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## Cucumber Mosaic Virus in Bangladesh

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As many as 92 different samples belonging to 15 botanical families, showing virus disease-like symptom were collected from various locations of Bangladesh in 1986-87. Plant samples were lyophilized or dried with calcium chloride and preserved at 4°C. Since inactivation of most of the samples was observed in mechanical inoculation to original or closely related host plants in 1989, the dried samples were subjected to double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and dot-immunobinding assay (DIBA) for detecting cucumber mosaic virus (CMV). Two, three, two and one of the samples of cucumber, chilli, pepper and tomato, respectively, were found to be positive against the antiserum of CMV in DAS-ELISA. Of these eight samples, six reacted positively in DIBA. Simplified double-diffusion (SDD) test was also applied to detect CMV from dried samples. All these three methods DAS-ELISA, DIBA and SDD test were found to be useful for the detection of CMV from dried samples. Results demonstrated that the antigenicity of CMV retained at least for 2-3 years after drying even if they had completely lost their infectivity. The results also suggested the occurrence of CMV on cucurbitaceous and solanaceous plants in Bangladesh, but its distribution frequency seemed not so high.

### INTRODUCTION

Bangladesh, a predominately agro-based country, grows many varieties of crops such as legumes, vegetables, fibres, spices etc. as major food and cash crops (Rabbani, 1984 ;Rashid, 1976 ;Rashid, 1981). High prevalence of plant diseases has been reported to limit the yield and quality of crops there (Talukder, 1974 ; Ahmed, 1984). Fakir (1984) reported that hot-humid-tropical climate is one of the main reasons which favours the catastrophic occurrence of plant diseases on various crops in Bangladesh. Among these, the diseases caused by viruses have been found to be high on the basis of the symptomatological observation (Ahmed, 1984). The actual cause of these diseases have not yet been identified due to lack of proper facilities and trained manpower to investigate the plant viruses. However, the identification of viruses infecting various crops is highly important to combat the diseases.

Cucumber mosaic virus (CMV) has been reported to be one of the most economically important aphid-borne viruses infecting a wide variety of hosts including herbaceous and woody plants all over the world (Franki *et al.*, 1979). It has been reported that 775 plant species belonging to 85 families are said to be susceptible to CMV and the highest number of susceptible hosts are listed in Cruciferae, Solanaceae, Compositae, Leguminosae and Cucurbitaceae (Lovisolo, 1981). The virus has been given special attention due to its complex epidemiology as it is transmitted by several

aphid species and through seeds of some hosts and also due to its serious damage to crop production (Cohen, 1982 ; Jones, 1988 ; Conti and Lovisolo, 1982 ; Martelli and Quacquarelli, 1982 ; Phillips and Burnt, 1985). Although, Lovisolo (1981) reported the severe occurrence of CMV on many crops specifically in the temperate zone, its occurrence on various crops has also been reported from the south and south east, tropical asian countries (Kajiware and Konno, 1986 ; TARC, 1977).

This study was, therefore, undertaken to make a serological survey on the occurrence of CMV on different crops in Bangladesh by using dried specimens.

## MATERIALS AND METHODS

### Plant sample used

In all 92 samples representing 39 plant species of 15 botanical families were collected from various fields located in seven different administrative districts of Bangladesh in 1986-87 (Table 1). The leaves of plants showing virus disease-like symptom were collected and dried by lyophilization or with calcium chloride. The dried samples were preserved at 4°C until use.

### Inoculation test

All the samples were inoculated to the original hosts and some common local lesion hosts such as *Chenopodium quinoa*, *C. amaranticolor*, *Gomphrena globosa*, *Nicotiana tabacum*, *N. glutinosa*, *Cucumis sativus*, *Pisum sativum* etc. following the

**Table 1.** List of the samples collected in Bangladesh.

| No.                  | Plant samples  | Symptom               | Location                                     |
|----------------------|----------------|-----------------------|--|
| <b>CUCURBITACEAE</b> |                |                       |  |
| *1                   | Bottle gourd   | Mosaic                | Nurbag <sup>1)</sup> , Gazipur <sup>2)</sup> |
| 2                    | Bottle gourd   | Vein-clearing         | Mouckak, Gazipur                             |
| 3                    | Bottle gourd   | Mosaic                | Gazipur, Gazipur                             |
| *4                   | Bottle gourd   | Mosaic                | Kashimpur, Gazipur                           |
| 5                    | Bottle gourd   | Yellowing             | Kashimpur, Gazipur                           |
| 6                    | Bittergourd    | Mosaic, Curl          | Joydebpur, Gazipur                           |
| 7                    | Bittergourd    | Mosaic                | Kashimpur, Gazipur                           |
| *8                   | Cucumber       | Mosaic                | Kashimpur, Gazipur                           |
| 9                    | Cucumber       | Yellowing             | Kashimpur, Gazipur                           |
| 10                   | Cucumber       | Mosaic                | Jessore, Jessore                             |
| *11                  | Pumpkin        | Mosaic                | Kashimpur, Gazipur                           |
| 12                   | Pumpkin        | Mosaic                | Salna, Gazipur                               |
| 13                   | Round cucumber | Mosaic                | Joydebpur, Gazipur                           |
| 14                   | Spongegourd    | Mosaic, Vein-clearing | Charpolisha, Jamalpur                        |
| 15                   | Sweetgourd     | Mosaic                | Katabari, Jamalpur                           |
| 16                   | Whitegourd     | Mosaic                | Kashimpur, Jamalpur                          |
| 17                   | Zucchini       | Mosaic                | Joydebpur, Gazipur                           |
| <b>SOLANACEAE</b>    |                |                       |  |
| 18                   | Chilli         | Curl                  | Kashimpur, Gazipur                           |
| 19                   | Chilli         | Yellowing, Curl       | Joydebpur, Gazipur                           |
| 20                   | Chilli         | Curl                  | Ragunathpur, Jamalpur                        |
| *21                  | Chilli         | Vein-clearing         | Kashimpur, Gazipur                           |
| 22                   | Chilli         | Mosaic                | Salna, Gazipur                               |
| 23                   | Eggplant       | Mosaic                | Nutonhat, Jessore                            |

|                      |                 |                       |                        |
|----------------------|-----------------|-----------------------|------------------------|
| 24                   | Eggplant        | Mosaic                | Kashimpur, Gazipur     |
| 25                   | Eggplant        | Yellowing             | Kashimpur, Gazipur     |
| 26                   | Eggplant        | Vein-clearing         | Kashimpur, Gazipur     |
| 27                   | Eggplant        | Mosaic                | Salna, Gazipur         |
| 28                   | Pepper          | Curl                  | Gazipur, Gazipur       |
| 29                   | Pepper          | Enation               | Kashimpur, Gazipur     |
| 30                   | Tomato          | Curl                  | Joydebpur, Gazipur     |
| 31                   | Tomato          | Purple                | Joydebpur, Gazipur     |
| 32                   | Tomato          | Yellowing             | Kashimpur, Gazipur     |
| 33                   | Tomato          | Curl                  | Salna, Gazipur         |
| 34                   | Tomato          | Necrosis              | Salna, Gazipur         |
| 35                   | Tomato          | Purple                | Salna, Gazipur         |
| 36                   | Tomato          | Crinkle               | Salna, Gazipur         |
| 37                   | Tomato          | Yellowing             | Salna, Gazipur         |
| <b>LEGUMINOSEAE</b>  |                 |                       |                        |
| 38                   | Asparagus bean  | Yellowing             | Salna, Gazipur         |
| 39                   | Blackgram       | Mosaic                | Pars, Jamalpur         |
| 40                   | Blackgram       | Mottle                | Pars, Jamalpur         |
| 41                   | Blackgram       | Yellow mosaic         | Rahmatpur, Barisal     |
| 42                   | Blackgram       | Mosaic, Chlorosis     | Nashopur, Dinajpur     |
| 43                   | Country bean    | Mosaic                | Salna, Gazipur         |
| 44                   | Cowpea          | Mosaic, Vein-clearing | Jessore, Jessore       |
| 45                   | Cowpea          | Mosaic                | Salna, Gazipur         |
| *46                  | Cowpea          | Mosaic                | Joydebpur, Gazipur     |
| 47                   | Hyacinth bean   | Mosaic                | Bhaorber, Jessore      |
| 48                   | Longbean        | Mosaic                | Kashimpur, Gazipur     |
| 49                   | Longbean        | Necrosis              | Kashimpur, Gazipur     |
| 50                   | Longbean        | Shrinking             | Kashimpur, Gazipur     |
| 51                   | Longbean        | Yellow spots          | Kashimpur, Gazipur     |
| 52                   | Longbean        | Yellowing             | Kashimpur, Gazipur     |
| 53                   | Mungbean        | Curl, Mottle          | Gabua, Patuakhali      |
| 54                   | Pigeonpea       | Mosaic                | Ragunathpur, Jamalpur  |
| 55                   | Soybean         | Mosaic                | Salna, Gazipur         |
| 56                   | Soybean         | Mosaic                | Ishurdi, Pabna         |
| 57                   | Soybean         | Mosaic                | Kashimpur, Gazipur     |
| 58                   | Yard long bean  | Vein-clearing         | Joydebpur, Gazipur     |
| 59                   | Yard long bean  | Mosaic                | Joydebpur, Gazipur     |
| <b>PAPILIONACEAE</b> |                 |                       |                        |
| 60                   | Groundnut       | Yellowing             | Rahmatpur, Barisal     |
| 61                   | Groundnut       | Mosaic                | Loknathpur, Chuadanga  |
| <b>CARICACEAE</b>    |                 |                       |                        |
| *62                  | Papaya          | Fern leaf             | Kashimpur, Gazipur     |
| *63                  | Papaya          | Mosaic                | Joydebpur, Gazipur     |
| 64                   | Papaya          | Mosaic                | Kashimpur, Gazipur     |
| *65                  | Papaya          | Vein-clearing         | Salna, Gazipur         |
| *66                  | Papaya          | Mosaic                | Patuakhali, Patuakhali |
| 67                   | Papaya          | Mosaic                | Salna, Gazipur         |
| <b>CRUCIFERAE</b>    |                 |                       |                        |
| 68                   | Cauliflower     | Mosaic                | Salna, Gazipur         |
| 69                   | Chinese cabbage | Mosaic                | Kashimpur, Gazipur     |
| 70                   | Chinese cabbage | Yellowing             | Kashimpur, Gazipur     |
| 71                   | Chinese cabbage | Curl                  | Kashimpur, Gazipur     |
| 72                   | Chinese cabbage | Chlorosis             | Kashimpur, Gazipur     |
| 73                   | Radish          | Mosaic                | Salna, Gazipur         |
| <b>MALVACEAE</b>     |                 |                       |                        |
| 74                   | Cotton          | Mosaic                | Basherhat, Dinajpur    |
| 75                   | Cotton          | Vein-clearing         | Basherhat, Dinajpur    |

|              |            |               |                       |
|--------------|------------|---------------|-----------------------|
| 76           | Cotton     | Chlorosis     | Basherhat, Dinajpur   |
| 77           | Okra       | Mosaic        | Kashimpur, Gazipur    |
| 78           | Okra       | Vein-clearing | Salna, Gazipur        |
| 79           | Okra       | Mosaic        | Rahmatpur, Barisal    |
| 80           | Okra       | Mosaic        | Salna, Gazipur        |
| TILIACEAE    |            |               |                       |
| 81           | Jute       | Mosaic        | Ragunathpur, Jamalpur |
| 82           | Jute       | Vein-clearing | Ragunathpur, Jamalpur |
| 83           | Jute       | Mosaic        | Charpolisha, Jamalpur |
| ARACEAE      |            |               |                       |
| 84           | Colocasia  | Vein-clearing | Ragunathpur, Jamalpur |
| 85           | Colocasia  | Mosaic        | Salna, Gazipur        |
| UMBELLIFERAE |            |               |                       |
| 86           | Carrot     | Necrosis      | Kashimpur, Gazipur    |
| 87           | Coriander  | Necrosis      | Joydebpur, Gazipur    |
| COMPOSITAE   |            |               |                       |
| 88           | Lettuce    | Chlorosis     | Kashimpur, Gazipur    |
| LILIACEAE    |            |               |                       |
| 89           | Garlic     | Mosaic        | Joydebpur, Gazipur    |
| ASTERACEAE   |            |               |                       |
| 90           | Safflower  | Mosaic        | Joydebpur, Gazipur    |
| PIPERACEAE   |            |               |                       |
| 91           | Betel leaf | Mosaic        | Rahmatpur, Barisal    |
| MUSACEAE     |            |               |                       |
| 92           | Banana     | Bunchy-top    | Kahmatpur, Barisal    |

<sup>1)</sup> : Name of collection site

<sup>2)</sup> : Name of administrative district

methods of Hill (1984). Inoculations were done in a temperature controlled greenhouse (20–25°C).

### Antiserum and virus

Polyclonal rabbit antiserum to the yellow strain of CMV (CMV-Y) was used in all the experiments. The antiserum was absorbed with insolubilized healthy tobacco leaf proteins before use. For DAS-ELISA,  $\gamma$ -globulin was purified from the absorbed antiserum by ammonium sulfate precipitation followed by DEAE cellulose column chromatography (Clark and Bar-Joseph, 1984). In DIBA test,  $\gamma$ -globulin solution was absorbed with extract of healthy tobacco leaves to eliminate trace amount of antibodies against healthy components. The antiserum of CMV-Z (serotype P) was also used in SDD test.

### Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

DAS-ELISA was performed essentially as described by Clark and Adams (1977) with slight modifications. Wells of polystyrene microtiter plates were coated with  $\gamma$ -globulin (2  $\mu$ g/ml) for 3 hr at 30°C. The crude extract was triturated ca. 0.05 g dried samples in 0.5 ml of sample buffer on Parafilm by using glass rods. An amount of 30  $\mu$ l of homogenate was poured in each well of plates containing 120  $\mu$ l of sample buffer and incubated overnight at 4°C. The enzyme conjugate (1 : 800 dilution) was reacted for 3 hr at 30°C. Artificially infected tobacco leaves with CMV-Y (dried

samples) were used as positive control. The ELISA values were measured by using an ELISA analyzer (Immuno Reader NJ-2000, Inter med) at the wavelength of 405 nm.

#### **Dot-immunobinding assay (DIBA)**

With some simple modifications, DIBA procedures were carried out following the method described by Powell (1987). Dried samples were macerated in TBS (0.02 M Tris-HCl, 0.5 M NaCl, pH7.5) on Parafilm with glass rods. Nitrocellulose membrane (NCM, Transfer Blot, Bio Rad) was immersed in distilled water and placed on filter paper for 5 min to dry. Two  $\mu$ l of extract was spotted on the NCM and dried for 10 min. The NCM was put in blocking solution consisted of 2 % BSA (bovine serum albumin) and 2 % Triton X-100 in TBS for 1 hr, followed by washing with TBST (TBS containing 0.05 % Tween 20). The NCM was placed in a plastic box and 20  $\mu$ l/grid of  $\gamma$ -globulin (2 $\mu$ g/ml) diluted with 1 % healthy tobacco leaf extract in TBST containing 0.2 % BSA and 2 % polyvinylpyrrolidone (antibody and conjugate buffer) was added and the membrane was incubated for 2 hr at room temperature. The NCM was washed three times with TBST. The membrane was then incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate diluted 2,000 times with the antibody buffer and washed as described earlier. The NCM was finally, incubated for 30-60 min in color development solution prepared by mixing fast red TR salt (Sigma) and naphthol AS-MX phosphate (Sigma) as recommended by Bantari and Goodwin (1985). The reaction was stopped by washing the NCM in distilled water after 30 min incubation and then air dried for visual observation.

#### **Simplified double-diffusion (SDD) test**

Some of the samples positive in DAS-ELISA and DIBA were tested by simplified double-diffusion test for sero-diagnosis of CMV. SDD test was performed as described by Shohara and Inoue (1978). The crude extract was prepared by macerating dried samples in 24 % Noble Agar (Difco), 0.85 % NaCl and 0.2 % sodium azide in 0.2 M Tris-HCl buffer (pH7). The antiserum used in the experiment was diluted to, 1 : 2. Results were recorded after 22 hr incubation at 30°C. Hexagonal arrangement of wells was applied.

#### **Virus purification**

Two infective samples-cucumber (8) and chilli (21) propagated on cucumber and chilli, respectively were used for the purification of the virus. The virus was partially purified following the method described by Maeda et al. (1983) with some modifications. Infected leaves of cucumber and chilli were separately used for purification. For each 100 g of leaves homogenized with mortar and pestle in 200 ml of 0.5 M citrate buffer (pH 6.5) containing 0.025 M EDTA. Homogenate was filtered through double-layered cheese cloth. The filtered extract was stirred for 20 min at 4°C with 1/5 volume of chloroform followed by centrifugation at 5,000 g for 15 min and the supernatant was collected. The supernatant was stirred with 1 % Triton-X 100 for 20 min and then 8 % PEG was added. The mixture was centrifuged at 8,000 g for 20 min and collected the pellet. The pellet was resuspended in 0.005 M sodium borate buffer (pH 8.0) containing 0.005 M EDTA followed by centrifugation at 8,000 g for 20 min and the supernatant was collected. A small amount of 20 % sucrose was layered onto the

borate-EDTA buffer following layering of virus suspension. This was then centrifuged at 87,650 g in Hitachi 65 P ultracentrifuge (RPS 65 T rotor) for 90 min. The pellet resuspended in the borate-EDTA buffer contained the partially purified virus.

### Electron microscopy

The partially purified virus was fixed with 2 % formaldehyde and negatively stained with 2 % uranyl acetate or 2 % phosphotungstic acid (pH 6.0). The particle size was measured by a transmission electron microscope (JEM-100 S, JEOL Ltd.).

## RESULTS

### Inoculation test

Ten different samples marked with asterisk in Table 1 were found to be infective when inoculated to their original hosts in the greenhouse. All others remained inactive in repeated inoculation. Among these ten infective samples, one cucumber (Sample No. 8) and one chilli (Sample No. 21) were later confirmed as CMV. All other infective samples were identified as viruses other than CMV (our unpublished data).

On sap inoculation, both the virus isolates (cucumber, Sample No. 8 and chilli, Sample No. 21) produced local lesions on *Chenopodium quinoa*, *C. amaranticolor*, *Gomphrena globosa* and *Pisum sativum* typical for CMV. The isolates also induced mosaic symptoms typical for CMV on the leaves of inoculated *Nicotiana tabacum* (White burley, Xanthi and Samsun NN), *N. glutinosa*, cucumber and chilli (Jones, 1988 ; Phillips and Runt, 1985 ; Tobias et al., 1982).

### DAS-ELISA

Out of 92 samples tested, eight samples were found to be CMV-positive in DAS-ELISA (Table 2). Positive samples included the samples representing the four plant species such as cucumber, chilli, pepper and tomato among the 39 different plant species tested.

### DIBA

All the samples tested by DAS-ELISA were included in the DIBA. The positive reactions were recognized by the appearance of well-defined purple red spot on the

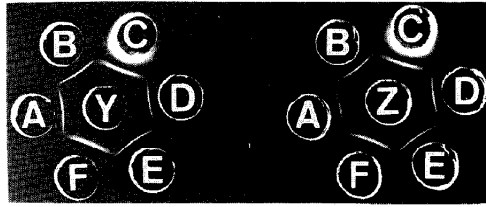
Table 2. Samples positive in DAS-ELISA, DIBA and SDD test.

| Plant samples               | DAS-ELISA       | DIBA | SDD test |
|-----------------------------|-----------------|------|----------|
| *Cucumber (8) <sup>a)</sup> | + <sup>b)</sup> | +    | +        |
| Cucumber (9)                | +               | +    | +        |
| Chilli (18)                 | +               |      | rlt      |
| Chilli (20)                 | +               | +    | +        |
| *Chilli (21)                | +               | t    | +        |
| Pepper (29)                 | +               | +    |          |
| Tomato (30)                 | +               | +    | nt       |
| Tomato (32)                 | +               |      |          |

\*) : Infective sample in inoculation test.

a) : Sample number in table 1.

b) : + : Positive reaction, - : Negative reaction, nt : Not tested.



**Fig. 1.** Simplified double-diffusion (SDD) test by using dried sample. Y, Antiserum of CMV-Y ; Z, Antiserum of CMV-P ; A, Chilli (Sample No. 20 ; B, CMV control sample; C, Cucumber (Sample No. 8); D. Chilli (sample No. 21), E, CMV control sample; F, Cucumber (Sample No. 9).

nitrocellulose membrane. Results of DIBA are summarized in Table 2. In total six samples were found to be positive in DIBA. Two samples positive in DAS-ELISA were reacted negatively in DIBA. Appearance of non-specific reactions has been reported as only problem in DIBA was successfully eliminated by absorbing antisera with 1 % healthy leaf extracts.

#### SDD test

As shown in Table 2 and Fig 1, four different samples out of six tested, produced distinct precipitin bands in SDD test. Two samples found to be positive in DAS-ELISA and DIBA did not produce any precipitin bands in SDD test.

#### Electron microscopy

The isometric virus particles measuring about 30 nm in diameter were observed under electron microscope from partially purified cucumber and chilli samples.

### DISCUSSION

The results of this study demonstrated the occurrence of CMV on cucurbitaceous (cucumber) and solanaceous (chilli, pepper and tomato) plants in Bangladesh. Several other samples of different plant species including Leguminosae and Cruciferae reacted negatively, though these two families reported to have the highest numbers of hosts susceptible to CMV (Franki *et al.*, 1979 ; Lovisolo, 1981). Moreover, the frequency of CMV positive samples even in cucurbits and solanaceous plants were not so high (8/37) in this study. Hot-humid-tropical climate of Bangladesh might be one of the reasons of low prevalence of the virus there, as Lovisolo (1981) reported the high prevalence of CMV specially in the temperate zone.

Three serological methods, DAS-ELISA, DIBA and SDD test, were found to be useful for the detection of CMV from dried samples. The loss of infectivity of the virus during storage did not interfere with the detection of antigenicity in serodiagnosis. The highest number of samples, eight were found to be CMV positive in DAS-ELISA followed by six in DIBA and four in SDD test. Results suggested DAS-ELISA was the highest in sensitivity of DAS-ELISA as compared to two other methods used. The superiority of DAS-ELISA as a serological method over any others has been reported



by many researchers (Clark, M. F. 1981; Clement *et al.*, 1986). However, Ozyanar and Sako (1987) reported the equal sensitivity of DAS-ELISA and DIBA in detecting CMV, when they used either purified virus or crude leaf extracts of artificially inoculated plants in which the antigen concentrations and specificity were expected to be high compared to field samples used in our experiments. SDD test was found to be less sensitive than either of the methods.

Six CMV positive samples out of eight were found to be inactivated in storage within 223 years. McKinny et al. (1965) reported that CMV remained infective up to 15 years in storage. However, it has been reported that the longevity of virus infectivity varied with many factors such as host plants, drying methods, storage conditions etc (McKinny and Silber, 1968). Moreover, in our case, drastic change of temperature condition for several days during transportation of the samples from Bangladesh to Japan might be one of the main cause of inactivation of the virus.

In our study, the samples were irregular in number and limited in respect of plant species and areas surveyed. The high prevalence of CMV in Bangladesh may be expected in winter crops as the virus was reported to be common in the temperate zone.

However, to draw a sound conclusion on the occurrence of CMV in Bangladesh requires extensive survey considering the epidemiology and host range of the virus. Results of our study was suggestive, rather than conclusive to take well-planned research on CMV in Bangladesh.

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