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Comparative Infectivity of Papaya Ringspot Virus (PRSV) Subjected to Mutagens and Heat Attenuation in *Carica papaya* L. (Solo Variety)

FRANCO G. TEVES

Abstract

Putative mild strains of the papaya ringspot virus (PRSV) were generated using nitrous acid treatment and heat attenuation based on cross-protection experiments on papaya seedlings. Ultraviolet light irradiation of the severe strain of the virus did not reduce its virulence as shown by the apparent absence of cross-protective capability. The presence of PRSV in papaya seedlings showing either no or mild symptoms after the cross-protection experiment was determined serologically using enzyme-linked immunosorbent assay (ELISA). This result is significant in that these locally-generated putative mild PRSV strains showed cross-protective properties on experimental papaya seedlings. The application of cross-protection technology along with the cultivation of tolerant or resistant papaya varieties would be the only means by which plantations in Mindanao can withstand the devastating effects of this virus already felt in the Visayas after successfully spreading from Luzon.

Key Words: papaya ringspot virus, *Carica papaya* L., cross-protection, ELISA

Intruduction

Papaya ringspot virus (PRSV or PRV) is a plant potyvirus that causes one of the most destructive diseases of papaya (*Carica papaya* L.), a fruit tree that abounds in tropical and sub-tropical regions (Conover, 1964). This virus has been reported to be a limiting factor for papaya production in such areas causing severe crop losses (Yeh, 1991). Members of three dicotyledonous families, namely, Caricaceae, Chenopodiaceae, and Cucurbitaceae, have been reported to be hosts of PRSV. The earliest literature reporting the destructive effect of PRSV on papaya was in Hawaii in 1945 by Lindner.

Papaya infected by PRSV exhibits mottling and distortion of leaves, ringspots on

FRANCO G. TEVES, Associate Professor, Department of Biological Sciences College of Science and Mathematics, MSU-Iligan Institute of Technology, Iligan City

fruits, and water-soaked streaks on stems and petioles (Figures 1 and 2). The plant becomes stunted drastically reducing fruit production (Purcifull *et al.*, 1984). Efficient transmission of this virus is through aphids in a non-persistent manner and spreads rapidly once it occurs in a papaya orchard. The virus can completely destroy a papaya orchard within a month if no control measures are done (Yeh, 1991). Control of the disease in the field is very difficult because of the absence of genetic sources of resistance to PRSV and the efficient dissemination of the potyvirus by several aphid species in a non-persistent manner (Rezende and Pacheco, 1998). Most of the proposed methods for control rely on the application of insecticide or mineral oil and the adoption of cultural practices especially the use of reflective mulches that are not effective where disease incidence is high. In addition, only a limited number of transgenic papaya strains resistant to PRSV have been developed although delivery systems for the transgenes and propagation techniques of papaya by tissue culture have already been perfected by several workers as reviewed by Yeh (1991). Because of the staggering cost and technical difficulty in producing PRSV-resistant papaya through such methods, the only valuable alternative at present would be cross-protection with mild strains of PRSV until resistant cultivars become available (Rezende and Pacheco, 1998).

Cross-protection is a phenomenon in which plants systematically infected with one viral strain prevents the expression of a second related strain of the same virus (Dodds, 1982). Aside from the classical works on tobacco mosaic virus (TMV), successful cross-protection experiments have been reported for the tomato strain of TMV since 1970 in the Netherlands and the United Kingdom (Fletcher and Rowe, 1975; Rast, 1975; Fletcher, 1978). Oshima (1975) used an attenuated mutant of TMV for protection against tomato mosaic virus. The same technique was used for the citrus tristeza virus (Price, 1970; Müller and Costa, 1977; Costa and Müller, 1980), for PRSV in papaya (Wang *et al.*, 1987), and PRSV in zucchini (Rezende and Pacheco, 1998). Multiple inoculation with three attenuated viruses for the control of cucumber virus disease has also been reported recently in Japan (Kosaka and Fukunishi, 1997).

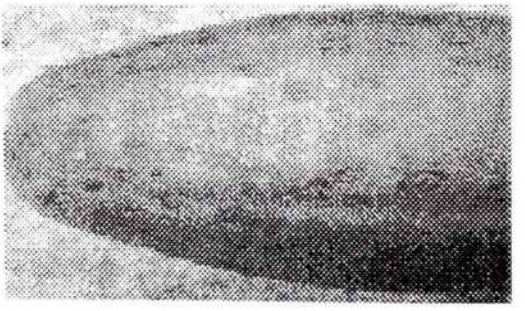

Figure 1. *Papaya fruit showing ringspots characteristic of PRSV infection (Queensland Government Department of Primary Industries, Australia).*

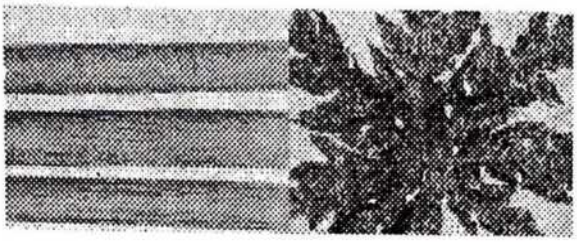

Figure 2. *Water-soaked streaks on petioles and mottling and distortion of leaves of papaya which are clear symptoms of PRSV infection (Queensland Government Department of Primary Industries, Australia).*

The primary strategy for solving this problem is to be able to obtain a source of mild strains of PRSV that could effectively cross-protect papaya against the severe strain. An attempt was made in Taiwan to isolate naturál mild strains of PRSV in which one has been reported (Lin *et al.*, 1989) but is not very stable because of its apparent high rate of mutation. Induced mutations particularly with the use of nitrous acid have been found to produce good results for plant RNA viruses (Gierer and Mundry, 1958; Mundry, 1959; Siegel, 1965). Its application for producing mild strains of PRSV for cross-protection purposes has been widespread (Yeh *et al.*, 1984; Yeh, 1991; Kosaka and Fukunishi, 1997) but quite limited in the Philippines.

Heat attenuation as a means of producing less severe infections has been known for years. The available data on thermal inactivation points (TIP) for several PRSV strains (50°C-55°C; 55°C-60°C) as reported by Lin *et al.* (1989) may be tried for cross-protection although no published data have been reported as yet.

Ultra-violet (UV) mutagenesis has been a traditional tool for producing mutant strains of various organisms (Gerhardt, 1995). However, like TIP, no literature could be found to date in the application of UV radiation for the production of mild strains of PRSV.

Materials and Methods

Growing of Host Papaya Seedlings

Fifty papaya seeds (solo variety) were obtained from the stock seed collection of the Virology Laboratory, Department of Plant Pathology, University of the Philippines Los Baños. The seeds were first grown in seedling boxes in the greenhouse of the Department of Plant Pathology and then individually transferred to separate pots until they reached the two- to three-leaf stage.

Mutagenesis

The method by Yeh and Gonzalves (1984) was followed for the extraction of sap from PRSV-infected papaya showing the typical symptoms, for mutagenesis. Approximately 200

grams of freshly obtained papaya leaves were ground using mortal and pestle with the aid of liquid nitrogen and distilled water (1 ml/g). The ground leaf material was then strained through cheesecloth, the crude sap collected and centrifuged in a Sorvall SS34 rotor at 8,000 rpm for 10 minutes. The supernatant was then collected and divided into three aliquots.

Nitrous Acid Treatment of Papaya Leaves

Approximately 50 ml aliquot of the supernatant collected above was mixed with 0.1 M sodium acetate and 0.4 M sodium nitrate (effective pH of 6.0) and incubated at 20°C for 30 minutes. The reaction was stopped by adding an equal volume of 0.1 M potassium phosphate buffer (ph of 7.0). Portions of Mt. Pinatubo ash was added to the mixture as an abrasive agent and immediately inoculated to leaves of five papaya seedlings using glass rods. Rinsing of inoculated leaves was done by spraying distilled water in wash bottle.

UV Treatment

The method by Gerhardt (1995) was used for UV mutagenesis. Five milliliter aliquots from the supernatant were placed in separate sterile Petri plates. The plates were exposed to UV irradiation (λ = 235 nm) for one, two, five, ten, and thirty minutes, respectively. The irradiated samples were used as inocula which were then applied to leaves of papaya seedlings using Mt. Pinatubo ash as abrasive agent. Five replicates were set up per treatment. Rinsing was done as previously described.

Heat Attenuation

Based on TIP data for three PRSV strains in Taiwan (Lin *et al.*, 1989), a 50-ml aliquot from the supernatant was placed in water bath set at 55°C for 10 minutes. This was then inoculated to five papaya seedlings following the inoculation method as described previously.

Buffer-treated and untreated plants were also grown using treated seedlings as controls. The seedlings were then allowed to continue growing until new leaves developed approximately two to three weeks later. New leaves were examined for characteristic symptoms compared to controls and the observations were then recorded.

Enzyme-linked Immunosorbent Assay (ELISA)

A modified ELISA for PRSV (Veracruz *et al.*, 1998) was performed on duplicate immulon 11 polystyrene micro-ELISA plates using sap from leaves of treated papaya seedlings. Positive and negative controls were also included. Result was assessed by visual estimation using color formation (pale yellow = weak reaction, dark yellow = strong reaction) and verified by using an ELISA microplate reader (absorbance = 405 nm).

Membrane Immunobinding Assay (MIBA)

In order to confirm results from ELISA and for improved sensitivity, MIBA or

immunoblot assay was performed using the same materials as for ELISA following the protocol of *Veracruz et al.*, 1998).

The schematic flow of methods is shown in Figure 3 below.

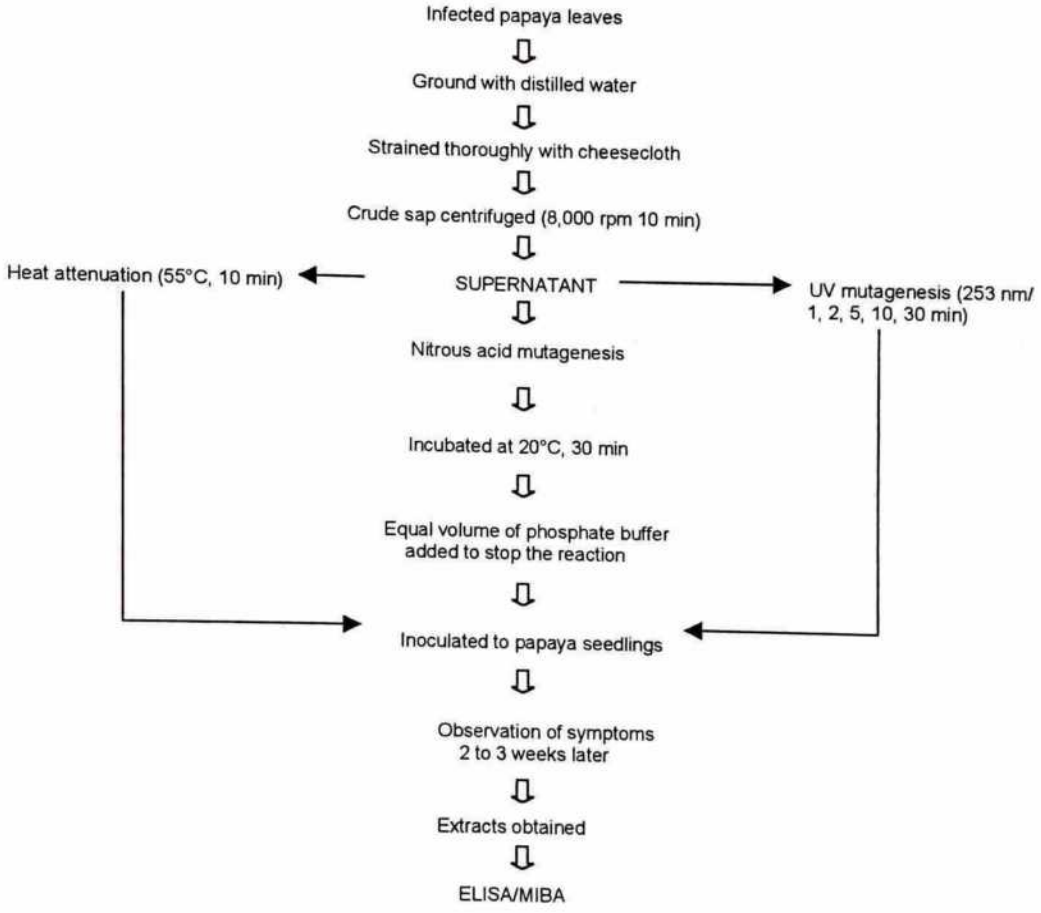

Figure 3. *Schematic flow of methods for the whole study.*

Results and Discussion

Table 1 shows the graded symptoms exhibited by papaya seedlings inoculated with extracts from PRSV-infected papaya with severe symptom which were UV-irradiated, nitrous acid-treated and heat-treated. Severe chlorosis was observed on leaves of UV-irradiated plants whereas mild chlorosis was seen on heat- and nitrous acid-treated plants. Mottling was also more severe on UV-irradiated plants than on heat- and nitrous acid-treated plants. Other types of leaf deformity aside from the typical curling and mottling was nil in all treatments.

A trend can be seen in that UV-irradiation did not show any attenuation of the severe symptoms. Papaya seedlings that were inoculated with UV-irradiated sap containing the severe strain of PRSV still exhibited the severe symptoms of PRSV. There was an apparent cross-protection effected by putative mild strains of PRSV generated by heat attenuation and nitrous acid treatment as shown in the table. Presence of PRSV in the treated papaya seedlings was confirmed by the results from serological tests (ELISA and MIBA) using plants with severe symptoms as positive control using cross-absorbed local, non-cross absorbed local and Taiwan (TSRI) polyclonal antisera (obtained either locally from the Virology Laboratory, Department of Plant Pathology, UPLB, or imported from Taiwan). Presence of PRSV was verified by a positive reaction between the positive control (severely-infected plant) and Taiwan antiserum on the microplates. Weak reactions with the extracts from heat-attenuated and nitrous acid-treated plants were observed. Nevertheless, the presence of PRSV in these plants cannot be ruled out because of the mild symptoms exhibited as shown in Table 1 and the positive serological tests. It is possible that these weak reactions were caused by changes in the antigenic properties of the PRSV resulting from induced mutations. Result of the ELISA is shown in Table 2 in absorbance units using a microplate reader.

The locally produced antisera gave negative reactions with sap from heat attenuated and nitrous acid-treated plants even though mild symptoms were exhibited by these plants. This could be due to loss of activity of these antisera caused by prolonged storage. Positive MIBA reactions were also observed for both the heat attenuated and nitrous acid-treated plants using the Taiwan antiserum indicating the presence of the virus in these plants that exhibited mild symptoms of the disease. These observations significantly point to only one thing, that is, heat-attenuated and nitrous acid-treated PRSV have successfully conferred cross-protection on papaya seedlings which were infected with the wild type severe strain of the virus.

It is interesting to note that no single replicate from any treatment developed the typical water-soaked streaks on the petioles and except for two (replicate 5/treatment A and replicate 4/treatment C), the same could also be said for leaf deformity or stunting of growth even if all positive control replicates did develop stunted growth and leaf malformation. The variations in severity of symptoms particularly shown by UV irradiation could imply the generation of mutant viruses whose mutations also produced modifications in their virulence on papaya plants.

Table 1. *Cross-protective effect of putative PRSV mutants (M)/attenuated strains (A) on papaya seedlings after inoculation with PRSV severe strain (S).*

TREATMENT	REPLICATE	WATER-SOAKING	CHLOROSIS	MOTTLING	STUNTED GROWTH/ LEAF DEFORMITIES
Negative Control (No M/A, No S)	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	-	-
Positive Control (No M/A, Infected with S)	1	-	+	+	+
	2	-	+	+	+
	3	-	+	+	+
	4	-	+	+	+
	5	-	+	+	+
A (No M/A+Buffer)	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	-	+
B (M/UV-1 min + S)	1	+	-	+	-
	2	-	-	++	-
	3	++	-	+++	-
	4	++	-	-	-
	5	+	-	+++	-
C (M/UV-2 min + S)	1	++	-	+	-
	2	+	-	-	-
	3	++	-	+	-
	4	++	-	++	+
	5	+	-	++	-
D (M/UV-5 min + S)	1	+	-	+	-
	2	++	-	++	-
	3	++	-	++	-
	4	-	-	++	-
	5	++	-	++	-
E (M/UV-10 min + S)	1	++	-	++	-
	2	-	-	+	-
	3	++	-	+	-
	4	++	-	++	-
	5	++	-	++	-
F (M/UV-30 min + S)	1	+	-	+++	-
	2	++	-	++	-
	3	+	-	+++	-
	4	-	-	+	-
	5	-	-	+	-
G (Heat attenuation, 55°C, 10 min)	1	+	-	+	-
	2	-	-	+	-
	3	-	-	+	-
	4	+	-	+	-
	5	+	-	+	-
H (Nitrous acid)	1	+	-	+	-

Note: - Asymptomatic
+ Mild symptom
++ Moderate symptom
+++ Severe symptom
++++ Very severe symptom

Table 2. *Sample micro-ELISA reading using a microplate reader.*

ASSAY #: 1
PLATE #: 1
NOTES: PRSV/ Franco G. Teves
REPORT: SINGLE WAVELENGTH: 405 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.002	0.001	-0.002	-0.007	-0.008	-0.010	-0.007	-0.011	-0.005	-0.123	-0.119	-0.119
B	-0.007	-0.012	-0.007	-0.012	-0.011	-0.011	-0.020	-0.020	-0.012	-0.122	-0.121	-0.122
C	-0.023	-0.013	-0.013	-0.018	-0.018	0.003	-0.017	-0.022	-0.011	-0.121	-0.123	-0.119
D	0.000	-0.006	-0.017	-0.021	-0.012	-0.016	-0.014	-0.021	-0.016	-0.122	-0.123	-0.122
E	0.000	-0.004	-0.012	-0.017	-0.018	-0.020	-0.023	-0.021	-0.013	-0.123	-0.121	-0.116
F	-0.004	-0.009	-0.009	0.020	0.011	0.048	0.161	0.185	0.233	-0.121	-0.121	-0.118
G	-0.017	-0.017	-0.023	-0.025	-0.013	-0.015	-0.031	-0.031	-0.027	-0.123	-0.123	-0.122
H	-0.009	-0.008	-0.016	-0.021	-0.010	-0.016	-0.023	-0.029	-0.022	-0.120	-0.122	-0.120

NOTE: F/4 and F/5 = Absorbance for heat-attenuated PRSV
F/6 and F/7 = Absorbance for nitrous acid-treated PRSV
F/8 and F/9 = Absorbance for positive control

Conclusion and Recommendations

Based on the results of this study, it can be concluded that mild strains of PRSV have been successfully generated through nitrous acid treatment and heat attenuation. Ultraviolet light treatment did not effectively produce mild strains of the virus as shown by the lack of cross-protection on papaya seedlings. Although putative mild strains generated through heat attenuation probably are difficult to maintain, the simplicity, cost-effectiveness and safety aspects of the procedure is worth considering. However, because of the predicted stability of the mutation(s) in the mild PRSV strains generated through nitrous acid treatment, the latter would still be the most practical for application on a commercial scale.

It is recommended that quantitative methods for measuring infectivity be used for nitrous acid- and heat-treated plants by inoculation on local lesion hosts such as *Chenopodium quinoa* L. Further, DNA sequencing and protein profile studies will have to be conducted to determine any correlation with the production of mild symptoms for nitrous acid treatment and heat atttenuation, and weak serological reactions. The result will be invaluable for proving that the weak serological reactions in asymptomatic or mildly affected papaya seedlings were due to altered antigenic properties of PRSV.

Multi-mild strain inoculation can also be conducted using two or more mild PRSV strains similar to studies on zucchini in Japan. In the absence of resistant papaya cultivars against PRSV, it is further recommended that an integrated approach be set forth for papaya production in the Philippines. These are (1) enhancement of the quality and productivity of PRSV-tolerant papaya cultivars; (2) use of pathogen-derived resistance for production of transgenic papaya cultivars; and (3) cross-protection of papaya using single or multiple mild

strains of PRSV.

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