Environment and Ecology 36 (2) : 489—494, April—June 2018
Website: environmentandecology.com ISSN 0970-0420

Occurrence, Molecular Characterization, Physiological Study and Yield Loss Assessment of Broad-Leaved Mustard (*Brassica juncea* var *rugosa)* Infected with *Turnip mosaic virus* in Arunachal Pradesh

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Received 24 December 2017; Accepted 13 January 2018; Published on 30 January 2018

Abstract *Turnip mosaic virus* (TuMV), member of the genus *Potyvirus,* is the most important virus of commercially grown cole crops in many Asian countries.Cole crops are an important part of daily diet in India. The study revealed that the maximum mean disease incidence was in serrated leaf type (63.67%). The maximum yield loss was recorded in green leaf type (19.46%). The occurrence of TuMV in broad-leaved mustard was confirmed by symptomatology, transmission electron microscopy (flexuous filamentous particles of 800 × 12 nm), DAS-ELISA, RT-PCR and partial characterization of cytoplasmic inclusion (CI) protein and coat protein (CP) domains. Phylogenetic analysis of the partial CP sequences of the new TuMV isolate (AR-BrLM;KP876503) revealed their closest relationship with the members of the World-B genogroup of TuMV. Significant physiological changes were observed in diseased leaves in terms of chlorophyll, total sugar, reducing sugar, total phenol and total proteins.

Keywords TuMV, Transmission electron microscopy, DAS-ELISA, RT-PCR, Phylogeny.

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Introduction

Broad-leaved mustard (*Brassica juncea* var *rugosa*) is a leafy vegetable and locally known as *laaii patta* in North-eastern states of India. In Arunachal Pradesh the crop is usually grown in *rabi* season as the most important leafy vegetable crop. Despite the ability of broad-leaved mustard to grow under a very wide range of climatic and soil conditions, problems such as diseases and insect-pests reduce its leaf yield significantly. Among the diseases, *Turnip mosaic virus* (TuMV) is one of the most economically important plant virus worldwide having wide host range over 300 species. In India, reports on TuMV of broad-leaved mustard are very few and it may be considered as an emerging threat for its cultivation [1]. Keeping this in view, this paper highlights the potential of the disease, yield loss assessment, physiological study, molecular characterization, transmission electron mi-

croscopy of broad-leaved mustard infected with TuMV.

Materials and Methods

Disease survey and yield loss assessment

This study was carried out at ICAR research complex for NEH Region, Arunachal Pradesh Center, Basar, India. It lies in between $27^0 59´$ N latitudes, $94^0 40´$ E longitudes with 650 m altitude from MSL. During 2013-14, intensive surveys were conducted for the presence of the TuMv disease in the farmers' vegetable fields. The incidence of the TuMV was recorded through random sampling from 10 vegetable fields. The disease incidence was based on the visual observation of the characteristic symptoms. Ten healthy and diseased leaves (3rd and 4th leaf from the base of plant) were randomly collected from each location and further brought to the laboratory for the estimation of yield loss.

Transmission electron microscopy (EM)

The symptomatic leaf samples were collected from field and examined under EM following the leaf dip method. The preparations were negatively stained using 2% aqueous uranyl acetate (pH 4.5). The grid was placed on a drop of extract from petiole of leaves. After 1 min the grid was washed with 10 drops of distilled water and stained with 2% uranyl acetate. Immediately after drying the grid was examined under EM.

Double antibody sandwich (DAS)-ELISA

The symptomatic and non-symptomatic leaf samples were collected from fields. They were tested for the presence of TuMV by DAS-ELISA protocols [2].

Molecular characterization

The symptomatic and non-symptomatic leaf samples were collected from fields. Total RNA extracts (RNeasy Plant Mini Kit, Qiagen Inc, Valencia, CA) from symptomatic, as well as non-symptomatic samples were subjected to reverse transcription (RT)-PCR assays using One-Step RT-PCR kit (Qiagen Inc, Valencia, CA), one set of *Potyvirus*-specific degenerate primer (CIFor/CIRev) targeting the cylindrical inclusion (CI) protein domain and coat protein (CP) specific primer (TuMV CP-F/TuMV CP-R). The RT-PCR amplicons were gel purified (GeneJET, Fermentas, India) and each fragment was sequenced bi-directionally (Biolink, New Delhi, India).

Physiological changes in broad-leaved mustard infected with TuMV

For estimation of chlorophyll, healthy and diseased leaves of different symptoms were taken randomly of 3rd and 4th leaf from the base of plant. The leaves were washed with distilled water and the water was soaked by filter paper. Then, fresh leaf samples were weighed accurately (50 mg) on an analytical balance and chlorophyll was extracted by a non macerated method. The chlorophyll extract was transferred to a cuvette and the absorbance was read in a spectrophotometer (Genesys, 10 uv) at 645 nm and 663 nm against DMSO blank. Chlorophyll-*a, b* and total were calculated by using following formula :

Chlorophyll-*a* (mg/g tissue) =
$$[12.7\ (D\ 663) - 2.69\ (D\ 645)] \times V/1000 \times W$$
Chlorophyll-*b* (mg/g tissue) =
$$[22.9\ (D\ 645) - 4.68\ (D\ 663)] \times V/1000 \times W$$
Total Chlorophyll (mg/g tissue) =
$$[20.2\ (D\ 645) + 8.02\ (D\ 663)] \times V/1000 \times W$$

Where : D = Optical density at respective nm, V= Final volume of chlorophyll extract (i.e. 10 ml), W = Fresh weight of the tissue extracted (i.e. 50 mg).

For estimation of total sugar and reducing sugar, healthy and diseased leaves of different symptoms were taken randomly of 3rd and 4th leaf from the base of plant. The total sugar content was estimated by using the method of Hedge and Hofreiter. The reducing sugar content was estimated by using the method of Somogyi.

For estimation of total phenol, healthy and diseased leaves of different symptoms were taken randomly of 3rd and 4th leaf from the base of plant. Total phenol content was estimated by using the method of Folin and Ciocalteu.

For estimation of total protein, healthy and dis-

Table 1. Reaction of different type of broad-leaved mustard against TuMV under natural field condition of Basar, Arunachal Pradesh. [a]Mean disease incidence (%) of 10 farmers' vegetable fields at 60 Days after sowing (DAS), [b]Mean fresh leaf weight of 100 healthy (H) and diseased (D) leaves, [c]Mean fresh leaf yield of 100 plants.

Type	Mean disease incidence (%)	Mean fresh leaf weight (g)[b] H	D	Yield loss (%)	Mean fresh leaf yield/plant (g)[c]	Symptoms
Serrated leaf type	63.67	3.9	3.31	15.13	97.08	Mild puckering, Mottling and Interveinal chlorosis
Green leaf type	40.43	10.69	08.61	19.46	138.30	Severe puckering, Interveinal chlorosis, Leaf distortion and Mottling
Purple leaf type	22.14	11.95	10.77	09.87	165.06	Mild puckering and Mottling
Mean	42.08	08.85	07.56	14.82	133.48	

eased leaves of different symptoms were taken randomly of 3rd and 4th leaf from the base of plant. Total protein content was estimated calorimetrically by using Lowry method.

Results and Discussion

Disease survey and yield loss assessment

The first symptoms of the disease were noticed during the 2nd week of Dec, 2013 which prevailed upto Feb month. Symptoms of the disease were vein clearing and vein banding on the young leaves followed by mild and severe puckering, mosaic, mottling, interveinal chlorosis and irregular chlorotic patches (Table 1). Severally affected plants showed stunted growth and reduced size of leaves which appeared thickened leathery and brittle in texture. Mean aphid vector population (193.75 aphids /10 cm of inflorescence) was also recorded at the time of flowering. Intensive surveys of the different farmers' vegetable field revealed that the maximum mean disease incidence was in serrated leaf type (63.67%) in Basar, Arunachal Pradesh. Similarly, Devi et al. [3] also reported disease incidence from 63.25 to 90.5% in Manipur, India. In the farmers' fields the maximum yield loss was recorded in green leaf type (19.46%). Guo et al. [4] showed that 30-100% yield loss in stem mustard in China. Therefore, it may emerge as a potential threat for broad-leaved mustard cultivation in Arunachal Pradesh, India.

Transmission electron microscopy (EM)

The symptomatic leaf samples when examined under electron microscope revealed the presence of flexuous filamentous virus particle of 800 × 12 nm (Fig. 1a), indicating the possibility of a *Potyvirus.*Haq et al. [5] also reported to have similar particle morphology of TuMV infecting leaf mustard.

Double antibody sandwich (DAS)-ELISA

DAS-ELISA using poty-group specific antisera con-

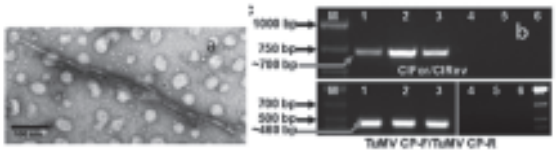

Fig. 1. (a) Transmission electron micrograph of flexuous filamentous *Potyvirus particles* in infected leaf tissue, (b) RT-PCR detection of *Potyvirus* using degenerate primer (CIFor/CIRev) and revalidation of TuMV infection through RT-PCR using TuMV CP-F/TuMV CP-R specific primer; M=1kb DNA ladder; lane 1-3 template from symptomatic; lane 4-6 template from non-symptomatic plants.

Table 2. Effect of TuMV infection on chlorophyll, total sugar, reducing sugar, total phenol and total protein contents in green leaf type of broad-leaved mustard at 60 Days after sowing (DAS). [a]Mean of 15 samples, [b] Percent increase (+) or decrease (-) = Mean contents of healthy leaves- Mean contents of diseased leaves /Mean contents of healthy leaves × 100.

	Chlorophyll					
Symptoms	a (mg/g fresh weight)	% increase (+) or decrease (-) [b]	b (mg/g fresh weight)	% increase (+) or decrease (-) [b]	Total (mg/g fresh weight)	% increase (+) or decrease (-) [b]
Light to mild mottling						
Mean [a]	1.52	-22.45	0.18	-48.57	1.70	-26.41
Mild to severe mottling						
Mean [a]	1.06	-45.92	0.27	-22.86	1.33	-42.42
Severe puckering and deformed leaf lamina and midrib						
Mean [a]	1.91	-2.55	0.33	-5.71	2.24	-3.03
Healthy leaf						
Mean [a]	1.96	-	0.35	-	2.31	-
CD (5%)	0.482	-	NS	-	0.481	-

Table 2. Continued.

	Total sugar		Reducing sugar		Total phenol		Total protein	
Symptoms	Content (mg/g fresh weight)	% increase (+) or decrease (-) [b]	Content (mg/g fresh weight)	% increase (+) or decrease (-) [b]	Content (mg/g fresh weight)	% increase (+) or decrease (-)[b]	Content (mg/g fresh weight	% increase (+) or decrease (-)[b]
Light to mild mottling								
Mean [a]	0.04	+ 33.33	0.11	+ 22.22	0.30	+ 42.86	1.19	0.00
Mild to severe mottling								
Mean [a]	0.04	+ 33.33	0.08	- 11.11	0.18	- 14.29	1.25	+ 5.04
Severe puckering and deformed leaf lamina and midrib								
Mean [a]	0.02	- 33.33	0.03	- 66.67	0.12	- 42.86	1.64	+ 37.81
Healthy leaf								
Mean [a]	0.03	-	0.09	-	0.21	-	1.19	-
CD (5%)	0.009	-	0.037`	-	0.05	-	0.053	-

firmed the association of *Potyvirus* and 38% of tested samples were positive with infected sample showing absorbance values of greater than 2.5 folds compared to healthy samples.

Molecular characterization

Transmission EM and DAS-ELISA observation indicated the possibility of *Potyvirus* infection. There-

fore, attempt was made to identify and characterize the virus species at molecular level by applying RT-PCR. The symptomatic leaf sample showed virus-specific amplification of ~700 bp (*Potyvirus* specific degenerate primer, ClFor/CIRev) of cylindrical inclusion (CI) protein domain (Fig. 1b). The RT-PCR amplicon from the sample was gel purified and fragment was sequenced bi-directionally. The partial sequence was assembled and submitted in National Center for Biotechnology Information (NCBI) GenBank (KP876500). A total of 33 sequences were aligned using Clustal W algorithm of MEGA6 and the phylogenetic tree was constructed on the matrices of aligned sequences with 1000 bootstrap replicates following neighbor-joining phylogeny of MEGA6.

The initial BLAST analysis showed that the partial CI domain of the new isolate (KP876500) shared 91-95% nucleotide identity with previously reported TuMV isolates available in GenBank (KF246570). However, the maximum nucleotide identity of 95% was shared with TuMV isolate ZHI from China (KF246570). The corresponding protein identity was 98.9-99.5% with the same isolate (protein id AGX26124). Findings were revalidated by screening the same samples with TuMV coat protein (CP) specific primers (TuMV CP-F/TuMV CP-R). Only the infected samples gave specific amplicon of ~460 bp. Further, the direct sequencing of the eluted amplicons (368 bp) generated from TuMV CP specific primers (KP876503: AR-BrLM) showed 100% identity with previously reported TuMV isolates both at nucleotide and protein level. Thus, TUMV was identified as the causal agent of mosaic disease in broad-leaved mustard. The partial CP sequence (KP876503) was compared with 33 TuMV isolates representing all genogroups of TuMV. Phylogenetic analysis of the partial CP sequences (AR-BrLM; KP876503) of the TuMV isolate revealed their closest relationship with the members of the World-B genogroup of TuMV.

Physiological changes in broad-leaved mustard infected with TuMV

The minimum amount of chlorophyll-*a* (1.06 mg/g fresh tissue) was observed in mild to severe mottling type of symptom and the maximum (1.96 mg/g fresh tissue) was recorded in healthy leaves (Table 2). Most of the viral and bacterial infection leads to chlorosis [6].

There was a significant increase in the total sugar and reducing sugar contents in light to mild mottling symptom (33.33% and 22.22%, respectively) and then found significantly decreased in severe puckering, deformed leaf lamina and midrib (33.33% and 66.67%, respectively) type of symptoms (Table 2). One of the metabolic functions of the sugars is the formation of phosphate esters which serve as substrate for respiration and release of energy. Due to retarded photosynthesis activity, less starch may have been synthesized in viral infected leaves. An enhanced rate of respiration was observed in pigeonpea leaves infected with pigeonpea sterility mosaic virus [7].

Similarly, total phenol content was also observed significantly increased in light to mild mottling symptom (42.86%) and then found decreased in mild to severe mottling (14.29%) type of symptoms (Table 2). Reason for the decrease of phenol content in severe mottling type could be due to enhanced synthesis of viral components in the host cells competing with normal biosynthetic pathways [8].

Total protein content was also observed significantly increased in severe puckering, deformed leaf lamina and midrib (37.81%). This results shows that virus infection leads to increased total protein content due to accumulation of viral proteins.

Physiological and photosynthetic properties and growth of plants infected by virus have been shown negatively influenced by several researchers [9, 10]. It is often found that fitness of virus-infected plants was lower than that of healthy plants. The low productivity of infected plants has been probably due to physiological stress with low photosynthetic rate of chlorotic leaves. Actually decrease in photosynthetic rate of the infected leaves is often associated with development of the symptoms [11]. The present findings supported the physiological changes observed in broad-leaved mustard infected with TuMV.

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