MOLECULAR DIAGNOSIS OF CUCUMBER MOSAIC VIRUS OF CHILLI BY DAC-ELISA AND RT-PCR

V. BHUVANESWARI, M. SUBBA RAO, K.V.M. KRISHNA MURTHY, P. RAJA SEKHAR, Y. KOTESWARA RAO

Abstract: Survey carried out in farmers' fields in the major chilli growing areas of Krishna – Godavari Zone of Andhra Pradesh revealed that the incidence of cucumber mosaic virus ranged from 0.77 to 71.11 per cent. Of the total 270 fields surveyed, the incidence was less than 5.0 per cent in 175 fields, 5.0 – 15.0 per cent in 64 fields, 15.0 – 25.0 per cent in 22 fields, 25.0 – 50.0 per cent in seven fields and above 50.0 per cent in two fields indicating its wide prevalence on chilli in the zone.The symptoms of chilli cucumber mosaic virus disease was characterized by thesymptoms such as mosaic mottling, puckering, yellow discoloration, vein-clearing, curling, inward rolling, distortion, filiform, rat tailing and reduction in inter nodal length, leaf and fruit size and stunted growth. Samples showing typical CMV symptoms were collected and identified by Direct Antigen CoatingEnzyme Linked Immunosorbent Assay (DAC-ELISA). Samples tested positive by ELISA were further confirmed for the presence of CMV by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Key words: Cucumber mosaic virus, chilli, DAC-ELISA, RT-PCR.

Introduction: Chilli (*Capsicum annuum* L.) is an important commercial spice and vegetable crop belonging to the family Solanaceae. India's chilli production stood at around 12 lakh tonnes while the global out put was 27.7 lakh tonnes. Chilli is the second largest traded spice in the world with a 22% contribution in the world spice trade. India contributes 25% of the global chilli exports. Chillies are cultivated mainly in the states of Andhra Pradesh, Orissa, Maharashtra, West Bengal, Karnataka, Rajasthan and Tamil Nadu. Andhra Pradesh is the foremost accounting for 50% of the production in the country.Andhra Pradesh occupies a pivotal position in chilli cultivation with an area of 2.36 lakh ha and an annual production of 7.48 lakh t, which ranks first in production in the country (Statistical Abstract of Andhra Pradesh, 2005).

Chilli is susceptible to a wide variety of diseases caused by fungi, bacteria, viruses and phytoplasmas, out of which viruses cause heavy losses to the crop yields (Satya Prakash, 2001). Over 72 viruses have been reported to infect chilli crop all over the world (Satya Prakash and Singh, 2004) and 19 viruses including cucumber mosaic virus (CMV), pepper veinal banding virus (PVBV), potato virus Y (PVY), pepper veinal mottle virus (PVMV), pepper mottle virus (PMV), tobacco etch virus (TEV) and tobacco mosaic virus (TMV) were reported to occur in India. However, the knowledge on the occurrence of these viruses in Andhra Pradesh is not fully known and at present detection of viruses in field level is difficult. Thedetailed information in this regard is needed to identify the viruses by molecular tools and to evaluate efficient management strategies. Hence, an attempt was made to characterize the CMV of Krishna – Godavari Zone of Andhra Pradesh.

Materials and Methods

Survey, Symptomotology and sample collection: Survey was undertaken in farmers' fields in the major chilli growing areas of Guntur, Prakasam, Krishna, East and West Godavari and parts of Khammam district pertaining to the Krishna – Godavari Zone of Andhra Pradesh to assess the incidence of cucumber mosaic virus. Samplings were made at flowering and podding stages of the crop in two consecutive seasons during *kharif*, 2002-03 and 2003-04.A total of 270 fields were surveyed during both years. In each field, five plots, each of a size of 2.0 x 2.0 m were laid out diagonally with the help of a wooden quadrangle. In each plot, the number of healthy chilli plants and the number of plants showing the typical symptoms of CMV were recorded and the per cent incidence was estimated using the following formula.

$$\text{Per cent incidence of CMV} = \frac{\text{Number of plants infected}}{\text{Total number of plants}} \times 100$$

Diseased samples showing the typical symptoms of CMV were collected from different plants in the field and enclosed in polyethylene bags. Care was taken to make the bag airtight removing the air inside. Labels furnished with the information on place of collection, date of collection and the variety were tagged on to the bags. These samples were carried to the laboratory in ice buckets and transferred immediately to the refrigerator at 4^{o}C for carrying out further studies.

Isolation and Maintenance of Virus Isolates: The virus isolates sampled from farmers' fields were inoculated on to the chilli cultivar 'Sindhur' (CA-960) and tobacco plants *Nicotiana tabacum* cv. Gautham by sap inoculation method adopting the following procedure.

Sap Inoculation: The infected chilli leaves made into small pieces were weighed and ground in the presence of 0.01 M phosphate buffer (pH 7.0) containing 0.2% 2-mercaptoethanol.. The entire process of grinding was done under cold conditions in an ice bucket with the help of pre-chilled mortar and pestle. The crude extract was filtered through a double layered muslin cloth in a beaker. A pinch of carborundum powder (600 mesh) was added to the filtrate.Young and actively growing chilli plants were used for inoculation purpose. The leaves of the experimental plants to be inoculated were washed with distilled water prior to inoculation to remove the dirt. The extracted sap was rubbed gently over the leaves for 2 to 3 times in one direction followed by rinsing with distilled water. The test plants were labelled and kept under observation for symptom expression.

Purification of virus: The cucumber mosaic virus maintained on chilli plants in the laboratory was inoculated to tobacco plants (*Nicotiana tabacum* var. Gautham). Young tobacco leaves showing the typical symptoms of CMV were harvested at 14 to 18 days after inoculation and washed in tap water to remove the soil and dirt. The veins of the leaves were removed and the leaf lamina was made into small pieces. The leaf sample was subjected to purification as per the procedure described by Walkey (1985) and Kiranmai *et al.* (1997) with suitable modifications.

Immuno-diagnosis

Enzyme Linked Immuno Sorbant Assay (ELISA): The Assay was performed in 96 well polystyrene plates using the protocol described by Hobbs *et al.* (1987). Tests were carried out to determine the relationship of the virus with the antisera of Cucumber mosaic virus and Peanut bud necrosis virus. Young leaves showing the symptoms of CMV of chilli isolates maintained on tobacco (*Nicotiana tabacum* var. Gautham) were harvested and folded separately between the blotter paper sheets enclosed in polyethylene covers. These samples were carried to the laboratory in ice buckets for carrying out the tests. Crude extracts of test samples were prepared by grinding the infected leaf material in presence of coating buffer at 1:10 ratio (1g of leaf sample/ 9ml buffer) at 4°C with the help of pre-chilled, sterilised mortars and pestles. The extracts were dispensed in the respective wells of the micro titre plate @ 200 µl per well with a micropipette. The plates were incubated at 37°C for 1 h or at 4°C for overnight.The contents of the plates were drained off and washed for three times in PBS – tween buffer for 3 min each.Antisera containing antibodies (CMV & PBNV) diluted in conjugate buffer to a concentration of 1:1000 dilution was added to each of the well @ 200 µl per well. Then the plates were incubated at 37°C for 1 h and washed in PBS – tween as in step – 3.Alkaline phosphatase (ALP) labelled with anti rabbit IgG was diluted in antibody buffer to a concentration of 1:1000 dilution and added to the wells @ 200 µl per well. The plates were incubated at 37°C for 1 h and washed in PBS-tween as in step- 3.Substrate buffer containing 0.5 –1.0 mg /ml of p-nitrophenyl phosphate was then dispensed to each of the well @200µl/well and incubated at room temperature for 10-30 minutes. The production of P-nitrophenol was stopped by addition of 50 µl of 3.0 M NaOH/well.Light orange to yellow colour development indicated a weak to strong positive reaction and the results were recorded by visual observation.

Total RNA isolation: CMV being RNA virus, total nucleic acid was extracted from 100 mg of infected and healthy tissue using RNeasy Plant Mini Kit (Qiagen, Gmbh, Hilden, Germany) according to protocol provided by the manufacturer and was used as template for amplification in the reverse transcriptase polymerase chain reaction (RT-PCR).

The total RNA from the tissues of chilli and tobacco infected with CMV was extracted in the laboratory for running the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The RNeasy kit consisting of RLT, RW1 and RPE buffers was used for this process. The protocol as per the catalogue (Cat. No.74904) prescribed by the manufacturer M/s. Qiagen Inc., USA (ChatsworthCA 9131) with slight modifications was adopted as furnished here under.Fresh leaves from chilli and tobacco plants infected with CMV were harvested and stored for overnight at 4°C. A sample of 100 mg each was ground separately in presence of liquid nitrogen in a set of pre-chilled sterilised mortar and pestle. The powdered samples were immediately soaked in 450 µl of lysis buffer (RLT buffer) containing 1% β-mercaptoethanol and vortexed for 10 sec. The mixture was incubated in water bath at 56°C for 2 min. This clear lysate was then passed through spin column (lilac) and centrifuged at 12,000 rpm for 2 min in a bench top centrifuge at room temperature. The flow through was transferred to an eppendorftube. To this a half volume of (225 µl) chilled ethanol (absolute) was added and mixed thoroughly by pipetting. The same was then passed through another spin column (pink) and centrifuged at 12,000 rpm for 15 sec at room temperature. The flow through was discarded and 700 µl RW1 buffer was added to the same column. This mixture was again centrifuged at 12,000 rpm for 15 sec. Then RN easy column was transferred to new collection tube and 500 µl of RPE buffer was added. The mixture was centrifuged at 12,000 rpm for 15 sec. The flow through was discarded. Another 500 µl of RPE buffer was added and again centrifuged at 12,000 rpm for 15 sec. After that RN easy column was placed

in a new 2.0 ml collection tube and further subjected to centrifugation at 12,000 rpm for 1 min. The flow through in the tube was discarded. Finally, RNA was eluted with 50 µl of sterile RNase free water by centrifuging at 12,000 rpm for 1 min. The eluted RNA was collected in an Eppendorf tube. This was further used as a template in reverse transcription-polymerase chain reaction (RT-PCR). Another sample consisting of healthy leaf material from chilli and tobacco were performed in parallel to the infected samples as checks.

PCR amplification: RT-PCR amplification was carried out for CMV.For detection of CMV by RT-PCR, amplifications were carried out using coat protein (CP) gene specific primers (Forward-5'-GCCGTAAGCTGGATGGACAA-3')andReverse-5'-TATGATAAGAAGCTTGTTTCGCG-3') (Table 1)described by Singh *et al.* (1995)in a thermal cycler. Prior to running of the RT-PCR, the isolated RNA from the infected samples of chilli and tobacco were used as templates. These templates were incubated at 75°C for 5 min and snap-cooled on wet ice for 5 min. The primers as listed above were used to prime the amplification of complete CP genes from isolates. A 30 µl amplification mixture the composition of which as presented in Table 2was prepared in a 50 µl thin walled microfuge tube. The tube was loaded to the PCR machine. Then the RT-PCR was performed in a single tube in an automated thermal cycler (Power Block II, Ericomp INC., San Diego, CA, USA). The mixture was pulse centrifuged and placed in a thermal cycler set at the thermal cycling profile chosen for reaction presented in Table 3.The PCR products of CMV were analyzed by loading the products on 1% agarose gel containing ethidium bromide. The gel was visualized under Gel documentation system (UVP Germany).

Results and discussion

Survey and symptomtology: Survey undertaken in farmers' fields in the major chilli growing areas of Guntur, Prakasam, Krishna, West and East Godavari and parts of Khammam districts pertaining to the Krishna – Godavari (KG) zone of the Andhra Pradesh presented in Table 3 revealed that The incidence of CMV ranged from 0.77 to 71.11 per cent. The results indicated that of the total 270 fields surveyed in both the consecutive seasons 2002-'03 and 2003-'04, 175 fields recorded less than 5.0 per cent, 64 fields recorded 5.0-15.0 per cent, 22 fields recorded 15.0-25.0 per cent, seven fields recorded 25.0-50.0 per cent and two fields recorded more than 50.0 per cent incidence of CMV.Under field conditions, the disease was characterized by the visual symptoms such as mosaic mottling, puckering, yellow discoloration, vein-clearing, curling, inward rolling, distortion, filiform, rat tailing and reduction in inter nodal length, leaf and fruit size and stunted growth (Fig. 1).

Purification: The chilli isolate of cucumber mosaic virus was purified as per the procedure described,the virus fraction obtained after sucrose gradient centrifugation was diluted in citrate buffer (0.05 M & pH 7.0) and was further subjected to centrifugation @ 16,000 rpm at 4° C for 2 h. The virus pellet thus obtained was stored in 200 µl of citrate buffer (0.05 M & pH 7.0) for further studies.

Direct Antibody Coating Enzyme Linked Immunosorbant Assay (DAC-ELISA): The direct antibody coating enzyme linked immunosorbant assay (DAC-ELISA) was carried out using the antisera of CMV and PBNV at 1:1000 dilution. DAC-ELISA of symptomatic samples from field showed positive reaction for antisera against CMV and negative reaction to PBNV (Fig 2). These results are supported by earlier reports by Salamon and Kolber (1986), Khalil and Mikhail (1987), Kearneyet al. (1990) and Akanda et al. (1991).

Total RNA isolation and Reverse Transcriptase – polymerase Chain Reaction (RT-PCR): The total RNA from both chilli and tobacco tissues infected with CMV was isolated in the laboratory using RN easy kit.The RT-PCR product of ELISA positive samples which were visualized on Gel Doc after electrophoresis showed a distinct band at 450 bp corresponding to the band of CMV positive (Fig3).The results revealed that RT-PCR of the two tobacco and one chilli sample infected with CMV produced an expected band of 450 bp from leaves. This has confirmed that RT-PCR was found as an appropriate test for the detection of CMV. This result confirmed the similar findings of Singh et al. (1995) who reported that RT-PCR assay with primers designed in a conserved region of the 3' end of the CMV coat protein gene amplified a 486 to 488 bp DNA fragment from the infected banana samples.

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Table1. Specific primers used for amplification of coat protein genes of cucumber mosaic virus isolates

Primers	Orientation	Position	Sequence	Tm (°C)
1	Upstream	CP region	5'-GCCGTAAGCTGGATGGACAA-3'	62
2	Downstream	CP region	5'-TATGATAAGAAGCTTGTTTCGCG-3'	64

Table2.Amplification mix used for reverse transcription-polymerase chain reaction

Reagents	Volume required (µl per reaction)
Template (RNA)	10.0
Omniscript reverse transcriptase	1.0
5 X buffer	6.0
Taq DNA polymearase (3 U/µl)	1.0
10 X PCR buffer (10 mM)	3.0
Primer (Upstream) (200 ng)	0.2
Primer (Downstream) (200 ng)	0.2
dNTPs (10 mM)	1.0
$MgCl_2$ (25 mM)	2.5
Sterile distilled water	5.5
Total	30

Table3. The thermal cycling profile chosen for reverse transcription polymerase chain reaction

Steps involved (°C)	Temperature (min)		duration		Cycle number			
Reverse transcription			42	45				1
Denaturation			94	4				1
Primer annealing	94			0.5				1
Primer 1 Primer 2	56				1	40sec	30	
Synthesis	72							
Final extension			72	10				

Figures in parenthesis indicate the number of chilli fields surveyed

Table4. Incidence of CMV district wise in Krishna -Godavari Zone of Andhra Pradesh

District	Disease incidence (%)				
	<5	5-15	15-25	25-50	>50
Guntur (50)	31	14	4	1	-
Prakasam (50)	34	11	2	2	1
Krishna (50)	26	18	6	-	-
West Godavari (50)	32	10	6	1	1
East Godavari (50)	32	11	4	3	-
Khammam (20)	20	-	-	-	-
Total (270)	175	64	22	7	2

Fig 1: Mosaic mottling, puckering, filiform, rat tailing and reduction in leaf size on chilli leaves induced by CMV

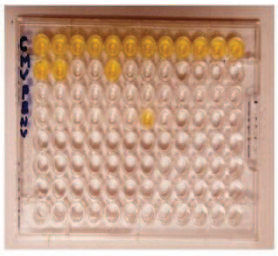

Fig 2: DAC-ELISA test carried for CMV

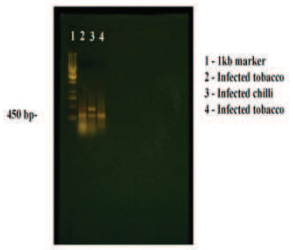

Fig 3: RT- PCR for CMV

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Andhra Pradesh Rice Research Institute & Regional Agricultural Research Station,
Maruteru-534 122, Acharya N.G.Ranga Agricultural University,
Andhra Pradesh, India.
Email: bhuvanavk2001@gmail.com