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Molecular Detection of *Cucumber Mosaic Virus* Infecting Chilli (*Capsicum annuum* L.)

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Abstract

Cucumber mosaic virus (CMV) is widely distributed having a wide host range, affects seriously both hot and bell peppers. Chilli plants showing symptoms typical to CMV infection such as mosaic, mottling, yellow discolouration, vein clearing, leaf deformation, shoestring or leaf narrowing were collected from the chilli fields of Guntur, Andhra Pradesh, India. The samples showed positive reaction only to the CMV in DAC-ELISA were taken to purify the CMV biologically from field infected chilli samples through mechanical sap inoculation to cucumber and *Nicotiana glutinosa*. The Inoculated cucumber *N. glutinosa* expressed the symptoms characteristic to CMV such as chlorotic spots followed by mild mosaic and leaf distortion. Molecular detection of CMV Guntur isolate was done by using the primer designed for RNA1 with the expected amplicon size of 800 bp.

Keywords: Cucumber mosaic virus, Detection, Reverse transcriptase PCR, ELISA, Chilli

Chilli (*Capsicum annuum* L.) is the most important vegetable, spice, medicinal herb, and ornamental plant grown across the world and ranked after potato and tomato. The major chilli growing countries are India, China, Pakistan Nigeria, Mexico, Indonesia and the Korean Republic. India and China contribute close to 87 per cent of the world's production. India alone contributes to an extent of 68 per cent of the world's production and also the largest exporter of chilli. India ranks first in the production and export of dry chillies in the world and grown over an area of 0.915 million ha area with a production of 6 million tonnes. The five major cultivated species of Capsicum are *C. annuum, C. frutescens, C. chinense, C. pendulum* and *C. pubescens.* Among these *C. annuum* is most widely grown throughout the world. Chilli is a rich source of vitamin A, C and E. Recently, Russian scientists have identified vitamin "P" in green chillies which are considered to be important as it protects from secondary radiation injury (Verghese, 1999). The pungency and colour in chilli are due to an alkaloid capsaicin and pigments capsanthin and capsorubin, respectively. Capsaicin has significant medicinal value and used in the preparation of balms, ointments for cold, sore throat and chest congestion. Capsanthin is being extensively used in the cosmetics, perfumes, paints, dyes and for colouring foods, which is extracted from the red chillies (Verghese, 1999).

The crop has been widely distributed in the world and prone to many diseases caused by different agents. Among these, virus diseases are known to be a major threat for the production of chilli resulting in low yields and poor fruit quality (Alanso *et al*., 1989). Nearly, 40 viruses were reported to infect chilli worldwide. Among them, *Cucumber mosaic virus* (CMV), *Chilli veinal mottle virus* (ChiVMV) and *Chilli leaf curl virus* (ChLCV), were reported as the most destructive viruses affecting chilli cultivation in terms of incidence and causing loss up to 100 per cent with respect to marketable fruit yield. Further, in some areas, virus diseases resulting in growing chillies uneconomical in turn resulting in the abandonment of fields' prior to harvest.

Mosaic disease in chilli caused by CMV is occurring frequently leading to severe damage to the chilli crop every year in India (Khan *et al*., 2006). The virus has wide host range infecting over 1,200 species belonging to hundred plant families. The CMV belongs to the genus *Cucumovirus*, family *Bromoviridae* and has isometric particle morphology with approximately 28-30 nm in dia. The virus is transmitted by more than eighty different aphid species in a non-persistent manner under natural conditions (Palukaitis *et al.,* 1992). The CMV is a tripartite virus with a genome comprising of three plus sense, ssRNA molecules

encased in separate particles and designated as RNA1, RNA2 and RNA3 in decreasing order of molecular weight. RNA1 and RNA2 encode for the protein 1a and 2a, respectively, which forms the replicase complex necessary for viral replication (Palukaitis *et al.,* 1992). N-terminal region of 1a protein contains the putative methyltransferase domain (Rizos *et al.,* 1992) and C-terminal region shows sequence similarity to viral helicases. The RNA2 also encodes a second protein, 2b, which functions in host-specific long-distance movement, which is expressed from the subgenomic RNA 4A and acts as suppressor of gene silencing (Li *et al*., 1999) and also behave as pathogenicity determinant (Soards *et al.,* 2002). A recent finding suggested that this suppressor protein is indirectly involved in aphid transmission (Ziebell *et al.,* 2011). A study on the mechanism of 2b action showed that the suppressor specifically recruits AGO4 small RNAs and directly interacts with PIWI and PAZ domains of AGO4 affecting its slicer activity, causing hypomethylation of its loci (Hamera *et al.,* 2012). The RNA3 encodes two proteins; the 3a protein involved in cell-to-cell movement (MP), and the 3b protein which forms the capsid protein (CP), which is also involved in cell-to-cell movement and aphid-mediated CMV transmission from plant to plant (Conti *et al.,* 1997). CMV upon infection induces various symptoms in chilli such as mosaic, mottling, yellow discolouration, vein clearing, leaf deformation, shoestring or leaf narrowing, stunted growth, reduced fruit size and whitish streaks on green fruits (Arlisokmen *et al.,* 2005). The recombination and re-assortment of viral genome components are leading to the emergence of novel viral strains resulting in more devastation of the crops and spilling of newly emerged strains into new hosts with expanding host range.

Development of protocols for virus detection will play a key role in studying the epidemiological and management aspects of the viral disease. In this backdrop, an attempt was made to detect cucumber mosaic virus infecting chilli through reverse transcriptase-polymerase chain reaction (PCR) in the present study.

Material and Methods

Collection of isolates

For conducting research on virus diseases pure virus cultures are essential. The initial virus isolation has to be done from the field infected samples. In order to isolate CMV, leaf samples from virus infected chilli plants showing symptoms typical to CMV infection *viz.* mosaic, mottling, yellow discolouration, vein clearing, leaf deformation, shoestring or leaf narrowing were collected from farmers field at Guntur Andhra Pradesh. Since there is the probability of mixed infection of viruses in the field collected plant samples, in order to know the viruses present in the samples, the collected leaf samples were tested by DAC-ELISA **(**Lakshminarayana Reddy, 2006**)** using polyclonal antisera of CMV, ChiVMV, *Tobacco mosaic virus* (TMV), Capsicum chlorosis virus (CaCV) and Groundnut bud necrosis virus (GBNV).

Mechanical transmission and biological purification

The samples which showed positive reaction only to the CMV in DAC-ELISA were taken for mechanical sap transmission. The infected leaf sample was washed with tap water to remove the dust particles adhering to them and dried between the folds of blotting paper. The infected leaves were then macerated with chilled mortar and pestle using 0.05M potassium phosphate buffer (pH 7.0) added with 0.02 per cent 2-â-mercaptoethanol at the rate of 1ml/g of leaf tissue and the crushed sap was filtered through double-layered muslin cloth and filtrate was mixed with celite powder (600mesh at 0.025 g per ml). The inoculum was applied gently on the upper surface of the cotyledon leaves of cucumber (variety Infinity) and rubbed uni-directionally, with a small piece of absorbent cotton wool. After 3-5 minutes, the excess inoculum on the leaves was washed with a fine jet of distilled water. The inoculated plants were maintained under insect-proof glasshouse for symptoms expression. The leaf sample from infected cucumber was collected and then inoculated to *Nicotiana glutinosa* through mechanical sap inoculation as described above. The inoculated *N.glutinosa* are maintained under the glasshouse for symptoms

expression and maintenance of CMV culture. In all these mechanical inoculations of viruses, a set of plants were mock inoculated without the virus, which was used as negative control.

Viral cDNA synthesis, PCR amplification of CMV

Total RNA was extracted from both CMV infected leaf sample and healthy leaf sample collected from *N. glutinosa* maintained in the glasshouse using RNeasy Plant Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The integrity and quality of the total RNA were checked on 1 per cent agarose gel and quantified by Nanodrop (Thermo Fisher Scientific, USA). First strand viral cDNA synthesis was carried out with 5µg total RNA. Total RNA was denatured along with 1µl Oligo (dT) (20pmol/µl) at 72 ^{0}C for 5 min, followed by addition of 4µl of 5X first strand buffer, 0.2µl ribonuclease inhibitor (40 U/µl), 2 µl of 10mM dNTPs and 0.1µ1 MMLV-RT (200 U/µl) (Fermentas) to make total reaction volume to 20 µl. The reaction was performed at 42 ^{0}C for 60 min followed by incubation at 75 ^{0}C for 5 min.

The PCR amplification was performed with the GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler using specific primers designed (Table 1) to RNA1 segment of CMV by retrieving the CMV isolates sequences available in the NCBI, GenBank. The PCR reactions were carried out in a volume of 25iL containing 100ng of DNA template 0.5U *Taq* DNA polymerase (Fermentas, Germany), 25mM $MgCl_2$ (Fermentas, Germany), 2mM dNTPs (Fermentas, Germany) and 25 pmol of each primer. The thermocycler was set for 35 cycles of denaturation at 94 °C for 45 secs, annealing at 58 °C for 45 seconds and extension at 72 °C for 90 seconds with initial denaturation and a final extension at 94 °C for 3 mins and 72 °C for 10 mins, respectively. The PCR products were electrophoresed on 0.8 per cent agarose gels stained with ethidium bromide (10ug/mL) and were viewed in the gel documentation system (Alpha Innotech, USA).

TABLE 1

Specific primers designed for amplification of RNA 1 genome segment of CMV

Primer set	Sequence	Annealig temperature	Expected size
Forward Primer: CMRN1248	5'GAAGGCYGTG CGAGGTATAT3'	58 ^{0}C	0.8 kb
Reverse Primer: CM3R1	5'GGTCTCCTTTT GGAGACCCCCAC3'		

RESULTS AND DISCUSSION

Collection and maintenance of CMV isolates and symptomatology

Chilli plants infected with *Cucumber mosaic virus* expressing symptoms such as mild mosaic, mosaic mottling, leaf distortion, shoe string/rat tail/filiform leaves, yellowing, stunted growth, systemic chlorotic lesions and light green streaks on the fruits was well documented by several researchers across the world (Sevik 2003, Khan *et al.,* 2006). For the isolation of CMV, the leaf samples from the chilli plants showing symptoms described by earlier workers were collected from the farmer's field of Guntur district of Andhra Pradesh. The chilli plants were known to be infected by more than 40 viruses belonged to diverse groups (Green and Kim, 1991). In India, most frequent viral infections are ChiVMV (Lakshminarayanareddy, 2006), CMV (Ashwathappa, 2014), CaCV (Krishnareddy *et al*., 2008) and GBNV. (Anjaneya Reddy *et al.,* 2008). Because of this, there will always be a chance of mixed infections among the field collected chilli leaf samples. In order to know the status of virus infection in the chilli samples collected in the current study, DAC-ELISA using CMV, ChiVMV, PBNV specific antibody was performed. The CMV is mechanically sap transmitted virus (Dooiittie and Zaulneyer 1953) and known to infect several host plants such as tomato, chilli, banana, *C. annuum, tobacco, N. glutinosa, N. rustica,* and various cucurbits (Kumari *et al.,* 2013,). Therefore, an attempt was made to biologically purify the CMV from field infected chilli samples through mechanical sap inoculation to cucumber and *N. glutinosa*. Initially, the samples showing positive to only to CMV were mechanically sap inoculated to cucumber as described

A . Mock control B. Inoculated

Fig. 1: Cucumber plants were mechanically sap inoculated with *Cucumber mosaic virus* (CMV) inoculum prepared in potassium phosphate buffer pH 7.00.

in the material and methods. The inoculated plants started expressing the symptoms from the sixth day after inoculation with the mild mosaic and progressed towards severe mosaic symptoms on these plants by fifteen days after inoculation (Fig. 1). Even though, the samples used for inoculation have shown negative to other viruses upon inoculation to cucumber will help to get rid off viruses infecting chilli (ChiVMV) and not able to infect cucumber, if any probable escape of viruses being not detected by ELISA because of low titre. Then the CMV infected leaf sample from cucumber plant was collected and virus isolate was inoculated to the *N. glutinosa* for further maintenance of virus culture. The *N. glutinosa* expressed the symptoms from twelve to fifteen days after inoculation with the induction of systemic chlorotic spots followed by mild mosaic and leaf distortion (Fig 2). The virus isolate maintained on the *N. glutinosa* collected for the study was designated as CMV Guntur isolate. For

A . Mock control B. Inoculated

Fig. 2 : *Nicotiana glutinosa* plants were mechanically sap inoculated with CMV inoculum prepared in potassium phosphate buffer pH 7.00. Mock inoculation without virus was used as negative control. The mock inoculated *N.glutinosa* plants expressed no symptoms(A) and the virus inoculated plants expressed mosaic motttling, leaf distortion, stunted growth (B).

TABLE 2

Detection of CMV by DAC-ELISA in field collected chilli sample

Virus detected by DAC ELISA	OD @ 405nm for healthy sample	OD @ 405nm for infected sample
CaCV	0.100	0.157
ChiVMV	0.173	0.154
CMV	0.109	0.933
GBNV	0.152	0.108
TMV	0.107	0.134

CaCV : Capsicum chlorosis virus
ChiVMV : *Chilli veinal mottle virus*
CMV : Cucumber mosaic virus
GBNV : Groundnut bud necrosis virus
TMV : Tobacco mosaic virus

TABLE 3

Confirmation of CMV in the biologically from purified host plants by DAC-ELISA

Sample	OD @ 405nm
Buffer	0.109
Positive control	0.721
Healthy cucumber	0.094
CMV inoculated cucumber	1.244
Healthy *Nicotiana glutinosa*	0.100
CMV inoculated *N. glutinosa*	0.933

the maintenance of virus cultures and further use of infected plant material, the plant species which are having more lifespan, can grown easily, free from sucking pests such as mites (which pose a problem in glasshouses) and minimal inhibitors in the extracted sap are preferred. The *N. glutinosa* in this study served the above-said purpose. The biological purification of plant viruses from the mixed infection of viruses in the field collected samples by passing of through different hosts of virus isolates is a well-established procedure for deriving the pure virus cultures (Ashwathappa, 2014). The leaf sample from

the biologically purified CMV and maintained on *N. glutinosa* in the glasshouse was used for all further studies.

Molecular detection of *Cucumber mosaic virus* isolate

Amplification of part of the viral genome was extensively exploited for the routine detection of plant viruses from the various crops (Geetanjali *et al.,* 2011; Dong *et al.,* 2008; Hareesh *et al.,*2006; A similar attempt was made in the present study by designing the specific primers to RNA 1 segment of CMV as described in material and methods with expected

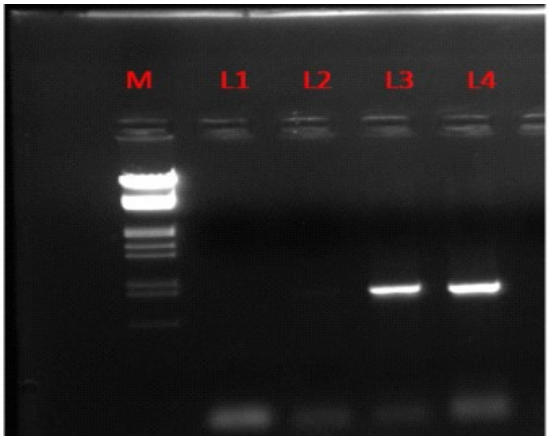

Fig. 3: Amplification of RNA 1 gene of CMV maintained on *Nicotiana glutinosa* by reverse transcriptase polymerase chain reaction.

M – 1 KB marker

Lane 1and 2 *N. glutinosa* healthy sample

Lane 3 and 4 *N. glutinosa* infected sample

amplicon size of 800 bp (Fig. 3). The PCR amplification from the CMV- Guntur isolate maintained on the *N. glutinosa* resulted in the expected amplicon. There was no amplification seen in the healthy samples which served as a negative control. The utilization of readily available viral sequences for designing primers to conserved genomic segments of viruses is the key to the development of genus/species/strain specific PCR based detection protocols.

The primers designed in the study and the reverse transcriptase PCR protocol given in the material and methods can be utilized for the routine detection of CMV in the plant samples.

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