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DETECTION OF TOBACCO MOSAIC TOBAMOVIRUS IN CIGARETTES THROUGH RT-PCR

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TMV was tried to recover from a variety of branded cigarettes and cigars. Tobacco from six different brands of cigarettes and cigar were processed and reverse transcriptase polymerase chain reaction was employed for the detection of TMV. RT-PCR confirmed the presence of TMV in tobacco from one brand of cigarette and one brand of cigar. Bean plants (*Phaseolus vulgaris)* were inoculated manually with tobacco sap of cigarettes resulting in the production of localized disease lesions. Together, these results showed that tobacco used to make cigarettes and cigars can function as an effective disease vector, potentially aiding the movement of infectious TMV between countries. This is an important finding prompting a need to test smoking tobacco for other virus particles that infect tobacco plants and survive processing as well as considering biosecurity measures to limit virus transmission

Keywords: TMV, indexing, smoking tobacco, virus transmission, electrophoresis

INTRODUCTION

Viruses (plant, animals and phages) have been detected in surface waters, in spray irrigation and atmospheric cavity. Some animal viruses have the ability of aerosol transmission. Tobamoviruses can be spread through the atmosphere (Castello *et al.,* 1995). Tobamoviruses are stable, infectious and can be mechanically transmitted from plant to plant. They can be survived in plant debris for long time. Tobacco mosaic tobamovirus (TMV) is a major viral pathogen across 200 hosts from 30 families, especially in solanaceoeus plants (including the important resource crop tobacco (*Nicotiana tabacum* L.) as well as food crops like tomato and bean. TMV has been a bench mark and always regarded as model organism for understanding the genetic code, protein structure and functions not only in plant viruses but also in animal viruses (Agrios, 2005). The pathogen is a very well characterized rod shaped virus particle, measuring 18 x 300 nm. The single stranded viral RNA (ssvRNA) is comprised of about 6400 nucleotides (Scholthof, 2004). TMV is very stable regarding its survival in host tissues for long time. Four proteins have been translated in TMV; one for coat protein, two are associated with polymerase enzyme production and the fourth one encodes movement protein (Agrios, 2005; Anonymous, 2009; Kawamura-Nagaya *et al.,* 2014).

Tobacco plants are frequently infected by TMV in all major growing areas of the world. The average annual loss due to this disease has been estimated in the USA about 40,000,000 pounds tobacco (Scholthof, 2004). Moreover losses due to TMV in tomato and bell pepper up to 90% (Vinayarani *et al*., 2011; Kumar, 2012). Transmission of this virus is usually through mechanical transmission such as use of infected tools in the field or can be transmitted by vectors, such as sucking insetcs like aphids; some successful trials of aphid (*Myzus persicae*) transmission have been reported, but control of transmission is not yet feasible (Lojek and Orlob, 1972; Mughal *et al.,* 2006). Contaminated soils play an important role for the survival and transmission of TMV (Yang *et al.,* 2012)

TMV can bear adverse conditions much better than hosts and can survive in infected tissue, plant debris, seeds and even in cigarettes for many years (Anonymous, 2009; Mughal *et al.,* 2006). This could therefore pose a threat to socio economic status of the growers and tobacco industry if viral particles are being transmitted geographically through smoking tobacco. As a first step to understanding whether TMV can be found in smoking tobacco, we designed primers for PCR to analyse tobacco for detection of TMV in branded cigarettes and cigars.

MATERIALS AND METHODS

***Primer design*:** A combination of forward and reverse primers was designed from the TMV complete genome (accession code AF273221 GenBank) to bind to conserved regions from the C-terminus of the polymerase domain and the movement protein. The primers 4800f (ACACACAGTTGGACGACG) and 5470r (CTCATCAACGACTTCTTCTG) of 18 and 20 nucleotides respectively were designed using the Primer3 Input (Version 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/).

***Extraction of RNA*:** Four brands of cigarettes (B, M, M1, W) and two brands of cigar (K, H) were used as sources of

processed tobacco from around the world; further information on the brands is available on request. Total RNA was extracted from tobacco of cigarettes using the QIAGEN total RNA extraction kit. A bead beater was used to grind 100 mg of tissue in 450µl of RLT buffer (provided with the kit). The homogenized tissue was centrifuged at 13000 rpm for 2 min. The supernatant was collected in a sterile 1.5 ml Eppendorf tube and 0.5 volumes of 96-100% ethanol was added and mixed immediately by pipette. The sample was transferred to an RNeasy spin column placed in a 2ml collection tube and centrifuged for 30 sec at 10,000rpm. The flow through was discarded followed by addition of 700µl RW1 buffer to the spin column and centrifuged for 30 sec at 10,000rpm. RPE Buffer (1000µl) was added to RNeasy spin column and centrifuged for 30 and 120 seconds respectively. The RNeasy spin column was placed in a new 2ml collection tube and centrifuged at 13000rpm for 1 minute, and the spin column transferred to a new 1.5ml collection tube. 40µl RNase free water was applied directly to the column and centrifuged for 1 minute at 10,000rpm to collect the purified RNA. RNA was quantified by spectrophotometry.

***Reverse transcription (RT) system*:** After extraction of RNA from tobacco, first strand cDNA was synthesized using GoScript™ Reverse transcription system (Promega, USA). The reaction mixture was prepared as follows. Final volume 1 (Vf1= 5µl) and Final volume 2 (Vf2= 15µl) were prepared sequentially. Vf1 (Experimental RNA (3µl each; Reverse Primer 0.25 µl each and Nuclease free water make the volume up to 5 µl) was kept in water bath at 70°C for 5 minutes followed by chilling on ice for 5 minutes. Vf1 was then centrifuged for 10 seconds and kept on ice. Vf2 (GoScript™ 5X reaction buffer @4µl; $MgCl_2$@3µl; GoScript™ Reverse transcriptase @1µl; PCR nucleotide mix @1µl and Nuclease free Water to make the volume up to 15µl was prepared and mixed with Vf1 to make the volume 20µl. The formulation was kept in heat block at 25°C for 5 minutes for annealing. Extension was carried out by keeping the mixture of Vf1 and Vf2 in a heat block at 42°C for 1hr.

***Polymerase chain reaction (PCR)*:** PCR kit (GoTaq, Promega, USA) was used for the reverse transcriptase (RT) products to amplify TMV cDNA. The total volume of 50 µl PCR mixture contained 5X buffer (Go Taq® Flexi buffer @10µl), $MgCl_2$ solution (25mM) @4µl, PCR nucleotide mix (10mM) @1µl, Upstream primer @0.25µl, Downstream primer @0.25µl, Go Taq® DNA polymerase @0.25µl, Template DNA @1µl and Nuclease free water @32.25µl. PCR was run under the conditions of initial denaturation at 95ºC for 2 min (1 cycle) followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 45 sec, final extension at 72°C for 5 min and finally holding at 4°C. The PCR was carried out in a (Biorad iCycler) thermal cycler.

***Gel electrophoresis*:** PCR products were run through 1% agarose gel in 0.5X TBE buffer containing X 10µl Gel Red. The electrophoresis conditions for the gel were 100 volts for 70 minutes. The bands on the gel were measured with the help of Hyper ladder 1 (Fermentas, USA) and visualized using gel document system.

***Pathogenicity tests*:** Pathogenicity tests in Beans (*Phaseolus vulgaris* L) plants were done by extracting sap from tobacco in 0.02M Phosphate buffer, pH 7.4. Plants were inoculated by applying sap to the forefinger and gently abrasing the abaxial surface of bean leaves at stages 4-6. Plants were incubated under natural light lamps at 22°C, 50% humidity for 72 hrs.

RESULTS

***PCR detection of TMV*:** We first aimed to use the PCR primers to check whether we could detect TMV from six different brands of cigarette and cigar tobacco sold in the UK. The PCR amplifications were performed as described above section. The reverse transcriptase products were used as templates to amplify specific virus genomic fragments, which were electrophorased on 1% agarose gel. PCR confirmed the presence of TMV in tobacco of one cigarette brand and one cigar brand. The sizes of these bands were ~ 688nt (Fig. 1), as expected from the genome sequence.

***Pathogenicity tests*:** A second approach involving

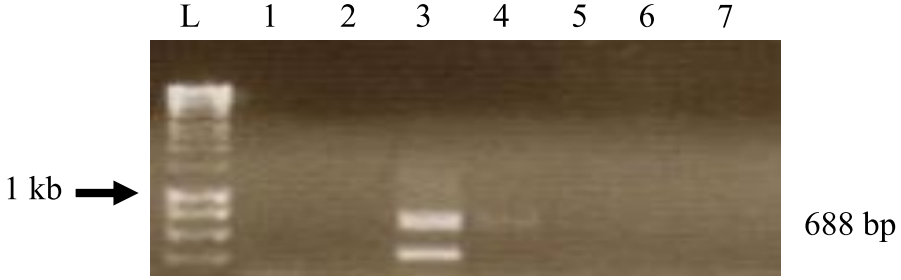

Figure 1. PCR amplification of TMV genomic fragments from cigarette and cigar tobacco. PCR products were electrophoresed through a 1% agarose gel for 1 h at 100 V. Samples in lane 1: Tobacco from M1; 2, Tobacco from B; 3, Tobacco from M; 4, Tobacco from K; 5, Tobacco from H; 6, Tobacco from W; 7, Control (No template).

application of tobacco sap to bean leaves was used to determine the presence of TMV in tobacco, we chose bean plants because it is susceptible to TMV and can be cultivated quickly and inoculated easily. Five bean plants were tested for each tobacco sap. Only tobacco from PCR-confirmed samples produced the local lesions on bean leaves (Fig. 2A); sample M caused lesions in leaves of all 5 bean plants tested whereas sample K produced lesions in 4 of the 5 inoculations done.

***Confirmation of TMV in infected bean leaves*:** The infected leaves of bean plants from TMV were collected and processed to extract sap in Phosphate buffer (0.02M) to isolate the TMV from the tissues. The cDNA was synthesized through RT system and subjected to PCR as described in the materials and methods. We postulated that the disease lesions observed in bean were caused by TMV introduced from the tobacco sap. We therefore homogenized infected bean tissue and used the RT-PCR approach to amplify TMV genomic DNA (Fig. 2B).

DISCUSSION

Tobamoviruses, such as TMV and ToMV, have broad host ranges and have been found in diverse environments including soils, forest trees and water samples. Given the stability of the virus particles, it is important to assess the likelihood of virus survival in plant products for consideration of quarantine rules of pathogens across geographic zones. PCR primers were designed to amplify TMV and found the virus to be detectable from tobacco

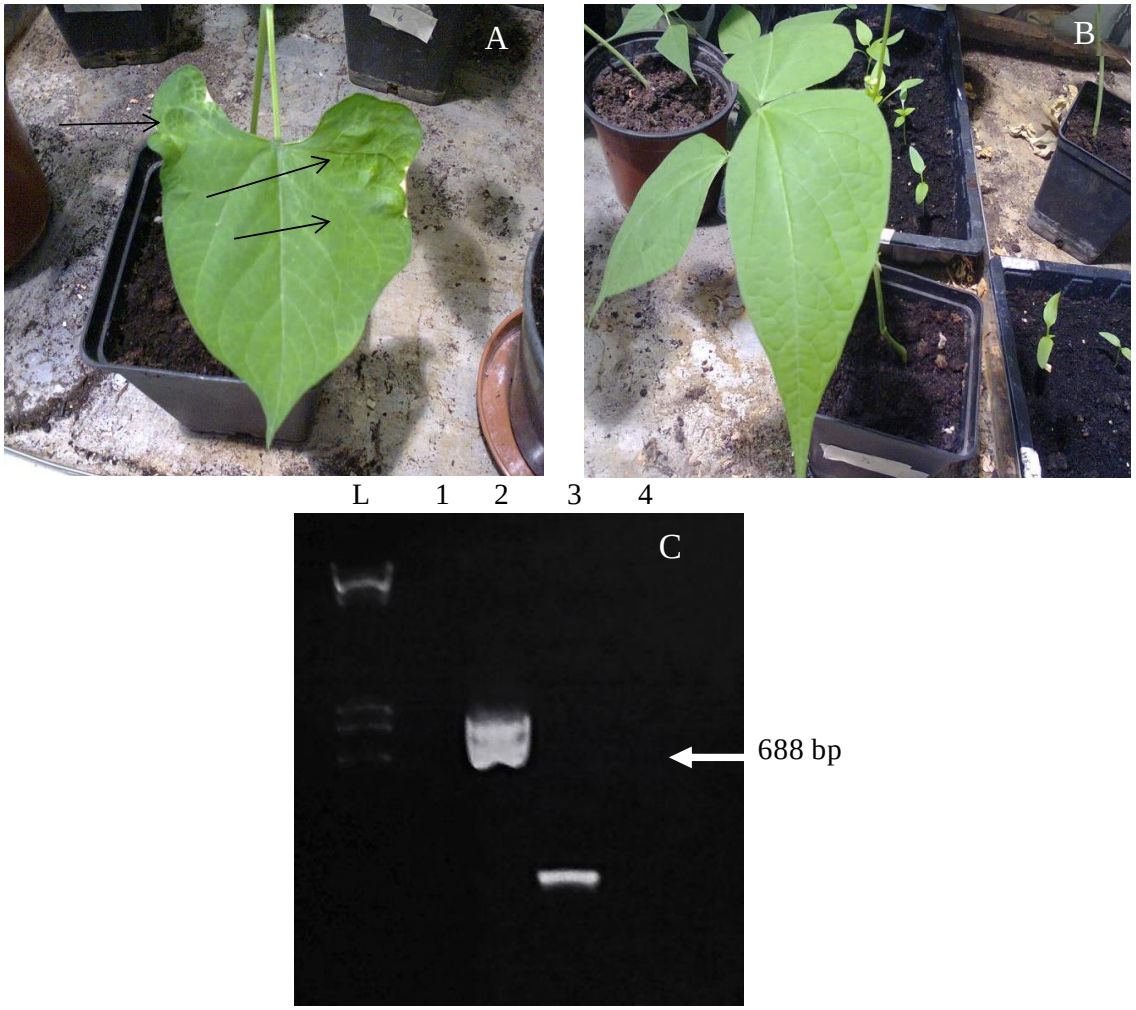

Figure 2. Detection of TMV using bean inoculation and recovery. A) Disease lesions (arrows) caused by application of tobacco sap on *Phaseolus vulgaris* leaf; B) Control bean leaf sample with tobacco sap free from TMV showed no symptoms; C) PCR detection of TMV from infected bean tissue: Lane L, Hyperladder 1; 2, tissue exhibiting TMV disease symptoms using tobacco sap on bean plant; 1 and 3, tissue from control plant tissue; 4, PCR control (No template).

samples in branded cigarettes. Moreover, indicator plants were also inoculated with TMV derived from cigarette/cigar tobacco sap, and subsequently isolated and detected the virus using the RT-PCR detection method. Biological assay using the beans as indicator plants was performed to confirm the mechanical transmission of TMV and purification of virus as well. The tobacco seedlings were out of season in February and could not be available anywhere in Reading, UK. Therefore culture of TMV was tried to maintain in bean plants and confirmed thorough PCR. The result for the mechanical transmission was in accordance with Kumar *et al.* (2011). Diagnosis of viral diseases through serological and molecular techniques has always been helpful for incidence and monitoring of emerging and established viral diseases so that effective management strategies could be formulated. Sero-diagnosis and biological assays are time-consuming processes with some limitation, whereas molecular techniques like RT-PCR can rapidly detect plant viruses such as TMV. Low concentration of virus in soil or plant samples requires high sensitivity that cannot be attained through ELISA (Yang *et al.,* 2012). The results of PCR detection of TMV were in accordance with Kumar *et al.* (2011). They used multiplex RT-PCR for the detection of two tobamovirus TMV and ToMV in pepper and tomato other than tobacco plants. Similarly Jacobi *et al.* (1998) used the immune-capture virion for PCR reaction through immunocapture reverse transcriptase-polymerase chain reaction (IC) RT-PCR to detect TMV and considered that PCR is more reliable to distinguish TMV and Tomato mosaic virus (ToMV) than ELISA. They also observed that ELISA has some limitations regarding low virus concentration in plant tissues and does not facilitate the serological differentiation between two viral isolates or members of same virus group because of cross-reactivity between antisera. Therefore multiplex IC-RT-PCR was developed for simultaneous detection of different members of tobamovirus group with greater specificity. As TMV can be survived in processed tobacco for many years, therefore tobacco fields where workers usually smoke during agronomic practices are at high risk for the transmission of TMV. Fog, clouds and water are also the reservoir for tobamovirus (Castello *et al.,* 1995)

The detection of TMV in tobacco of cigarettes and cigars sold in the UK highlights the likely transmission of this virus from where the tobacco plants were originally grown and thus represents a route for the transmission of TMV, and potentially other virus plant pathogens, between geographical zones. This has important implications for biosecurity control of pathogen spread.

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