparable with viruses of the potato virus Y group to which PRSV belongs (Table 2).

Table 2. Comparison of the $A_{260/280}$ values of the viruses belonging to potato virus Y group* and PRSV

Virus	*$A_{260/280}$*
1. Bearded iris mosaic	1.12
2. Carnation vein mottle	1.15
3. Carrot thin leaf	1.18
4. Clover yellow vein	1.29
5. Dasheen mosaic	1.09-1.19
6. Henbane mosaic	1.10
7. Hippeastrum mosaic	1.21
8. Iris mild mosaic	1.25
9. Pea seed-borne mosaic	1.14-1.18
10. Peanut mottle	1.24
11. Pepper veinal mottle	1.25
12. Papaya ringspot virus	1.19

*Values were obtained from the C.M.I./A.A.B. Descriptions of Plant Viruses.

Fig. 1. Symptoms of papaya ringspot virus on papaya leaves collected fron the field and used as source of inoculum.

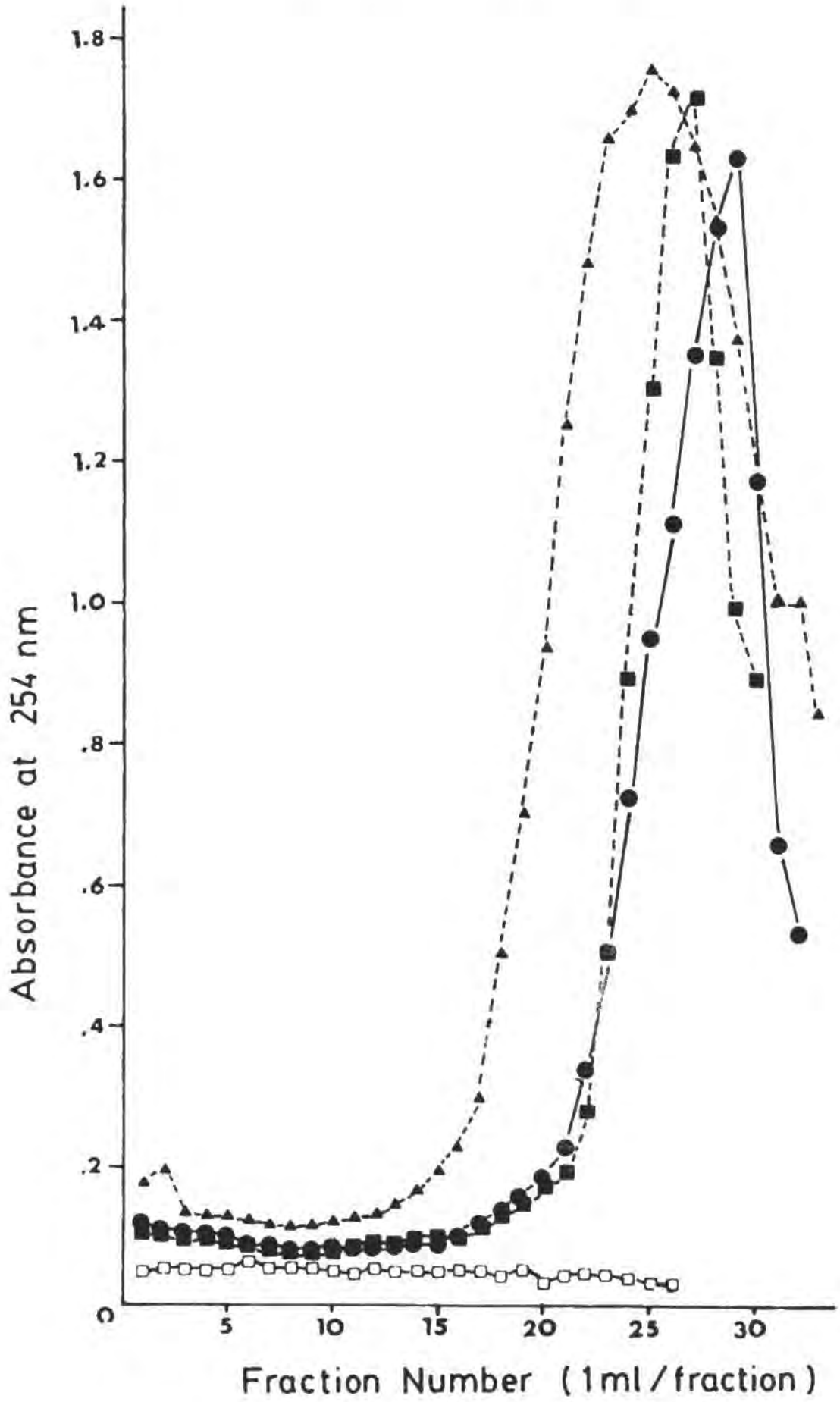

Fig. 2. Scanning patterns of a PRSV preparation after centrifugation in 10-40 percent sucrose gradient, △, □, ○, PRSV purified preparation; □, phosphate buffer (0.1 M containing 0.01 M EDTA, pH 7.0).

Fig. 3. Electron micrograph of papaya ringspot virus particles purified by sucrose density gradient centrifugation (72,000x).

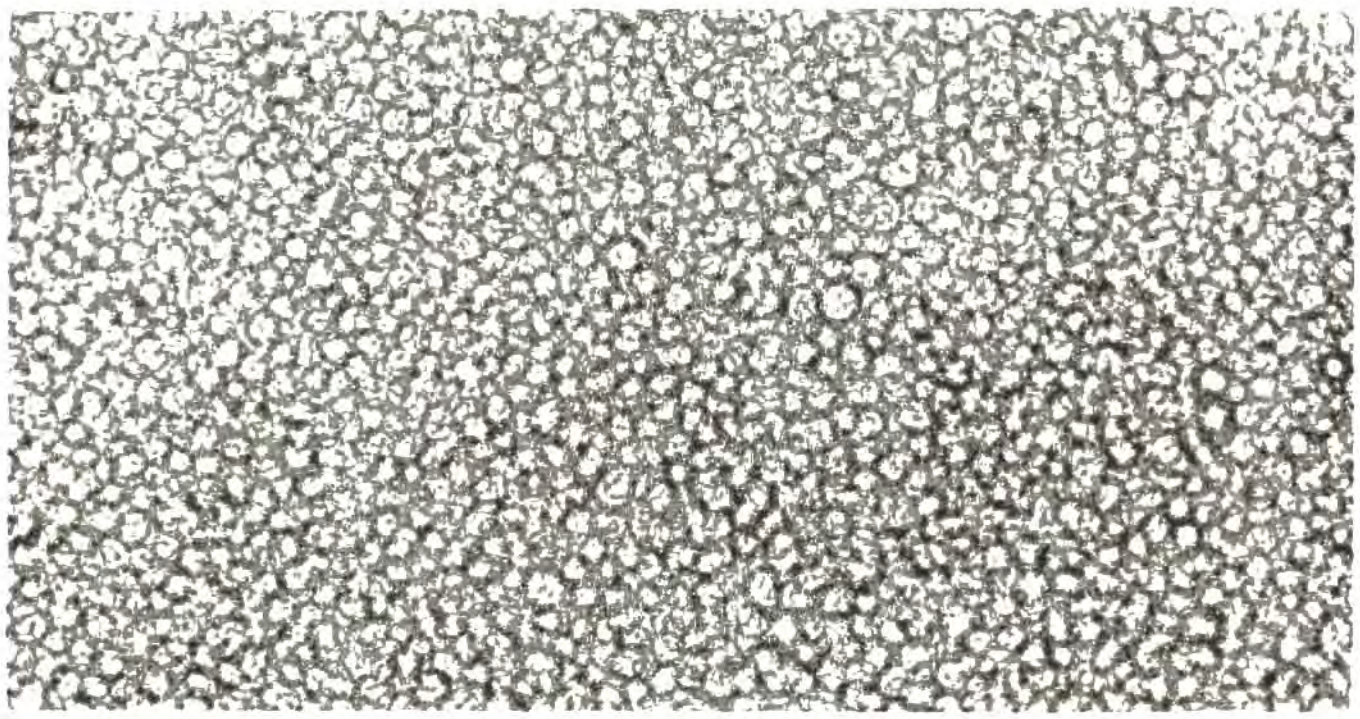

Fig. 4. Myeloma and spleen cells after fusion (200 x).

Production of antiserum and hybridomas against PRSV

Ninety six well plates containing fused cells were examined under the microscope (Fig. 4). The myeloma cells are bigger than the spleen cells as shown in Figs. 5 and 6. On the 3rd to 5th day, both myeloma and spleen cells were dying out while the hybridomas started to grow. A colony of PRSV hybridomas is presented in Fig. 7. After 12 days, the 3-96 well plates were assayed using the ELISA double antibody sandwich technique. The appearance of yellow color due to p-nitrophenol

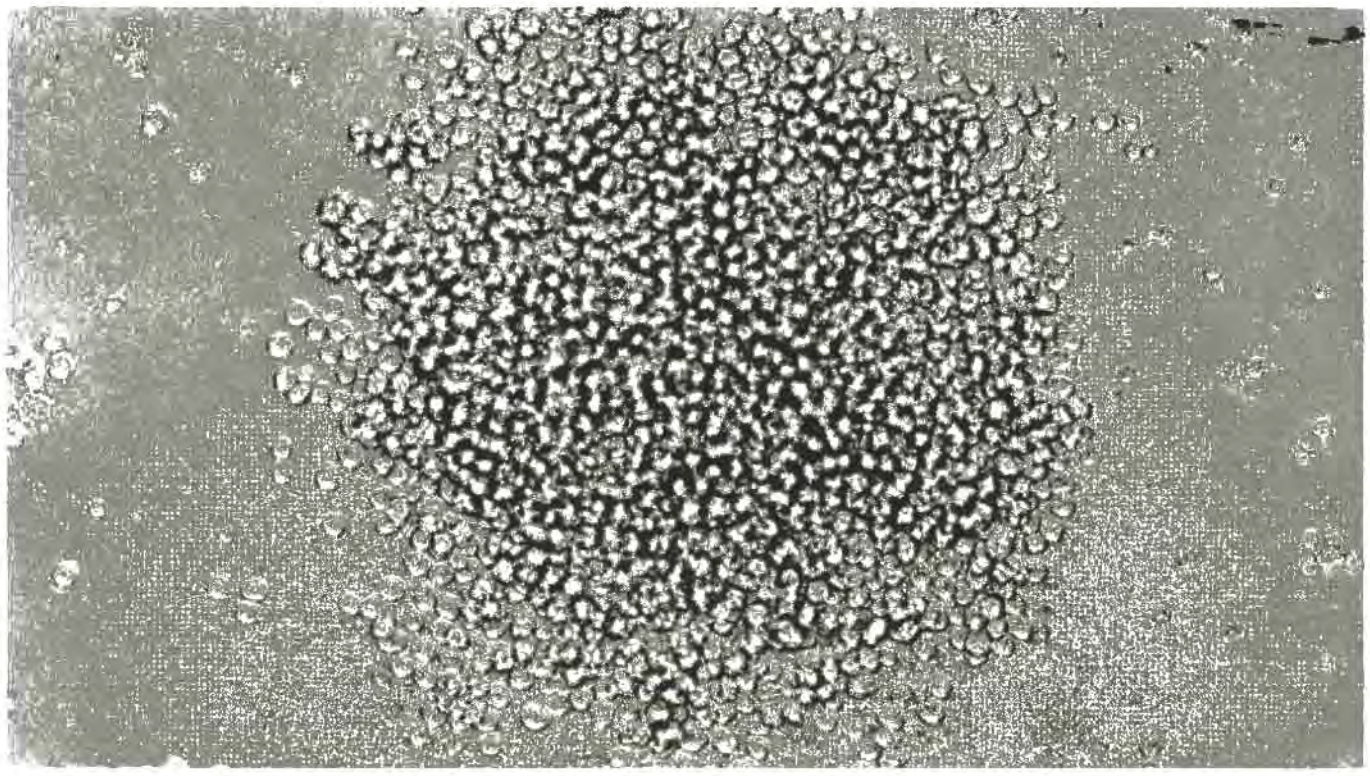

Fig. 5. Photomicrograph of myeloma cells cultured in RPMI 1640 + HT + 12% FCS + 8 – azaguanine (200 x).

Fig. 6. Spleen cells from immunized mice seen under the phase contrast microscope (200 x).

formation indicated positive reaction. Results of the assay are shown in Figs. 8 and 9. Thirteen positive hybridomas were obtained in the first screening with 2 hybridomas showing high antibody activities as indicated by the dark-yellow color. A second and third assays of the positive hybridomas reduced the number to 8 and 2, respectively. Only those hybridomas that gave consistent positive reaction and fast grower were multiplied and stored in liquid nitrogen for the cloning experiments. Table 3 summarizes the results obtained for the 2 fusion experiments. Four positive

hybridomas (Table 4) were obtained from the 2 fusion experiments with their corresponding absorbance readings at 410 nm. The negative controls were PBS buffer, healthy papaya sap and mouse normal serum while the positive control was PRSV antiserum.

Fig. 7. PRSV hybridomas in a 96-well plate seen under the phase contrast microscope (200 x).

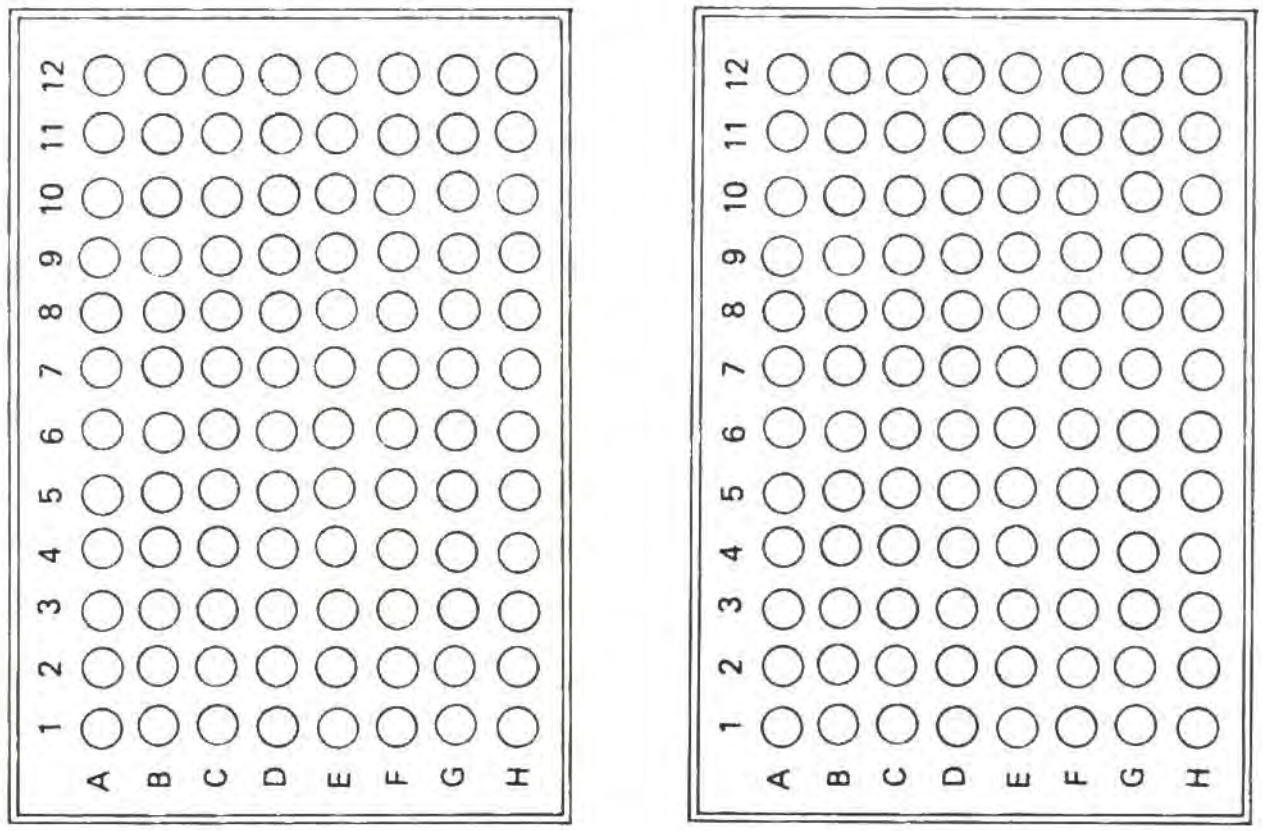

Fig. 8. Initial screening of nos. 1 and 2 PRSV – 96 well plates for positive hybridomas using the double antibody sandwich ELISA technique.

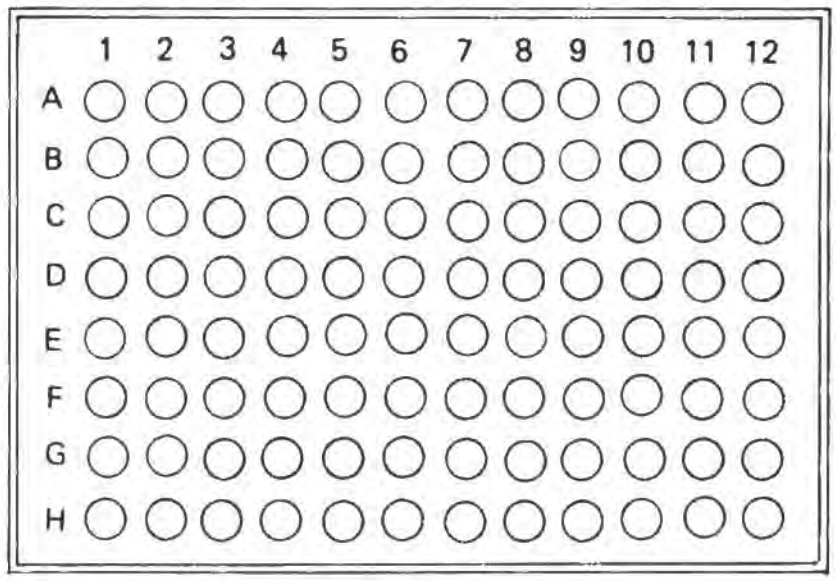

Fig. 9. Initial screening of no. 3 PRSV – 96 well plates for positive hybridomas using the double antibody sandwich ELISA technique.

Table 3. Production of stable PRSV hybridomas after 2 cell fusion experiments

Fusion No.	*Number of wells tested*	*Number of well containing antibody producing hybridomas*			*Total number of hybridomas obtained for cloning experiments*
		1st screening	*2nd screening*	*3rd screening*	
1	285	13	8	2	2
2	380	17	6	2	2

Table 4. Absorbance readings at A_{410} nm of PRSV positive hybridomas with PBS buffer, healthy papaya sap, mouse normal serum and antiserum as controls

	$A_{410\ nm}$
Negative control	
1. PBS buffer	0.057
2. Healthy papaya sap	0.049
3. Normal serum	0.065
Positive control	
1. Antiserum	0.991
Hybridoma samples	
1. $PRSV_J - 3$	0.249
2. $PRSV_J - 5$	0.606
3. $PRSV_B - B$	0.447
4. $PRSV_B - C$	0.291

Table 5. Rapid screening of the crude saps of infected papaya plants collected from different areas with PRSV antiserum using the ELISA double antibody sandwich technique

Sample source	A_{410nm}
Tanauan, Batangas	0.374
San Pablo City, Laguna	0.297
Tagaytay City, Cavite	0.335
Healthy papaya sap	0.100
PBS buffer	0.097

The antiserum against PRSV was then used for rapid screening of infected papaya plants in Cavite, Batangas and Laguna. Results in Table 5 indicate that the papaya plants from the 3 provinces were positive for PRSV as shown by their high absorbance readings which were 3x higher than the PBS buffer and healthy papaya sap (negative controls).

With the development of the rapid screening technique using PRSV antiserum and eventually monoclonal antibodies from PRSV hybridomas for detection of virus infection of papaya plants, we can now look forward to screening for tolerant papaya varieties under Philippine condition. Furthermore, the use of tolerant papaya varieties may lead to the control of the disease.

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