Original Research Article

Wilting of Bean Plants from Tobacco Mosaic Virus from Smoking Tobacco in Pakistan

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ABSTRACT

Tobacco mosaic virus (TMV) was detected in one out of three samples of different commercial brands of cigarettes by RT-PCR. A PCR product between 650-680bp in size was observed on 1% agarose gel, which was confirmed as TMV through sequencing. Mechanical inoculation of sap from the PCR positive samples showed local lesions on bean (*Phaseolus vulgaris*) plants used as an indicator plant. In addition, symptoms of wilting were also observed in the bean plants. RT-PCR of extract from the inoculated bean plants reconfirmed the presence of TMV in bean plants. This study shows that TMV can be prevalent and has virulence in commercial tobacco (*Nicotiana* spp.). The wilting in bean plants upon mechanical inoculation of TMV was observed for the first time and can be reported as a new symptom of TMV infection in beans. This wilting associated to TMV opens the discussion for presence of different isolates of virus, symptomatic expression on differential hosts and methods of mechanical inoculation.

Key words: Beans plants, Mechanical transmission, RT-PCR, Extraction of RNA.

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INTRODUCTION

Tobacco mosaic virus (TMV) belonging to *tobamovirus* genus is one of the devastating widely distributed viruses infecting several vegetables, ornamental, leguminous and other solanaceous crops, especially tobacco around the world (*Nicotiana* spp.). This virus has been known as one of the most stable viruses and can survive without planting material in soil and smoking tobacco for many years, sometimes up to 100 years.[1] This virus causes local infection as well as systemic infection in different hosts. TMV can be transmitted easily through mechanical means in differential hosts[2] with crop debris and contaminated equipment.[1,3-8] Different methods including biological, serological and molecular assays have been used for the detection of TMV in plants with great ease and precision. Molecular detection through PCR variants is not only easy but also has been found to be sensitive, quick and reliable.[2,6-8] As TMV is stable and can be found in dried form tobacco,[3] there are chances for the transmission of TMV through the hands of man in the form of cigarettes as cigarettes are traded between countries. Thus, the following study was conducted to extract RNA for the detection of TMV in smoking tobacco and its transmission to indicator plants.

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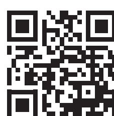

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MATERIALS AND METHODS

Extraction of RNA

Dried tobacco samples were collected from three (P1-3) different commercial brands of cigarettes for the detection of TMV. Total RNA from the samples was extracted following standard protocol using RNA extraction kit (Qiagen, USA) with some modification.[3] Dried tobacco sample of 3g from each brand of cigarettes and was pulverized in liquid nitrogen using pestle and mortar. Extraction buffer [0.13 M Sodium chloride, 0.0083 M

Sodium dibasic hydrogen phosphate (Na_2HPO_4), 0.0014 M Potassium dihydrogen phosphate (KH_2PO_4), 0.0026 M Potassium chloride, 0.13% sodium sulfite, 2% PVP K-40, 0.02% Sodium azide, 0.2% Egg albumen and 0.05% Tween-20] was prepared in one litre of distilled water with the pH of 7.4. Each sample was added in a screw cap tube with 5ml extraction buffer and homogenized. After homogenization, the samples were put on ice for 2 min and then transferred to spin column in 2 ml collection tube provided with RNA extraction kit (Qiagen, USA). The samples were centrifuged for 5 min at 12,000 rpm. Supernatant was transferred to 1.5 ml micro-centrifuge tube and suspended in 0.5 volume of absolute ethanol by pipetting. 600µl of mixture was transferred to spin column and centrifuged at 10,000 rpm for 15 s. Flow through was discarded and about 700 µl RW1 buffer was added and placed in centrifuge again. Again, flow through was discarded and 1000 µl RPE buffer was added to spin column in two steps and centrifuged for 3 min at 12,000 rpm. The spin column was placed in a 1.5 ml tube and 50 µl RNase free water was added and centrifuged at 12,000 rpm for 2 min. All the centrifugation steps were carried out at 4°C. The extracted RNA was preserved at -80°C for further use.

Reverse transcriptase polymerase chain reaction (RT-PCR)

A set of forward and reverse primers were designed from the TMV isolate (accession code AF273221) for RT-PCR amplification of TMV; 5895f GACAGTGACTTTAAGGTG and 6395r GAACCCCTCGCTTTATTACG. RT-PCR was performed[3] using the RT kit (Promega, USA). cDNA was subjected to PCR in a thermal cycler for 35 cycles under the conditions (provided with the PCR kit) of initial denaturation at 95°C for 2 min followed by denaturation at 95°C for 1 min. Annealing was done at 55°C for 1 min. Temperature for extension was set to 72°C at 1 min. A final extension was carried out at 72°C for 5 min. PCR product was run on 1% agarose gel with 0.1% SDS at 90 V for 70 min. The bands were observed in gel documentation system and images were captured. The bands were extracted and sent for direct sequencing.

Mechanical inoculation

Two grams of dried tobacco from PCR confirmed samples was ground and homogenized using pestle and mortar in 5 ml 0.02 M phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.6). The sap was passed through double layer muslin cloth and placed on ice for mechanical inoculation. Ten seedlings of three-week-old beans were used as indicator plants. Five seedlings were dusted with carborandum powder and sap was applied with the forefinger. Water was applied to other five seedlings as control. Excess sap was immediately washed under tap water and plants were kept in insect-free cages. Total RNA was extracted from the infected beans after one week and subjected to RT-PCR as described above.

RESULTS

In this study, TMV was recovered and partially sequenced from smoking tobacco of commercial cigarette brands and bean plants upon mechanical inoculation through RT-PCR with TMV specific primers. RT-PCR confirmed the presence of TMV in the samples. Nucleic acid extracted from one out of three commercial cigarette samples showed a band of the expected size, approximately 650-700bp in agarose gel electrophoresis (Figure 1A). Sequencing of the amplicon confirmed the similarity of the PCR product with 99% identity to the region of coat protein gene of complete sequence TMV isolates (Genbank accession number AF273221). Mechanical inoculation of sap from the RT-PCR TMV positive sample on bean plants (indicator plants) showed local lesions followed by quick wilting after three days of inoculation in four out of five plants (Figure 2 A-D). RT-PCR of the nucleic acid extract from the inoculated bean plants confirmed the presence of TMV. The size of band and sequence information was the same as before (Figure 1B). Therefore, mechanical inoculation coupled with PCR confirmation revealed that quick

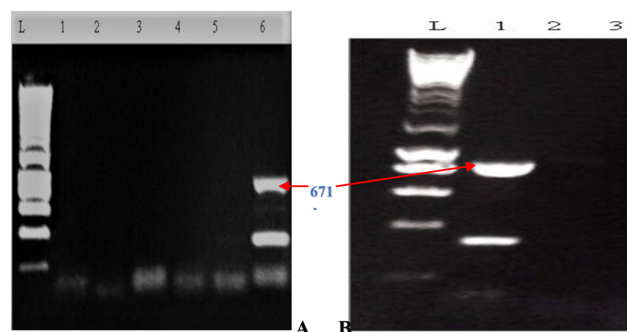

Figure 1: (A) PCR amplification of Tobacco mosaic virus (TMV) on 1% agarose gel from smoking tobacco. Lane 1: Control (No template); lane 2, Sap from healthy bean plant; lane 3, Sap from healthy tobacco leaves; lane 4, tobacco from P1; lane 5, tobacco from P2; lane 6, tobacco from P3. (B) Reconfirmation of Tobacco mosaic virus (TMV) on 1% agarose gel from Inoculated bean (*Phaseolus vulgaris*) Lane 1: wilted bean sample inoculated from tobacco sample (PCR confirmed); lane 2, Sap from healthy bean plant; lane 3, Control (No template).

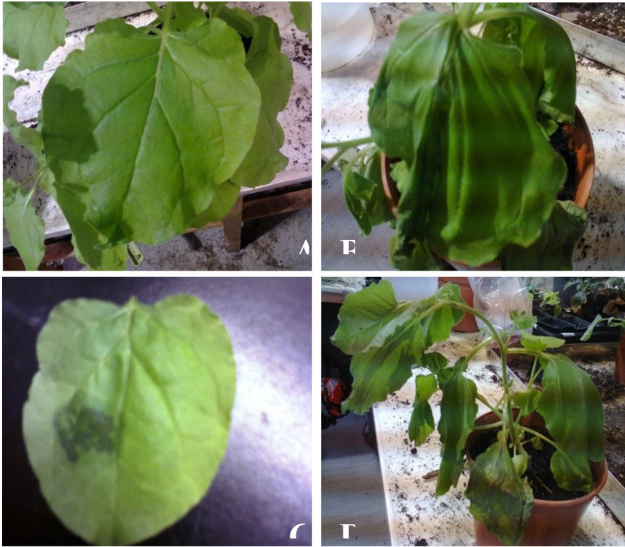

Figure 2: A-D Symptoms on bean (*Phaseolus vulgaris*) plants after mechanical inoculation with isolate of Tobacco mosaic virus (TMV). (A) Bean plant just after inoculation (B) Bean showing wilting after 3days of inoculation (C) Leaf showing necrotic lesion (D) wilted plant after one week of inoculation.

wilting was also associated with TMV. Our results also showed the infection of TMV on indicator plants in the form of local lesions and quick wilting.

DISCUSSION

TMV is stable and can be found in dried form of tobacco[1-4,6] and the trading of cigarettes between countries enhances the chances for the transmission of TMV. The results of this study show that TMV could be prevalent in cigarette tobaccos in Pakistan. RT-PCR with TMV specific primers were found to be an effective method to detect TMV from dried tobacco. RT-PCR can detect rapid plant virus like TMV even in low concentration from plant and soil samples.[8] The presence of *tobamoviruses* has also been reported in fogs and clouds through RT-PCR.[5] RT-PCR is very effective to detect and identify virus in different hosts.[5] Mechanical inoculation of sap from the TMV positive tobacco sample on bean plants showed local lesions followed by quick wilting after three days of inoculation. Therefore, results for wilting were significant and needs investigation. It was detected[7] that the infectivity of TMV in compost extract of cigar tobacco debris in Indonesia. Indicator plants also produced necrotic lesion when sap from compost was applied mechanically. These findings are also in favor of our findings related to detection and infectivity of TMV in smoking tobacco. The symptoms on differential hosts can characterize the virus isolate.[4] It was observed[9] that mechanical inoculation on differential hosts for the transmission of TMV and confirmed the local and systemic infection. Moreover,[2] reported the successful mechanical inoculation of TMV on bean plants. They used different indicator plants to show different types of local and systemic symptoms. They only found local lesions not the wilting upon mechanical inoculation of TMV on beans. It has been reported that wilting can occur in some cases based on buffer used for the extraction of TMV and washing of the indicator plants. The TMV infection increased the wilting if indicator plants were not washed with water after inoculation in the presence of potassium phosphate buffer.[10] But again, it is the concentration of inoculum that needs to be determined for the different types of symptomatic expression. Our observation of quick wilting in beans is not common when inoculated by TMV infected samples and has not been reported yet. Therefore, this aspect of quick wilting, which might be due to different isolate of TMV, needs to be investigated in the future.

CONCLUSION

It concluded that TMV can induce a variety of symptoms from necrosis to wilting depending upon the nature of isolates and host plants. symptomatic expression on indicator plants are being used for the strain differentiation and characterization of different isolates of viruses. Moreover, as per previous literature, different types of symptoms are associated with TMV isolates on different hosts. Wilting of bean plants opens a new link for the investigation of symptomatic expression due to virus infection for the future studies.

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