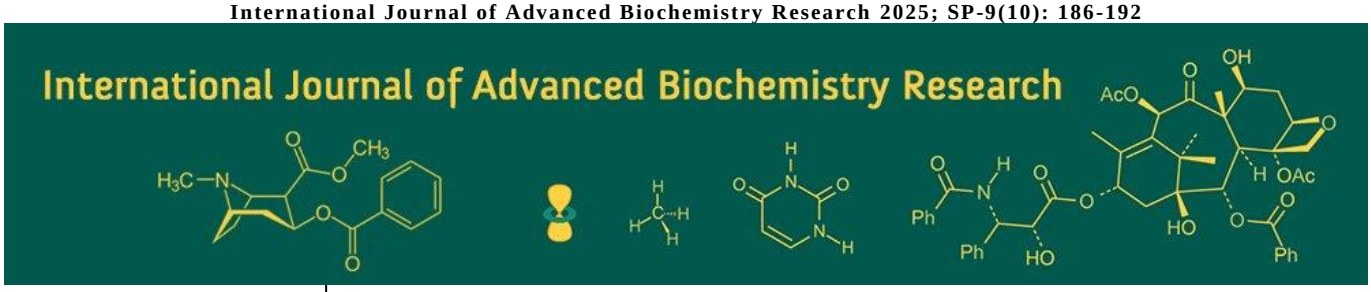

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Symptomatology, transmission and molecular characterization of *Rustica tobacco mosaic virus* (RTMV) in *nicotiana rustica*

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Abstract
Tobacco (*Nicotiana* spp.) is the most widely grown commercial non-food crop in the world. Among the numerous diseases, *Rustica* tobacco mosaic disease is the most concerning and destructive. In recent years, it has become one of the most severe disease, causing substantial yield loss and quality degradation in tobacco across many tobacco producing regions of the world. The virus was successfully isolated and maintained through mechanical transmission using sap inoculation under controlled glasshouse conditions. Symptomatology studies revealed characteristic mosaic patterns of light and dark green patches, leaf distortion, chlorosis, stunted growth, yellowing, puckering and a distinct erect growth habit with significant reduction in both the number and size of leaves. These symptoms were consistently observed in both naturally infected plants and those artificially inoculated, validating the pathogenic nature of *Rustica tobacco mosaic virus*. Molecular confirmation of the virus was achieved through PCR amplification using two pairs of coat protein (CP)-gene specific primers. A ~478 bp amplicon was consistently obtained from infected samples, corresponding to the CP gene of RTMV. The amplified CP-gene sequences were confirmed as *Tobacco mosaic virus* and submitted to NCBI GenBank under accession number PV368839, thus validating the virus identity at the molecular level.

Keywords: *Rustica tobacco mosaic virus*, mechanical transmission, RT-PCR

Introduction
Tobacco is a self-pollinating, short-day, C_3 plant. It is the most widely grown commercial non-food crop in the world and is one of the most important commercial crops in India due to its significant role in the country's economy and its substantial contribution to agriculture and commerce, including export. Nicotine, a secondary metabolite of tobacco, is the primary reason it is grown (Smith and Smith, 1942) [23]. As early as the 18th century, tobacco was one of the first substances to be employed as an insecticide. It is referred regarded as a "miracle crop" due to its characteristics and uses, which include stimulants, narcotics, pesticides and therapeutic applications (Charlton, 2004) [7]. Although tobacco is a tropical crop, it is currently produced in subtropical and temperate regions of the world. It occupies 4.29 million hectares worldwide, produce 7.49 million tonnes of output and 1.75 tonnes of productivity per hectare. China, India, United States, Brazil, Turkey, Russia, Italy and Zimbabwe are the world's leading tobacco-growing nations. Among them China is the world's largest producer of tobacco. After China, India is the second-largest producer (Anonymous, 2024) [4]. Tobacco is grown in India having 0.45 million hectares of land, produce 761 million kg with a productivity of 1.7 tonnes per hectare (Anonymous, 2023a) [2]. The states of Gujarat, Andhra Pradesh, Karnataka, Bihar, Uttar Pradesh, West Bengal and Tamil Nadu are the main producers of tobacco. The majority of the nation's *Rustica* types are produced in north India's tobacco growing regions. Gujarat, West Bengal, Odisha, Uttar Pradesh, Karnataka, Tamil Nadu and Bihar are the primary producers of snuff, chewing tobacco and hookah tobacco. Gujarat is home to the cultivation of bidi tobacco, hookah (Gadaku), chewing (lal and kala chopadia) and *Rustica* tobacco. Mehsana, Banaskantha and Sabarkantha districts in north Gujarat while Anand and Kheda districts in middle Gujarat are the primary locations for Rustica tobacco growing, which occupies 33,500 hectares and yields 66,188 million kg of tobacco with a productivity of 1.975 tonne/ha (Anon., 2023b) [3].

Numerous nematodes, bacterial, viral and fungal diseases can affect tobacco that is grown under the *N. tabacum* and *N. rustica* species. These diseases affect the cured leaf's quality metrics in addition to lowering tobacco production. The average crop loss from illnesses is thought to be between 5 and 10 per cent in typical years. Important nursery and field diseases now a days include bacterial wilt (*Ralstonia solanacearum*), sore shin (*Rhizoctonia solani*), brown spot (*Alternaria alternata*), frog eye leaf spot (*Cercospora nicotianae*), damping-off (*Pythium aphanidermatum*), black shank (*Phytophthora parasitica* var. *nicotianae*) and hollow stalk (*Erwinia carotovora* sub sp. carotovora), Broomrape (Orobanche spp.), *Tobacco leaf curl virus* (TLCV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV) and *Rustica tobacco mosaic virus* (RTMV). "Mosaic of Rustica tobacco" is a naturally occurring viral disease that seems to be the most prevalent (Pimpale & Summanwar, 1982) [20]. In Gujarat, Badi (1982) [5] noted a high prevalence of mosaic on Rustica. Based on serological research, they hypothesized that the causative agent was the *Rustica tobacco mosaic virus* (RTMV). Over the past 20 years, *N. rustica* infected with RTMV have experienced significant losses as a result of mosaic. Rustica tobacco's "mosaic" disease is typified by mosaic and widespread leaf chlorosis, which results in dark green blisters and plant stunting. Numerous researchers have documented that under experimental conditions, a variety of viruses including *Alfalfa mosaic virus* (AMV), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Tobacco distortion mosaic virus* (TDMV), *Cucumber mosaic virus* (CMV), *Tobacco distorting virus* (TDV), *Petunia mosaic virus* (PMV), *Tobacco etch virus* (TEV), *Primula mottle virus* (PrMoV), *Tobacco mosaic virus* (TMV), *Tomato aspermy virus* (TAV), *Tobacco chlorotic mottle virus* (TCMoV) and *Chilli mosaic virus* (CMV) are capable of infecting tobacco plants and producing visible symptoms. (Patel and Patel, 1987; Murayama *et al*., 1953; Verma and Lal, 1967 and Biswas *et al*., 2005) [18, 16, 25, 6].

Material Method

Symptomatology and mechanical transmission of *Rustica tobacco mosaic virus*

The virus culture was obtained from severely infected plants of *Rustica* tobacco in the experimental fields of the BTRS, AAU, Anand. The characteristic symptoms of Rustica tobacco mosaic disease under natural field conditions and mechanical inoculated plants of Rustica tobacco were visually observed from disease initiation to development and recorded.

The *Rustica tobacco mosaic virus* infected leaves were ground in a chilled pestle and mortar with phosphate buffer (0.1 M) in the ratio of 1:10 to maintain the stability of virus. Later, 2 µl/ml of β-mercaptan ethanol was added to remove the host impurities in the sap. The sap obtained was filtered with muslin cloth or absorbent cotton to remove the impurities.

Young *Rustica* tobacco seedlings of GCT 3 were raised in pots in the glasshouse at BTRS. Virus inoculation was done by swabbing the sap on the leaves in one direction. The inoculum was applied on the upper surface of the cotyledonary leaves using a cotton swab (Fig. 1). To create abrasion, carborundum powder was dusted on the cotyledonary leaves before inoculation. The plants were washed with water after five to ten minutes to remove the abrasive. The inoculation was carried out three times at two days intervals after each inoculation to prevent the chances of disease escape. The inoculated plants were kept in under observation and the uninoculated plants were kept as control. The plants were later observed for symptoms expression and transmission (%) as per the following formula.

$$\text{Transmission (\%)} = \frac{\text{Total number of infected plants}}{\text{Total number of inoculated plants}} \times 100$$

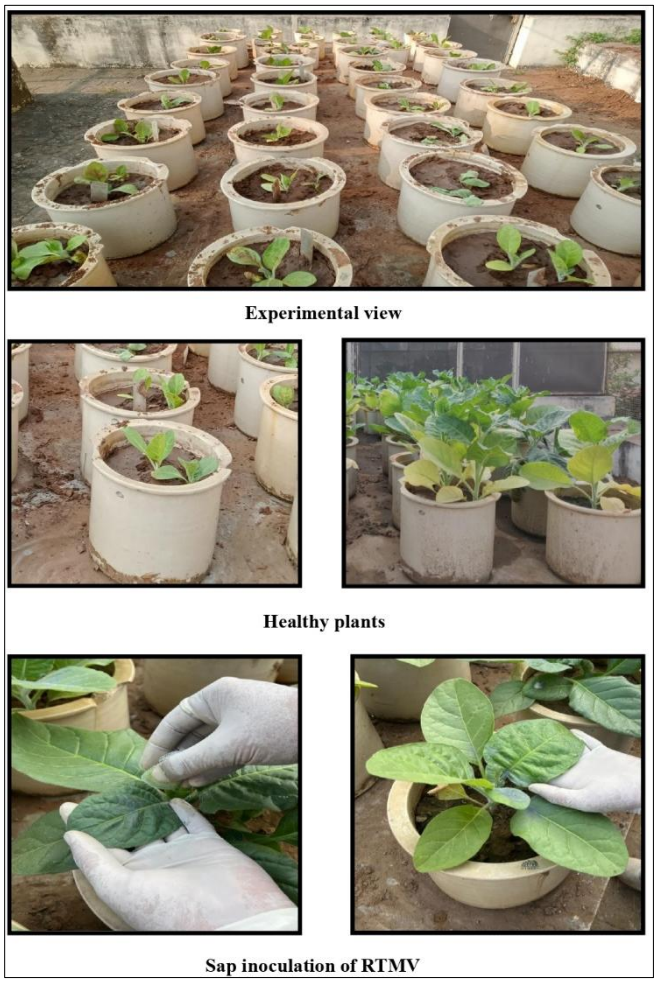


Fig 1: Mechanical transmission of RTMV by cotton swab method

2.2 Detection and characterization of *Rustica tobacco mosaic virus* through molecular technique

Procedure for RNA extraction

TRIzol reagent is an acid-guanidium-phenol based reagent that is used for the isolation of total RNA from biological samples. TRIzol (Invitrogen) was used for total RNA isolation from plant leaf samples of T_1 generation of tobacco plants. The protocol for RNA isolation is mentioned below.

1. Plasticware, glassware *etc*. were treated with 0.5 per cent DEPC followed by drying at 80 °C overnight and autoclaved. While mortar and pastels were cleaned with chloroform and autoclaved.
2. Tender upper leaves of tobacco plants were taken for RNA isolation, chilled with liquid nitrogen and stored at -80 °C until isolation.
3. The samples were placed in mortar and crushed in liquid nitrogen to obtain white powder.

4. One milliliter of TRIzol reagent was added to mortar and waited till thawed and transferred to a 2 ml Eppendorf tube.
5. Chloroform (0.5 ml) was added to the tube and centrifuged at 12,000 rpm for 15 min at 4 ºC.
6. Supernatant was transferred to a 1.5 ml tube and an equal volume of chilled isopropanol was added and centrifuged at 10,000 rpm for 10 min at 4 ºC.
7. The remaining isopropanol was discarded and 100 µl of 70 per cent ethanol diluted in RNAse-free water was added and centrifuged at 10,000 rpm for 5 min at 4 ºC.
8. The ethanol was discarded and waited until remaining ethanol was dried
9. Finally, RNase-free water (20 µl) was added and kept till pellet was dissolved.
10. Obtained RNA was subjected to quantification by TECAN nanoquant machine and quality was checked by running on agarose gel electrophoresis.

Qualitative assessment of total RNA by gel electrophoresis

The quality of isolated RNA samples was checked through 1.2 per cent agarose gel electrophoresis. The gel was prepared in 1X Tris Borate EDTA (TBE), which was also use as electrophoresis buffer. RNA with two bands (18S and 28S) of r-RNA was observed in gel image. Diffused band besides r-RNA indicates presence of good amount of m-RNA.

Procedure for gel electrophoresis

1. The gel casting tray and comb were thoroughly cleaned with 70 per cent alcohol with tissue paper.
2. The ends of the gel casting tray were sealed with spacers; the comb was inserted.
3. Agarose gel (1.2%) was prepared by adding 2.4 g agarose to 200 ml of TBE (1x) buffer.
4. The solution was boiled by putting the flask in the microwave oven and cooled to 45 °C temperature.
5. Ethidium bromide (10 µl of conc. 10 mg/ml) was added to the gel and mixed gently.
6. The gel was poured into the gel casting tray. After the gel was completely set, the tape was removed and the gel was placed into the electrophoresis tank.
7. Five hundred ml of TBE (1x) buffer was poured into the electrophoresis tank enough to cover the gel to a depth of 5 mm.
8. The comb was removed carefully.
9. 1/10th volume of loading Bromophenol blue dye (6x) was added to DNA samples and mixed by gentle tapping and spinning for 2-3 sec. in a microcentrifuge.
10. Samples were loaded onto the wells and the power supply of about 80 V was provided to run the gel.
11. The power supply was switched off when the dye was about 2 cm from the positive end and the gel was removed from the gel apparatus.
12. The gel was viewed using the Gel Documentation System.

Quantitative assessment of total RNA by nanodrop spectrophotometer

To estimate the quantity and quality (in terms of protein and DNA contamination) of isolated RNA samples, spectrophotometry was performed. One µl RNA sample was loaded into the well of the Nanodrop spectrophotometer (NanoDrop 1000, USA) and the concentration and purity of total RNA extracted was determined as the A260 nm/A280 nm and A260 nm/A230 nm ratio automatically calculated by the instrument. The UV absorbance was checked at 260 and 280 nm for sample concentration and purity.

cDNA synthesis

The extracted total RNA was subjected to cDNA synthesis using the Revert Aid First Strand cDNA Synthesis Kit - Thermo Fisher.

Protocol

All the components of the kit were thawed, mixed, briefly centrifuged and stored on ice for further use.

1. The following reagents were added into a sterile, nuclease-free PCR tubes on ice in the indicated order:

Template RNA	**Total RNA**	**0.1 ng - 5 µg**
Primer	Random Hexamer primer	1 µL
	or gene-specific primer	15-20 pM
Water, nuclease-free		Up to 12 µL
Total volume		12 L

2. The RNA template is GC-rich or contains secondary structures. So, the reagents were mixed gently by tapping against the tube and centrifuge briefly. The reaction incubates at 65 °C for 5 min. After completion of incubation time, tubes were chilled on ice, spin down and placed back on ice.
3. The following reagents were added into the indicated order into PCR vials having RNA and primer:

Fghjkgh

5X Reaction Buffer	4 µL
RiboLock RNase Inhibitor (20 U/µL)	1 µL
10 mM dNTP Mix	2 µL
RevertAid M-MuLV RT (200 U/µL)	1 µL
Total volume	20 L

4. Total 20 µl reaction was prepared for individual vial. Content was mixed gently by tapping against the tube and centrifuged briefly.
5. In gene-specific primed cDNA synthesis, the vials were incubated for 60 min at 42 °C.
6. The reaction was terminated by heating at 70 °C for 5 min. The reverse transcription reaction product was directly used in PCR applications or stored at -20 °C for less than one week. For longer storage, -70 °C is recommended.

Amplification and sequencing

The amplified product was sequenced by ABI genetic analyser.

PCR Amplification

Synthesized cDNA was subjected to PCR (Polymerase Chain Reaction) using the RTMV CP gene specific primers for the confirmation of viral infection. Primers for coat protein genes were selected from standard scientific literature (Li a*et al*., 2018) [14]. Table 1 shows sequences of the primers used to amplify and detect coat protein gene. The oligonucleotides were synthesized at SLS Research Pvt. Ltd., Surat, Gujarat, India. The pair of primers were highly specific and gave a PCR product of known size that was easily identified by electrophoresis on agarose gel.

Separation of amplified product by agarose gel electrophoresis

From about 10 µl of amplified product, 3 µl was resolved on 1.8 per cent agarose gel which was pre-stained with ethidium bromide (1 µg/ml) using 1X TBE buffer prepared from 10X TBE buffer at pH 8.0 along with 100 bp and 1 Kb as DNA molecular weight marker. Electrophoresis was done at 80V for 40 minutes. The gel was observed in a transilluminator over UV light and the image was documented using the Alpha EaseFC4.0.0 Gel Documentation System.

Nucleotide sequence of the amplified product

The sequencing of the amplified product was carried out at SLS Research Pvt. Ltd., Surat, India.

Table 1: Sequence of oligonucleotide primers used for coat protein gene

Sr. No.	Primer	Sequence (5'→ 3') (3'→ 5')	Reference
1.	TobamodF2	TGGACGGTGTGCCTGGTTGYGG	Li *et al*., (2018) [14]
2.	TobamodR2	ACTGAATCTACCTGTAATTGCTAT	

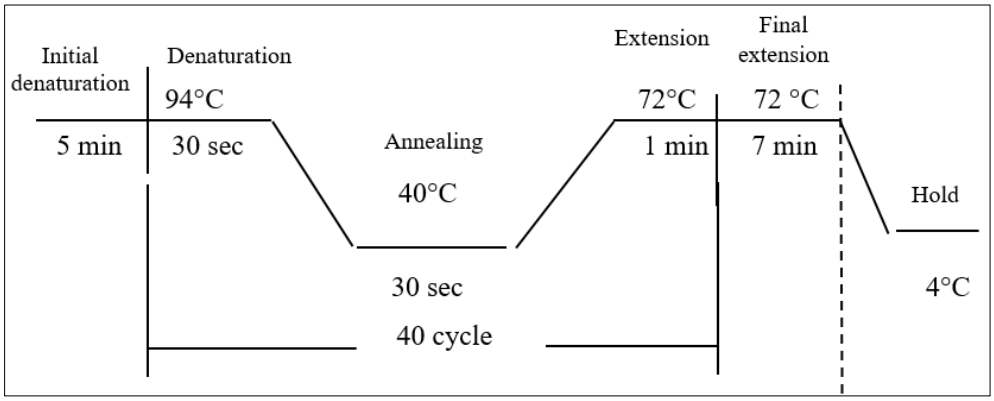
PCR reaction mixture was prepared as mentioned in table 2 from the stock solutions of each individual component. The reagents were mixed thoroughly by a short spin using microcentrifuge.

Table 2: PCR reaction mixture

Sr. No.	Reagents	Quantity (µl/reaction)
1.	DreamTaq green PCR master mix (MBI, Fermentas AG)	25.00
2.	Forward primer (10pM/µl)	2.00
3.	Reverse primer (10pM/µl)	2.00
4.	Nuclease free water	13.00
5.	Template DNA (50 ng/µl)	8.00
Total		50.00

The tubes were placed in the Thermal Cycler (Eppendorf, Germany) for cyclic amplification. The conditions for amplification were programmed in the machine as mentioned below.

Programming for PCR



Analysis of PCR products

About 10 µl of amplified products from each tube along with 2 µl dye were resolved on 1.8 per cent agarose gel, pre-stained with ethidium bromide (1 µl/ml) using 1X TBE buffer prepared from 10X TBE buffer at pH 8.0 along with 100 bp as DNA molecule weight marker. Electrophoresis was done at 80V for 2 hrs. The gel was observed in a transilluminator over UV light and the image was documented using the Alpha EaseFC4.0.0 gel documentation system**.**

Sanger DNA sequencing

The sequencing of the amplified product was carried out at and SLS Research Pvt. Ltd., Surat, Gujarat, India.

Homology analysis of the sequence and phylogenetic analysis

The RTMV nucleotide sequence was analyzed using the BLASTn (Basic Local Alignment Search Tool, nucleotide version) algorithm via the GenBank database of the National Center for Biotechnology Information (NCBI), USA, to identify homologous sequences (Altschul *et al*., 1997) [1]. Subsequent multiple and pairwise sequence alignments were performed using BioEdit version 5.09. To assess the percentage sequence similarity with closely related species identified through BLAST, a phylogenetic tree was constructed in MEGA XI software using the CLUSTALW (Thompson *et al*., 1997) [24] algorithm. The neighbor-joining method with 1000 bootstrap replicates was employed to infer evolutionary relationships among the aligned sequences.

Results and Discussion

Symptomatology and Mechanical transmission of *Rustica tobacco mosaic virus*

In case of natural infection in field, plant at younger stage showed mosaic pattern of light and dark green area on leaves, stunted growth, leaf distortion, yellowing of leaves, puckering and erect growth of plants (Fig. 2). In RTMV infected tobacco plants, there was fewer production of leaves and leaves were smaller in size.

More or less similar symptoms were consistently observed in most tobacco plants infected with RTMV. One of the most prominent symptoms was the development of mosaic patterns on the leaves, which agrees with the findings of Pazarlar *et al*., (2013) [19]. Infected plants also showed noticeable stunted growth, a common indicator of viral infection (Mochizuki *et al*., 2014) [15]. Leaf chlorosis or yellowing was frequently observed as well.

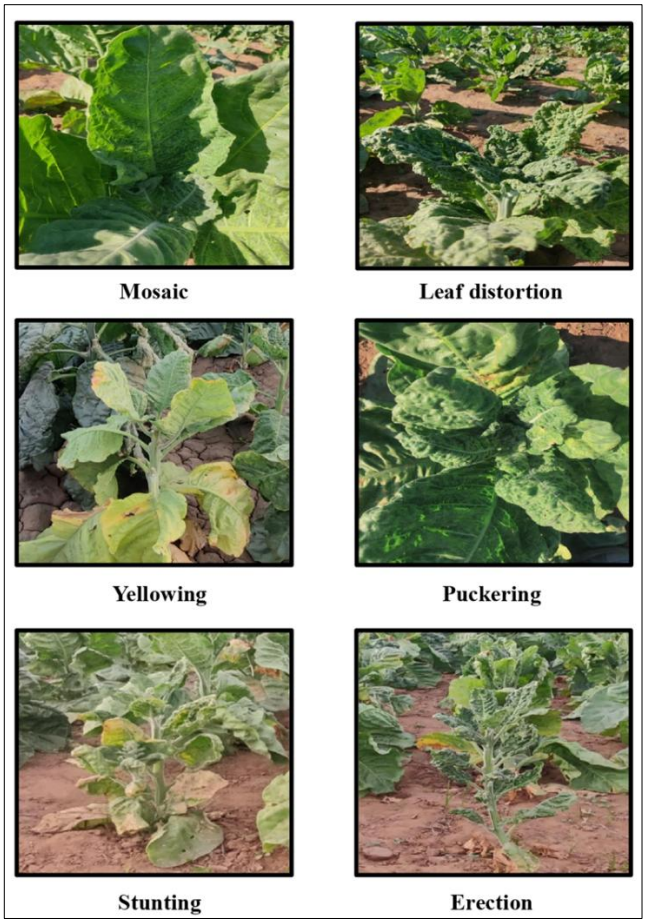


Fig 2: Symptoms expressed by RTMV under field conditions

Mechanical inoculation of RTMV was successfully carried out on the GCT 3 variety of tobacco using the cotton swab method. A total of 50 healthy and uniform plants were selected for inoculation to ensure experimental consistency. The inoculation process was performed under controlled conditions and all plants were maintained under identical environmental parameters to minimize variability in symptom expression.

Following inoculation, the plants were regularly observed for the appearance of typical RTMV symptoms. Within a few days post-inoculation, visible symptoms began to develop in a majority of the treated plants. Out of the 50 inoculated plants, more than 35 exhibited clear and characteristic symptoms associated with RTMV infection. These symptoms included light and dark green mottling (mosaic), chlorotic patches, leaf distortion, puckering and in some cases, a reduction in overall plant vigor and stunted growth (Fig. 3).

The high incidence of symptomatic plants, accounting for over 70 per cent of the total inoculated population, indicates a successful mechanical transmission of the virus using the cotton swab method. This also demonstrates a considerable level of susceptibility of the GCT 3 variety to RTMV under experimental conditions. The results affirm the effectiveness of mechanical inoculation for experimental virus transmission studies and provide important baseline data for evaluating host-pathogen interactions and varietal resistance in tobacco.

The findings of this study are align with earlier research on the mechanical transmission and symptom expression of TMV in susceptible tobacco varieties (Scholthof, 2004) [22]. Several previous studies have reported high efficiency of TMV inoculation through mechanical methods such as rubbing or swabbing with an abrasive agent. For instance, Sacristan (2011) [21] demonstrated the ease of TMV transmission via mechanical means and emphasized the role of direct contact between viral particles and wounded epidermal cells for successful infection. Similarly, Kheyrodin (2017) [11] showed that even a mild abrasive application can lead to systemic infection in susceptible hosts within a short period.

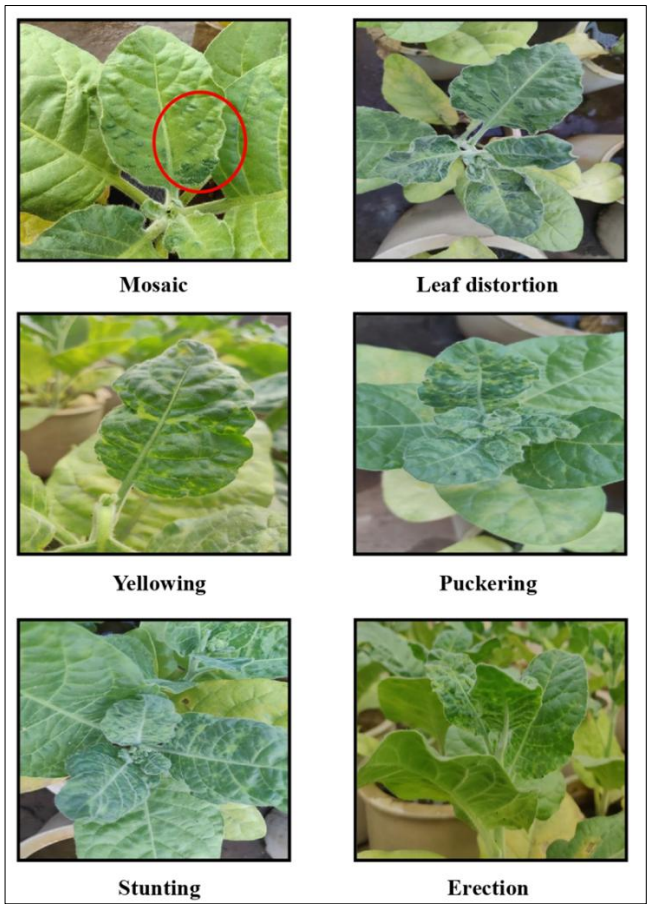


Fig 3: Symptoms expressed by mechanical transmission of RTMV

Detection and characterization of *Rustica tobacco mosaic virus* through molecular technique

Qualitative Assessment of Total RNA by Gel Electrophoresis

In present investigation, the quality, quantity and integrity of total RNA extracted from RTMV infected leaves was checked by nanodrop spectrophotometer. The quantity of total RNA extracted was 932 ng/µl (Table 3). The ratio of 260/280 and 260/230 was 2.01 and 2.20, respectively which suggested purity of RNA (Table 3) which is agreement with the normal purity standards of RNA as described by Chomczynski and Mackey (1995) [8] and Gallup (2011) [10].

Table 3: Assay of the DNA sample obtained through NanoDrop Spectrophotometer

Sr. No.	Sample	A_{260nm}/A_{280nm}	A_{260nm}/A_{230nm}	Concentration (ng/µl)
1	RTMV	2.01	2.20	932

cDNA Synthesis

Random primers/Oligo-(dT) primers/gene-specific primers and reverse transcriptase enzyme are key component to obtain cDNA. Single stranded cDNA template was observed

when cDNA was run on 1.8 per cent agarose gel to check integrity of cDNA, which specified that the cDNA prepared was solely from mRNA and free of any contamination of other RNAs. Synthesized cDNA was used for amplifying viral CP-gene through RT-PCR.

Amplification and Sequencing

The amplified product was sequenced by ABI genetic analyser.

PCR amplification

Synthesized cDNA was subjected to PCR (Polymerase Chain Reaction) using the RTMV CP gene and other gene-specific primers for the confirmation of viral infection.

Analysis of PCR Products

The integrity of isolated RNA samples was confirmed on 1.8 per cent agarose gel electrophoresis. Gel image showed amplicon of ~478 bp (Fig. 4).

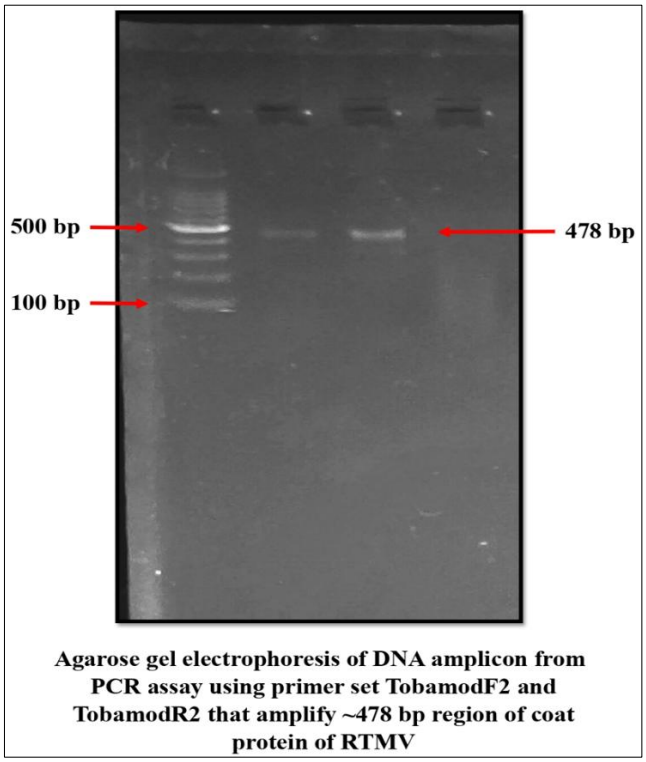


Fig 4: Agarose gel electrophoresis of PCR product of RTMV

Sequencing result

The sequences were aligned and assessed (Table 4) in the NCBI nucleotide BLAST to determine the similarity of newly sequenced samples to the GenBank databases and they were matched at diverse global similarity levels. The viral CP-gene sequences from infected leaves were confirmed to be *Rustica tobacco mosaic virus* and submitted to the NCBI GenBank.

A BLAST search for similarities of RTMV identification showed that the percentage of similarity of the isolates ranged from 93.10 to 93.93 per cent (Accession No. PV368839). As a result, the species name was assigned based on the closest match found through the BLAST search with already reported TMV (Accession No. PP471647) from New Delhi.

The RT-PCR based detection assay have also been used by many workers for the detection of RTMV and the method was found more effective and sensitive for the detection of RTMV in *rustica* tobacco leaves. (Kimaru *et al.*, 2020; Dai *et al*., 2012; Kumar *et al.*, 2011) [12, 9, 13].

Homology Analysis of the Sequence and Phylogenetic Analysis

The phylogenetic tree was constructed with a nucleotide sequence of the sequenced RTMV and compared with other similar worldwide viral isolates available in the NCBI database (Fig. 5).

Our findings do not support the hypothesis proposed by Badi (1982) [5] and Naveen Kumar (2005) [17] that RTMV arose from TMV via a straightforward genotypic transformation. Instead the genetic divergence, phylogenetic separation and conserved sequence variations suggest that RTMV should be treated as a distinct species or at least a divergent subgroup within the Tobamovirus genus. This challenges previous evolutionary assumptions and underscores the need for a taxonomic reevaluation of RTMV.

Table 4: Nucleotide sequences of CP-gene of RTMV

***Rustica tobacco mosaic virus* (Accession No. PV368839)**
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCT
TGTCATCAGCGTGGGCCGACCCAATACAGTTACTTACTTT
ATGTACTACTGCCTTACGTACTCAGTTTCAGACACAACA
AGCTCGAACTGTCGTTCAAAGACAATTCAGTCAGGTGTG
GAAACCTTCACCACAAGCAACTGTTACGTTCCCTCACGG
TCACTTTACGGTGTACAGGTACAATGCGGTATTACACCC
GTTAGTCACAGCACTGTTACGTGCATTTCACACTACAAAT
ACAATACTACAAGTTCAAAATCAGGCGAACCCCACGACT
GCCGAAACGTTACACGCTACTCGTACAGTACACGACGCA
ACGGTAGCCATACGGAGCGCTATACATACTTTACTACTA
CAATTCATCAGAGGAACCGGATCTTATAATCGGAGCTCT
TTCGAGAGCTCTTCTGGTTTGGTTTGGACCTCTGGTCCCG
CAACTTCA

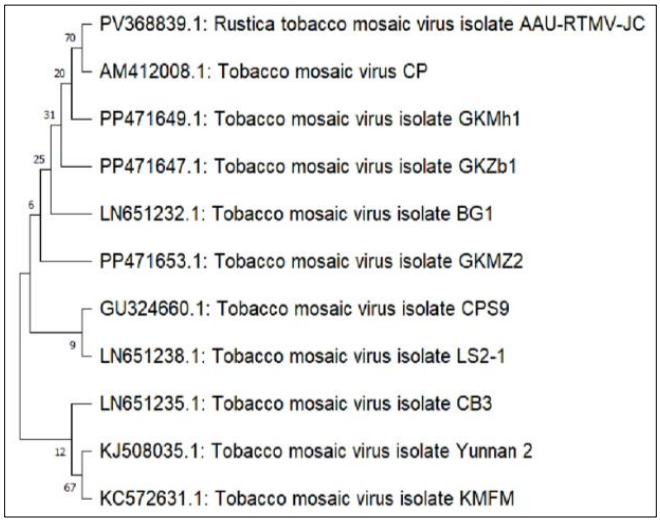


Fig 5: Phylogenetic tree based on sequence of cDNA viral CP gene of RTMV

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