Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(6): 2004-2008
Received: 12-09-2017
Accepted: 15-10-2017

Ng Taibangnganbi Chanu
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India

Y Herojit Singh
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India
College of Agriculture, Central Agricultural University, Imphal, Manipur, India

Ph Sumitra
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India

Surjit Singh
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India

S Rakesh Singh
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India

SS Roy
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India

N Prakash
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India

SK Sharma
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India

Correspondence
SK Sharma
ICAR Research Complex for NEH Regions, Manipur Centre, Lamphelpat, Imphal, Manipur, India

Molecular based indexing of viral disease complex of king chilli (*Capsicum Chinense* J.) in North Eastern Region of India

Ng Taibangnganbi Chanu, Y Herojit Singh, Ph Sumitra, Surjit Singh, S Rakesh Singh, SS Roy, N Prakash and SK Sharma

Abstract
King chilli (*Capsicum chinense* J.) also known as "Umorok" in Manipur or "Naga chilli" in Nagaland or "Bhut Jolokia" in Assam is the most important spice crop of North East India. It is one of the hottest chillies in the world. In spite of its importance, cultivation of king chilli is limited by numbers of biotic and abiotic factors. Among the various biotic factors, viral disease complex is considered to be the most significant, destructive and devastating factors. Various surveys were conducted at different king chilli growing areas of Manipur and its neighbouring states to record the prevalence of viral complex in order to characterize the associated viruses. Varying kind of symptoms ranging from leaf mottling, puckering shoe- string, vein banding, severe curling, mosaic and smaller leaf lamina were observed under field conditions. Out of 79 samples tested for four viruses (*Cucumber mosaic virus*: CMV; *Chilli veinal mottle virus*: ChiVMV; begomoviruses and tospoviruses) in PCR or RT-PCR using specific primers targeting CP regions of respective viral genome, 30 samples were found positive for CMV (37.97%) and 17 samples for ChiVMV (21.51%) were found positive. Further, it was also observed that 3 samples were found mix infection.

Keywords: Coat protein, Disease, King Chilli, Symptoms, Viruses

Introduction
King chilli (*Capsicum chinense* J.) is widely grown in North Eastern (NE) region of India and consumed in many preparations both spice and medicines. India is the largest producer of chillies in the world with a production of 1492.14MT. The average national productivity of chillies in India is 1.92MT/ha. Area under chilli cultivation in North East India is 43.10 thousand hectare with a production of 39.25 MT (IHB, 2014). Among NE states, the productivity of chilli is highest in Tripura (1.57 t/ha) and production in Assam with16.48 MT (IHB, 2014). It is a remunerative crops being grown in the states of Manipur, Nagaland, Mizoram, Meghalaya and Assam etc. King chilli is one of the hottest chillies worldwide, being grown and consumed in the NE states. In addition, many other types of chilli landraces are also being grown in the region. The various chilli variants existing in this region were most likely derived from natural inter-specific crosses (Kumar *et al*., 2011) [10] and known under diverse local names (Sanatombi *et al*., 2010) [15]. Different landraces of king chilli grown in the state of North East India are infected by viral disease complex exhibiting various symptoms ranging from leaf mottling, vein banding, leaf distortion, narrowing of leaf lamina, severe curling, necrosis and stunted growth. Due to the infection of viral disease complex, production and productivity of king chilli in North East has been reduced to greater extent. A high incidence of viral diseases (up to 100%) in local chilli cultivars from Darjeeling and Sikkim was reported and associated viruses, *Cucumber mosaic virus* (CMV) and a *Potyvirus* was identified (Biswas *et al*., 2005) [3]. Among the various potyviruses infecting chilli, *Chilli veinal mottle virus* (ChiVMV) is the most important and destructive virus present throughout Eastern Asia and in some African countries. It is also one of the most widely distributed *Potyvirus* associated with devastating viral disease in NE India (Banerjee *et al*., 2014) [2]. *Potyvirus* causes about 30% of the prevailing plant viral diseases and their extent of infection is economically dangerous to various crops including pepper. CMV is a plant pathogenic virus in the family *Bromoviridae*, genus *Cucumovirus*. This virus was first reported in cucumbers (*Cucumis sativus*) showing mosaic symptoms in 1934. CMV and ChiVMV can be transmitted from plant to plant both mechanically by sap and by aphids in a stylet-borne fashion. Seed transmission of CMV has been reported from 0 to 100% in various host species, including weed species (Neergaard, 1977) [13]. The large number of aphid vector species and natural host

reservoirs accounts for the high incidence of CMV in field plants (Cerkauskas Ray *et al*, 2004). Infection of CMV often leads to the losses of 10–20% in the yield and even if harvested, crops are commonly found in poor condition. CMV infects more than 1200 plant species in 100 families (Edwardson and Christie, 1991) [5], and has the largest host range of any RNA virus, making it one of the most economically important plant viruses infecting chillies and pepper. The ubiquitous nature of CMV and ChiVMV makes the management difficult. Viral complex like symptoms were commonly associated with the lower productivity of king chilli in NE India, however the associated viruses and their prevalence has not been identified. In the present study a number of virus infected king chilli were sampled from different king chilli growing region of Manipur and other parts of NE states and characterized biologically as well as by molecular methods.

Materials and Methods

Field Survey and Sample Collection

A systematic survey was conducted at different King chilli growing pockets of Manipur and its neighboring states during the year 2016-17. Ideally 5-10 symptomatic or asymptomatic samples were collected from each location surveyed and samples were stored in plastic bags and brought to the laboratory after which they were processed immediately for virus testing. Collected symptomatic leaves were kept in RNA later and stored at '- 80 °C' for future purpose.

Mechanical Inoculation

Mechanical transmission was undertaken using king chilli plants. Infected leaf samples of king chilli collected from different pockets of Manipur were finely ground in a snap chilled mortar and pestle using sodium phosphate buffer pH 7.5 (1:1 w/v). The finely ground sap was filtered through double layer cheese cloth and filtrate was mixed with a pinch of celite powder. For sap inoculation a piece of sterile cotton pad dipped in a filtrate was rubbed unidirectional from petiole towards the margin of the leaves on the test plants (20 days old). The leaves were supported with a piece of cardboard under the lower surface to avoid injury. The inoculated leaves were washed with a jet of water to remove the traces of celite. The plants were labelled and kept for observation under insect proof conditions and were observed at weekly intervals post-inoculation.

RNA isolation, cDNA synthesis and (RT-PCR) based detection for CMV and ChiVMV

Total RNA were extracted from leaf samples using commercial kit. 8.0 µl of total RNA for each sample was mixed with 1.0 µl (100pM/µM) of specific reverse primer targeting CP regions of CMV and ChiVMV. Template mixture was heated at 70 °C for 5 min, followed by a short spin for 5 s after snap chilling on ice. The reaction mixtures for cDNA synthesis were prepared using reverse transcriptase (Promega, Madison, USA), added with 5.0 µl template (100-120 ng) mixtures prepared as above to it and mixed well. The first strand cDNA were synthesized by subjecting the reaction mix to 65 °C for 5 min followed by 42 °C for 6 0min for reverse transcription and then to 70 °C for 15 min in an automated thermal cycler. The PCR reaction was performed in 25 µl reaction mixture containing 2.5 µl of 5x taq buffer, 1 µl of $Mgcl_2$,1µl dNTP mix (2.5 Mm each), 1µl each of forward primer and reverse primer and 0.5 µl of Taq. Polymerase and sterile distilled water to make up the volume. Primer pair CMV2F 5′-TTAAGAAATATACCGCTTTTTT-3′; CMV2R 5′-AGTCCTTCCGAAGAAACC-3′ targeting CP gene of CMV and primer pair ChiVMV-CPF-5′-CAGGAGAGAGTGTTATGCTG-3′; ChiVMV-CPR-5′-TTTTTTTTTTTTTTTTAACGCCAACTATTG-3′ targeting CP gene of ChiVMV were used in RT-PCR. The amplifications were carried out in an automated thermal cycler programmed for specific amplification of CP genomic region of CMV and ChiVMV (Table 1 and 2).

Table 1: PCR profiles used to amplify CP genomic region of CMV

Stage	Coat Protein (CP)
Stage-I	Initial denaturation – 5 min at 94°C
Stage-II (30 cycles)	Denaturation- 30 s at 94°C Annealing- 30 s at 50°C Synthesis- 45 s at 72°C
Stage-III	Final extension -10 min at 72°C

Table 2: PCR profiles used to amplify CP genomic region of ChiVMV

Stage	Coat Protein (CP)
Stage-I	Initial denaturation – 5 min at 94 °C
Stage-II (35 cycles)	Denaturation- 45 s at 94 °C Annealing- 2 min at 59 °C Synthesis- 2.5 min at 72 °C
Stage-III	Final extension -10 min at 72 °C

Analysis of PCR products by Agarose gel electrophoresis

Following PCR, the expected amplicons were analyzed using 1.0% agarose gel electrophoresis prepared in 1X Tris-acetate EDTA (TAE) buffer. An aliquot of 5 µl of 250 bp step up DNA ladder (GeNei, Bangalore) was electrophoresed simultaneously along with the PCR products in each gel to serve as molecular weight marker. Samples were loaded and subjected for electrophoresis at 60 Volts for 1 h. After the electrophoresis, the gel was observed under ultraviolet transilluminator (GelDoc XR, Biorad, Germany) and photographed.

Result

A) Survey

Field survey and sample collection

Surveys were conducted in different King chilli growing pockets of Manipur and its neighbouring states to study the viral disease complex of king chilli. Typical symptoms of yellow mosaic, leaf mottling, puckering, shoe-string, vein banding, and severe curling of leaves were observed during the survey on the infected king chilli plants. Based on the characteristic symptoms of disease, symptomatic leaf samples from different regions were collected (Fig.1). Severe symptoms were mainly observed in the older king chilli plants. Overall stunting, small leaf size with deformed fruits were seen in king chilli plantations which were a year old (Fig. 2). A total of 79 numbers of symptomatic and asymptomatic chilli leaf samples were tested for ChiVMV, CMV, begomoviruses as well as tospoviruses infection through RT-PCR using primers targeting coat protein (CP) region of viral genomes.

Fig 1: Symptoms of viral infection observed on king chilli leaf samples collected from different parts of Manipur

Fig 2: Severe leaf deformation and distorted fruits observed on severely infected king chilli plants; 1: Leaf mottling (Nambol), 2; Mosaic (Purul), 3; Leaf curling (Kotlen), 4; Mosaic, 5; Deformed fruits of King Chilli, 6; Deformed fruits of King Chilli, 7; Deformed fruits of King Chilli.

B) Biological characterization of CMV and ChiVMV isolates

In order to characterize the selected representative CMV and ChiVMV isolate biologically, isolates showing yellow mosaic, leaf mottling, puckering, shoe-string, vein banding, and severe curling of leaves collected from different regions of Manipur were mechanically inoculated to host (king chilli) plants. Out of 30 king chilli samples inoculated, 20 samples showed symptoms of typical CMV and ChiVMV infection after 19-24 days of inoculation (Fig.3; a, b, c and d). Inoculated plants were confirmed by RT- PCR. Mock-inoculated plants did not show any symptoms (Fig 3; e & f)

Fig 3: (a & b) Symptom Expression of CMV

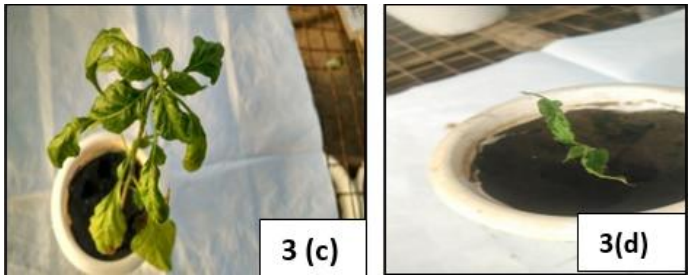

Fig 3: (c & d) Symptom Expression of ChiVMV

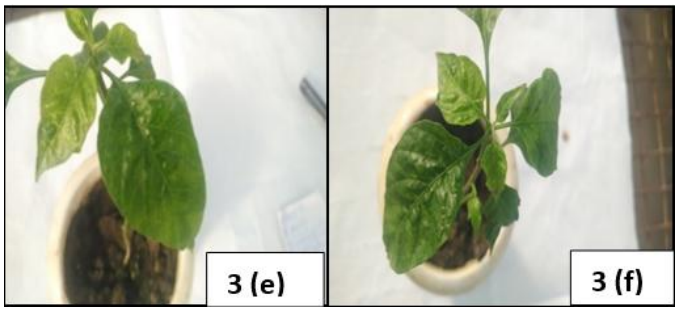

Fig 3: (e & f) Mock test

C) RT-PCR based detection of ChiVMV and CMV

ChiVMV and CMV were successfully detected using reverse transcription PCR based method. ChiVMV was detected in 6 locations while CMV was detected in 9 locations. Out of 79 samples tested for four viruses in RT-PCR using specific primers targeting CP regions of each viral genome, 30 samples were found positive for CMV (37.97 %) and 17 samples for ChiVMV (21.51%). None of the above samples were found positive for begomoviruses (chilli leaf curl viruses) and tospoviruses (capsicum chlorosis virus: CaCV). The study established the existence of ChiVMV and CMV in King Chilli plantation sites both in hills and plains of Manipur and other neighbouring states like Nagaland. The present study also conclusively reported the association of ChiVMV and CMV in bringing havoc to the chilli plantations. There were three from Ahallup and Heingang which were confirmed positive for both CMV and ChiVMV. Thus RT-PCR based analysis has been found useful in detection of both the viruses. The PCR amplicon obtained for both the viruses were in the band size typically obtained for CMV and ChiVMV. The viral origin of specific amplicons was confirmed by sequencing.

Table 3: Detection of collected king chilli samples for CMV and ChiVMV virus along with their mix infection using RT-PCR

Sl no.	Locality	District	No. Of sample collected	CMV (+ve)	ChiVMV (+ve)	Mix infection of CMV & ChiVMV	Symptoms
1	Mongjam	Imphal East	6	4	-ve	-ve	Leaf mottling, yellow mosaic
2	Canchipur	Imphal east	4	-ve	4	-ve	Shoes string
3	Singhat	Churachanpur	6	2	-ve	-ve	Smaller leaf size, yellow mosaic
4	Ngarian	Senapati	3	-ve	3	-ve	Mottling and shoe-string
5	Ahallup	Imphal East	6	2	2	2	Leaf mottling
6	Phunal Maring	Chandel	2	-ve	2	-ve	Thin lamina, small leaf
7	Mana ingkhol	Imphal West	8	6	-ve	-ve	Mottling and shoes string
8	Heingang	Imphal East	10	6	1	1	Mottling, mosaic
9	Maibakhul	Imphal East	12	4	-ve	-ve	Vein bending, shoes string
10	Maibakhul	Imphal East	12	-ve	5	-ve	Vein bending, shoes string
11	Medziphema	Nagaland	4	3	-ve	-ve	Leaf mosaic and smaller size
12	Andro	Imphal East	4	2	-ve	-ve	Vein bending and curling
13	Kotlen	Senapati	2	1	-ve	-ve	Smaller leaf size

Symptomatic variation among the king chilli leaves was recorded under field conditions. In all together samples from twenty different locations covering 12 district of Manipur has been indexed using reverse transcription PCR (RT-PCR) technique. Presence of 657bp and 1160 bp (Fig. 4a & 4b) on gel electrophoresis of amplified PCR product indicates the presence of CMV and ChiVMV on the respective samples respectively.

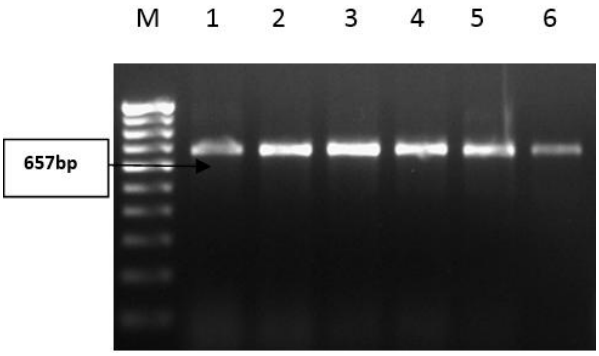

Fig 4(a): Gel electrophoresis of PCR amplicon of coat protein of CMV, showing bands at 657 bp which is positive for CMV; M symbolises the 100bp Ladder

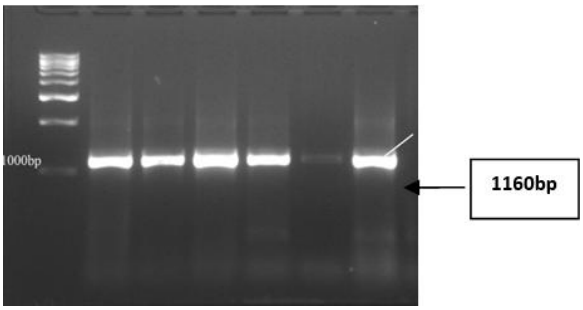

Fig 4(b): Gel electrophoresis of PCR amplicon of coat protein of ChiVMV, showing bands at 1160 bp which is positive for ChiVMV; M symbolises the 1kbladder Ladder

Discussion

Presence of ChiVMV and CMV has come to light from the present molecular based indexing of king chilli samples. King chilli which is an important spice crop has been seriously hampered by these two viruses. RT-PCR based detection exhibited higher incidence of CMV (37.57 % of the tested samples were positive) as compared to ChiVMV (21.51% samples positive). We also recorded the mixed infection of 3 samples for CMV and ChiVMV from the samples showing severe decline symptoms and distorted fruits. Thus, the present RT-PCR based viral indexing using coat protein specific primer was found convenient for routine detection. Biswas *et al.* (2005) [3] reported high incidence of viral diseases in local chilli culivars from Darjeeling and Sikkim. Using immunoassay based methods association of CMV, ChiVMV and CaCV was detected from *Capsicum annuum* in Karnataka. (Manyam and Byadgi, 2015) [12]. In the present study, out of 30 king chilli samples mechanically inoculated, 20 samples showed symptoms of typical CMV and ChiVMV infection after 19-24 days of inoculation. Based on the symtomology, host range, transmission, electron microscopy and serological studies, infection of chilli veinal mottle virus (ChiVMV) detected in Uttar Pradesh (Prakash *et al*., 2002). The virus was successfully transmitted by mechanical transmission and aphids.

Conclusion

Study of viral disease complex in king chilli in Manipur remains unknown. It is a prerequisite requirement to identify the associated viruses of king chilli before going for characterization and development of fast, easy and reliable diagnostics. From the analysis it has also been come to a conclusion that ChiVMV and CMV is associated with viral disease complex in king chilli plantations of Manipur and its neighbouring states. The incidence of CMV (37.97%) and ChiVMV (21.51%) were observed from samples collected from Manipur. Further research work can be done to identify the genetic diversity of viral complexes of king chilli found in North East India particularly Manipur.

Aknowledgement

The first author is grateful to DST (Department of Science and Technology, Govt. of India) for financial support under WOS-A scheme. Further, authors are also very thankful to Director, ICAR NEH regions and Joint Director, ICAR RC NEH region, Manipur centre for providing facilities in conducting research works.

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