Pak.J.Bot., 27(1): 233-241, 1995.

SOME BIOLOGICAL PHYSICAL AND SEROLOGICAL PROPERTIES OF POTATO LEAF ROLL VIRUS (PLRV) IN PAKISTAN

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Abstract

Mean incidence of potato leaf roll virus (PLRV) in the plains of Pakistan was recorded as 44% in general, 53% in the market or uncertified seed and 11% in certified or improved seed. The virus was successfully transmitted by side cleft grafting of infected potato to *Datura stramonium* and *Physalis floridana* which proved useful hosts for the maintenance and propagation of the virus. The virus was purified through extraction in phosphate or citrate buffer and precipitation with polyethylene glycol followed by 2-3 cycles of differential centrifugation. The average yield of virus was 0.265 mg per kg of source tissue. The virus sedimented as single component in sucrose density gradients, reacted positively against potato leaf roll virus (PLRV) antiserum in gel immunodiffusion tests and absorbed UV light with a 260/280 ratio of 1.7. Isometric particles of 25 nm in diameter were observed under electron microscope (EM). The virus identity was also confirmed by Enzyme Linked Immunosorbent Assay (ELISA) and serological specific electron microscopy (SSEM).

Introduction

Potato leaf roll virus (PLRV) is an economically important viral disease of potato throughout the world. It was first reported in Germany and Denmark and its viral nature was confirmed through vector transmission (Rich, 1983). Potato is the principal host of PLRV, but some other Solanaceous hosts are occasionally infected. The infected plants manifest upright growth, rolling and yellowing of leaves which under secondary infection become stiff, dry, leathery, crispy and papery when touched. The virus is responsible for degeneration of seed stocks and yield losses sustained are quite high.

PLRV is not mechanically transmissible. It is a persistent, circulative, phloem-limited and aphid transmitted virus. More than 10 aphid species are involved in its transmission. Green peach aphid (*Myzus persicae* Sulz) being the most efficient vector, whereas potato aphid (*Macrosiphum euphorbiae* Thos.) also plays an important role (McCarthy, 1954). Infected seed tuber, volunteer potato and randomly distributed infected plants in the field combined with favourable environment for the vector, provide potential source of infection and spread of PLRV (Wright & Bishop, 1981).

Low concentration of PLRV in the phloem tissue presents many problems in its isolation and very few workers have reported successful purification of the virus (Kojima & Murayama, 1972). Rowhani & Stace-Smith (1979) and Hepp & de Zoeten

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(1978) were able to purify small amount of virus and characterize it. Some other purification procedures for PLRV and leutoviruses have also been reported. Enzyme-linked immunosorbent assay (ELISA) is widely and effectively used for the detection of PLRV in potato tubers, indicator and propagative host and insect vector (Casper, 1977; Gugerli, 1980; Clark *et al.*, 1980). Serologically specific electron microscopy (SSEM) was used by Braun & Opgenorth (1987).

PLRV is one of the major potato diseases in Pakistan (Mughal *et al.*, 1988) where infected seed serve as source of infection, abundance of aphid population and favourable climatic conditions particularly in the spring and summer (Mirza *et al.*, 1982). The virus is associated with degeneration of seed stocks (Hussain *et al.*, 1978). However, the causal virus has not been characterized to any extent. The present study presents some of the biological, physical and serological properties of PLRV isolate commonly occurring in Pakistan.

Material and Methods

Virus source and culturing: Potato crop was surveyed at 12 locations in 6 fields at each and incidence of PLRV was recorded in 5x5 sq. meter area taken at random (Barnett, 1986). Diseased specimens were collected and indexed for other potato viruses i.e., potato virus X (PVX), potato virus S (PVS), potato virus M (PVM), potato virus A (PVA) and potato virus Y (PVY) through ELISA (Clark & Adams, 1977). Infectivity of naturally infected material was assayed on *Datura stramonium* L., and *Physalis floridana* Rydb. Plants and tubers specifically infected with PLRV were selected and planted in 23cm clay pots. Seeds of test plants were grown in sterilized mixture consisting of equal parts of soil, peat, farm yard manure and sand. Seedlings were transplanted in 13 cm diam., plastic pots at 2-3 leaf stage. All plants were raised and maintained in an insect-free glass house maintained at 18-23°C.

Virus transmission: For mechanical inoculation, infected material was ground in 0.02 M phosphate buffer, pH 7.2 (1 g: 2 ml) in a pestle and mortar and sap was rub-inoculated on carborundum dusted leaves of *D. stramonium* L., *P. floridana* Rydb., *Solanum nigrum* L., *S. tuberosum* L., *Lycopersicon esculentum* Mill, *Nicotiana tabacum* L., *N. rustica* L., *Chenopodium amaranticolor* Coste & Reynier and *C. quinoa* Willd. Inoculated plants were kept under observation for one month. For cleft graft transmission (Hill, 1984), shoots from PLRV-infected tubers or plants were removed as scion and grafted on potato, tomato, tobacco, *S. nigrum, D. stramonium* and *P. floridiana*. The cuts were covered with parafilm. Grafted plants were placed in a humid chamber at 90% R.H and 27-30°C for 4 days, checked by ELISA, maintained and used as virus source. The infected material was further multiplied by planting cuttings of *P. floridana* in the soil mixture applied with Hoagland solution and transferring them in pots.

Virus Purification: Some known methods were attempted but with little success. Conditions for the purification of PLRV were therefore, standardized through stepwise series of experiments on extraction and suspension huffers, emulsifying agents, antioxidants and differential cycles of centrifugation. The following method was routinely applied:

Infected leaves of *P. floridana* were harvested 4-5 weeks after grafting, cooled to 4°C and homogenized in 0.5 M potassium phosphate buffer, pH 7.4 containing 0.1% Mercaptoethanol (MCE) and 0.1 % sodium diethyldithiocarbamate (Na-DIECA) or 0.01 M EDTA and 1% Triton X-100 (1 g/ml). The homogenate was emulsified with 10-15% (v/v) chloroform: butanol (1:1) and sieved through two layers of muslin cloth. The emulsion was centrifuged at 75000 rpm for 15 min, Polyethylene glycol (PEG 6000) @ 8% and sodium chloride @ 1% were added to the supernatant and stirred for 1 h. Virus precipitate was collected by centrifugation at 8000 rpm for 15 min., and dispersed in 0.5 M phosphate buffer containing 0.75 M urea, or in citrate buffer (0.1 M, 0.01 M EDTA) heated to 45°C and kept over night. After clarification at 8000 rpm for 10 min., the supernatant was subjected to high speed centrifugation at 28000 rpm for 2 h, and the pellets suspended in 0.05 M phosphate or citrate buffer containing 0.01 M EDTA. After clarification, a second cycle of differential centrifugation was repeated. Two ml of partially purified virus was layered on 10-40% sucrose density gradients and centrifuged at 23000 rpm for 2 h. The virus band was collected and dialyzed against respective buffers of low molarity over night and concentrated by ultracentrifugation. All purification was done in Beckman centrifuges (J-2-21 and L-8-80) using standard rotors. UV spectrum and virus concentration were measured in a Beckman Spectrophotometer Model 34 using quartz cuvette of 1 cm path length.

Electron Microscopy: Carbon-coated copper grids of 200-mesh were floated on drops of samples for 30-60 seconds. The grids were negatively stained with 2% potassium phosphotungstic acid (PTA), pH 6.8 for one minute (Hitchborn & Hills, 1965). The excess fluid was drained off with a piece of filter paper and the grids were examined in JOEL 100 CX-II electron microscope. A total of 300 particles were measured from the electron micrographs and mean diameter of PLRV particles were calculated.

Serological Methods: The virus isolate was specifically identified and confirmed through serological techniques such as gel immunodiffusion (Ouchterlony, 1962), ELISA as described by (Clark & Adams, 1977) and serologically specific electron microscopy or immune electron microscopy (Derrick, 1873).

Results

Distribution on PLRV: The incidence of PLRV in the potato fields surveyed ranged between 6-64% with an average of 44%. High disease incidence (53%) was encountered with the uncertified seed used by more than 50% of the farmers as against 11% with certified seed or seed produced by the farmers under phytosanitary conditions (Table 1). Although disease was randomly distributed in fields, its incidence was comparatively higher near the margins than in the centre of field. Typical symptoms associated with primary and secondary infection were noted, the latter was more evident (Fig.1).

Virus Transmission: PLRV failed to transmit mechanically to any of the indicator host species tested. However, side cleft grafting was highly successful in transmitting the virus to *D. stramonium* and *P. floridana* (Table 2). Transmission was efficient when stocks and scions united perfectly and the best cambial connection usually took 3-5 days. A temperature of 27°C and 90% RH were very conductive for graft transmission

Table 1. Distribution of potato leaf roll virus in different seed source.

Seed source	Seed condition	Average of 72 observations % Frequency of farmers	% PLRV	Symptoms
Market Seed	Uncertified unspecified	58	53	Complex and severe, primary and secondary infection present
Certified Seed	Second multiplication of imported seed	16	13	Primary and secondary infection
Imported Seed	First multiplication	4	6	Primary infection
Farmer's own Seed (rogued)	Third multiplication	10	15	Primary and secondary infection
High Hill Seed	First multiplication	12	7	Primary infection

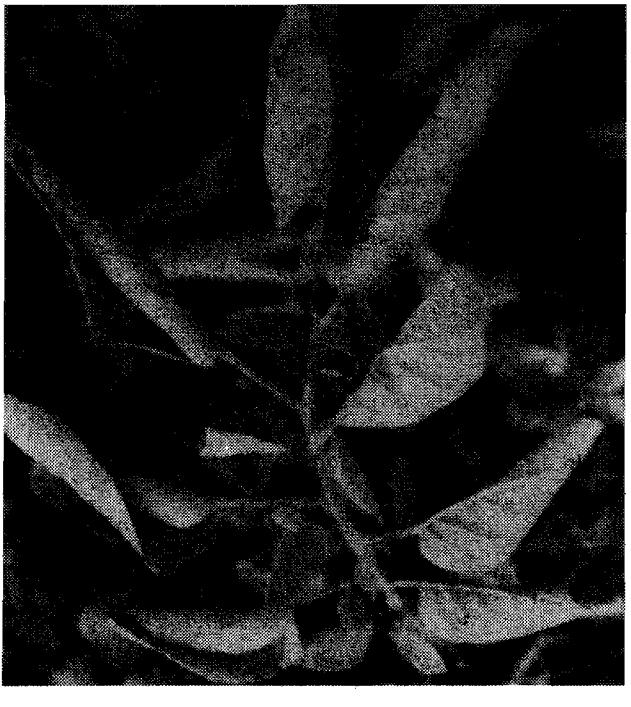

Fig. 1. A typical PLRV-infected potato leaf after secondary infection.

Table 2. Transmission of PLRV in different scions and stocks.

Scion Infected	Stocks Healthy	No. of plants Grafted	Successful	Infected	% PLRV	Symptoms
Potato	*D. stramonium*	10	7	3	42.8	VC.Ch.
	P. floridana	12	8	4	5.0	VC.Ch.St
	S. nigrum	5	3	0	0.0	---
	Potato (Cardinal)	8	7	2	28.5	Ch. St.
	Tomato	8	5	2	40.0	Ch. LR.
	Tobaccó	8	2	0	0.0	---
Potato	*D. stramonium*	7	5	3	60.0	Ch.VC.
	P. floridana	15	12	10	83.0	Ch.St.
	S. nigrum	4	3	0	0.0	---
	Potato	7	6	2	25.0	Ch.St.LR.
	Tomato	7	4	1	25.0	Ch.St.
	Tabacco	6	2	0	0.0	---
D. stram-	*D. stramonium*	10	8	4	50.0	Ch.St.VC.
onium	*P. floridana*	20	16	14	87.0	Ch.VC
	S. nigrum	4	2	0	0.0	---
	Potato	8	4	1	25.0	Ch.St.
	Tomato	7	4	1	25.0	Ch.
	Tobacco	6	2	0	0.0	---
P. florid-	*D. stramonium*	10	8	7	85.5	Ch.St
ana	*P. floridana*	80	74	70	94.5	Ch.St.LR.
	S. nigrum	5	3	0	0.0	---
	Potato	8	4	2	50.0	Ch.St.
	Tomato	7	3	1	33.3	Ch.St.
	Tobacco	8	3	0	0.0	---

*PLRV from potato fields.

**PLRV from green house grown potato plants.

Key: (No. symptoms). Ch = Chlorosis, St = Stunting, VC = Vein clearing, LR = Leaf rolling.

and disease development in the indicator and propagative hosts (Fig.2). Infected shoots of *D. stramonium* and *P. floridana* proved better scion than potato or any other tissues. ELISA tests indicated that the infected plants attained the highest concentration of PLRV 25-30 days after grafting, but the virus was detectable 6-10 days after graft inoculation.

D. stramonium developed interveinal chlorosis with young leaves slightly rolled and bent downward. Yellowing of leaves started from the petioles and spread along the leaf margins. Symptom developed rapidly in *P. floridana* when grafted from *D. stramonium* or *S. tuberosum*. Chlorotic patches started from the base of petioles and spread along the margins followed by interveinal yellowing. In general, *P. floridana* appeared to be the best host for the propagation and maintenance of PLRV. Cuttings

Fig.2. Symptoms of PLRV in *P. floridana* after grafting (right), with healthy control (left).

from infected *P. floridana* were planted in fine sand in small wooden trays which were covered by polythene sheet for rooting. These were then transferred to 10 cm diam., pots after 14 days and maintained at 20-25°C. Application of Hoagland solution stimulated regeneration of new shoots. This method provided a continuous and consistent supply of infected material for virus purification.

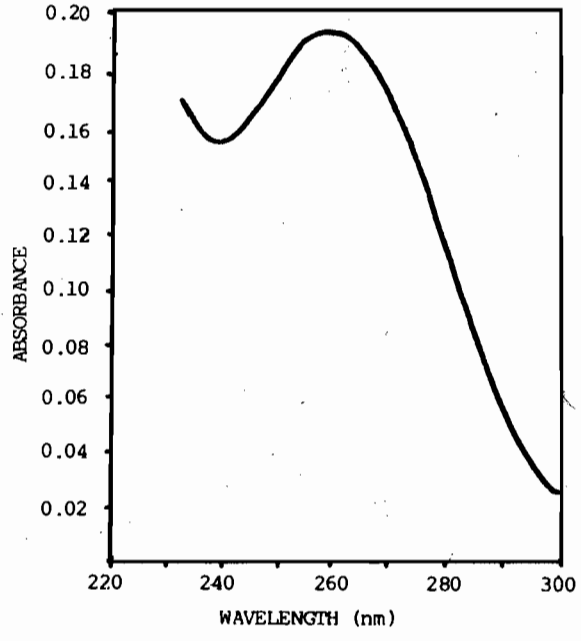

Fig.3. UV absorption spectrum of purified preparation of PLRV.

Virus Purification and Characterization: The method standardized for the purification of PLRV was routinely and consistently used. It was observed that extraction buffer of high molarity and suspension buffer of low molarity, and longer incubation of extracts and pellets at appropriate temperature gave better release of virus. A yield of 0.265 mg virus/Kg of infected tissue was obtained. The virus sedimented as a single component in the gradients with a sharp band. The UV absorption spectrum of purified virus preparation was typical of nucleoprotein, maximum at 260 nm with 260: 240 and 260: 280 ratio of 1.4 and 1.7, respectively (Fig.3). The negatively stained virus preparations contained isometric particle with average diameter of 25 nm (Fig.4).

Serology: Sap extracted from infected potato and other indicator hosts did not react with high titer PLRV antiserum (1:256) in the immunodiffusion tests. However, purified and concentrated virus preparations reacted faintly. The virus was easily detected by ELISA from the potato tubers and sprouts and indicator hosts. Heel ends of potato tubers contained more virus than rose end. SEEM was more sensitive than ELISA and this technique recognized and trapped large number of virus particles.

Discussion

Potato leaf roll virus remains the most acute and serious problem in potato production in Pakistan. Both the primary and secondary phases of PLRV are of common occurrence in potato fields. Secondary infection affect yield and serve potential source of contamination and infection. Market seed used by more than 50% of the farmers carried high incidence of PLRV although other seed sources including imported one were not entirely free from virus. Degeneration of seed stocks due to

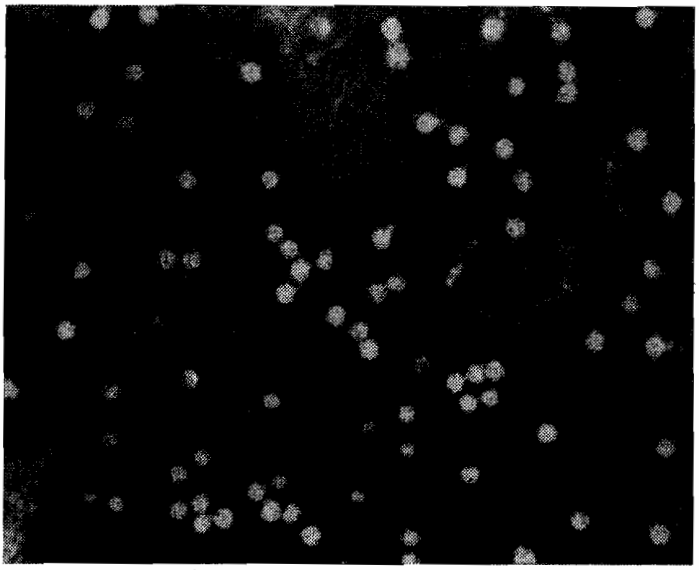

Fig.4. Electron micrograph of PLRV particles, stained with 2% phosphotungstic acid. X 96000.

PLRV has been widely recognized and studied (Hussain *et al.*, 1978; Mirza, 1978). Strenuous efforts are therefore, required to produce large quantities of virus-free certified seed in aphid free areas and season.

Non-availability of adequate quantity of PLRV infected material had been a problem in virus isolation. Previously, green peach aphids, *M. persicae* were used as virus transmission agent (MacCarthy, 1954) which was laborious because of rearing viruliferous aphid colonies, acquisition and inoculation feedings and danger of transmitting stylet-borne viruses as PVY and PVA by the aphid in shorter period than PLRV. This problem was overcome by propagating infected tissue through grafting on *P. floridana* and its further multiplication through vegetative cutting which provided a continuous supply of source tissue. This aspect represents a marked improvement on previously known methods of propagating the virus.

Physical and biochemical characterization of PLRV and its antiserum production require substantial quantities of purified virus which are generally difficult to obtain. Absence of reaction between infected sap with high-titer antiserum confirmed low virus concentration in the host. Similar difficulties were experienced by many workers including Kojia & Murayama (1972) and Hepp & de Zoeten (1978). Conditions for the purification of virus were standardized through step wise series of experiment and finally a routinely applicable method was developed. Virus preparations obtained were free from host impurities, showed UV spectrum characteristics of nucleoprotein and contained large number of isometric particles. However, yield was still low i.e., 0.265 mg/kg to produce antiserum.

PLRV was specifically identified serologically. Only concentrated virus reacted positively against PLRV antiserum in gel immunodiffusion test. The virus was quickly detected in the infected hosts, potato tubers and other preparations through ELISA and SSEM. The virus could not be detected by ELISA in freshly harvested tubers, but after one month storage at room temperature, encouraging results were obtained especially when heel ends of potato tubers were tested. Similar observations were made by Gugerli (1980). However, this aspect needs detailed investigation.

Acknowledgements

We are thankful to Dr. D.Z. Maat, Research Institute for Plant Protection, Wageningen for PLRV antiserum, to Dr. Alan Smith, Pakistan Swiss Potato Development Project, PARC, Islamabad for ELISA kits of potato viruses and to Mr. Asif Mahmood, National Agricultural Research Centre for his help in electron microscopy.

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(Received for Publication 24 March 1992)