Seed transmission of an Indian isolate of Cucumber mosaic virus infecting chilli (Capsicum annuum L.)

ABSTRACT: Chilli (*Capsicum annuum.* L) is an economically valuable spice crop worldwide and *Cucumber mosaic virus* (CMV) is the major constraint for its production. To study its seed transmissibility nature, the chilli seedlings were sap-inoculated with CMV isolate (CMV-TSWA) from Warangal district, Telangana and raised under insect proof glasshouse condition until seed production. The CMV-infected plants developed mosaic, mottling, deformation, systemic infection, stunted appearance, delayed flower formation, deformed fruit production and reduced seed production compared to uninfected healthy plants. The virus was detected in the leaves, flowers, green fruit, dry pericarp and seeds of sap-inoculated chilli plants by DAC-ELISA and the percentage of detection was 83.33% in leaves, 46.66% in flowers, 20% in fruits, 16.66% in pericarp of dried chilli and 13.33% in seeds. Seedlings emerged from the infected seeds initially showed mottling and wrinkling of leaves, which further intensified as leaf deformation and stunted appearance compared to healthy ones, and the presence of CMV was confirmed by DAC-ELISA. CMV is seed-borne in chilli and transmitted from seed to seedlings and the rate of transmission was 9% as determined by grow-out test and DAC-ELISA.

Key words: *Cucumber mosaic virus, chilli, seed-borne, seed transmission, grow-out test, DAC-ELISA*

1. INTRODUCTION

Chilli (*Capsicum annuum.* L) is one of the most valuable cultivated spice and vegetable crops worldwide. It is cultivated globally for its fruits that are used for various purposes like vegetable, pickles, spices and condiments. There are approximately 25 recognised species in the genus *Capsicum* globally and *C.annuum* is the most widely and commonly cultivated species for its pungent and non-pungent types [1]. India is the world's largest producer, consumer and exporter of chilli and Indian chilli is well recognized in the global market due to its colour and pungency.

Chilli cultivation is under constant threat by many pests and diseases and about 35 viruses are known to infect chilli globally [2]. Among the viruses, *Cucumber mosaic virus* (CMV) is the most significant constraint to chilli production worldwide [3]. CMV has a broader host range infecting more than 1241 species [4]. It is transmitted by aphids in a non-persistent manner and in some hosts through infected seeds. Vertical transmission through seed from one season to another plays a major role in survival of the virus [5] as the plant grown from infected seed acts as a primary source of inoculum from which the virus is disseminated by aphids. Even though, very low rate of transmission through seed is reported in many crops, it is important for perpetuation, overwintering and long-distance dissemination of the virus [6]. CMV transmission through seeds has been described in few species based on biological or serological techniques [3] and polymerase chain reaction [6, 7]. Embryo transmission of CMV has been reported in bean, spinach [8], lentil [9], lupin [10] and pepper [6, 7]. The detection of CMV either in seed lots or in plants germinated from seeds has been reported in common bean, *Vigna unguiculata* and *V. radiata* [11, 12]; pea and faba bean [13]; chickpea, vetch, and several clovers [14]; tomato [15]; alfalfa [16] and other few crops. The present study was undertaken to evaluate, whether CMV is transmitted though seeds in chilli under Indian conditions.

2. MATERIALS AND METHODS

2.1 Collection and maintenance of CMV isolate: The young chilli leaves showing typical CMV symptoms were collected from Warangal district, Telangana and subjected to Direct Antigen Coating - Enzyme Linked Immunosorbent Assay **(**DAC-ELISA) using polyclonal antiserum. The sample, which reacted positive to CMV antiserum, was chosen and designated as CMV-TSWA (*Cucumber mosaic virus* - Telangana state Warangal) isolate. The seeds of Pusa Jwala variety were sown in pots

containing sterilized soil and vermicompost mixture at 3:1 ratio. The young leaves (3-4 leaf stage) of 20 days old seedlings (30 in number) were sap-inoculated with CMV-TSWA isolate and the uninoculated healthy plants served as control.

2.2 Sap inoculation and production of CMV infected seed:
The mechanical sap inoculation was carried out by extracting sap from the symptomatic leaf sample in chilled 0.1 M phosphate buffer (pH 7.2) containing 0.1% 2-mercaptoethanol in a pestle and mortar kept in an ice tray. The extracted sap was rubbed gently on the young leaves of cucumber (*Cucumis sativus*) and chilli seedlings dusted with carborundum, which were then washed off with tap water after 5-7 min to remove excess inoculum. The inoculated plants were maintained under insect proof glasshouse conditions for further studies. All the plants were kept under observation for development of symptoms under insect proof glasshouse until seed production. The observation on symptom expression and number of flowers produced were taken on alternate days while the number of fruits produced and its shape were recorded once in a week. The leaves, flowers, green fruit, pericarp of dry chilli and seeds were collected from both CMV-infected and healthy plants at various time and analysed for the presence of CMV.

2.3 Direct Antigen Coating – Enzyme Linked Immunosorbent Assay (DAC-ELISA): The samples taken from CMV inoculated and healthy plants such as leaves, flowers, green fruit, pericarp of dry chilli and seeds were subjected to DAC-ELISA [17] using 96-well Tarson microtitre plates. Each sample was technically replicated thrice and tested. The polyclonal antibody against CMV was obtained from DSMZ, Germany (product number AS-0929). Two fifty microgram sample taken from different plant parts (leaves - 2 in numbers, flowers - 4-5 in numbers, green fruit - 1 in number, pericarp of dry fruit - 2 in numbers and seeds - 25-30 in numbers pooled as one sample) was homogenized in 500 µl of coating buffer using pestle and mortar and 100 µl of the extract was loaded in each well of ELISA plate. Extracts from 30 inoculated samples, positive control, negative control, and buffer control were added to the respective wells. The plate was covered with aluminium foil and incubated at 37 ˚C for 2 hours and washed three times with PBS-T buffer. Then, blocking solution of PBS-T with 5% skimmed milk was added to the plate and incubated at 37˚C for 2 hours. After washing three times with PBS-T, CMV polyclonal antibody, diluted in antibody buffer at1:200 dilution, was added to the wells and kept at 37˚C for 2 h for incubation. Then, the plate was washed three times with PBS-T and anti-rabbit IgG (produced in goat) conjugated with alkaline phosphatase enzyme (1:7000 dilution) was added at 100 µl per well and kept for 2 h at 37 ˚C. Finally, the wells were washed three times with PBS-T and 100 µl of 0.5 mg substrate *p*-nitrophenyl phosphate dissolved in one ml of diethnolamine substrate buffer (pH 9.8) was added in each well and the plate was kept under dark for 30 minutes for colour development. The reaction was stopped by addition of 50 µl of 3 M NaOH to each well and the absorbance was read at 405 nm in Biotek - ELISA micro plate reader.

2.4 Grow-out test: The seeds were collected from CMV-infected chilli plants and sown in disposable cups having sterile soil and vermicompost for grow-out test and maintained in insect-proof cages. One hundred germinated seedlings were observed periodically for symptom appearance. The leaves from all the seedlings both from symptomatic or non-symptomatic were collected 60 days after sowing and analysed for the presence of virus through DAC – ELISA.

3. RESULTS AND DISCUSSION

3.1 Symptoms and detection of CMV in sap-inoculated vegetative tissues: The sap-inoculated chilli seedlings were grown under insect proof glasshouse condition until seed production. The seedlings expressed symptoms 15-20 days after inoculation and the symptoms observed were mosaic, mottling, deformation, and systemic infection. The visual observation of CMV infected plants showed stunted appearance; delayed flower formation and fruit set compared to uninoculated healthy plants. Also, there was a noticeable decrease in the number of flowers and fruits produced in inoculated plants (Table 1). The CMV infected plants produced deformed fruits which were smaller than usual and there was a drastic reduction in number of seeds per fruit, but the seeds produced were of normal in shape and appearance. The result of the present study is in line with the findings of Ali and Kobayashi (2010) [6], who observed severe mosaic and mottling in CMV inoculated chilli plants and reported a significant reduction in number of fruits and seeds in infected plants compared to control. Crowley (1957) [18] investigated the effect of *Tobacco mosaic virus* (TMV) and CMV on the

fertility of pungent peppers and reported that CMV intervened the growth of pepper plants by upsetting the normal hormonal control. He also stated that the infected plants were stunted in appearance; produced fewer flowers over a longer period; distorted fruits and reduced seed production.

Table 1: Effect of mechanically inoculated CMV on the production of flower, fruit, and seed in chilli

Plant No.	Number of flowers/plant	Number of fruits/plant	Average number of seeds/fruit
1	31	11	123
* 2	62	29	171
3	72	30	179
4	41	12	85
5	25	5	134
6	45	19	115
7	34	13	92
8	38	16	141
9	77	34	188
10	37	13	154
11	41	17	129
12	71	31	184
13	29	9	111
14	42	18	82
15	36	13	125
16	41	15	132
17	30	9	141
18	24	8	78
19	37	15	154
20	29	10	94
21	40	17	89
22	29	9	93
23	45	18	107
24	33	12	139
25	36	13	127
26	26	8	102
27	39	15	128
28	69	28	167
29	32	11	119
30	35	12	103
Uninoculated healthy*	73	32	183

***Average of five plants**

3.2 Detection of CMV in different plant parts by DAC-ELISA: Samples were collected from the leaves, flowers, green fruit, pericarp and seeds of CMV inoculated and healthy plants and subjected to DAC-ELISA. The absorbance values of the samples tested, healthy, positive, and negative control were presented in Table 2. The value twice that of negative control is considered as positive. Interestingly, CMV was detected in all the plant parts tested including seeds. In some cases, CMV was detected only in leaves and in few plants, it reached the seeds as well.

Table 2: Detection of *Cucumber mosaic virus* (CMV) in different plant parts of chilli by DAC-ELISA

Inoculated plant	OD at 405 nm				
	Leaves	Flowers	Green fruit	Pericarp of dry fruit	Seeds
1	**0.776±0.06**	**0.498±0.05**	**0.376±0.03**	**0.294±0.08**	**0.265±0.05**
2	0.135±0.05	0.035±0.03	0.015±0.06	0.045±0.07	0.027±0.08
3	0.127±0.02	0.027±0.02	0.007±0.02	0.113±0.02	0.125±0.05
4	**0.463±0.09**	0.161±0.07	0.111±0.09	0.100±0.06	0.097±0.02
5	**0.513±0.02**	**0.403±0.02**	**0.367±0.03**	**0.313±0.08**	**0.294±0.04**
6	**0.713±0.01**	**0.413±0.05**	0.119±0.04	0.103±0.05	0.041±0.02
7	**0.318±0.07**	**0.298±0.03**	0.170±0.07	0.128±0.09	0.091±0.05
8	**0.674±0.05**	**0.454±0.07**	**0.391±0.06**	**0.334±0.03**	**0.274±0.01**
9	0.193±0.03	0.182±0.04	0.119±0.05	0.135±0.04	0.110±0.02
10	**0.911±0.02**	**0.339±0.02**	0.175±0.06	0.131±0.05	0.112±0.01
11	**0.293±0.01**	0.123±0.09	0.068±0.05	0.123±0.09	0.114±0.08
12	0.123±0.06	0.197±0.05	0.162±0.08	0.127±0.05	0.029±0.03
13	**0.512±0.01**	**0.321±0.04**	0.194±0.06	0.012±0.03	0.001±0.01
14	**0.329±0.06**	0.143±0.03	0.096±0.03	0.120±0.04	0.111±0.07
15	**0.296±0.09**	0.126±0.01	0.085±0.07	0.103±0.08	0.102±0.02
16	**0.720±0.05**	**0.235±0.04**	0.174±0.01	0.106±0.02	0.111±0.07
17	**0.340±0.02**	0.107±0.03	0.149±0.09	0.144±0.05	0.091±0.01
18	**0.438±0.06**	0.153±0.02	0.081±0.05	0.025±0.09	0.103±0.03
19	**0.613±0.07**	0.154±0.01	0.186±0.02	0.129±0.03	0.106±0.07
20	**0.295±0.06**	**0.235±0.09**	**0.229±0.04**	0.175±0.05	0.084±0.08
21	**0.315±0.09**	0.153±0.03	0.103±0.02	0.092±0.03	0.084±0.04
22	**0.715±0.05**	**0.497±0.08**	**0.321±0.07**	**0.297±0.09**	0.059±0.02
23	**0.428±0.04**	**0.249±0.04**	0.127±0.05	0.109±0.03	0.062±0.05
24	**0.413±0.08**	0.103±0.03	0.099±0.07	0.023±0.06	0.019±0.03
25	**0.337±0.05**	0.123±0.06	0.130±0.01	0.044±0.09	0.013±0.04
26	**0.213±0.02**	0.139±0.08	0.130±0.05	0.132±0.02	0.132±0.02
27	**0.480±0.05**	**0.242±0.04**	0.178±0.02	0.124±0.03	0.108±0.09
28	0.194±0.03	0.171±0.03	0.116±0.07	0.071±0.02	0.084±0.07
29	**0.297±0.08**	**0.257±0.04**	0.149±0.04	0.121±0.01	0.111±0.04
30	**0.546±0.05**	**0.436±0.01**	**0.394±0.05**	**0.309±0.03**	**0.298±0.05**
Positive control	0.942±0.04	0.973±0.09	0.921±0.05	0.965±0.02	0.990±0.03
Healthy control	0.133±0.01	0.127±0.08	0.109±0.06	0.116±0.03	0.132±0.04
Negative control	0.101±0.09	0.120±0.05	0.113±0.06	0.115±0.03	0.107±0.08

**A*405 *absorbance values recorded half an hour after the addition of substrate. Value represents average of three replications*.

Out of 30 sap-inoculated plants, CMV was detected in the leaves of 25 plants; flowers of 14; green fruits of 6; dry pericarp of 5 and lastly in the seeds of 4 plants and the percentage of detection was 83.33% in leaves, 46.66% in flowers, 20% in fruits, 16.66% in pericarp of dried chilli and 13.33% in seeds.In all the cases, when the leaf was positive for CMV, the reproductive parts of the same plant were also positive; however, the concentration of the virus was gradually reduced from the leaves to seeds. Similar observation has been reported by Ali and Kobayashi (2010) [6], who reported the

presence of CMV in the leaves, petals, fruit flesh (pericarp), whole seed, embryo, and seed coat of chilli in USA.

3.3 Assessing the seed transmission of CMV in chilli by grow-out test: In order to determine seed transmissibility of CMV in chilli, the seeds collected from infected plants were sown in disposable containers and one hundred germinated seedlings were observed periodically for symptom appearance. Four weeks after emergence, 9 infected seedlings started to show mottling and wrinkling of leaves, which was further intensified to leaf deformation. The infected seedlings showed reduction in growth and stunted appearance compared to healthy ones (Plate 1). The leaf samples were collected from all the seedlings (60 days post-germination) and analysed for CMV through DAC-ELISA. Nine seedlings, which developed symptoms only reacted positive to CMV antiserum (data not shown). Interestingly, the virus was detected only in symptomatic plants and the rate of seed transmission was reported as 9%. In USA, Ali and Kobayashi (2010) [6] observed the seed-borne nature of CMV in chilli and reported that CMV can be transmitted from seed to seedlings and the percentage ranged from 10-14%.

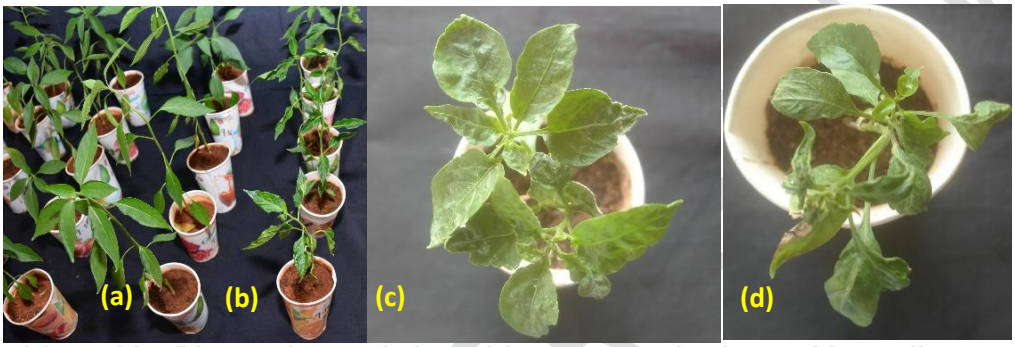

Plate 1 (a). Healthy (b). CMV infected plants (c). Symptomatic plants with mottling, mosaic and leaf deformation (d).Severe leaf deformation and stunted apperance

Arogundade *et al*., (2018) [7] collected 22 chilli accessions from 8 states in Nigeria and sap-inoculated them with CMV and reported its transmission to fruits as well as seeds in four accessions, *viz*., NHCrB/09/059, NCr/AA/MAY/09/015, NCr/SA/01/09/050 and NCr/AA/MAY/09/051. The CMV was detected in the seedlings raised from the infected seeds of the above accessions with transmission percentage of 73.33%, 66.67%, 66.67% and 16.67%, respectively. In another experiment, they subjected the seedlings raised from the seeds of pepper cultivars Tatase, Rodo and Sombo to Antigen Coated Plate ELISA (ACP-ELISA) for CMV and reported the natural seed transmission incidence as 57%, 86% and 71% respectively for the above cultivar. The result of the present study is in consonance with the findings of Ali and Kobayashi (2010) [6] and Arogundade *et al*., (2018) [7]. These studies confirm that CMV can be seed-borne in chilli and vertically transmitted to next generation.

4. CONCLUSION: This preliminary study indicated that CMV infected chilli plants showed stunted appearance; delayed flower formation; deformed fruit production and reduced seed production. CMV was detected in the leaves, flowers, green fruit, dry pericarp and seeds of sap-inoculated chilli plants by DAC-ELISA. CMV is seed-borne in chilli and transmitted from seed to seedlings and the rate of transmission was 9% as determined by grow-out test and DAC-ELISA. The infected chilli seed serves as a potential inoculum source for local field spread by vector as well as long- distance movement.

REFERENCES:

1. Kenyon, L., Kumar, S., Tsai, W. S., and Hughes, J. D. A. (2014). Virus diseases of peppers (*Capsicum* spp.) and their control. *Advances in Virus Research, 90*, 297-354. doi: 10.1016/B978-0-12-801246-8.00006-8
2. Vijeth, S., Sreelathakumary, I., Aiswarya, C.S., and Prashant Kaushik. (2020). Screening of popular Indian chilli pepper (*Capsicum annuum* L.) genotypes against the Chilli leaf curl virus disease. *Plant Pathology Journal 19*, 121-131.
3. Palukaitis, P., Roossinck, M. J., Dietzgen, R. G., and Francki, R. I. B. (1992). *Cucumber mosaic virus*. *Advances in Virus Research,41*, 281- 348. doi: 10.1016/S0065- 3527(03) 62005-1
4. Edwardson, J. R. and Christie, R. G. (1991). Cucumoviruses. In CRC Handbook of Viruses Infecting Legumes, pp. 293–319. CRC Press, Boca Raton, FL.
5. Johansen, E., Edwards, M.C., and Hampton, R.O., (1994). Seed transmission of viruses: current perspectives. *Annul Review of Phytopathology 32*, 363- 386.
6. Ali, A. and Kobayashi, M. (2010). Seed transmission of *Cucumber mosaic virus* in pepper. *Journal of Virological Methods, 163*, 234- 237.
7. Arogundade, O., Samuel Balogun, O. and Lava Kumar, P. (2018): Seed transmissibility of *Cucumber mosaic virus* in Capsicum species. *International Journal of Vegetable Science,* (DOI: 10.1080/19315260.2018.1487498).
8. Yang, Y., Kim, K. S., and Anderson, E. J. (1997). Seed transmission of *Cucumber mosaic virus* in spinach. *Phytopathology*, *87*, 924- 931.
9. Makkouk, K. M., and Attar, N. (2003). Seed transmission of *Cucumber mosaic virus* and *Alfalfa mosaic virus* in lentil seeds. *Arab Journal of Plant Protection*, *21*,49- 52.
10. O'Keefe, D. C., Berryman, D. I., Coutts, B. A., and Jones, R. A. C. (2007). Lack of seed coat contamination with *Cucumber mosaic virus* in lupin permits reliable, large-scale detection of seed transmission in seed samples. *Plant Disease, 91*,504- 508.
11. Babovic, M., Bulajic, A., Delibasic, G., Milijic, S. and Todorovic, D. (1997). Role of bean seed in transmitting *Bean common mosaic virus* and *Cucumber mosaic virus*. *Acta Horticulturae, 462*,253- 258.
12. Abdullahi, I., Ikotun, T., Winter, S., Thottapilly, G., and Atiri, G. I. (2001). Investigation on seed transmission of *Cucumber mosaic virus* in cowpea. *African Crop Science Journal, 9*, 677- 684.
13. Latham, L. J., and Jones, R. A. C. (2001). Incidence of virus infection in experimental plots, commercial crops, and seed stocks of cool season crop legumes. *Australian Journal of Agricultural Research, 52*,397- 413.
14. Latham, L. J., Jones, R. A. C., and McKirdy, S. J. (2001). *Cucumber mosaic* cucumovirus infection of cool-season crop, annual pasture, and forage legumes: Susceptibility, sensitivity, and seed transmission. *Australian Journal of Agricultural Research,52*,683- 697.
15. Park, K. H., and Cha, B. J. (2002). Detection of TMV, ToMV and CMV from tomato seeds and plants. *Research in Plant Disease, 8*,101-106.
16. Jones, R. A. C. (2004a). Occurrence of virus infection in seed stocks and 3-year-old pastures of lucerne (*Medicago sativa*). *Australian Journal of Agricultural Research 55*,757- 764.
17. Hobbs, H.A., Reddy, D.V.R., Rajeshwari, R., and Reddy, A.S. (1987). Use of direct antigen coating and protein a coating ELISA procedures. *Plant Disease*, *71*(8):747-749.
18. Crowley, N. C. (1957). Studies on the seed transmission of plant virus diseases. *Australian Journal of Biological Sciences*, *10*(4), 449-464.