Serological Detection of Four Plant Viruses in Cucurbitaceous Crops from Bangladesh

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Abstract

In all 21 samples of various cucurbitaceous crops showing virus disease-like symptoms were collected in 1986-87 from different locations of Bangladesh and preserved at 4°C after lyophilization or with calcium chloride. These dried samples were used for serological detection of different cucurbit-viruses, viz., cucumber mosaic virus (CMV), papaya ringspot virus (PRSV/WMV 1), watermelon mosaic virus 2 (WMV 2), zucchini yellow mosaic virus (ZYMV), cucumber green mottle mosaic virus (CGMMV) and squash mosaic virus (SqMV) by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and dot-immunobinding assay (DIBA) in 1989. Out of the 21 samples tested, two, nine, one and three were found to be positive against the antisera of CMV, PRSV, WWV 2 and SqMV, respectively. None of the samples reacted positively against the antisera of ZYMV and CGMMV. Some samples of bottlegourd, bittergourd, cucumber and pumpkin did not react with any of the antisera used.

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Key words: cucurbit-virus, dried sample, DAS-ELISA, DIBA, Bangladesh.

INTRODUCTION

Cucurbitaceous plants include very important crops all over the world^{9,10,19,20}. Among various diseases of cucurbitaceous crops caused by different pathogens such as fungi, bacteria, viruses, nematodes *etc.*, the virus diseases have been given special importance due to non-availability of resistant species, epidemiological hazards and lack of effective control measures^{13,19}. Lovisolo¹⁹ reported that as many as 50 different viruses have been found to be able to infect cucurbits naturally or experimentally. Among these viruses, such as cucumber mosaic virus (CMV), papaya ringspot virus (PRSV/WMV 1), watermelon mosaic virus 2 (WMV 2), zucchini yellow mosaic virus (ZYMV), cucumber green mottle mosaic virus (CGMMV), squash mosaic virus (SqMV) *etc.* cause serious damage to the crops throughout the world^{3,4,11,12,16,17,19,20,22,23,32,33}. Most of these viruses have also been reported from different tropical Asian countries including India and Sri Lanka which are two neighbours of Bangladesh^{12,30,31}. So, it is likely to be thought that some of these important viruses may be prevalent on various cucurbitaceous crops in Bangladesh.

In Bangladesh, several species of cucurbits, viz., cucumber (*Cucumis sativus*), sweetgourd/summer squash (*Cucurbita moschata*), bottlegourd (*Lagenaria siceraria*), whitegourd/waxgourd (*Benincasa hispida*), pumpkin (*Cucurbita maxima*), bittergourd/balsam pear (*Momordica charantia*), spongegourd (*Luffa cylindrica*) and round cucumber (*Cucumis sativus*) are cultivated as major vegetable crops^{25,26}. Some of them are considered to be very important as they provide year round vegetables in Bangladesh²⁶. It is observed that these crops suffer from diseases showing symptoms similar to the virus diseases causing tremendous yield loss every year. However, the virus diseases of crops in Bangladesh remain uninvestigated until now, due to lack

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of proper facilities and trained manpower¹⁾. In the present study, the serological detection of viruses infecting various cucurbitaceous crops in Bangladesh was emphasized.

MATERIALS AND METHODS

Samples. In total 21 samples comprised of nine different species of cucurbitaceous crops were collected from different locations of Bangladesh in 1986–87 (Table 1). The leaves of the plant showing virus disease-like symptoms in the field were collected in small polyethylene bags and immediately processed for drying by lyophilization or with calcium chloride. These dried samples were carried to Japan and stored at 4°C until testing in 1989.

Antisera. Polyclonal antisera of CMV-serotype Y (CMV-Y), PRSV-watermelon strain (PRSV-W), PRSV-papaya strain (PRSV-P), WMV 2, ZYMV, CGMMV-yodo strain (CGMMV-Y), CGMMV-watermelon strain (CGMMV-W) and SqMV were used in the experiments. PRSV-W, WMV 2 and ZYMV antisera were provided by Dr. N. Sako, Saga University, Japan. PRSV-P antiserum was generously given by Dr. D. Gonsalves of Cornell University, USA. Antisera of CMV-Y and SqMV were obtained from by Dr. T. Maeda, Okayama University, Japan and CGMMV (Y and W) antisera were also provided by Dr. M. Kameya, National Agriculture Research Center, Tsukuba, Japan.

Inoculation test. All the 21 cucurbitaceous samples were inoculated separately to the respective host plants and also to some common local lesion hosts several times, following standard inoculation procedures described by Hill¹⁶⁾. The inoculated plants were maintained in an air-conditioned greenhouse, temperature of which was controlled at 20–25°C. The buffer used for inoculation was 0.02 M phosphate containing 0.5% sodium sulfite, pH 7.2.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Purified anti-CMV-Y, -PRSV-W, -WMV 2 and -ZYMV γ -globulin and γ -globulin conjugated to alkaline phosphatase used in the experiment were provided by Dr. N. Sako of Saga University, Japan. The protocol of ELISA was as outlined by Clark and Adams⁹⁾ with slight modifications.

Table 1. List of the cucurbit-samples collected in Bangladesh

No.	Plant samples	Symptoms	Location
1.	Bottlegourd	Mosaic	Nurbag, Gazipur
2.	Bottlegourd	Vein-clearing	Mouchak, Gazipur
3.	Bottlegourd	Mosaic	Gazipur, Gazipur
4.	Bottlegourd	Mosaic	Kashimpur, Gazipur
5.	Bottlegourd	Mosaic	Kashimpur, Gazipur
6.	Bottlegourd	Mosaic	Kashimpur, Gazipur
7.	Bottlegourd	Yellowing	Kashimpur, Gazipur
8.	Bittergourd	Mosaic, Curl	Joydebpur, Gazipur
9.	Bittergourd	Mosaic	Kashimpur, Gazipur
10.	Cucumber	Mosaic	Kashimpur, Gazipur
11.	Cucumber	Mosaic	Kashimpur, Gazipur
12.	Cucumber	Mosaic	Kashimpur, Gazipur
13.	Cucumber	Mosaic	Jessore, Jessore
14.	Round cucumber	Mosaic	Joydebpur, Gazipur
15.	Pumpkin	Mosaic	Kashimpur, Gazipur
16.	Pumpkin	Mosaic	Jessore, Jessore
17.	Pumpkin	Mosaic	Salna, Gazipur
18.	Spongegourd	Mosaic, Vein-clearing	Charpolisha, Jamalpur
19.	Sweetgourd	Mosaic	Katabari, Jamalpur
20.	Whitegourd	Mosaic	Kashimpur, Jamalpur
21.	Zucchini	Mosaic	Joydebpur, Gazipur

Wells of microtiter plate (Nunc, Denmark) were coated with γ -globulin (2 μ g/ml, 200 μ l/well) in 0.05 M carbonate buffer (pH 9.6) containing 0.02% sodium azide and incubated at 37°C for 3 hr. The small amount (*ca.* 0.05 g) of dried samples was macerated with glass rod on Parafilm in a few drops of PBS (0.02 M phosphate buffer, 0.15 M NaCl, 0.02% sodium azide) containing 0.05% Tween 20 and 2% polyvinylpyrrolidone (PVP Av. Mol. Wt. 40,000). An amount of 30 μ l aliquot was poured in each well containing 120 μ l sample buffer (PBS-Tween-PVP) and incubated for 4°C for 24 hr. The enzyme conjugated γ -globulins were applied at 1:1,000 dilution in PBS-Tween-PVP with 2% bovine serum albumin (BSA). After adding conjugated γ -globulin (100 μ l/well), the plate was incubated for 37°C for 3 hr. In each well an amount of 100 μ l of substrate (*p*-nitrophenylphosphate, 1 mg/ml) in 10% diethanolamine containing 0.02% sodium azide (pH 9.6) was applied and incubated for 1 hr at 30°C. The plant leaves infected with each virus were used as positive control. The reaction was stopped by adding 50 μ l of 3 N NaOH per well. Unless otherwise stated, the microtiter plates were washed at least four times with PBS-Tween after each incubations. Absorbance values were measured by using an ELISA analyzer (Immuno Reader NJ-2000) at 405 nm. The experiments were designed so that there was two observations for each sample per plate.

Dot-immunobinding assay (DIBA). In DIBA, polyclonal antisera of CMV-Y, PRSV-W, PRSV-P, WMV 2, ZYMV, CGMMV-Y, CGMMV-W and SqMV were used. DIBA was essentially performed as described by Hibi and Saito¹⁴) with some modifications. Nitrocellulose membrane (NCM, Bio-Rad) was dipped in distilled water for 10 min and dried on filter papers for 5 min. They were then dotted with 2 μ l sample extracts prepared by homogenizing *ca.* 0.05 g dried samples in 0.5 ml TBS (0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5) and dried for 15 min. The NCMs were incubated in blocking solution consisted of 2% BSA, 2% Triton X-100 in TBS for 1 hr, then briefly washed with TBST (Tween 20 in TBS). Antiserum (1:4,000) diluted with 1% healthy leaf extract in TBST containing 0.2% BSA and 2% PVP (polyvinylpyrrolidone) was spotted on the NCM at the rate of 20 μ l/grid and incubated for 50 min. The NCM was rinsed in TBST for three times with gentle shaking for 30 min. Membranes were incubated for 1 hr in conjugate solution prepared by diluting with antibody buffer (1:8,000) and washed in TBST as described previously. Finally, the NCM was reacted with color development solution for 30–60 min as recommended by Bantari and Goodwin⁹). The reaction was stopped by washing the NCM in distilled water and then air dried for visual observation and storage. Unless otherwise stated, the NCMs were incubated at room temperature and all the operations were performed in plastic box. The experiments were repeated at least twice to confirm the results.

RESULTS

DAS-ELISA

As shown in Table 2, three viruses *viz.*, CMV, PRSV and WMV 2 were detected by DAS-ELISA from different cucurbitaceous samples. Among these viruses, PRSV was detected from 9 samples, CMV from two cucumber samples (Sample No. 10 and 11) and WMV 2 from one pumpkin sample (Sample No. 15). None of the samples were reacted positively against ZYMV in ELISA. Although the dried samples stored for 2–3 years were used, viruses were successfully detected without any difficulties due to non-specific reactions in DAS-ELISA. The absorbance values less than 0.1 were regarded as negative in all cases.

DIBA

The results of DIBA are summarized in Table 2. In all PRSV, SqMV, CMV and WMV 2 were detected from 9, 3, 2 and 1 samples, respectively. The mixed infection of PRSV with SqMV was detected from two samples of cucumber and sweetgourd (Sample No. 13 and 19), while round cucumber (Sample No. 14) was found to be singly infected with SqMV. The positive reaction of DIBA was graded by the appearance of distinct purple red spot on the NCM. In

Table 2. Detection of viruses from cucurbit-samples by DAS-ELISA and DIBA a)

No.	Plant sample	Reaction against antibodies of different viruses b)							
		CMV-Y e)	PRSV-P d)	PRSV-W e)	WMV 2 e)	ZYMV e)	CGMMV-Y d)	CGMMV-W d)	SqMV d)
*1.	Bottlegourd	-	-	-	-	-	-	-	-
2.	Bottlegourd	-	+	+	-	-	-	-	-
3.	Bottlegourd	-	+	+	-	-	-	-	-
*4.	Bottlegourd	-	-	-	-	-	-	-	-
*5.	Bottlegourd	-	-	-	-	-	-	-	-
6.	Bottlegourd	-	-	-	-	-	-	-	-
7.	Bottlegourd	-	-	-	-	-	-	-	-
8.	Bittergourd	-	-	-	-	-	-	-	-
9.	Bittergourd	-	+	+	-	-	-	-	-
*10.	Cucumber	+	-	-	-	-	-	-	-
11.	Cucumber	+	-	-	-	-	-	-	-
12.	Cucumber	-	-	-	-	-	-	-	-
13.	Cucumber	-	+	+	-	-	-	-	+
14.	Round cucumber	-	-	-	-	-	-	-	+
*15.	Pumpkin	-	-	-	+	-	-	-	-
16.	Pumpkin	-	+	+	-	-	-	-	-
17.	Pumpkin	-	-	-	-	-	-	-	-
18.	Spongegourd	-	+	+	-	-	-	-	-
19.	Sweetgourd	-	+	+	-	-	-	-	+
20.	Whitegourd	-	+	+	-	-	-	-	-
21.	Zucchini	-	+	+	-	-	-	-	-

a) Same results were obtained both in DAS-ELISA and DIBA.

b) CMV-Y (cucumber mosaic virus-serotype Y), PRSV-W (papaya ringspot virus-watermelon strain), PRSV-P (PRSV-papaya strain), WMV 2 (watermelon mosaic virus 2), ZYMV (Zucchini yellow mosaic virus), CGMMV-Y (Cucumber green mottle mosaic virus-Yodo strain), CGMMV-W (CGMMV-water-melon strain), SqMV (Squash mosaic virus).

c) Samples tested by DAS-ELISA and DIBA.

d) Samples tested by DIBA.

* Infective sample in inoculation test.

general, the appearance of non-specific reactions was regarded as the main problem of DIBA as reported by many researchers^{2,18,24,27}). The antisera used in this study was absorbed with the healthy leaf extracts of the propagation host plant for each virus antigen, and could successfully eliminate this problem.

Inoculation test

Five samples (marked with asterisk in Table 2) out of 21 were found to be infective in inoculation tests. Among these samples three bottlegourd samples (Sample No. 1, 4 and 5) produced mosaic symptoms on bottlegourd and cucumber plants. These isolates also induced necrotic local lesions to *Gomphrena globosa* and chlorotic local lesion on *Chenopodium quinoa* and *C. amaranticolor*. The three isolates produced identical symptoms on the inoculated plants. The cucumber sample (Sample No. 10) also produced similar type of yellow mosaic symptoms on cucumber. The necrotic local lesion on *Pisum sativum* and chlorotic local lesion on *C. quinoa* and *C. amaranticolor* were induced by the cucumber isolates. The only one sample of pumpkin (Sample No. 15) produced systemic mosaic symptoms on pumpkin and sweetgourd and also chlorotic local lesion on *C. amaranticolor* and *C. quinoa*.

DISCUSSION

The results of this study demonstrated that the cucurbitaceous crops in Bangladesh have been infected with CMV, PRSV, WMV 2, SqMV and one unidentified virus isolated from bottlegourd. Among these viruses PRSV was found to be common in nine different cucurbitaceous species tested and distributed to the all areas surveyed (Tables 1 and 2). WMV 2 with a wide host range which has been reported to cause severe damage to cucurbits in India³¹) was detected only from one pumpkin sample. SqMV was detected from cucumber, round cucumber and sweetgourd samples of three different locations, though the virus was not reported from other tropical countries like India, Sri Lanka, Philippines and Thailand^{12,31}). PRSV-W and PRSV-P have been reported as serologically indistinguishable²²). Therefore, we could not identify the strains of PRSV distributed in Bangladesh whether they belong to PRSV-P or PRSV-W.

We failed to detect two important cucurbit-viruses, ZYMV and CGMMV from our samples. However, ZYMV has also been found to be absent in the list of virus diseases of crops in India, Sri Lanka, Philippines and Thailand^{12,30}). So, this virus might not be present in Bangladesh and those tropical Asian countries. But the failure of detecting CGMMV from any samples is of interest as it has been reported to be quite common in India³⁰).

The viruses propagated from the cucumber (Sample No. 10) and pumpkin (Sample No. 15) were detected as CMV and WMV 2, respectively, by both DAS-ELISA and DIBA. The dip preparations of the leaves inoculated with three bottlegourd samples (Sample No. 1, 4 and 5) showed filamentous virus particles identical with the viruses of potyvirus group under the electron microscope. But they were found to be serologically unrelated to all the viruses tested. Further experiments on the other properties of the virus are necessary for identification. Another important aspect of inoculation test is that the viruses in most of the samples were found to be inactivated within 2–3 years storage. PRSV, the most common virus in Bangladesh seemed to be very unstable as all the samples were inactivated during preservation. The transportation of the samples from Bangladesh to Japan which changed the physical conditions of the samples for several days may be one of the reasons of loosing infectivity.

DAS-ELISA has been reported by many researchers as highly sensitive serological method over any other serological methods^{5,7,8}). However, some researchers^{21,28}) reported that DIBA and DAS-ELISA were equal in sensitivity as serological methods. In our study, in which dried samples were used, both methods were found to be equally effective in detecting viruses. These informations suggest that serological methods are very useful to detect plant viruses distributed in an area or country more conveniently even after preservation for long time.

The number of samples used in this study were limited in respect to different cucurbitaceous

species and collection sites. The kinds of anti-virus-sera used were also limited. Therefore, it is difficult to draw a sound conclusion on the virus diseases of cucurbitaceous crops in Bangladesh from this study. However, the results in our study revealed that four viruses serologically identical to PRSV, CMV, WMV 2 and SqMV are distributed on various cucurbitaceous crops in Bangladesh. Some other viruses including bottlegourd isolates which did not react with any antisera used also exist there. These facts emphasize the importance of taking in depth and well-planned research on the cucurbit-viruses in Bangladesh.

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和 文 摘 要

Abdul Mannan AKANDA・津野和宣・脇本 哲：バングラデシュ国に分布するウリ科作物からの4種植物ウイルスの血清学的検出

バングラデシュ国内各地からウイルス性と思われる病徴を呈した9種のウリ科植物から罹病葉21試料を採集し、真空凍結乾燥あるいは塩化カルシウムを用いた乾葉法により乾燥し、4°Cで保存した。約2年間保存後、大部分の試料ではウイルスの感染性を認めることができなかったため、ウリ科植物に寄生性をもつ7種のウイルスに対する8種の抗血清を試し、これらの試料中のウイルスを double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) および dot-immunobinding assay (DIBA) によって検出した。その結果、zucchini yellow mosaic virus と cucumber green mottle mosaic virus は検出されなかったが、cucumber mosaic virus, papaya ringspot virus, watermelon mosaic virus 2 および squash mosaic virus が、それぞれ2, 9, 1 および3点の試料から検出され、papaya ringspot virus がバングラデシュ国のウリ科植物にとくに広く分布するものと考えられた。ユウガオ、ツルレイシ、キュウリおよびカボチャの試料のなかには、用いたいずれの抗血清とも反応しないものが認められた。