CP 553

THE OCCURRENCE OF YELLOW SPOT VIRUS, A MEMBER OF TOMATO SPOTTED WILT VIRUS GROUP, ON PEANUT (Arachis hypogaea L.) IN INDIA*.

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ABSTRACT

A virus that induced yellow leaf spots which later coalesced and became necrotic, was isolated from peanut (Arachis hypogaea) in India and named peanut yellow spot virus (PYSV). PYSV was sap-transmissible to eleven species of Chenopodiaceae, Leguminosae, and Solanaceae. The virus induced local lesions in all the hosts. V. unguiculata was a good local lesion host. The infection in Pisum sativum, Vigna radiata and V. unguiculata became systemic when they were maintained at temperatures between 20 and 30°C. In thin sections of peanut leaves spherical membrane-bound particles of 70 to 100 nm in diameter were observed to occur in clusters. Purified virus preparations contained five polypeptides of 90000, 54000, 44000, 40000 and 31000 dalton polypeptides. When virus particles were treated with Nonidet P 40, the polypeptides were removed with the exception of that of 31000 daltons. In both ELISA and western blots, PYSV did not react with antisera to two tomato spotted wilt virus isolates. On the basis of these properties, PYSV is regarded as a previously undescribed virus belonging to the tomato spotted wilt virus group.

INTRODUCTION

Peanut (Arachis hypogaea L.) is affected by several virus diseases in India (Reddy, In press). One virus disease which is characterized by yellow leaf spots which later coalesce and become necrotic was reported in 1978 from several locations (ICRISAT, 1978). The causal virus was named "peanut yellow spot virus" (PYSV). In thin sections of peanut leaflets, membrane-bound virus particles of 70 to 100 nm, similar in morphology to tomato spotted wilt virus (TSWV), were observed. But in host range and symptoms, the virus differed from the TSWV reported from India by Ghanekar et al. (1979). In disease surveys PYSV was found to be widely distributed in India. A similar disease was also observed in peanut in Thailand (Wongkaew et al., 1985).

We report here host range, symptomatology, purification, serological relationships and protein analysis of the PYSV.

MATERIALS AND METHODS

Virus culture and maintenance

Peanut leaflets showing small chlorotic spots (initial symptoms) were collected from the field. Extracts prepared in

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0.05 M phosphate buffer, pH 7.0, containing 0.075% thioglycerol (PBT) were inoculated onto peanut plants (cv. TMV 2). The virus later isolated from these plants was maintained either in peanut or in Vigna radiata (cv. HY 45).

Assay host:

Since the virus consistently produced distinct chlorotic local lesions on Vigna unguiculata (cowpea, cv. C 152), this cultivar was chosen as a local lesion assay host.

Host range studies:

Extracts from 0.5 - 1 g of young peanut leaflets showing initial symptoms were inoculated onto six plants of each of 17 test species and these were maintained in a glasshouse for 45 days at 20-30°C. Extracts from inoculated and non-inoculated leaves of each test plants were checked for infection by sap inoculation onto V. unguiculata, and by protein - A coating (PAC) enzyme-linked immunosorbent assay (ELISA) (Hobbs et al. 1987) using a homologous antiserum.

Properties of the virus in sap:

The physical properties of the virus were determined in extracts from peanut leaflets prepared in PBT. For determining thermal inactivation point (TIP), one ml aliquots of sap diluted to 10^{-1} were heated at a range of temperatures from 40 to 75°C in a water bath for 10 minutes. Longevity in vitro (LIV) was determined using a 10^{-1} dilution incubated at 25°C for up to 2 days. The dilution end point (DEP) was determined by diluting the sap from 10^{-1} to 10^{-8}.

Electron microscopy:

Portions of peanut leaflets showing initial symptoms were prefixed overnight in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and then washed thrice in the buffer. The pieces were then post-fixed in 2% aqueous osmium tetroxide for 6 hours, followed by three washings in distilled water. Dehydration was done in a graded series of acetone, and the pieces were then embedded in Spurr's medium (Spurr, 1969). Ultrathin sections were cut and stained in uranyl acetate and lead citrate, then examined in an electron microscope (Philips 201 C).

Virus purification:

All purification steps were carried out at, or near to, 4°C. The procedure was similar to that developed for purification of TSWV from peanut (M.R. Sudarshana, I.K. Kumar, A.S. Ratna, R. Rajeshwari, and D.V.R. Reddy, in preparation). Peanut leaflets showing initial symptoms of light green or pale yellow spots (green areas of leaflets removed) were frozen at -80°C. Frozen leaflets were triturated in a blender in an extractant containing 0.02 M phosphate buffer, pH 7.5, containing 0.01 M diethyl- dithiocarbamate and 0.02% monothioglycerol; at a rate of 4 ml for each gram of tissue. The homogenate was filtered through two layers of cheese-cloth and subjected to 10000 rpm for 20 minutes. The resulting pellet was suspended (1 ml per gram tissue) in 0.01 M phosphate buffer, pH 7.5, containing 0.01 M sodium sulfite (PBSS), stirred for 1 hour at 4°C and clarified at 10000 rpm for 10 minutes. The supernatant was subjected to rate zonal density gradient centrifugation for 1 hour at 20000 rpm in a Beckman SW28 rotor in gradients made of 8.0 ml each of 20, 30, 40% sucrose, and 10 ml of 50% sucrose

(w/v) in PBSS. A light scattering zone at a height of 4.3-5.5 cm was collected, diluted in PBSS and centrifuged at 30000 rpm for 1.5 hours in a Sorvall T865 rotor. The resulting pellets were suspended in PBSS and centrifuged at 10000 rpm for 5 minutes. The supernatant was subjected to another cycle of rate zonal gradient centrifugation. The major light scattering zone (4.3-5.5 cm height) was collected, diluted in PBSS, and pelleted.

Preparation of nucleocapsids:

Purified virus from 100 gram tissue was suspended in 10 ml of PBSS and Nonidet P-40 was added to give a final concentration of 1%. After stirring for 15 minutes it was subjected to rate zonal centrifugation in a Beckman SW 28 rotor as described above. A light scattering zone which appeared between 4.0 to 4.5 cm height was drawn, diluted in PBSS, and centrifuged at 30000 rpm for 1.5 hours in a Beckman R 40 rotor.

Serology:

Purified virus obtained from 50 gram tissue was suspended in 1.0 ml of a 0.01 M phosphate buffer, pH 7.0, containing 0.85% NaCl, and emulsified with 1.0 ml of Freund's incomplete adjuvant. Emulsion was injected intramuscularly into the hind leg of a New Zealand White inbred rabbit at three different sites. Five such injections were given at weekly intervals. Bleeding began 1 week after the last injection, and was carried out at weekly intervals. Serum was lyophilized and stored at -20°C.

For testing serological relationships a protein A coating method of ELISA (PAC-ELISA) was used (Hobbs et al. 1987). Protein A at 1 ng/ml concentration, prepared in 0.05 M sodium carbonate buffer, pH 9.6, was used to coat wells of microtitre plates. Healthy and infected leaf extracts were prepared in an antigen buffer (Clark and Adams, 1977). Antisera were cross-adsorbed with healthy peanut leaf extracts (Hobbs et al. 1987). Anti-rabbit Fc-specific immunoglobulins from antiserum produced in goats (Cappel Laboratories Inc., Pennsylvania) were conjugated to penicillinase (B-lactamase) and used at 1:10,000 dilution (Sudarshana and Reddy, 1989). Sodium salt of penicillin-G was used as a substrate. Absorbance values were recorded at 620 nm in a Titertek Multiscan MCC ELISA plate reader for three replications of each sample.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE analysis of proteins of the purified virus was done according to the procedure of Laemmli (1970). Purified virus samples were suspended in a sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 1% SDS, 15% glycerol, 0.5% 2-mercaptoethanol, and 0.001% bromophenol blue, and boiled for 5 minutes prior to loading. Samples were electrophoresed with markers at 35 V for 15 hours. Gels were stained with silver nitrate as follows. The gel was first fixed in a solution containing 50% methanol and 12% acetic acid for 30 minutes, then given three washes in distilled water, allowing 20 minutes for each wash. It was then treated for 30 minutes with 0.0005% dithiothreitol (DTT) in distilled water (a stock solution of 0.05% DTT was diluted to 1:100 before use). The DTT was then removed, and the gel treated with 0.2% silver nitrate, prepared in distilled water, for 30 minutes. The gel was then rapidly rinsed in distilled water and immersed in a developer (containing 3% sodium carbonate and 0.05% formaldehyde prepared in distilled water)

until the polypeptides were clearly visible, which usually took about 10 minutes.

The following protein markers from Bio-rad Laboratories were used: Phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000) and soybean tripsin inhibitor (21500). Molecular weights were determined from linear scans of gels on a LKB Ultroscan XL densitometer.

Electro-blot immunoassay:

Purified virus was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher and Schull BA 85, 0.45 um pore size) utilizing a semi-dry transfer unit (TE 70 Semiphor, Hoefer Scientific Instruments, California). Nitrocellulose membranes presoaked in 0.192 M glycine, 0.025 M tris, pH 8.3, containing 0.0013 M SDS and 20% methanol (Towbin et al. 1979) were used and transblotting was done at 15 V for 1 hour. After transfer of proteins the membrane was incubated while shaking, for 2 hours in a solution of 5% (w/v) spray-dried milk (SDM, "Everyday" by Nestle) in tris-buffered saline (TBS, 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5). The membrane was then incubated for 1 hour in a 1/500 dilution of antisera in TBS with 0.05% tween-20 (TTBS) containing 5% SDM (TTBS-SDM). After washing three times for 10 minutes each in TTBS, the membrane was incubated for 1 hour in horseradish peroxidase-labelled anti-rabbit Fc-specific immunoglobulins, produced in goats, and prepared in TTBS-SDM. After the membrane was washed three times in TTBS, it was treated with a TMB membrane peroxidase substrate and an enhancer (Kirkegaard and Perry Laboratories, Maryland). Color (blue) development was recorded visually. The membrane was washed with distilled water, air-dried and photographed immediately.

RESULTS

Symptomatology and host range:

Initial symptoms in mechanically inoculated plants appeared as chlorotic spots, which enlarged and became pale yellow. As leaflets aged, spots became dark yellow and ultimately necrotic (Fig. 1). Coalescence of adjacent lesions sometimes led to death of leaflets.

Eleven of 17 plants tested were infected (Table 1). In all the hosts the virus induced local lesions (Fig. 2). Systemic infection in Pisum sativum, Vigna radiata and V. unguiculata occurred only when ambient temperatures were between 20 and 30°C. The following hosts were not infected by the virus: Brassica campestris, Cyamopsis tetragonolobus, Glycine max (cv. Bragg), Lycopersicum esculentum (cvs. Marglobe, Pusa Ruby), Nicandra physaloides and Solanum melongena.

Properties of the Virus in sap:

The DEP was between 10^{-2} and 10^{-3}, the TIP was between 45° and 50°C, and LIV was for 5 hours at 25°C.

Electron microscopy:

Spherical membrane-bound particles, ranging from 70 to 100 nm in diameter, were observed in the cytoplasm (Fig. 3). Numerous clusters of enveloped virus particles were also observed in cytoplasm.

Virus purification:

Preparations of purified virus were pale green. The purification method adopted yielded intact membrane-bound particles of 70-80 nm in diameter. Scattered membranous host contaminants were also present (Fig. 4).

Serology:

Cross-adsorption with healthy peanut leaflet extracts was necessary to avoid non-specific reaction presumably due to presence of antibodies to host material. In PAC-ELISA, PYSV reacted with homologous antiserum. It did not react with antisera to two TSWV isolates (Table 2).

SDS-PAGE:

Five major proteins of molecular weights 90000, 54000, 46000, 40000 and 31000 daltons (average of five determinations) were consistently resolved in 10% acrylamide gels. Several minor polypeptides, which varied from sample to sample, were also observed. These proteins were probably derived from host material.

Following treatment with Nonidet-40, the major polypeptide retained was that of 31000 daltons, while low proportions of 54000 and 40000 dalton polypeptides were also observed.

Electro-blot immunoassay:

The proteins separated by SDS-PAGE, were transferred to nitrocellulose by electrophoresis, and tested by a homologous and by two TSWV antisera. Using the homologous antiserum, all the five viral polypeptides could be detected. No reaction was observed with any of the polypeptides when TSWV antisera were used.

DISCUSSION

From the above results it is evident that PYSV resembles TSWV in particle morphology, polypeptide composition and physical properties in crude plant sap. Both viruses are transmitted by thrips (Amin and Mohammad, 1980). In host range studies, PYSV produced local lesions on Chenopodium amaranticolor, C. quinoa, Petunia hybrida, Phaseolus vulgaris, and Vigna unguiculata. However when TSWV and PYSV were assayed under similar conditions on V. unguiculata, PYSV took 9-12 days to produce lesions as compared with 4-5 days for TSWV. Also the lesions produced by PYSV were uniformly chlorotic and surrounded by a halo, whereas those produced by TSWV were of concentric chlorotic rings. This feature can be used to distinguish PYSV from TSWV. Unlike PYSV, TSWV infects Brassica campestris, Cyamopsis tetragonoloba, Glycine max, Lycopersicum esculentum, Nicandra physaloides and Solanum melongena (Reddy and Wightman, 1988). While TSWV induces systemic infection in Datura stramonium, Nicotiana benthamiana, and N. glutinosa, PYSV induces only local lesions.

In ELISA tests PYSV was shown to be serologically distinct from two TSWV isolates infecting peanuts, while none of the PYSV polypeptides reacted with the two TSWV antisera in electro-blot immunoassay.

In the morphology of virus particles, and in the presence of clusters of enveloped virus particles, PYSV closely resembled

TSWV (Francki and Grivell 1970; Ie, 1982).

The molecular weights of PYSV polypeptides differed from those of TSWV as reported by Milne and Francki (1984). The 54000 dalton polypeptide is considered to be envelope glycoprotein G1 and 46000 and 40000 polypeptides are considered to be glycoproteins G2. Evidence was presented to show that NP 40 treatment removed all major polypeptides except the 31000 dalton polypeptide. Thus, as in TSWV (Mohamed et al. 1973; Tas et al. 1977), this polypeptide is considered to be associated with the nucleocapsid.

Since TSWV is known to resemble members of the family bunyaviridae (Milne and Francki, 1984; de Haan et al: 1989) it is appropriate to group TSWV and any similar virus in the family "Phytobunyaviridae". To our knowledge, TSWV has been the sole member of this group, but based on the data presented in this paper, we consider that PYSV could be regarded as a distinct member of the "Phytobunyaviridae".

We are currently analyzing nucleic acids of PYSV and extent of homology between PYSV and TSWV nucleic acids.

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Table 1. Host range of peanut yellow spot virus as determined by mechanical sap inoculations.

Host species	Symptoms[1]	
	Local	Systemic
Canavalia ensiformis	NL	-
Chenopodium amaranticolor	CL[2]	-[3]
C. quinoa	NL	-
Datura stramonium	CCL	-
Nicotiana benthamiana	CL	-
N. glutinosa	CL	-
Petunia hybrida	NL	-
Phaseolus vulgaris (cv. Topcrop)	CL[2]	-
Pisum sativum	NL	NL and VN[4]
Vigna radiata	NL	NL and VN[4]
V. unguiculata (cv C-152)	CL[2]	CCL[4]

1 Virus infection was confirmed in ELISA tests CL chlorotic lesions; CCL chlorotic lesions with concentric rings; NL necrotic lesions; VN veinal necrosis.

2 Older lesions had necrotic centers.

3 Neither symptoms were observed nor virus was recovered in ELISA tests.

4 Systemic infection observed only when ambient temperatures were between 20° to 30°C.

Table 2. Serological relationships of peanut yellow spot virus in PAC-ELISA.

Dilutions of peanut leaf extracts[1]		Antisera to		
		PYSV	TSWV (Indian isolate)	TSWV[2] (Texas isolate)
Healthy	10^{-1}	0.09[3]	0.07	0.09
	10^{-2}	0.06	0.05	0.12
	10^{-3}	0.08	0.06	0.10
Infected with PYSV	10^{-1}	0.88	0.01	0.18
	10^{-2}	0.44	0.03	0.15
	10^{-3}	0.09	0.05	0.15
Infected with TSWV	10^{-1}	0.03	1.10	0.11
	10^{-2}	0.03	0.34	0.13
	10^{-3}	0.04	0.09	0.09

[1] Dilutions are based on original weight of tissue.

[2] Supplied by Dr. J.W. Demski.

[3] Absorbance at 620 nm. Values are mean of absorbance values for three replicate wells, obtained after deducting from value for three buffer controls.

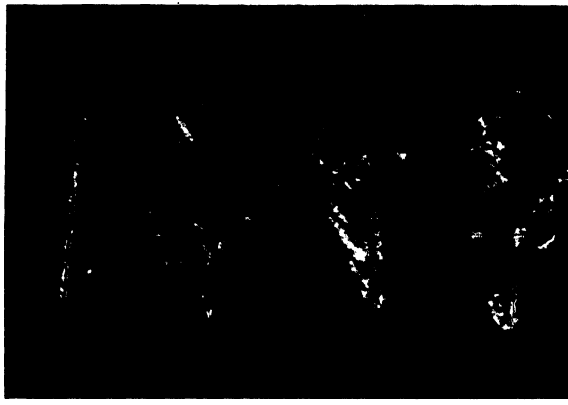

Fig. 1 Symptoms of peanut yellow spot virus on peanut leaflets. Symptoms development from initial (leaflet on left side) to advanced stage of disease development (leaflet on right side) is represented.

Fig. 2 Lesions produced on *Vigna unguiculata* cv. C 152.

Fig. 3 Thin section of a peanut leaflet infected with peanut yellow spot virus. Bundles of virus particles enclosed in a membrane are shown by arrows Bar = 1000 nm.

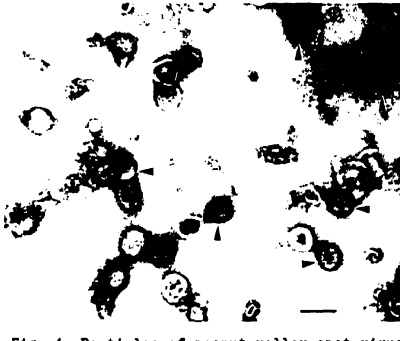

Fig. 4 Particles of peanut yellow spot virus. Bar = 140 nm.