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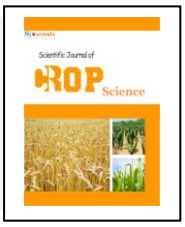

Original article

Physical properties of virus causing cotton mosaic disease

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ABSTRACT

The physical properties were found to be thermal inactivation point (TIP) 55–600C, dilution end point (DEP) 10-2 -10-3, dilutions and longevity in vitro (LIV) 5 hrs. In ELISA test it was found that the virus showing positive test only with anti serum of TSV of cowpea and cotton but negative reaction with PBNV of cowpea and cotton which clearly denied possibility of presence of PBNV in cotton producing these kinds of symptoms. ELISA report clearly showed that TSV antiserum of cowpea was showing positive results with clear chlorotic types of symptoms.

1. Introduction

Cotton belongs to genus Gossypium of the family Malvaceae and has several different species but Cotton varieties grown in India belongs to four distinct species viz. G. arborium, G. herbaceum, G. hirsutum and G. barbadence. It is said to have two centers of origin viz.old world India Indo-China or tropical Africa and new world Mexico or Central America. G. arborium and G. herbacium belongs to old world are known as deshi cotton, where as G. hirsutum and G. barbadense are new world cotton. G. arborium is indigenous to India while G. herbacium seems to have been introduced from Central Asia, and G. hirsutum constitutes the American uplands or Compodia cottons. Cotton is one of the most important fibre crops playing a key role in economic and social status of the world. Cotton locally known as “white gold ”is also a king of cash crops. Cotton contributes nearly seventy per cent of the raw material for the textile industry which earned over ten billion dollar in foreign exchange during 1996-97 (Kairon, 1998).

Viral diseases were reported initially causing significant damage or yield losses but with passage of time some viral diseases began to appear on crop. Of these, a virus disease imparting veinal chlorosis, leaf and stem necrosis extending to midveins and petioles, chlorotic and necrotic spots, leaf distractions resulting in stunting was observed in early stage of crop development (NCIPM -2006) And also mosaic type of symptoms are observed in Punjab and Pakistan which has been caused by Tobacco streak virus (TSV). The cotton mosaic disease is reported in various countries by Martelli & Carvalho (1961) in Brazil, Cauquil and Folin (1983) in USA and Ahmed and nelson (1997) in Pakistan and Punjab. Disease is identified as Cotton mosaic disease caused by Tobacco streak virus of Ilar group. The virus has shown epidemic from in Punjab recently. The virus causing cotton mosaic disease has not been characterized before therefore the present investigation is undertaken to study the characterization of cotton mosaic disease virus.

2. Materials and methods

The studies on physical properties viz. thermal inactivation point(TIP), dilution end point (DEP) and longevity in vitro (LIV) were carried by following the methods outlined by Bos et al.(1960) using cotton infected leaves as source and cowpea cv. Pusa Komal as an assay host.

2.1. Thermal inactivation point

Young infected cotton leaves with mosaic disease showing veinal chlorosis were used to prepare the extract. The standard extract was prepared in 1:1 ratio of tissue to buffer. Aliquots of 2 ml standard extract were pipetted in each glass test tube. The sap was subjected to different heat treatments from 35O C to 9OC with an interval of 5OC for 10 minutes in a thermostatic water bath. After heat treatment, sap was cooled by running tap water on the outside of tubes. Ten uniform sized primary leaves of assay host, of cowpea cv. Pusa Komal were inoculated with sap. No heat treatment served as control. Plants inoculated with sap with no heat treatment served as a control. Inoculated leaves of assay host were washed immediately with water. All inoculated plants were maintained in an insect proof glasshouse. Numbers of local lesions produced were recorded 7 days after inoculation and average numbers of local lesions per leaf were calculated.

2.2. Dilution end point (DEP)

An experiment was conducted to know upto what dilution the sap extracted from infected cotton leaves with Cotton mosaic disease retained infectivity at room temperature. The standard extract was prepared in 1:1 ratio of tissue to buffer and was further diluted in 10 fold (10X) series upto 10-10 dilution. Four replications of each treatment were taken. Ten primary leaves having uniform size of assay host cowpea cv. Pusa Komal were inoculated with sap from different treatments. The primary leaves of assay host inoculated with standard extract served as control. Plants were maintained in insect proof glasshouse. The local lesion count and number of local lesions per leaf were calculated.

2.3. Longevity in vitro (LIV)

An experiment was conducted to know how long the virus retained the infectivity in crude sap extracted from cotton infected leaves with cotton mosaic disease at room temperature. The standard extract prepared in 1:1 ratio of tissue to buffer was kept in test tube with rubber stopper. Ten uniform sized primary leaves of assay host, cowpea cv. Pusa Komal were inoculated at an interval of 5 hrs upto 50 hrs the plants of assay hosts inoculated immediately after extraction of sap served as control. Each treatment was replicated for 4 times. The inoculated plants were maintained in insect proof glasshouse. Local lesions count was taken five days after inoculation and average number of local lesions per leaf was calculated.

2.4. Virus purification

The inoculated leaves of the cowpea cv. Top Crop showing necrotic lesions and veinal necrosis are most suitable for purification.

2.5. Purification procedure

Collect 100g inoculated primary leaves showing necrotic lesions. Blend the leaves in 400ml 0.1M potassium phosphate (KPO4) buffer (pH 8.0), containing 0.75 percent monothioglycerol (V/V) and 0.17per cent

dithiocarbamate(W/V).Use 4 ml buffer for each g of tissue. Filter through two layers of cheese cloth. To the filtrate, add cold chloroform to give 10 per cent (V/V) and emulsify by thorough mixing for 5 to10 min. Centifuge at 6000 x g for 10 min at 50c. Collect the aqueous phase (upper clear layer). Add sodium chloride (NaCl)to the aqueous phase to give 0.2 M,and to this solution ,add polyethylene glycol (PEG, molecular weight 6000-8000) to give 8 per cent (V/W).Stir at 50cfor 90 min. Collect the pellet by centrifuging at 11,000 x g for 10 min. Resuspend the pellet in150 ml of 0.05 M KPO4 buffer (pH 8.) Containing 0.2 per cent Triton X-100. Clarify at 6000 x g for 10 min at 50c. Layer 26 ml of virus suspension on 12 ml of 30 per cent sucrose cushion solution in SW 28 rotor tubes. Centrifuge for 90 min at 95,000 x g. Discard supernatant .Dissolve pellet in 20 ml of 0.05 M KPO4 buffer (pH 8.0) Centrifuge for 10 minute 10,000 x g. Prepare sucrose gradients in a Beckman SW 28 rotor tube by layering 6, 9, 9, 9 ml of 10, 20, 30 and 40 per cent sucrose, respectively, in 0.01 M KPO4 buffer .Allow sucrose solution to form a gradient by leaving them overnight in a refrigerator at 40c. Layer 5 ml of supernatant from step 12 on each sucrose gradient and centrifuge at 40C at 90,000 x g for 1.5 hours. Draw light scattering zones at a depth of 50 to 56 mm and 58 and 64 mm from the bottom of tube. Dilute the zones in 0.01m KPO4 buffer and centrifuge in a Beckman R50 rotor at 96,000xg for 1.5h to pellet the virus. Resuspend the pellets in small quantity {150-200ml} of 0.01m KPO4 buffer for further use.

2.6. Antiserum production

Suspend 100 to 150mg of purified virus in 03 ml of mM phosphate buffer (pH 8.0) with an equal volume of Freunds complete adjuvant. Obtain a thick emulsion by repeatedly drawing into a syringe and ejecting with force. Inject this emulsion sub-cutaneously at multiple sites to a New Zealand white inbred rabbit. Subesquently give 4 intra-muscular and sub-cutaneous injections alternatively at multiple sites at weekly intervals using Freunds incomplete adjuvant. Bleed the rabbit 2 weeks after the last injection and subsequently at weekly intervals. Test the titer of the antiserum using ELISA. A rabbit can be led 6-8 times and each bleeding usually yields 10-15 ml of blood serum. Lyophilize the antiserum in small portions (1ml) and store at 700C.

2.7. Disease Diagnosis

The symptoms of TSV infection are often confused with those caused by PBNV. Therefore identification of infection by TSV should not be based solely on observation of necrotic symptoms. The following methods can be used for the detection of TSV.

2.8. Enzyme linked immuno sorbent assay (ELISA) technique for detection of TSV

A direct antigen coating enzyme linked immunosorbent assay[DAC-ELISA] described by Hobbs et al.[1987] was carried out using goat anti rabbit IgG alkaline phosphate conjugate to detect TSV in cotton using standard dilution of TSV antibody [1:1000].

2.9. Direct antigen coating [DAC] procedure

Do all incubations in a humid chamber. A small rectangular plastic box with moist paper towel is adequate. Take care not to contaminate the glass water intended for storing penicillin BTB solution with buffers salts because the reaction between penicillin and penicillinase is detected by changes in pH due to production of penicillionic acid. Unless the antiserum used is of very high quality, cross adsorption of crude antiserum with healthy plant extracts is recommended. This can be done by grinding healthy leaves in antibody buffer to give 1: 50 dilutions, then filtering through two thickness of chess cloth. Prepare antiserum dilution in healthy plant extracts suspended in antibody buffer. Incubate at 370C [for a minimum of 45 min],prior to adding them to ELISA plates. Cross adsorption of antisera substantially reduces the non specific reaction due to precipitation of antigens of plant origin. Always include appropriate controls, such as comparable dilutions of healthy plant extracts and well coated with coating buffer alone.

2.10. Procedure utiliising alkaline phosphate:

Prepare plant extracts in coating buffer. It is preferable to use dilutions of 1: 10 and above. Use appropriate controls such as a buffer, healthy leaf tissue, leaf tissue from TSV infected plant (positive control) and leaf tissue from any other virus infected plant (negative control). Incubate the plate at 370C for 1h. Dispense 200 ml into each well using a micropipette. Incubate the plate at 370C for 1 hour. Pour out the plant extract and rinse the ELISA plate in PBS-Tween. Follow this by washing the plate in PBS Tween, Allowing 3 min for each wash. It is always

preferable to use antiserum (TSV) cross-absorbed with healthy plant extract. Grind healthy leaves in conjugate buffer to give a 1:20 dilution of antiserum {high tier antisera can be used at 1:10,000 dilution and low tier antisera are used at 1:500 to 1:2000 dilutions}. Incubate the extract containing antiserum for 45 min at 370C. Add 200 µl of the above cross-absorbed antiserum to each well and incubate the ELISA plate at 370C for 1h. Wash the plate in PBS-Tween as in step 2 dilute alkaline phosphate labeled anti- rabbit Ig G or Fc to 1:5,000 or 1:10,000 in antibody buffer. Dispense 200ml into each well and incubate the ELISA plate at 370C for 1h. Wash the plate in PBS-Tween as in step 2. Dissolve 15mg tablet of p-nitrophenyl phosphate (5, 15 or 40mg tablets available) in 30 ml of diethanolamine solution (0.5mg ml-1). Dispense 200µl of this substrate mixture into each well and incubate at room temperature,for 30 min to 1 h. Record the yellow color development visually. Further development of yellow color due to the production of p-nitro phenol can be stopped by the addition of 50 µl of 3 M NaOH well-1. Take absorbance of yellow color of p- nitrophenol at 405 nm in an ELISA reader.

2.11. Substrate buffer for alkaline phosphate system

Dissolve 97ml of diethanol amine in 800ml distilled water. Add conc. HCL to adjust the pH to 9.8. Then make up the volume to 1000ml. Dissolve 10mg tablet of p-nitrophenyl phosphate in 20ml of substrate buffer.

3. Results and discussion

3.1. Physical properties of virus causing cotton mosaic disease

The results on physical properties viz.thermal inactivation point (TIP), dilution end point (DEP) and longevity in vitro (LIV) of the virus causing cotton mosaic disease are given in Table 1, 2 and 3, respectively. In the present investigation physical properties of the virus causing cotton mosaic disease are found to be mostly identical to the physical properties of the virus TSV causing stem necrosis in ground nut and sunflower necrosis disease (Ghanekar et al.; 1979 and Reddy et al.; 2002).

3.2. Thermal inactivation point (TIP)

Results (Table 1) revealed that virus of cotton mosaic disease was inactivated between 50 to 550C for TIP. All the leaves inoculated with cotton mosaic disease on cowpea c.v Pusa Komal produced local lesions at the temperature of 500C and not upto 550C.Number of local lesions decreased progressively as temperature was increased.

Table 1
Thermal inactivation point of virus causing cotton mosaic disease.

Sample no.	TIP (0C)	No of lesions/leaf
1.	Control	45
2.	45	8
3.	50	2
4.	55	0
5.	60	0
6.	65	0
7.	70	0
8.	75	0
9.	80	0
10.	85	0
11.	90	0

3.3. Dilution end point (DEP)

The data (Table 2) has revealed that the virus causing cotton mosaic disease was inactivated in between dilution of 10-2-10-3. For DEP, inoculated leaves of cowpea cv. Pusa Komal produced local lesions up to dilution of

10-2 but not beyond 10-3. At lesser dilution, local lesions produced were more. The local lesions decreased as dilution was increased.

Table 2
Dilution end point of virus causing cotton mosaic disease

Sample no.	DEP	No of lesions/leaf
1.	Control	45
2.	10-1	4
3.	10-2	2
4.	10-3	0
5.	10-4	0
6.	10-5	0
7.	10-6	0
8.	10-7	0
9.	10-8	0
10.	10-9	0

3.4. Longevity in vitro (LIV)

Results (Table 3) reveled that the virus causing cotton mosaic disease was viable for 4 hours but not up to 5 hours at room temperature (27-300C). For LIV all inoculated leaves of assay host cowpea cv. Pusa Komal produced local lesions up to 4 hrs. However the number of local lesions decreased progressively as aging of sap is increased at room temperature.

Table 3
Longevity in vitro of virus causing cotton mosaic disease

Sample No.	LIV (Hours)	No. of lesions/leaf	LIV (Hours)	No. of lesions/leaf
1.	Control	45	Control	45
2.	1	28	5	0
3.	2	16	10	0
4.	3	10	15	0
5.	4	6	20	0
6.	5	0	25	0
7.	6	0	30	0
8.	7	0	35	0
9.	8	0	40	0
10.	9	0	45	0

3.5. ELISA test

ELISA test for virus isolate of cotton mosaic disease was carried out to detect the virus in present given samples. About 10 expected cotton mosaic disease samples were tested by ELISA test. The test reactions are compared with cowpea healthy, cotton healthy, cowpea TSV and cowpea PBNV for check purpose. The results shown by this test are given below in Table 4 and 5.

Ten samples of cotton mosaic disease were tested for TSV and PBNV by ELISA test. ELISA is a rapid method and gives precise results. However, if the necessary expertise and facilities are not available for ELISA, reaction on diagnostic host such as cowpea cv. C-152 and cotton cv. MRC-6301 can help to differentiate TSV from PBNV .Both of these viruses produced characteristics local lesions. The procedure for inoculation of diagnostic host was divided into two phases On inoculated primary leaves of cowpea and cotton, TSV produced necrotic lesions and veinal necrosis within three days whereas PBNV produced only concentric chlorotic/necrotic local lesions five days after inoculation Tobacco streak virus causing cotton mosaic disease can be diagnosed using an ELISA system (Ahmad and Nelson, 1997). The sample of cotton mosaic were tested with two different kinds of antiserums i.e. antiserum

of TSV and antiserum of PBNV. Only Antiserum of TSV of cowpea of cotton has shown positive results .This shows that the virus producing chlorotic or mosaic symptoms in cotton are TSV but not the PBNV.

Table 4
ELISA test by using antiserum of TSV

Sr. No.	Symptoms	ELISA report
1.	Chlorotic and necrotic concentric circles	---
2.	New chlorotic one for the same leaf	---
3.	Leaf showing mite attack spots	---
4.	Necrotic lesions	+
5	New necrotic one on same leaf	+
6	Chlorotic spots	++
7	Chlorotic spots	++
8	Clear chlorotic spots	+++
9	Chlorotic spots	+
10	Necrosis like spots	---
Cowpea healthy		---
Cotton healthy		---
Cowpea TSV		+++
Cowpea PBNV		+++
Buffer		---

Table 5
ELISA test by using antiserum of PBNV

Sr. No.	Symptoms	ELISA report
1.	Chlorotic and necrotic concentric circles	---
2.	New chlorotic one for the same leaf	---
3.	Leaf showing mite attack spots	---
4.	Necrotic lesions	---
5	New necrotic one on same leaf	---
6	Chlorotic spots	---
7	Chlorotic spots	---
8	Clear chlorotic spots	---
9	Chlorotic spots	---
10	Necrosis like spots	---
Cowpea healthy		---
Cotton healthy		---
Cowpea TSV		+++
Cow pea PBNV		+++
Buffer		---

4. Conclusion

Tobacco streak virus (TSV) has not caused disease epidemics in cotton crop but by the observations of TSV in crops such as Ground nut, Sunflower and Soybean it has taken epidemic form so needs to be carefully studied. Many a times it is difficult to distinguish between TSV and PBNV .Techniques have been developed for precise diagnosis of the disease by ELISA and by the reaction of indicator hosts. TSV infects many host plant which are economically very important and survive on many weed hosts under field conditions. Parthenium, a widely distributed and symptom less carrier of TSV, plays a major role in perpetuation and spread of the disease.

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