COMPLEMENTARY DNA PROBE FOR THE DETECTION OF ISOLATES OF INDIAN PEANUT CLUMP VIRUS

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IN
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CERTIFICATE

This is to certify that the thesis entitled "**Use of complementary DNA probes for the detection of isolates of Indian Peanut Clump Virus**" being submitted by Miss K.V.Preetha Balan for the award of the degree of Master of Technology (Applied Botany) of the Agricultural Engineering department, Indian Institute of Technology, Kharagpur is a record of bonafide research work carried out by her under our supervision and guidance. The thesis in our opinion, worthy of consideration, for the award of the degree in accordance with the regulations of the Institute. The results embodied in the thesis have not been submitted to any other University or Institute for the award of any degree or diploma.

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ABBREVIATIONS

ALP	:	Alkaline phosphatase
B-IPCV	:	Bapatla- IPCV isolate
BPB	:	Bromophenol blue
BPU	:	Borate phosphate urea
BSA	:	Bovine serum albumin
BTB	:	Bromo thymol blue
CIAA	:	Chloroform isoamyl alcohol mixture
C-IPCV	:	Chinnaganjam-IPCV isolate
DAC	:	Direct antigen coating
ELISA	:	Enzyme linked immunosorbent assay
EtBr	:	Ethedium bromide
H-IPCV	:	Hyderabad- IPCV isolate
HRP	:	Horse raddish peroxidase
IPCV	:	Indian peanut clump virus
L-IPCV	:	Ludhiana-IPCV isolate
NaAc	:	Sodium acetate
PBS	:	Phosphate buffer saline
PEG	:	Polyethylene glycol
PNC	:	Penicillinase
SDS	:	Sodium dodecyl sulphate
SSC	:	Saline sodium citrate
STE	:	Sodium chloride, Tris, EDTA
TBE	:	Tris, Borate, EDTA
TE	:	Tris, EDTA
T-IPCV	:	Talod-IPCV isolate
WAPCV	:	West African peanut clump virus

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INTRODUCTION

INTRODUCTION

Peanut clump (PCV), a soil-borne viral disease which causes severe stunting and clumping of groundnut (Arachis hypogea L.) plants, is known to occur in West Africa and in India. The causal virus was shown to be rod shaped with two predominant particles of lengths 190 and 245 nm. The genome consists of two RNA species (Thouvenel and Fauquet 1981 and Reddy et al.,1985) of 1.35 $X10^6$ (5.1kb) and 1.9 $X10^6$ (6.3kb). The PCV belongs to Furovirus group, is soil-borne and transmission tests have shown that the natural vector is Polymyxa graminis (Plasmodiophorales). PCV is also seed-borne. Soil solarization and treatment with soil biocides were shown to control clump disease (Reddy et al., 1988). Although several groundnut genotypes have been screened under natural conditions none were found to be resistant to PCV.

In India, Reddy et al. (1985) and Nolt et al. (1988) have demonstrated the occurrence of isolates of peanut clump virus differing in symtomatology on certain host species and in serological properties. The Indian peanut clump virus (IPCV) collected at different geographic locations were B-IPCV collected in Bapatla, C-IPCV collected in Chinnaganjam and H-IPCV collected in Hyderabad (Andhra Pradesh); L-IPCV collected in Ludhiana (Punjab); T-IPCV collected in Talod (Gujrat). The PCV isolates of India and West Africa were serologically unrelated. Hence, serological tests such as ELISA, for dectecting several PCV isolates with an antiserum raised against a single isolate are not suitable.

In order to detect the clump virus in disease surveys, for screening seed lots in quarantine and for virus detection in epidemiological studies, it is essential to be able to detect various PCV isolates. To accomplish this it may be useful to prepare a polyclonal or monoclonal antibody with broad specificity, or alternatively a c-DNA probe which can detect (PCV) nucleic acids of several isolates may be devised. Since Reddy et al. (1985) have shown that a c-DNA probe prepared from L-IPCV isolate can detect several Indian isolates and a West African PCV isolate, it should be possible to use this technique to prepare a single probe for detection of all IPCV isolates.

The main aim of the present study was to use cloned PCV genome and select a clone which would hybridize with RNA of several PCV isolates. The clones of PCV genome could subsequently be utilized in studies on sequencing viral genome, and in the identification of nucleic acid sequences which are homologous in different isolates, and in the preparation of non-radioactively labeled highly specific probe.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Clump disease of groundnut (Arachis hypogea L.) was first observed in Madras (India) by Sundararaman (1927). The first observations in clump disease in Africa were those by Trochain (1931) at Bambey in West Africa, and called as 'Peanut clump' (Bouhot 1967). Clump disease was subsequently reported in other West African places as Burkina Faso (Germani and Dhery 1973) and Cote de Ivoire (Thouvenel et al., 1976). This disease was shown to be widespread in groundnut crops grown in sandy or sandy loam soils in Punjab (Reddy et al., 1979) and in Andhra Pradesh, Gujrat and Rajasthan (Reddy et al., 1988).

It was originally proposed that clump disease was physiological in origin or else resulted from attack by insects or pathogens (Trochain 1931). Chevalier (1934); suggested that it may be a physiological disorder resulting from injury at the time of planting. Bouhot (1967) was the first to confirm that the disease was definitely linked to some factor in the soil, as the disease always reappeared at the same sites even after crop rotation, but soil quality clearly was not the causal factor. Soon after, Bouhot (1968) suggested the disease to be of viral origin which was later confirmed by Thouvenel, Germani & Pfiffer (1974), who reported that clump disease is caused by a soil and seed borne virus. This virus has properties typical of the furo virus group (Thouvenel et al., 1988). Clump disease of groundnut in India is caused by a virus which is

serologically distinct from that occurring in West Africa (Reddy et al., 1983).

DISTRIBUTION AND SYMPTOMS

Bouhot (1967) gave the following description for clump disease from Bambey (Senegal) : "The entire plant is affected, overall it appears healthy but stunted, dark green in color and densely bushy. All parts of the plants are stunted, in leaves the number remains unchanged, they are small in size with a reduced length width ratio and a darker shade of green than usual. Petiole length is reduced as is shoot length, diameter and internode distance. The root system is equally stunted with fewer secondary roots and root nodules. Flowers form as normal but are reduced both in number and size. Fruits begin to form but rarely reach maturity and never contain more than one seed".

Indian peanut clump virus is widely distributed in India. It was found on almost every farmers field examined during the years 1979-1981 in Sangru district in Punjab and was also found in Jullandhar and Ludhiana districts. In Gujrat state it occurred in Talod district, while in Tamilnadu it was found near Pondicherry. In Rajasthan it was found near Badagaon and in Andhra Pradesh it occurred in Guntur, Prakasham, Cuddapah and Medak districts (Reddy et al., 1988).

Naturally infected plants characteristically occur in patches and recurs in the same patches in succeeding groundnut crops. Infected plants shows severe stunting with

small green leaflets. The virus is restricted to crops raised in sandy and sandy loam soils. Infected plants are conspicuous because of severe stunting and have dark green leaflets and produce small pods. Roots are dark colored and have cortical tissue which are easily sloughed off (Reddy et al., 1979).

The disease causes severe reduction in yield which has been estimated to be 40-70 % in West Africa (Germani and Dhery, 1973). In India early infected plants failed to produce pods of commercial value and from the late infected plants yield loss may go upto 60% (Reddy et al., 1983).

DIAGNOSIS OF CLUMP

Studies on in vitro properties of clump virus in West Africa (WAPCV) showed the dilution end point to be between 10^{-3}-10^{-4} and thermal inactivation point to be between 60°C - 65°C, and the longevity from 22- 27 days at about 27°C (Thouvenel et al., 1976). The virus was infective for over 20 days at 25-30°C. Chenopodium quinoa has been found to be a good diagnostic host (Reddy et al., 1983). The virus has been found in the cells of roots, stems and leaves of systemically infected plants, whereas the virus particles seemed to be located in cytoplasm near the nucleus or along the plasmalemma (Thouvenel et al., 1988).

WAPCV was purified from Chenopodium amaranticolor leaves (Thouvenel et al., 1976) frozen for at least 3 weeks. Borate buffer, pH 8.0, was found best for the initial extraction and for storing the purified virus. The detailed

method of purification has been provided by Thouvenel et al., (1976) who estimated virus yeild to be 10-15 mg/Kg leaf tissue using $E^{0.1,\,1cm}_{260}$, 3.1, soil-borne wheat mosaic virus (Brakke 1971). Ultra violet absorption spectrum of purified virus WAPCV had a maximum of 267nm and a minimum of 250nm and was typical for a nucleoprotein. The maximum /minimum ratio was 1.08±0.01 and the E_{280}/E_{260} ratio of 1.09±0.01, which suggested a nucleic acid content of 4.5-5%. Electron microscopy of leaf dip preparations by Thouvenel et al., (1976) showed two rod shaped particles of 245±15nm and 190±10 nm length, both having 23±3 nm width. The sedimentation coefficients of these particles were found to be 183S and 224S and buoyant density in cesium chloride was 1.32g/ml (Thouvenel et al., 1980).

The existence of particles of two lengths (190 and 240 nm), as well as transmission in soil, are points of similarity between PCV, tobravirus (tobacco rattle and pea early browing viruses) and several unclassified viruses, but no serological relationship between PCV and tobraviruses or other soil-borne viruses such as wheat mosaic or beet necrotic yellow vein viruses.

Indian peanut clump virus also contains two rod shaped particles with two predominant lengths of 249± nm and 184±nm, with a diameter of 24± nm (Reddy et al., 1983). They observed that virus particles stained with phosphotungstate were 20±nm in diameter and 239± and 169±nm in length. Phosphotungstate stained IPCV particles showed a distinct central canal, clear

cross banding with a periodicity of 2.5±nm when stained with uranyl acetate. It has also been shown by them through studies in serological properties using precipitin ring test, ELISA and ISEM that WAPCV or IPCV are serologically unrelated to one another and to any of the tobraviruses tested . The capsid protein of PCV is a single polypeptide with a molecular weight of 23,000d (Gibbs et al., 1983) while WAPCV polypeptides have been found to contain 197 and 224 amino acid residues (Thouvenel et al., 1980).

PCV has a bipartite ssRNA genome that is apparently not polyadenylated. Unfractionated purified preparations of WAPCV were found to contain two ssRNAs with molecular weights 2.1 x 10^6d and 1.7 x 10^6d (Thouvenel et al., 1980) and IPCV were found to contain ssRNA with molecular weights 1.9 x 10^6d and 1.65 x 10^6d (Reddy et al., 1988).

Glyoxalated L-IPCV RNA run on agarose gels (Reddy et al., 1985) gave molecular weights of two components as 1.83 x 10^6d and 1.35 x 10^6d lower than the estimates obtained in non-denaturing conditions. It was assumed that the 249 nm particles contain the larger RNA species RNA-1, and that the 184 nm particles contained the smaller RNA species RNA-2, which occurred in slightly larger amounts. When RNA-1 and RNA-2 were separately inoculated only few lesions were produced in Phaseolus vulgaris. When both the RNAs were mixed the number of lesions were greatly enhanced suggesting that both are different parts of the IPCV genome and that both are needed for infectivity.

IPCV RNA when (Mayo et al., 1985)translated produced protein of mol. wt. 14300, 19500 and 24500 daltons. It was observed that the 24500d polypeptide co-migrated with IPCV coat protein and was the only product that reacted with antiserum to IPCV particles. Further translation of separated RNA species showed that the 195000d mol. wt. and 143000d mol. wt. polypeptides were predominant when RNA-1 was used and only 24500d mol. wt. polypeptide were predominant when RNA-2 was used. It was considered that translation products of some smaller, less abundant RNA which gave small polypeptide are possibly subgenomic messengers or of satellite RNA species.

HOST RANGE AND SYMPTOMATOLOGY AND ISOLATES OF PCV

Clump virus has a wide host range of which Chenopodium quinoa and Phaseolus vulgaris are two good diagnostic host. WAPCV produced local chlorotic spots which become ring spots with line patterns in Chenopodium amaranticolor, C. quinoa, and C. album (Thouvenel et al., 1976). IPCV is also known to have a wide host range (Reddy et al., 1983). In Chenopodium quinoa it produces chlorotic rings but in other hosts it may produce necrotic or chloritic leisions or systemic mosaic symptoms.

In West Africa at least two different isolates 'common' strain 'yellow' strain ,have been found, which are closely related serologically but produce slightly different coloration in groundnut leaves (Thouvenel et al., 1988).

IPCV isolates collected from five different geographical locations were distinguished by host symptoms

and serological tests but could not be distinguished on the basis of particle size or morphology (Nolt et al., 1988). The geographical locations and the corresponding isolates are: B -IPCV from Bapatla (A.P.), C-IPCV from Chinnaganjam (A.P.), H-IPCV from Hyderabad (A.P.), L-IPCV from Ludhiana (Punjab) and T-IPCV from Talod (Gujrat). They differed in symptomatology on Canavalia ensiformis and Nicotiana clevelandii X N. Glutinosa hybrid. (Reddy et al., 1985) (Nolt et al., 1988).

B-IPCV, H-IPCV and L-IPCV produces systemic mosaic symptoms in N. benthamiana. L-IPCV produced severe mosaic in N. clevelandii whereas other two isolates cause almost symptomless infection, although these two can show necrotic lesions in P. vulgaris, and chlorotic lesions in C. amaranticolor (Nolt et al., 1988). Serological studies such as ELISA and ISEM of IPCV isolates showed that B-IPCV and C-IPCV are very similar on the basis of host range and serology. T-IPCV though is related serologically to B-IPCV and C-IPCV, could be distinguished on the basis of symptom expression. H-IPCV were serologically and symptomatologically distinct to all other isolates. The details on these five isolates have been reported by Nolt et al.(1988).

A panel of monoclonal antibodies produced against PCV (Senegal) was used to characterize five serotypes of virus by ELISA (Huguenot et al., 1989). They used biotinylated mAb with enzyme labeled streptavidin along with the rabbit antiserum in ELISA and noticed the ability of mAb to detect

confirmational changes in PCV protein by slight changes in pH or adsorption to plastic surface, which clearly underline the neccessity of having same type of antigen preparation in all serological comparisons between different isolates.

Although no serological relationships were detected between IPCV isolates, extensive nucleotide sequence homologies between these and also between WAPCV were detected (Reddy et al., 1985). The three IPCV isolates which failed to show relationship by ISEM, showed 51-86% RNA sequence homology in tests with unfractionated DNA to one isolate. H-IPCV and B-IPCV are more closely related to each other, compared to L-IPCV. Nevertheless these three IPCV isolates share 23-41% sequence homology with WAPCV.

TRANSMISSION AND CONTROL OF THE DISEASE

WAPCV was transmitted upto 24% of seed from artificially infected plants and upto 14% of seed from naturally infected plants. Vector of PCV has shown to be Polymyxa graminis (Thouvenel et al., 1981).

WAPCV is seed-borne for two generations in groundnut but was not seed-borne in great millets (Sorghum arundinaceum), Phaseolus mungo or Nicotiana benthamiana. The patchy distribution of PCV in a crop was related to the resting spores of vector Polymyxa graminis in soils. Resting spores were detected in roots of S. arundinaceum, but not in root of groundnuts (Thouvenel et al., 1981).

There exists varietal difference in the transmission of virus e.g. in groundnut cv. M13, both L-IPCV and H-IPCV were

transmitted up to a maximum of 11% and the H-IPCV isolate was transmitted upto 9% in the groundnut cv. kadiri 3 (Reddy et al., 1988).

In some cases plants infected via seed carries the virus without any symptoms on the plant (Reddy et al., 1988) which makes it essential to test all the symptomless plants by infectivity or serological tests to asses seed transmission. A few plants infected via seed (<5%) carried the virus symptomlessly hence it becomes essential to test all the symptomless plants by infectivity or serological tests to asses seed transmission.

Clump can be prevented using selected seeds and by soil treatment with fungicides prior to planting the crop or by treating the soil with fumigants, and through appropriate crop rotation. Low relative humidity may not be the major limiting seasonal factor for IPCV transmission as it seems to be with WAPCV, but temperature plays an important role. IPCV has been shown to be controlled by applying biocides like carbofuran and aldicarb at nematicidal dosages. Solarization of infested soil, for a minimum of 70 days from April to June in Bapatla and Patancheru, showed that the diseases can be reduced.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. MECHANICAL INOCULATIONS OF PLANT VIRUSES

For mechanical innoculation of peanut clump virus (PCV), Phaseolus vulgaris was used because a) it is easier to maintain and handle than groundnuts. b) the symptoms appeared after 4-5 days of mechanical inoculation. The following precautions were taken in the process of innoculation:

1.1 Precautions:

All the glassware, mortars and pestles were soaked in 8% trisodium phosphate prior to cleaning with any laboratory detergent. Then was washed in tap water and rinsed several times in distilled water. These were thorougly dried either in a hot air oven or at room temperature. All pestles and mortars were sterilized in an autoclave. Inoculations was done either early in the morning or late in the evening especially when ambient day temperatures exceeded 30°C. Only chilled buffers were used in inoculations.

1.2 Preparation of Phosphate buffer:

0.05 M phosphate buffer was used and prepared with

KH_2PO_4 : 2.4 g
K_2HPO_4 : 5.4 g
Thioglycerol : 0.75 mL
water :to make 1 L
pH was adjusted to 7.00.

Note: Thioglycerol can be replaced with either :

0.01 M Na_2SO_3 (1.26 g/litre) or

0.02 M 2-mercaptoethanol (1.56 ml/litre)

1.3 Selection of hosts:

Bean (Phaseolus vulgaris) plants were raised in well fertilized pots. Fully expanded healthy primary leaves were only used for inoculation.

1.4 Prepration of infected leaf extracts:

Leaf tissue infected with virus was triturated in chilled 0.05 M phosphate buffer (using a per-chilled motor pestle) with 1:9 dilution i.e., 1 gm tissue in 9 mL buffer. Tissue were grinded to get a fine homogenate. The extract was filtered through two thickness of cheesecloth.

1.5 Procedure for mechanical sap inoculation:

Mechanical sap inoculation involved application of plant extracts containing viruses onto the surface of leaves of healthy bean plants permitting entry of the virus in plant cell. Leaves to be inoculated were dusted sparingly with 600 mesh carborundum (silicon carbide). The leaves were held with hand and plant extracts were applied uniformly on the leaf surface gently with the thick end of a pestle, or with the fingers. Leaves were rinsed with water immediately after inoculation. Every thing else was thoroughly washed with soap or trisodium phosphate to avoid contamination.

2.ENZYME-LINKED IMMONOSORBENT ASSAY (ELISA)

ELISA test was done keeping in view the basic principle of this assay to immobilise the antigen on to a solid surface and probing with specific immunoglobulin carrying an enzyme label. In the present investigation 'indirect' procedure direct antigen coating (DAC) was used with penicillinase as enzyme label.

2.1 Materials:

a) ELISA-plates :Both polystyrene and polyvinyl chloride (PVC) plates were used. PVC plates which could easily be cut to use a portion of a plate or individual strips containing 16 wells (2 rows of 8 wells) were also available for use.

b) Antisera.

c) Micropipettes (10-100 µL and 100-1000 µL). (The ones which provided adjustable volumes were preferred.

d) Repeatable micropipette (100 or 200 µL volume) or micropipettes which could hold eight microtips.

e) ELISA plate reader.

2.2 Solutions required:

a) Carbonate buffer (coating buffer): Na_2CO3 1.59 g and NaHCO3 2.93 g added to distilled water to make upto 1.0 L; pH of the buffer was adjusted to 9.6, and diethyldithiocarbamate (with sodium salt at 0.01 M) concentration was added 1.71 gm for 1 L.

b) Phosphate-buffered saline with Tween (PBS-tween): To 1L

PBS 0.5 ml Tween-20 was added.

[PBS: NaCl 8g, KCl 0.2g Na_2HPO_4 1.44g, Bovine serum albumin 1g was dissolved in 1 litre of water and pH was adjusted to 7.4].

c) Antibody buffer: PBS-tween 100 mL; Polyvinyl pyrollidone (40,000 MW) 2.0 g (2% final concentration); ovalbumin 0.2 g (0.2% final concentration).

d) Substrate buffer: 20 mg bromothymol blue (BTB) was dissolved in 50 mL of 0.2 M NaOH. The alkali was neutralized by adding conc. HCl dropwise. The volume was made upto 100 mL. Sodium penicillin-G (Potassium penicillin-G and procaine penicillin can also be used) was incorporated at 0.5 mg/mL and the pH was adjusted to 7.2 using either HCl or NaoH (0.01 to 0.1 M). The mixture was stored at 4°C.

Note: It is absolutely essential to adjust the pH to 7.2 before use. BTB solution alone is stable for several months at 4°C but with penicillin, it is stable for 2 to 3 weeks.

2.3. Procedure:

a) Plant extracts were prepared in coating buffer (1:10). 200 µL volumes were dispensed into each well using a micropipette. Plate was incubated at 37°C for 1 h.

b) Plant extracts were poured off and the ELISA plates were rinsed in PBS-tween. This was followed by washing the plates in three changes of PBS-tween, allowing 3 min for each wash.

Crude antisera diluted in antibody buffer were added. (It was essential to determine the appropriate dilution required. Since high titered antisera were used, 1:10,000 dilutions were used). 200 µL of antibody solution was added to each well and incubated at 37°C for 1 h.

d) Plates were washed in PBS-tween as in step (b).

e) Penicillinase-labeled antirabbit Ig G or Fc was diluted to 1:5,000 or 1:10,000 in antibody buffer. 200 µL was dispensed into each well and incubated at 37°C for 1 h.

f) Plates were washed as in (b) using 0.05% Tween-20 in distilled water.

g) 200 µL of substrate mixture was dispensed (Penicillin + Bromothymol blue) into each well and incubated at room temperature. The reaction was observed for 30 min to 2 h and results recorded.

h) **Measurements:** The blue color of bromothymol blue (at pH 7.2) first turned to light green, and then to light orange yellow to orange yellow color. A green color indicated weak positive while the orange yellow, a strong positive. Quantitative measurement was done by determining loss in absorbance of BTB at 620 nm. [Normally 0.2 mg/mL BTB gave an optical density (O.D.) of over 2 units and a strong positive reaction (orange yellow) gave less than 0.1 O.D. units].
(Results were recorded on Titertek Multiskan).

Caution: If the plates were washed in PBS-Tween, traces of buffer left in wells were adequate to buffer the reaction between penicillin and penicillinase and prevent the colour change from occuring. Therefore Tween-20 in distilled water must be used in (f).

3.PURIFICATION OF PLANT VIRUSES

The procedure of purification of PCV was developed at ICRISAT which could give 3-5 mg virus from 100 g plant material. The best host for maintaining PCV was found to be French bean (Phaseolus vulgaris cv Top crop). The virus produced chlorotic patches and veinal necrosis.

3.1 Materials:

a) Waring blender,

b) Refrigerated superspeed centrifuge and ultra centrifuge,

c) Rotor tubes,

d) 100 mL, 500 mL beakers. 500 mL and 1 L conical flasks,

e) Disposable 5.0 mL and 10.0 mL pipettes,

f) 250 mL and 1L measuring cylinders,

g) 250 mL or 500 mL glass bottles with stoppers,

3.2 Solutions required:

a) Phosphate buffer 0.1 M (0.1 M PB), containing sodium diethyl dithiocarbamate and thioglycerol.

KH_2PO_4	0.68 g
K_2HPO_4 H_2O	20.52 g
DIECA (0.1M)	17.1 g

Thioglycerol (0.2%) 2.0 mL

Distilled water to make upto 1L.

pH was adjusted to 8.0.

b) Borate-phosphate buffer containing urea (BPU):

KH_2PO_4	0.86 g
Borax	1.40 g
Urea	12.0 g
Distilled water	1L

pH adjusted to 8.3.

c) Sucrose (ANALA R) solutions prepared in BPU

10 g made up to 100 mL	10%
20 g made up to 100 mL	20%
30 g made up to 100 mL	30%
40 g made up to 100 mL	40%

d) 30 g sucrose was added to 50 mL BPU and after dissolving, the volume was made to 100 mL. NaCl was added to give 0.2 molarity, and polyethylene glycol (mol.wt. 6000-8000) was added to give 6% concentration.

e) Potassium phosphate buffer, 0.02 M (0.02 MPB):

KH_2PO_4	0.42 g
K_2HPO_4 $3H_2O$	3.84 g
Distilled water	1L

pH was adjusted to 7.5.

3.3 Purification procedure:

a) French bean leaves which showed symptoms (systemic veinal necrosis) after 4-5 days of mechanical inoculations were collected.

b) It was triturated in chilled 0.1 M PB (pH 8.0) containing Na DIECA and thioglycerol at the rate of 4 mL buffer for each gram of tissue, using a Waring Blender.

c) Filtered through two thickness of cheesecloth.

d) Chloroform was added to give 10% (V/V) final concentration, and was shaken for about 5 min.

e) The emulsion centrifuged at 5000 rpm for 10 min in a refrigerated centrifuge at 4°C.

f) The aqueous phase was drawn with a Pasteur pipette (green colored chloroform was avoided).

g) Aqueous phase volume was measured and NaCl added to give 0.2 molarity. Polyethylene glycol (PEG) of mw 6000-8000 was added to give 6% concentration. Stiring was done for approximately 10 min to dissolve NaCl and PEG.

h) Kept for 1.5 - 2 h in a refrigerator (at 4-6° C) for precipitation.

i) Precipitate was collected after centrifuging at 8,000 rpm for 10 min (in a refrigerated centrifuge at 4°C).

j) Pellets were resuspended in BPU. Triton X-100 was added to give a final concentration of 0.2% (V/V).

k) Centrifuged at 5000 rpm for 10 min in a refrigerated centrifuge at 4°C.

l) 13 mL of 30% sucrose prepared in BPU containing NaCl (0.2 M) and PEG (6%) was added in a Beckman SW 28 rotor tube. 25 mL virus suspension supernatant (step 'k') was layered on sucrose.

m) Centrifugation was done in SW 28 rotor at 25000 rpm for 90 min.

n) Pellets were resuspended in BPU (approximately at the rate of 1.0 mL BPU for each 5.0 gm starting plant material) and then left in a refrigerator overnight.

o) Sucrose gradients were prepared in Beckman SW 28 rotor tubes as follows: 6, 9, 9, and 9 mL of each of 10, 20, 30 and 40% sucrose in BPU were layered and stored in a refrigerator for at least 16 h in order to form a linear sucrose gradient.

p) Next morning, 5.0 mL of virus suspension (from step 'n') was layered on each gradient and centrifuged at 25000 rpm for 1.5 h in a SW-28 rotor (rate zonal centrifugation).

q) A clear light scattering zone at 5.6 - 6.0 cm height was visible. This zone was drawn with a bent needle attached to a syringe.

r) The virus zone was diluted in BPU and centrifuged in a Beckman R-50 rotor at 30,000 rpm for 2 hours.

s) Virus pellets appeared translucent depending on its purity.

Precautions:

All steps in the purification process need to be done in cold room (1-5^{o}C). If not possible, ice bath using chilled solutions must be used. All glassware must be thoroughly washed with distilled water, using high grade chemicals. Entire process need to be completed as quickly as possible.

4. EXTRACTION OF TOTAL NUCLEIC ACIDS FROM VIRUS-INFECTED TISSUE

4.1 Materials:

a) Chemicals

Agarose	Glycerol
Boric acid	Hydrochloric acid (HCl)
Bromophenolblue (BPB)	8-Hydroxyquinoline
CF-11	Isoamylalcohol
Chloroform	Phenol
m-Cresol	Sodium acetate (NaAc)
EDTA	Sodium chloride (NaCl)
Ethanol (EtOH)	Sodium dodecyl sulfate (SDS)
Ethidium bromide (EtBr)	Tris base (or Trizma)

b) mortars and pestles (or blender for larger quantities of tissue).

c) polypropylene centrifuge tubes with caps.

d) refrigerated superspeed centrifuge.

e) micropipettes (1-10 or 20 µL and 10-100 or 20-200 µL) with adjustable volumes.

f) microcentrifuge tubes - 1.5 mL, polypropylene (these are optional, but highly convenient).

g) horizontal gel electrophoresis system - including a tray for casting gels, combs and electrophoresis tank.

h) power supply - should be capable of delivering at least 100 V and 100 mA.

i) short wave UV-transilluminator (302 nm).

j) UV-protective goggles or face shields.

4.2 Solutions:

a) Sodium chloride, Tris, EDTA (STE):

Tris base	0.12 g	(0.01 M)

NaCl	0.35 g	(0.06 M)
EDTA	0.07 g	(0.002 M)
Distilled water	90.00 ml	

pH adjusted to 8.6 with HCl. Volume was measured and made up to 100 mL with distilled water. Transfered to 125 mL bottle and autoclaved. Stored at 4°C.

b) STE pH 7.0

Tris base	0.61 g	(0.05 M)
NaCl	0.58 g	(0.1 M)
EDTA	0.04 g	(0.001 M)
distilled water	to make 1L	

Preparation was done as in 4.3(a) and pH adjusted to 7.0.

c) 3 M NaAc

6.15 g NaAc was dissolved in 15mL of distilled water and the volume was made upto 25mL and autoclaved.

Stored at 4°C.

d) 10X Tris, Borate, EDTA (TBE)

Tris base	27 g	0.89 M
Boric acid	13.75 g	0.89 M
EDTA	1.86 g	0.002 M
Distilled water	200.00 mL	

pH adjusted to 7.6,the solution was autoclaved stored at room temperature.

e) 10% Sodium dodecyl sulfate(SDS):

0.2% g SDS was weighed and transfered in a sterile, disposable 5mL tube and added with 1.8mL sterile distilled water. They were dissolved by mixing and the volume was made upto 2mL, stored at room temperature.

Sterile distilled water:

Double distilled water was used in all cases in the procedure after it was autoclaved.

g) Glassware etc. :

25 mL cylinders - 1
50 mL cylinders - 3
250 mL cylinders - 1
25 mL beakers - 2
50 mL polypropylene centifuge tubes and caps - 8
15 mL Corex tubes - 2
100 mL flask - 1
small stir bars - 2
Yellow Eppendorf tips
Microcentrifuge tubes
All the above items were covered with aluminium foil before these were autoclaved.

4.3 Organic solvents:

a) Kirby's mixture

Phenol	35 mL (melted in water bath at 68°C)
m-Cresol	4.2 mL
STE pH 8.6	9.8 mL
8-hydroxyquinoline	0.04 g

Mixed in a 50mL polypropylene tube until the hydroxyquinoline got dissolved. The tube was wrapped with foil and kept at 4°C (in case of storing).

b) CIAA

Chloroform	48 mL
Isoamylalcohol	2 mL

Mixed in a 50mL polyproplyne tube. Stored at 4°C.

4.4Extraction procedure:

a) 5 g leaves, each of healthy and infected tissue were weighed and put in two separate mortars, which were sterilized.

b) To each mortar the following was added:

STE pH 8.6	5 mL
10 % SDS	0.5 mL
BME	25 µL
Kirby's solution	5 mL
CIAA	5 mL

c) It was triturated thoroughly until a fine extract was obtained.

d) Transferd to 50 mL centrifuge tube. The tubes were balanced to have equal weight (Kirby's solution was used for balancing).The tubes were capped and mixed well.

e) Centrifuged at 7000 rpm for 15 min.

f) After spinning, the emulsion got separated into an aqueous phase on top containing nucleic acids and an organic phase on bottom containing extracted protein. A prominent layer of white precipitate was visible at the interface, representing excess proteins that could not be solubilized by the organic phase, was removed by several successive extractions, till very clear aqueous phase has been obtained without a white precipitate at the interface. The aqueous phases was transfered to fresh tubes using pasteur pipettes avoiding interface as far as possible.

g) 5 mL each of Kirby's and CIAA was added to each tube.

h) Centrifuged at 5000 rpm for 10 min.

i) Aqueous phase was removed and transfered to fresh tubes.

j) 10 mL of CIAA was added, balanced using CIAA, capped, mixed well and centrifuged at 5000 rpm for 5 min.

k) Step (j) was repeated.

l) Volumes of aqueous material was measured and 2.5 volumes of cold EtOH was added in a tube and capped. Nucleic acid was easily visible as strands or balls of fibrous material. Kept at -20°C or below for at least 1 hr to obtain total NA.

m) EtOH-NA mixture was centrifuged at 7000 rpm for 15 min. At this stage pellet was easily visible.

n) Supernatants from the two tubes were poured off and the tubes were inverted, on clean Kimwipes to dry briefly. Vacuum drying was done to remove traces of ethanol.

o) In each case, the pellet was resuspended in 8 mL STE at pH 7.0. (Mixing was usually sufficient for this purpose but if pellets were not easily resuspended, tissue homogenizers or pipettes were used).

4.5 Preparation of samples for electrophoresis:

a) Tubes were put in adapters and into the centrifuge making sure that the tubes were aligned so that the position of the pellets were known (these pellets will be barely visible). Centrifuged at 10,000 rpm 15 for min in a refrigerated centrifuge.

b) The supernatant was poured off.

c) The tubes were placed with pellet in a vacuum dessicator 30-45 min or longer till no smell of EtOH was noticed in the pellets.

d) Pellets were resuspended in 25 µL TE (10 mM Tris, 1 mM EDTA, pH 7.6).

e) After mixing, the tubes were centrifuged at 1000rpm for a few seconds.

f) 5 µL sample buffer was added to samples.

Sample buffer (SB): In a microcentrifuge tube, 50 µL TE, 50 µL glycerol and a few grains of bromophenol blue (BPB) were mixed. The glycerol caused the NA solution to sink to the bottom of the well in the gel. The bromophenol blue (BPB) is a dye that runs with sample front and indicates how far the samples have run.

g) After mixing, 10 µL of total NA samples was transfered to fresh tubes and 2 uL SB was added.

4.6 Preparation of gel:

a) 50 mL sterile distilled water was taken in a flask the level of water in the flask was marked using a marker.

b) 0.5 g agarose was weighed and added to dH2O in flask.

c) Agarose suspension was boiled in a microwave oven until no more agarose particles were visible in the solution.

d) 5 mL 10X TBE was added. Sterile dH2O was added to bring the volume back to 50 mL. Swirled to mix the contents.

e) The gel temperature was allowed to come down to about 50-55^{o}C.

f) Ends of the casting tray was blocked with a tape, making sure it was well sealed. The comb was positioned.

g) When the gel was sufficiently cooled to 50-55^{o}C 2.5 µL of 10 mg/mL EtBr was added. 1/2 to 3/4 of the gel solution was poured into casting tray. Bubbles,

especially those around teeth of comb, were removed with a Pasteur pipette. Gel was allowed to solidify usually about 30 minutes was needed.

h) Comb and tape were removed from cooled gel.

4.7 Electrophoresis:

a) 250 mL TBE was taken in a cylinder.

b) Gel was placed on the tray of electrophoresis tank and TBE was poured till the gel was submerged.

c) Samples were loaded carefully into the wells using micropipettes, noting their positions.

d) Tank was covered and connected to power supply.

e) Electrophoresis was performed at 80 V for 1-2 hours. (It was possible to stop electrophoresis to visualize migration).

f) After completing the run the gel was removed (with the tray) and placed on a UV-transilluminator.

g) Result was recorded by using a polaroid camera (direct screen instant camera was used). Gels stored for long periods became unsuitable for photography.

5. EXTRACTION OF GENOMIC RNA FROM PURIFIED VIRUS AND ANALYSIS BY AGAROSE GEL ELECTROPHORESIS.

A purified preparation of PCV, stored in TE buffer at -20°C, was used to extract NAs.The nucleic acid was extracted by procedure described in section 4.

5.1 Extraction of genomic PCV RNA:

a) Purified virus was thawed and transfered to a sterile microfuge tube and the volume was measured.

b) SDS was added to obtain 0.5% concentration and Beta-mercaptoethanol to obtain 0.5% concentration (v/v) and was shaken at room temperature on a shaker for 15 min.

c) Equal volume of Kirby's mixture was added and thoroughly mixed.

d) This was centrifuged at 5,000 rpm (5 K) for 10 min in a microcentrifuge.

e) Aqueous phase was transfered to a fresh tube.

f) Equal volumes of CIAA was added and mixed well.

g) Centrifuged at 5 K for 5 min. Step (e) was repeated.

h) Sodium acetate was added to give a final concentration of 0.15 M ; and 2.5 volumes of cold EtOH was added because PCV has a ssRNA genome.

i) Kept for 1 hour or more at -20°C.

j) Centrifuged at 12 K for 15 min.

k) Supernatant was poured off, pellets were dried under a vacuum.

l) Pellets were resuspended in TE.

5.2 Quantitating the nucleic acid on a mini gel:

It was useful to run a quick minigel to ascertain whether both the RNA species were present and if their concentration in the sample was adequate. The procedure has been described below.

Preparation of samples for electrophoresis:

a) Pellets were resuspended in 25 µL TE (10 mM Tris, 1 mM EDTA, pH 7.6). Most of the pellets remained at the bottom of the tube, some along the sides.

b) After mixing, samples were centrifuged at 1000rpm for a few seconds.

c) Sample buffer (SB): In a microcentrifuge tube, 50 µL TE, 50 µL glycerol and a few grains of bromophenol blue (BPB) were mixed. The glycerol caused the NA solution to sink to the bottom of the well in the gel. The bromophenol blue (BPB) is a dye that runs with solvent front and indicates how far the samples have run.

d) 5 µL SB was added to samples.

The procedure for electrophoresis is described in section 4.

6.COMPLEMENTARY DNA SYNTHESIS

6.1 Materials :

a) Ameersham's c-DNA synthesis system plus (code:RPN.1256 Y/

b) 0.25M EDTA

c) Microcentrifuge

d) Water baths

e) Adjustable micropipettes

f) Microcentrifuge tubes and pipette tips (Autoclaved and dried before use).

6.2 Precautions:

Always sterile equipment used. Sterile disposable gloves were used to protect hands from organic solvents and to prevent RNase contamination. Radio-active material was handled carefully and wastes disposed properly.

6.3 Procedure:

H-IPCV RNA was used for c-DNA synthesis.

First strand synthesis:

a) One microgram of viral RNA was heat denatured at 65^{o}C for 5 min and immediately quenched in ice prior to use.

b) The reaction mixture was taken in a sterile microcentrifuge tube. The reaction mixture (20µl)contained 50mM Tris HCl, pH 8.3, 10mM magnesium chloride, 40mM potassium chloride, 80 mM dithiothretol, 25µg DNA primer mixture 0.67mM each of dCTP, dGTP and TTP, 20 um dATP, 10u Ci of(alpha 32-P) dATP (3000 Ci/n

mol ,Amersham UK) 50u human placental ribonuclease inhibitor, 4mM sodium pyrophosphate, 1µg viral RNA and 20U reverse transcriptase.

c) The reaction mixture was incubated for 40 min at 42 °C.

d) A small aliquot was processed for assaying first strand c-DNA. Prior to c-DNA synthesis sample was checked by electrophoresis on agarose gels.

Second strand synthesis:

a) To 20 µl of first -strand synthesis mixture , 5µl of 10 u Ci/µl (alpha 32-P)ATP, 0.8u E. coli ribonuclease H and 9 U E.coli dsDNA polymerase1 were added to a final volume of 100µl, incubated at 12° C for 1 h and then at 22°C for 1h.

b) The enzymes were inactivated at 70 ° C for 10 min, 2u of T4 DNA polymerase per ug of viral RNA template was added and incubated at 37° for 10 min .

c) The reaction was stopped by adding 10 µl of 0.25 M EDTA and 10 µl of 1% SDS.

d) This was subjected to chromatography through Sephadex G-50 to obtain only the incorporated c-DNA (as in section 9).

7. NICK TRANSLATION

7.1 Materials

Microcentrifuge tubes, tips; ^{32}P labeled dCTP/dATP,

DNase 1, Bovine serum albumin (BSA), DNA Polymerase 1, 1mM of dATP, dGTP, dCTP, TTP; 10X repair buffer (0.5M Tris.Cl (pH7.2), 0.1 $MgSO_4$, 1mM dithiothretol, 500µg BSA)

7.2 Procedure:

a) The reaction mixture was made as follows:

^{32}P labeled dATP/dCTP was evaporated in a microcentrifuge tube. To this the following were added keeping in ice.

water	2µL
dCTP,dTTP,dATP/dCTP 0.2mM	2µL
dCTP/dATP 25µM	2µL
10X repair buffer	2µL
BSA	2µL
DNA (0.1-0.5µg)(clones)	2µL
label 10uCi dATP	2µL
DNase 1 (0.01 ng/µL)	1.5µL
After 1 minute DNA Pol 1(3u/ml)	0.5µL

This was incubated for 90 minutes at 15°C.

b) 0.5µL EDTA(0.5M) was added and kept at -20°C.

c) This mixture was applied to Sephadex G-50 column (section 9) to separate the nick translated DNA from unincorporated dNTPs.

d) The sample was applied with 5µL sucrose dye and 10µL tRNA (10µg/ml)on to the column, equilibrated with TE and 0.1% SDS and 3drop fraction were collected.

e) The incorporation was measured using liquid scintillation counter.

This was used for hybridisation of PCV dot blots.

8. NORTHERN TRANSFERS

RNA fragments that have been separated according to size by electrophoresis through an agarose gel were denatured, transferred to a nitrocellulose filter and immobilized. The relative positions of the RNA fragments in the gel were preserved during their transfer to the filter. The RNA attached to the filter was then hybridized to 32P-labeled DNA. Autoradiography was used to locate the position of bands complementary to the radioactive probe .

8.1 Transfer of RNA from agarose gels on nitrocellulose paper:

a) After electrophoresis was completed RNA was stained with ethedium bromide and the gel was photographed. A ruler alongside gel was placed so that the distance that any given band of RNA had migrated could be measured directly from the photographic image.

b) Gel was transfered to a glass baking dish and any unused area of the gel was trimmed with a razor blade .

c) Denaturation of RNA was done by soaking the gel in several volumes of 1.5 M NaCl and 0.5 M NaOH for 1 hour at room temperature with constant shaking.

d) Gel was neutralized by soaking in several volumes of a solution of 1M Tris.HCl (pH 8.0) and 1.5 M NaCl for 1hour at room temperature with constant stirring or shaking.

e) A piece of Whatman 3MM paper was wrapped around a piece

of plexiglas or a stack of plates. The wrapped support was placed inside a large baking dish. The support was longer and wider than the gel. The dish was filled with 10X SSC almost to the top of the support and all air bubbles in the damp 3MM paper were removed by running a smooth glass rod.

f) The gel was inverted so that its original underside was exposed. All air bubbles between 3MM paper and the gel were removed.

g) Using a fresh scalpel and a paper cutter, a piece of nitrocellulose filter about 1-2 mm larger than the gel in both the dimensions was cut using gloves and blunt tipped forceps.

h) Nitrocellulose filter was floated on the surface of a solution of 2X SSC until wet, then it was immersed in 2X SSC for 2-3 min.

i) Wet nitrocellulose filter was placed on top of the gel, so that one edge extended just over the line of slots at the top of the gel. Air bubbles were removed.

j) Two pieces of Whatman 3MM ,exactly of same size of the gel in 2X SSC, was placed on top of the nitrocellulose filter.

k) A stack of paper towels (5-8cm high) was placed on 3MM paper. A 500g weight supported by a glassplate, was placed upon it. This was to facilitate flow of liquid from reservoir through the gel and the nitrocellulose paper, so that RNA fragments were eluted from the gel and deposited onto the nitrocellulose paper.

l) The transfer was allowed for 12-24 hours. As the paper towels became wet they were replaced.

m) The paper towels and the 3MM filters were removed from the gel. The dehydrated gel and filter was turned over and placed on a dry filter paper and the gel slots on the filter were marked with a soft pencil.

n) Gel was peeled and discarded. Filter was soaked in 6X SSC at room temperature for 5 min and then air dried.

o) The dried filter was placed between two sheets of 3MM paper and baked for 1hour at 80°C under vacuum. Such filters were used in hybridization studies with radioactively labeled probes.

9. CHROMATOGRAPHY THROUGH SEPHADEX G-50

This was used to separate high molecular weight molecules from low molecular weight particles. This method was used to separate DNA that was labeled by nick translation, and at several stages during the synthesis of c DNA. Two methods are available: conventional column chromatography and centrifugation through Sephadex G-50 packed in disposable syringes.

9.1 Materials

Sephadex G-50

STE (refer section 4)

Tris EDTA (refer section 4)

Disposable borosilicate glass pipette

Eppendorf tubes

Hand held Minimonitor/ Liquid scintillation counter

1 ml disposable syringes

Glass wool

Bench centrifuge

9.2 Preparation of Sephadex G-50

30g of Sephadex G-50 was slowly added to 250ml of TE (pH 8.0)in a 500ml beaker or bottle. It was allowed to stand at room temperature,autoclaved for 15 minutes at 15lb/in^2 on liquid cycle. Allowed to cool to room temperature. Supernatant was decanted and replaced with an equal volume of TE (pH 8.0), and stored at 4^{o}C in a screw capped bottle.

9.3 Column chromatography:

a) Sephadex G-50 column was prepared in a disposable 5ml borosilicate glass tube (or pipettes) plugged with sterile glass wool. The column was washed with several column volumes of TE (pH 8.0).

b) DNA sample was applied to the column (small volumes in 200µl). The tube was washed with approximately 100µl of TE(pH 8.0) and the washings applied to the column. A reservoir of TE was connected to the column so that the flow rate was about 0.5 ml/ min.

c) 15-20 fractions were collected (0.5ml) into eppendorf tubes. The radioactivity of ^{32}P labeled DNA in each of the tube was measured, by using a hand held minimonitor or by Cerenkov counting in a liquid scintillation counter. The fraction that showed peak radioactivity consisted of ^{32}P labeled nucleotides incorporated into DNA, while the trailing peak consisted of unincorporated [^{32}P] dNTPs.

d) The radioactive fractions in the leading peak were pooled and stored at -20°C.

Caution: Column was run behind lucite screens to shield personnel from exposure to radioactivity.

Note: A variety of matrixes (Sephadex G-75, G-100, Sephadex CL-4B, etc.) can be used.

9.4 Spun column procedure:

This method was useful when several preparations of DNA were labeled simultaneously or when needed to change the buffer in which the DNA was dissolved.

a) A 1 ml disposable syringe was plugged with a small amount of sterile glass wool. In the syringe a column (0.9ml bed volume) of Sephadex G-50 equilibrated in TE (pH 8.0), containing 0.1 M NaCl (STE) was packed.

b) The syringe was inserted into a glass centrifuge tube and centrifuged at 1600g for 4 minutes in a clinical centrifuge. Sephadex was added until the column volume was 0.9ml.

c) 0.1ml of STE was added and centrifuged at the same speed as in step (b).

d) Step (c) was repeated.

e) DNA sample was applied to the column in a total volume of 0.1ml (STE was used to make up the volume).

f) Centrifuged at exactly the same speed, for the same time as before, collecting 100ul effluent from the syringe in a decapped Eppendorf tube.

g) The unincorporated [^{32}P] dNTPs remained in the syringe, the DNA was collected from the Eppendorf tube.

Note: Spun column cannot be used with Sephadex G-100 since the beads would be crushed by centrifugation.

10. DOT BLOTS PREPARATION FOR NORTHERN HYBRIDIZATION

Dot blots were used considering it to be more advantageous and economical than the blotting of electrophoresed RNA. In dot blots nucleic acids are directly spotted using a dot blot apparatus onto the nitrocellulose filter and such blots have been used in hybridization studies. The advantages are that: a) the procedure is simple and consumes less time. b) require minimum amount of filter paper and materials, and c) small quantities of nucleic acids are sufficient for detection.

10.1 Materials:

a) Dot Blot apparatus

b) Vacuum pump

c) Sterile microcentrifuge tubes

d) Sterile microcentrifuge tips

e) 3MM Whatman filter paper

f) Nitrocellulose filter

g) Vacuum oven

h) Water bath

i) 20X SSC

k) Formaldehyde

l) Sterile distilled water

10.2 Procedure:

10.2.1 Preparation of samples:

a) Required amount of RNA was taken and made upto 200µL

with sterile distilled water.

b) Then, equal volumes of a mixture of 20X SSC and formaldehyde (6:4) was added to RNA and kept at 68°C for 10 minutes.

c) This was plunged into ice immediately, and centrifuged for a few seconds.

10.2.2 Preparation of blots:

a) Nitrocellulose filter was placed on the 3MM Whatman filter with proper orientation in the dot blot apparatus.

b) 200µL of sample (10.3.1) was spotted on to appropriate wells of the dot blot apparatus and then sufficient suction was applied from a vacuum pump.

c) The samples were washed three times. Each time 200µL of the mixture of 20X SSC: Formaldehyde (6:4) was used for washing.

d) Then the filter was dried on a filter paper.

e) Dried filter was placed in between the folds of 3MM Whatman filter and was baked in a vacuum oven at 80°C for 1h.

10.3 Precautions:

a) Nitrocellulose must be handled using forceps because if touched with bare hands, the finger prints will prevent proper blotting.

b) Vacuum drying must be done because if baked in an ordinary oven nitrocellulose may explode.

c) Blots once made are to be stored in a vacuum where it can remain very stable.

11. HYBRIDIZATION OF RNA IMMOBILIZED ON FILTERS TO RADIOACTIVE PROBES

11.1 Solutions:

a) Deionised formamide: Formamide stirred on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1h and filtered through Whatman 1mm paper and stored at 70°C.

b) Denhardt's solution 50X :

Ficoll	5g
polyvinylpyrrolidine	5g
BSA	5g

made upto 500mL with water.

c) Denatured, salmon sperm DNA: DNA of concentration 10 mg per mL (if needed stir it on a magnetic stirrer for 2-4 h at room temperature to help dissolve DNA) sheared by passing it several times through an 18-gauge hypodermic needle and boiled for 10 minutes and stored at -20°C. Just before use the DNA was heated for 5 min in boiling-water bath and chilled quickly in ice.

d) 10% SDS

e) 20X SSC

f) 0.5 M Sodium chloride

g) 1.75 M Sodium dihydrogenphosphate

h) 4M Sodium hydroxide

11.2 Materials:

Microcentrifuge tubes

Micropipette tips

Washing trays

Polythene bags to keep the filter

Polythene bag sealer

Sterile gloves

11.3 Procedure:

11.3.1 Hybridization:

a) The baked filters were floated on 6X SSC in a tray for 5 minutes.

b) The filter was placed in suitable size ploythene bags, sealed on three sides, sufficient to hold the solutions.

c) Prehybridization solution was made as follows:

50% Formamide	5ml of 100% Formaldehyde
5X SSC	2.5ml of 20XSSC
50mM Sodium phosphate pH 7	1ml of 0.5M
5X Denhardt's	1ml of 50X
100ug per mL ssDNA	0.5 ml of 2mg/ml
	10ml of pre hybridization solution.

d) This solution was carefully added into the polythene bag containing filter and then sealed leaving enough space to cut and reseal.

e) Incubated for 4-5 hours at 42^{o}C in a shaking water bath.

f) Pre-hybridization solution (without ssDNA) was made as follows.

50% Formamide
5X SSC
50mM Sodium phosphate
5X Denhardt's.

g) Denaturation of radio-active probe was done before hybridization as follows:
200µl of the labeled probe was added to 250 µl (100µg/ml) ssDNA. Then 11µl of 4M sodium hydroxide was added and kept at room temperature for exactly 10 minutes. To this 45 µl 1.75M sodium dihydrogen phosphate was added and immediately plunged into ice.

h) Solutions obtained from (f) and (g) were mixed to give the hybridization solution.

i) Prehybridization solution was drained completely from the sealed polythene bag by making a small incision at one end, thereafter, the hybridization solution was added and the bag was resealed and incubated at 42°C for 24 hours.

11.3.2 Hybridization wash:

a) After hybridization was complete the solution was discarded and the filters were washed. At every stage of washing the incorporation was checked using a hand held geiger counter.

b) Filters were washed 3-4 times, for 5-10 minutes of each wash, in a large volume (300-400ml) of 2X SSC and 0.1% SDS at room temperature. The filters were not allowed to dry.

c) The filters were washed twice, for 15 minutes each wash, in 100ml of 1X SSC and 0.1% SDS at 42°C.

d) Filters were air dried on a sheet of Whatman 3MM paper

at room temperature.

e) After marking the orientation of the blot the Whatman 3MM paper and filters were covered in a plastic wrap.

f) These were exposed to X-ray film for overnight at -70°C with an intensifying screen and then developed.

11.3 Precautions:

a) Filters were handled only with blunt tipped forceps.

b) Radioactive material was handled with extra care and wastes were disposed separately.

RESULTS

RESULTS

The distinct isolates of PCV were cultured from lyophilized tissue. Extracts from tissues were individually inoculated onto 5-6 days old Phaseolus vulgaris cv. Top crop plants. All these plants were maintained in a glass house at 20-25°C. Inoculated leaves showed veinal necrosis and necrotic spots after 5-6 days of mechanical inoculation. In case of systemic infection veinal necrosis and downward bending of leaves was observed in the subsequent leaves.

All leaf samples having disease symptoms were first tested by ELISA to confirm the presence of H-IPCV and then subjected to purification. H-IPCV isolate was used for obtaining adequate quantities of purified virus. The U.V. absorption spectrum (Fig.1) of purified H-IPCV showed a maximum value of 0.724 at 269.7 nm and a minimum value of 0.652 at 250.5 nm. The maximum/minimum ratio was 1.11. A_{260}/A_{280} ratio was found to be 1.01 indicating a nucleic acid content of 4.5-5% (Thouvenel et al., 1976). Yield of H-IPCV was 2.0± 0.5 mg/100g leaf tissue assuming $E^{0.1,\,1cm}_{260}$ of 3 O.D. where 3 O.D =1mg virus. Purified H-IPCV preprations were used for the following three studies:

Electron microscope study: Micrographs of purified H-IPCV preparation confirmed the existence of two types of rod shaped particles (Fig.2a). No host tissue could be seen (Fig. 2b) suggesting no contamination. At higher magnification (Fig. 3) the presence of a central canal with clearly discernible capsomeres could be seen.

ELISA: The ELISA test using purified H-IPCV preparations was

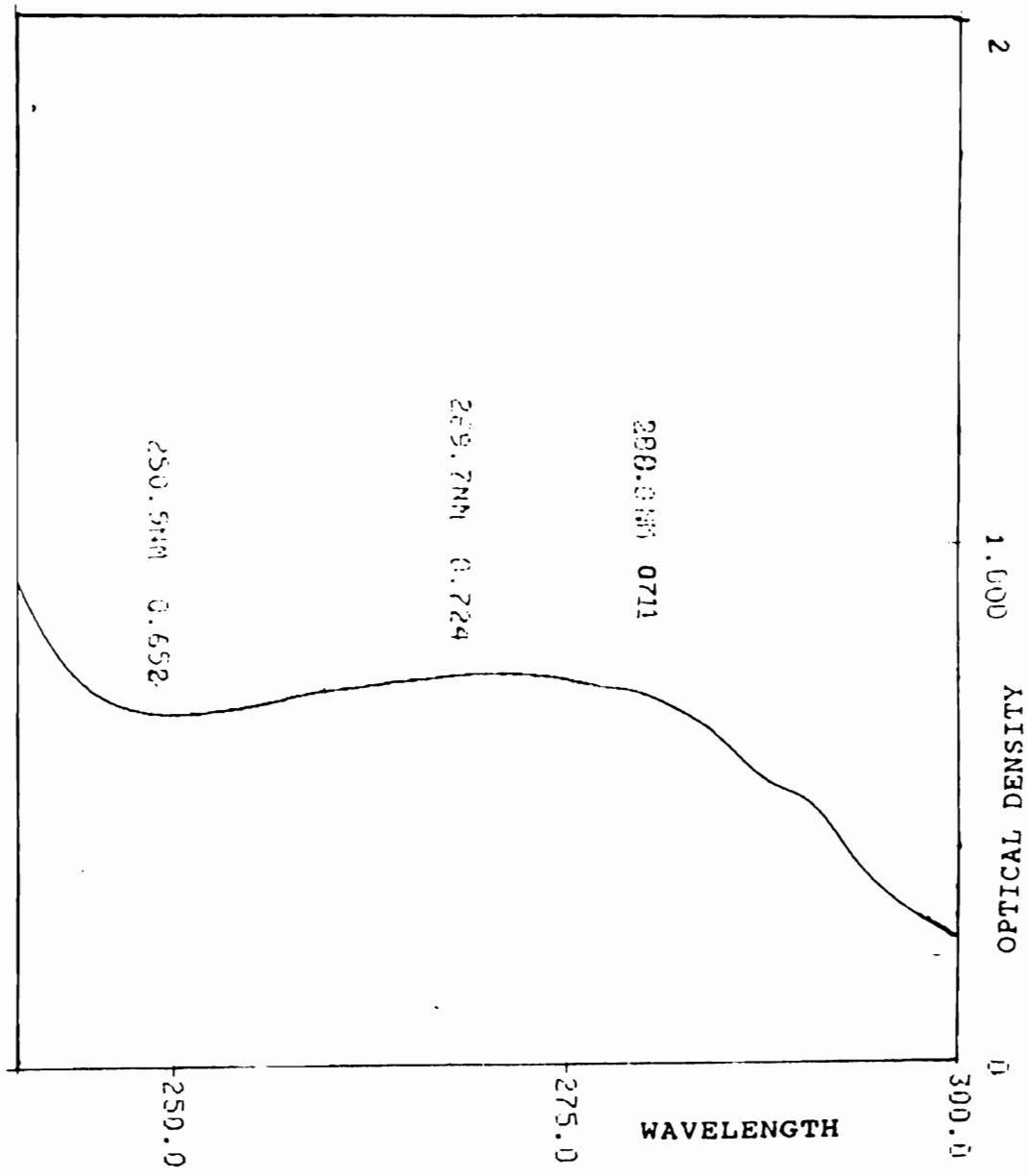
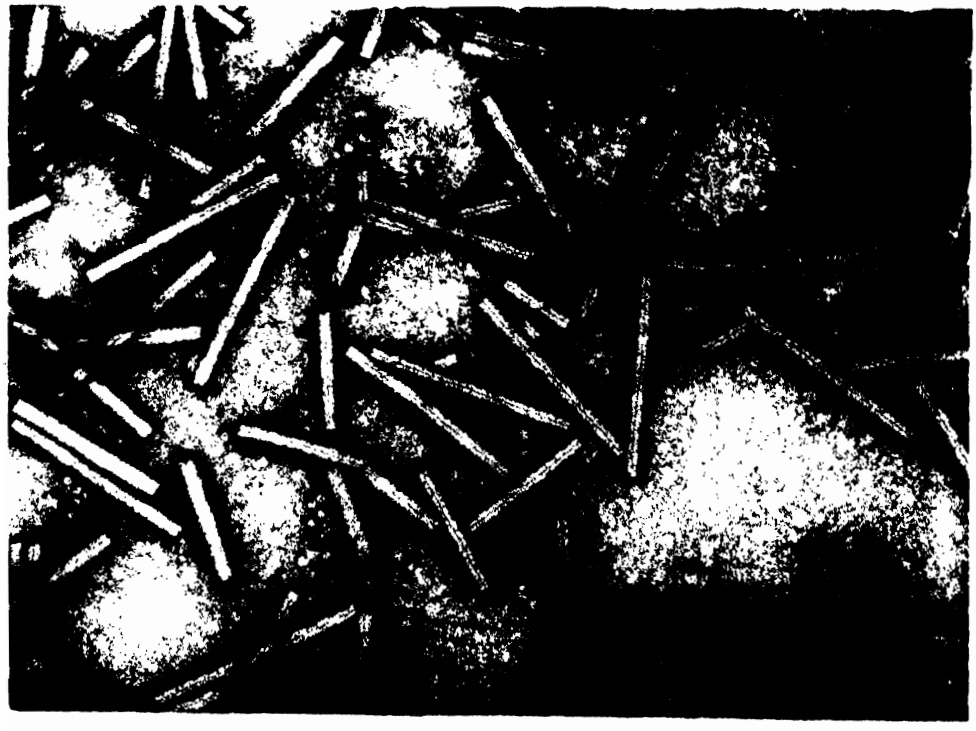
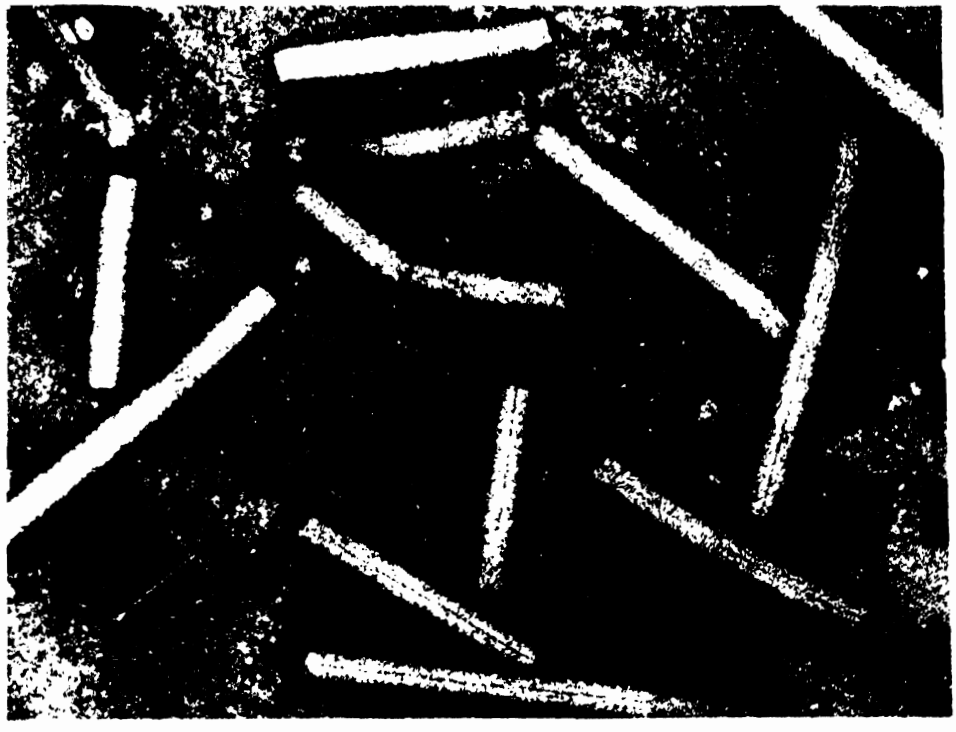


Fig.1: Ultra violet absorption spectrum of purified H-IPCV.



(a) magnification: 870,000 X



(b) magnification: 1,300,000 X (showing no host contamination).

Fig.2: Electron micrographs of purified H-IPCV (Uranyl acetate staining) showing two types with different lengths (indicated by arrows).

magnification: 2,990,000 X

Fig.3: Electron micrographs of H-IPCV showing the presence of central canal with clearly discernible capsomeres (indicated by arrows).

done using different antiserum of IPCV isolates, a WAPCV (N) and tobacco mosaic virus(TMV). The results showed strong reaction of IPCV with homologous antiserum and no reaction with any of the antiserum of other isolates or TMV . The significant difference of the reaction of H-IPCV with other antiserum at different dilutions can be clearly seen in Table 1, where extract from healthy plant was used as control.

Polypeptide analysis: The purified virus was subjected to electrophoresis. Viral polypeptide following electrophoresis was used in Western blots to test serological cross reaction with various antiserum for PCV isolates. The molecular weight of viral polypeptide has been estimated to be close to 24,000 daltons and found similar to the standardized H-IPCV polypeptide (24,000 d) when run together (Fig.4). The major single band observed (Fig.4b) corresponds to the coat protein of H-IPCV (24,000 d). A faint band which was also observed was a low molecular weight polypeptide of extremely low concentration, (shown with arrow) which might be of host origin. These results showed that the purified H-IPCV did not have contamination of high concentration of polypeptides from the host.

Serological tests were done by DAC-ELISA using crude extracts of H-IPCV (from green house) with antisera against different PCV isolates which clearly showed that H-IPCV reacted only with homologous antiserum (Table 2). It did not react with any of the other isolated PCV or TMV. These results demonstrated that H-IPCV is serologically distinct

TABLE 1: Purified H-IPCV isolate (antigen) against antiserum of different isolates. Absorption taken at 620 nm (Replicates of three trials). (@)

ANTISERUM USED	DILUTIONS OF PURIFIED H-IPCV 15mg	0.15mg	0.015mg	HEALTHY EXTRACT 10^{-1}
H-IPCV	1.720 ±0.017	1.555 ±0.079	1.434 ±0.029	0.032 ±0.029
L-IPCV	0.167 ±0.074	0.204 ±0.108	0.034 ±0.032	0.006 ±0.023
B-IPCV	0.075 +0.051	0.088 ±0.067	0.066 +0.012	0.054 +0.042
T-IPCV	0.173 +0.031	0.191 ±0.073	0.064 ±0.011	0.178 ±0.021
WAPCV (N)	0.182 ±0.029	0.126 ±0.096	0.255 ±0.062	0.028 ±0.026
TOBACCO MOSAIC VIRUS	0.179 ±0.072	0.083 ±0.074	0.081 ±0.068	0.059 ±0.036

(@): VALUES DEDUCTED FROM BUFFER CONTROLS.

a b

Fig.4: Electrophoresis of H-IPCV polypeptide on SDS-PAGE used in Western blotting with H-IPCV antiserum. (a) lane showing standardized H-IPCV polypeptide of 24,000 d (marker). (b) lane showing H-IPCV polypeptide from purified preparations. Single band corresponds to coat protein of H-IPCV (24,000 d). The faint band (arrow) of low molecular weight is suspected to be of host origin.

Table 2:Crude extracts of H-IPCV isolate antigen (*Phaseolus vulgaris*)against antisera of different PCV isolates. Absorption taken at 620 nm (Replicates of three trials).(@)

ANTISERA USED	DILUTIONS OF CRUDE H-IPCV EXTRACTS 10^{-1}	10^{-2}	10^{-3}	HEALTHY EXTRACT 10^{-1}
H-IPCV	1.185 ±0.039	1.115 ±0.083	1.062 ±0.035	0.032 ±0.039
L-IPCV	0.156 ±0.035	0.081 ±0.026	0.173 ±0.111	0.004 ±0.002
B-IPCV	0.086 ±0.034	0.144 ±0.083	0.057 ±0.001	0.058 ±0.116
T-IPCV	0.029 ±0.031	0.082 ±0.028	0.078 ±0.031	0.016 ±0.012
WAPCV (N)	0.104 ±0.064	0.166 ±0.132	0.192 ±0.066	0.018 ±0.021
TOBACCO MOSAIC VIRUS	0.034 ±0.021	0.055 ±0..016	0.019 ±0.021	0.027 ±0.036

(@): VALUES DEDUCTED FROM BUFFER CONTROLS.

from other PCV isolates. ELISA tests using crude extracts of H-IPCV (from field sample of groundnut), L-IPCV and WAPCV(N) isolates with anitsera for different PCV isolates. The results (Table 3) showed there was no reaction with non-homologous antiserum and that H-IPCV, L-IPCV and WAPCV(N) were unrelated to one another.

When RNA was extracted from the purified H-IPCV and checked on 1% agarose gel (minigel) two clear bands were observed (Fig. 5). These were RNA-1 and RNA-2 species without any degradation or without host plant nucleic acids. Higher molecular weight RNA (RNA-2) occured at a relatively higher concentration than the low molecular weight RNA (RNA-1). The extracted H-IPCV RNA was run on 1% agarose gel along with standardized RNA marker brome mosaic virus RNA which has a genome in four parts. Two clear bands of RNA were observed (Fig. 6) which were comparable to IPCV RNA of molecular weights 1.9×10^6 and 1.65×10^6 daltons. The H-IPCV RNA preparation was used for the preparation of PCV clones and used in hybridization studies.

The purified H-IPCV RNA was used for preparing first stand of radioactive c-DNA. The amount of incroporation of ^{32}P labeled nucleotides was checked with a Geiger Muller counter and this c-DNA was used in northern hybridization with cold H-IPCV RNA. H-IPCV RNA, total nucleic acids of healthy plant and tobacco mosaic virus (2 replicates) were run on an agarose gel. One set of the replica was stained with ethidium bromide to note the postion and size of the bands as shown in the flurogram (Fig. 7). The second set was

TABLE 3:Crude extracts of three different isolates against their different antisera. Absorption taken at 620 nm (Replicates of three trials).(@)

ANTISERA USED	DIFFERENT DILUTIONS OF CRUDE EXTRACTS 10^{-1}	10^{-2}	10^{-3}	HEALTHY EXTRACT 10^{-1}
USING H-IPCV ISOLATE ANTIGEN Groundnut tissue from field				
WAPCV (N)	0.020 ±0.024	0.024 ±0.026	0.040 ±0.017	0.027 ±0.024
L-IPCV	0.045 ±0.024	0.029 ±0.021	0.027 ±0.012	0.021 ±0.016
H-IPCV	0.190 ±0.033	0.168 ±0.020	0.125 ±0.016	0.013 ±0.011
USING WAPCV(N) ISOLATE ANTIGEN				
H-IPCV	0.029 ±0.012	0.028 ±0.030	0.016 ±0.014	0.006 ±0.012
L-IPCV	0.019 ±0.013	0.067 ±0.031	0.036 ±0.024	0.034 ±0.034
WAPCV (N)	0.194 ±0.041	0.139 ±0.028	0.081 ±0.029	0.023 ±0.019
USING L-IPCV ISOLATE ANTIGEN				
H-IPCV	0.024 ±0.038	0.021 ±0.012	0.034 ±0.021	0.009 ±0.002
WAPCV (N)	0.045 ±0.033	0.046 ±0.034	0.041 ±0.023	0.014 ±0.010
L-IPCV	0.165 ±0.037	0.142 ±0.018	0.129 ±0.014	0.016 ±0.009

(@) : VALUES DEDUCTED FROM BUFFER CONTROLS.

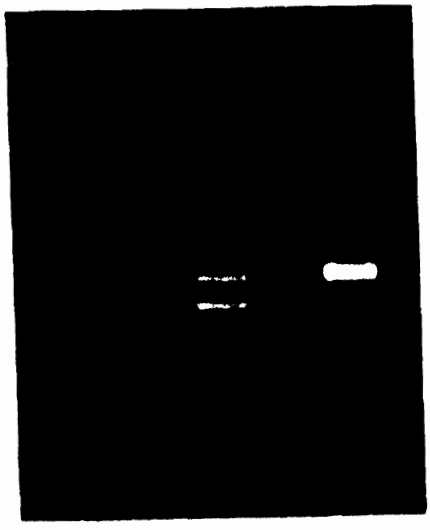


Fig.5: Flourogram of extracted H-IPCV RNA. RNA after extraction run on 1% agarose gel (mini gel) to check the presence and concentration of two RNA species (arrows). (a) 1ug H-IPCV RNA, (b) 2ug of H-IPCV RNA, (c) TMV RNA.

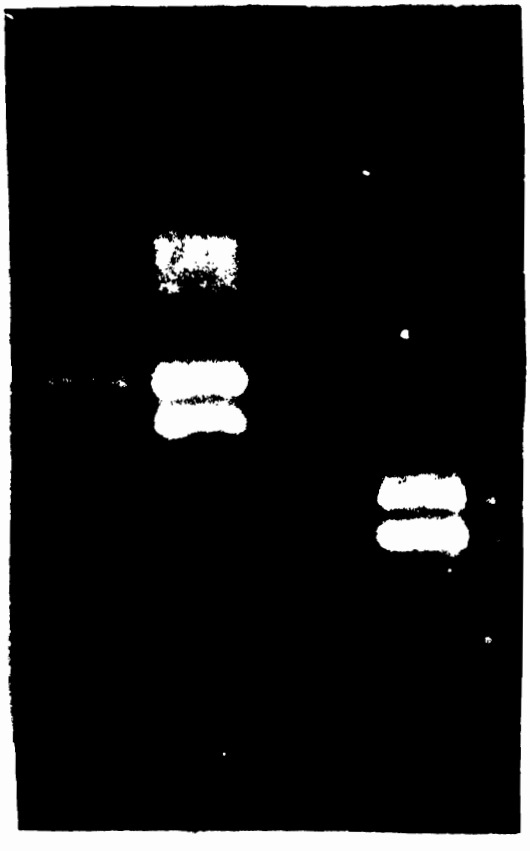


Fig.6: Flourogram of H-IPCV RNA with marker Brome mosaic virus RNA run on 1% agarose gel. (a) and (b) H-IPCV RNA of different concentrations. (c) BMV RNA with four RNA markers of $1.10X10^6$, $0.97X10^6$, $0.72X10^6$ and $0.30X10^6$ daltons

used for northern transfer on to nitrocellulose which was hybridized with radioactive c-DNA. Tobacco mosaic virus RNA was used as a negative control. Two clear bands were observed from the autoradiogram (Fig. 8) indicating complete hybridization of labeled c-DNA with both the RNA species of H-IPCV. No hybridization with TMV RNA or total nucleic acid of healthy plant was observed.

Few clones of H-IPCV were used to make radioactive c-DNA probes by nick translation. The fractions after column chromatography were collected and the level of ^{32}P incorporation was measured in liquid scintillation counter. Incorporated nucleotides showed a major peak. Fractions from the major peak were pooled and were used to probe dot blots prepared with purified virus, viral nucleic acid and with total nucleic acids from infected leaf tissue. Among all the H-IPCV clones analyzed, the 2 Kbp and 3.5-4 Kbp clones were employed in this study. Strong autoradiographic signals (Fig.9) were noticed with the purified H-IPCV and H-IPCV RNA. The signal obtained from partially purified L-IPCV RNA were considerably faint. The total nucleic acid blots showed hybridization with T-IPCV AND B-IPCV isolates. No detectable hybridization with nucleic acids extracted from healthy leaf extracts was observed. It was found that greater hybridization was achieved with 3.5-4 Kbp clone than with 2 Kbp clone used in the present experiment.

Fig.7: Flourogram before northern blotting. (a) H-IPCV showing two species (arrow), (b) total nucleic acid from healthy bean leaf and (c) TMV RNA.

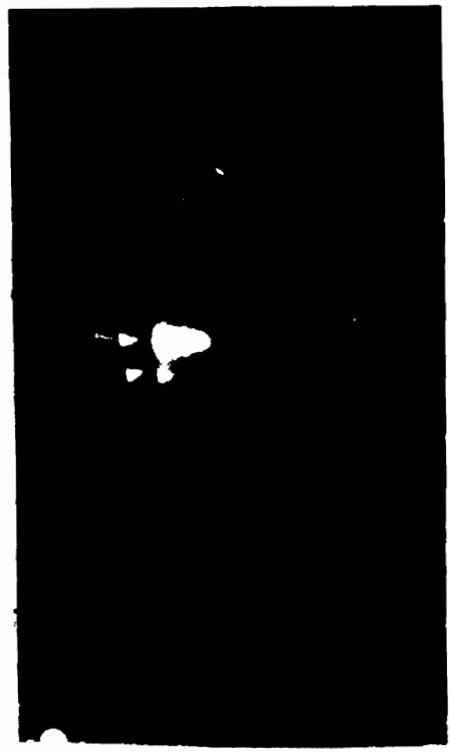


Fig.8: Autoradiogram after northern hybridization with first strand ^{32}P labeled H-IPCV c-DNA. (a) H-IPCV RNA, (b) total nucleic acid of healthy bean leaf, and (c) TMV-RNA

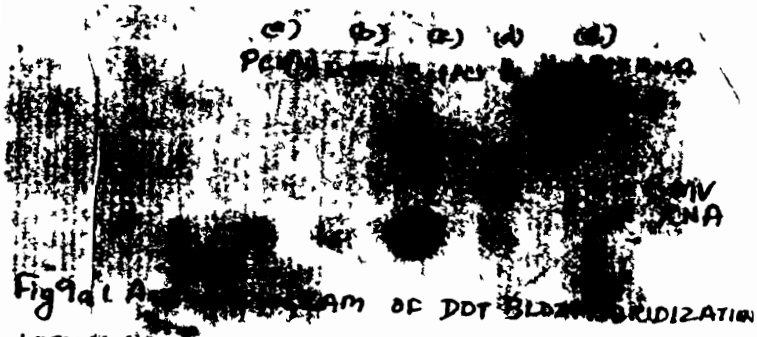


Fig 9a1 AUTORADIOGRAM OF DOT BLOT HYBRIDIZATION WITH H-IPCV C-DNA OF 3.5-4 Kbp CLONE AND TOTAL NUCLEIC ACID OF OTHER PCV ISOLATES.

He - Healthy

Fig 9a2. AUTORADIOGRAM OF DOT BLOT HYBRIDIZATION WITH H-IPCV C-DNA OF 3.5-4 Kbp CLONE AND RNA OF OTHER PCV ISOLATES.

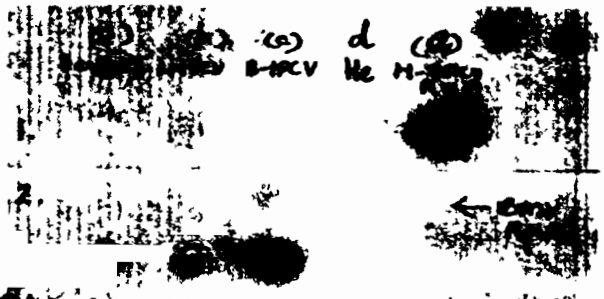


Fig 9 (I). AUTORADIOGRAM OF DOT BLOT HYBRIDIZATION WITH H-IPCV c-DNA OF CLONE AND TOTAL NUCLEIC ACID OF OTHER PCV. ISOLATES.
He - Healthy

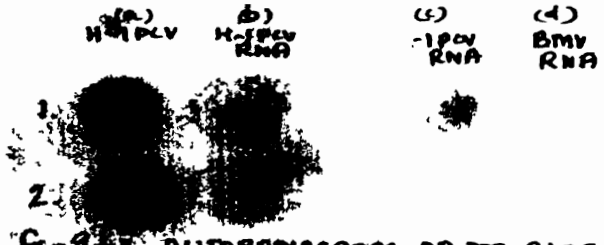


Fig 9 (II). AUTORADIOGRAM OF DOT BLOT HYBRIDIZATION WITH H-IPCV c-DNA OF CLONE AND RNA OF OTHER PCV ISOLATES.

DISCUSSION

DISCUSSION

Peanut clump disease, is a soil and seed-borne virus disease, caused by Indian peanut clump virus occuring in several parts of India and the isolates collected from different geographical locations are distinguishable by their host range and serological tests. One of the major problem is the early detection of this disease which is likely to help in reducing the disease incidence.

In the present investigation the disease symptoms as reported by Reddy et al, (1983) was confirmed after mechanical inoculations which showed both local infection as chlorotic and necrotic spots, and systemic infection evident from veinal necrosis and downward bending of leaves.

The ultra violet absorption spectrum of the purified virus obtained from H-IPCV was very close to that obtained by Thouvenel et al,(1967) working with WAPCV. The electron microscope study of H-IPCV confirmed the existence of two types of rod shaped particles as characterized earlier (Reddy et al., 1982). The ELISA test also suggested that the purification has been complete and there was no contamination. The polypeptide analysis showed coat protein of H-IPCV to be 24,000 d which was also observed earlier and confirmed that it was exclusively of the virus and there was no contamination of the virus or contamination of polypeptides from host.

For serological tests the 'indirect' ELISA procedure was preferred because it was considered that one enzyme

conjugate could be used with all the systems. In this test immobilized antigen was the target for unconjugated globulins in the unfractionated or crude antisera and the trapped antibody was detected by enzyme labeled anti-Fc or anti-IgG conjugates.

This test has already been considered (Reddy et al., 1982) useful for virus detection in field surveys for testing presence of virus in the seeds and determing serological relationships particularly when specific conjugates cannot be prepared.

Penicillinase system presently used has been preferred over the commonly used alkaline phosphate (ALP) and horse raddish peroxide system (HRP), it is economical and the substrate Penicillin-G is readily available which has longer shelf life, because of low molecular weight (2,400 d). It also has a high turn over rate. Penicillinase enzyme is not known to be present in higher plants and animals like peroxidases and other enzymes used in ELISA. The reaction kinetics are linear and can be detected by acid sensitive pH indicators.

The DAC-ELISA test showed strong reaction of H-IPCV with homologous antiserum and there was no rection with any of the antiserum to other four IPCV isolates used or with tobacco mosaic virus. The results clearly demonstrated that H-IPCV is serologically distinct from other PCV isolates. As isolates of PCV are serologiaclly unrelated, a single test like ELISA is not sufficient to detect the presence of these viruses.

It therefore becomes essential to device a simple but alternate method to ELISA which can be done easily in lesser time by using a single probe which can detect any isolate of PCV. Since c-DNA studies have shown that the isolates of PCV have homologies in nucleotide sequences (Reddy et al., 1985), finding out a particular nucleotide sequence which shows maximum hybridization with other PCV isolates, can be useful in making a single probe for PCV detection.

In the present investigation H-IPCV RNA was extracted and used for perparing first strand of radioactive c-DNA and this c-DNA was used in northern hybridization. It was also established that complete hybridization of labeled c-DNA with both the RNA species of H-IPCV RNA was possible. However northern bloting and hybridization of viral nucleic acid appears to be a time consuming procedure and may not be successfully used as a quick and simple method. It is evident that dot blot hybridization technique used can be a better alternative since it is not only quick but economical. It can detect homologous nucleic acid sequences with high sensitivity even when small quantity of nucleic acid is used.

In this present investigation nick translated ^{32}P labeled clones of H-IPCV were used. Though the three isolates H-IPCV, L-IPCV, WAPCV (N) were serologically unrelated, the different isolates of IPCV have been seen to posses nucleotide sequence homologies with each other. This clearly shows that if all the clones of H-IPCV are indeed screened by northern hybridization blots chances are excellent to locate

a clone which can detect several serologically distinct isolates of PCV occuring in India. It is also likely that these clones may hybridize with West African PCV isolates because it was earlier observed that extensive nucleotide sequence homologies exist not only between IPCV isolates but also between IPCV and PCV-WA (Reddy et al., 1985).

Lastly, it is felt that for virus detection in developing countries, especially in laboratories which are not equipped to handle isotopes, it could immensly help if simple non-radioactive labeled probes are made available. It is intended to develop a non-radioactive H-IPCV probe, using dioxigenin-labeled nucleotides which can be indispensable for dectecting clump virus in quarantine, disease surveys and in the studies in epidemiology.

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ABSTRACT

ABSTRACT

Peanut clump (PCV), is a soil-borne viral disease which causes severe stunting and clumping of groundnut plants incurring severe losses. It is very important to detect the clump virus in disease surveys, in screening seed lots in quarantine as well as for virus detection in epidemiological studies. To detect various PCV isolates either a polyclonal or monoclonal antibody with broad specificity may be prepared or a c-DNA probe can be deviced which would be able to detect nucleic acids of several isoloates of PCV

The main aim of the present study was therefore to use the clones of PCV genome and select a clone which would hybridize with RNA of several PCV isolates which could be used in the preparatiion of a c-DNA probe.

For this purpose Northern Dot Blot hybridization technique was used which was probed with nick translated ^{32}P labeled c-DNA clones of H-IPCV.

Among the H-IPCV clones analyzed the 2 Kbp and 3.5-4 Kbp clones were employed in this study to probe the IPCV isolates. Autoradiographic signals showed greater hybridization could be achieved with 3.5-4 Kbp clone than 2 Kbp clone using the pure virus and nucleic acid of H-IPCV and with RNA of partially purified preparations of L-IPCV and total nucleic acids of B-IPCV isolates.