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Evaluation of Multiplex Reverse Transcription Polymerase Chain Reaction (RT-PCR) for Simultaneous Detection of Potato Viruses and Strains

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A multiplex reverse transcription polymerase chain reaction (RT-PCR) protocol was evaluated for the simultaneous detection of *Potato leaf roll virus* (PLRV), *Potato virus X* (PVX) and *Potato Virus Y* (PVY). The multiplex RT-PCR detection of viruses was equally efficient whether RNAs were extracted using commercial kits or a sodium sulphite based nucleic acid extraction procedure. cDNAs were prepared either using a common primer (oligo dT) or specific antisense primer followed by specific primer pairs for PCR. The multiplex RT-PCR separation of strains of PVY was accomplished by using a competitive multiplex RT-PCR (with one antisense and two sense primers). The multivirus or multistrain detection approaches described here have potential application to other crop-virus combinations.

Keywords: detection of viruses, multiplex RT-PCR, potato viruses, strains

Introduction

Vegetative propagated agricultural and horticultural plants as well as trees generally harbour many viruses. Use of virus-free plant propagules is one of the strategies of disease management for these plants (Waterworth, 1998; Slack & Singh, 1998). To ensure post-harvest virus-free potato seed tubers, methods such as growing seed samples in off season and determining the virus content by visual symptoms, or testing the seed tubers in a laboratory by serological and nucleic acid based methods are being used successfully (Singh *et al.* 1999).

The reverse transcription polymerase chain reaction (RT-PCR) provides both narrow and broad specificity for the detection of RNA viruses (Singh, 1998). The availability of genome sequences of viruses and their strains has made possible the specific design of primers for RT-PCR for specific needs. RT-PCR for individual potato viruses have been developed (Singh & Singh, 1996, 1998; Singh *et al,* 1996), however, their use for the detection of individual viruses in a crop infected with several viruses is a costly affair. In order to minimize the cost, multiplex RT-PCRs for many crops and trees have been developed (Bariana *et al*; 1994; Hauser *et al,* 2002; Singh *et al,* 2000). Since multiplex RT-PCR for the detection of potato viruses and their strains has recently been made available (Nie and Singh, 2000, 2001, 2002 a,b), the objective of this study was to evaluate some of these protocols to detect potato viruses and their strains under Indian conditions.

Materials and Methods

Virus Cultures

Five virus isolates from Central Potato Research Institute, Shimla, consisting of an isolate each of *Potato leaf roll virus* (PLRV) and *Potato virus X* (PVX), and three isolates of *Potato virus Y* (PVY) were used. The PVY isolates were from *Physalis floridana*, *Datura stramonium* and *Solanum nigrum* plants.

Nucleic Acid Extraction

Two methods were used for the preparation of nucleic acid extract. In the first set, extracts were prepared, according to the manufacturer's instructions, using commercial RNeasy kit (Qiagen GmbH, Germany). In the second set, the RNAs were prepared by the sodium sulphite procedure (Singh *et al,* 2002). The procedure, in brief, was homogenizing 100 mg of leaf tissue in an extraction buffer (0.1 *M* Tris-HCl, *p*H 7.4, 2.5 m*M* $MgCl_2$, and 6 Units of DNase I) with a mortar and pestle. The homogenate was incubated at

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37°C for 10 min. RNA was extracted with phenol:chloroform:isoamyl alcohol (v:v:v; 25:24:1) using equal volume of sap and organic solvents. RNA was precipitated with equal volume of isopropanol containing 0.1 volume of 3 *M* sodium acetate, *p*H 5.2 at –20°C overnight. RNA was collected by centrifugation (12,000 g, 15 min at 4°C), then washed with 70% ethanol, dried at 37°C for 20-30 min and dissolved in 100 µl of sterile water.

Reverse Transcription

cDNA of PLRV, PVX and PVY were prepared using oligo (dT) common primer (Nie & Singh, 2000). For the separation of PVY strains, the cDNAs for all viruses and the PVY isolates were prepared using 50 ng of antisense primers (Table 1). cDNA was synthesized using reverse transcription system (Promega). The reverse trancription mixture had a final concentration of the following reaction components: 1 X buffer (10 m*M* Tris-HCl, 50 m*M* KCl, 0.1% Triton X-100), 5 m*M* $MgCl_2$, 1 m*M* of deoxyribonucleotides (dNTPs) mix, 20 units of RNasin, 0.5 ng random primers, 1 µg (9 µl) viral RNA (which was first denatured at 65°C and chilled on ice) and 15 U of AMV reverse transcriptase (AMV RT) were added to make 20 µl total reaction volume. The mixture was incubated at 42°C for 1 hr. Thereafter, AMV RT was inactivated upon incubation at 95°C for 2 min and the total volume was made up to 100 µl by adding 80 µl of nuclease free water.

The cDNA's of each virus were pooled in case of duplex and multiplex PCR.

PCR Amplification

The antisense and sense primers for each virus are given in Table 1. The primers for PLRV (4S and 5A) (Singh *et al.* 1995), PVX (Nie & Singh, 2001), PVY^o and PVY^N (Nie & Singh, 2002 a,b) have been described. The second set of PLRV (24F2 and 24A) primers are new and designed to be used in conjunction with the separation of PVY strains.

The PCR conditions varied depending on the number of intended viruses to be detected and the number of the primers involved. For uniplex PCR, 5 µl of the cDNA reaction mixture and the following reagents were added: 2.5 µl of PCR 10 X buffer [Perkin-Elmer] [100 m*M* Tris-HCl, 500 m*M* KCl, 0.01% (w/v) gelatin], 1.5 µl of $MgCl_2$ (25 m*M*), 2.5 µl of dNTPs mix (2 m*M* each), 50 ng (1 µl) each of specific sense and antisense primers, 0.5 µl AmpliTaq (5 U/µl, Perkin-Elmer, Applied Biosystems) and the reaction volume was made to 25 µl with nuclease free water. Cycling conditions were optimized to an initial denaturation at 94°C for 5 min and 30 subsequent cycles of amplification with 60 sec of denaturation at 92°C, 1 min of annealing at 60°C and 1 min of extension with 72°C followed by one cycle of final extension for 10 min at 72°C. In case of duplex and multiplex PCR the $MgCl_2$ conc was raised to 2.5 µl of $MgCl_2$ (25 m*M*). Ten µl of PCR products were electrophoresed in 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and photographed under UV light.

Results and Discussion

Using the published primers (Table 1) the amplification of the local isolates of PVX, PLRV and

Table 1—The primer pairs used for various potato viruses and PVY strains in RT-PCR

Virus/Viroid	Sequence		Fragment (bp)
Potato leaf roll virus (PLRV)	4 S	5′ – CGCGCTAACAGAGTTCAGCC –3′	336
	5 A	5′ – GCAATGGGGGTCCAACTCAT – 3′	
	24 F2	5′ – CACTGTGCTCCCTTAAATCC – 3′	613
	24 A	5′ – CTCACCTCCATATCATCCTCC- 3′	
Potato virus X (PVX)	10 S	5′ – TAGCACAACACAGGCCACAG – 3′	562
	10 A	5′ – GGCAGCATTCATTTCAGCTTC – 3′	
Potato virus Y° (PVY^0)	1 S	5′ – ACGTCCAAAATGAGAATGCC – 3′	480
	1A	5′ – TGGTGTTCGTGATGTGACCT – 3′	
Potato virus Y° (PVY^0)	S 7	5′ – GACAGTTGGACTTTTGCAACG – 3′	281
	A	5′ – CATTTGTGCCCAATTGCC – 3′	
Potato virus Y^N (PVY^N)	S 6	5′ – GGTGAAGCTAATCATGTCAAC – 3′	443
	A	5′ – CATTTGTGCCCAATTGCC – 3′	

All primer sequences, except PLRV 24 F2 and 24A have been published previously

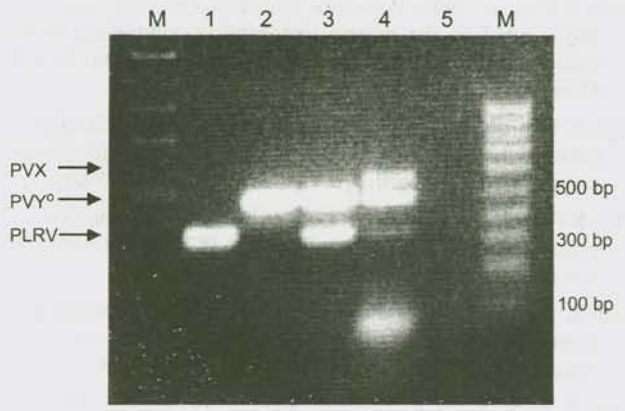

Fig. 1—Uniplex and multiplex RT-PCR of potato viruses. Lane M, molecular markers; lane 1, PLRV; lane 2, PVY^0 ; lane 3 PVY^0 + PLRV; lane 4, PVX +PVY^0 + PLRV; and lane 5, negative control

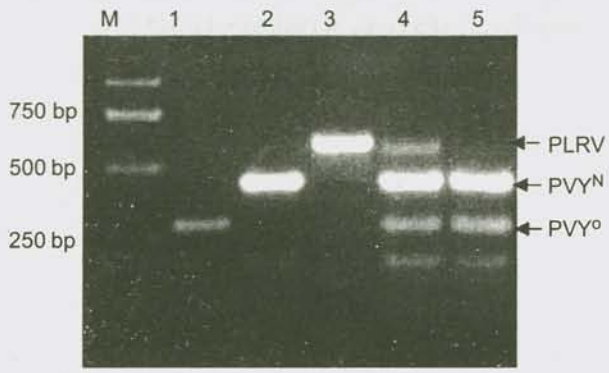

Fig. 2—Uniplex and multiplex RT-PCR of potato viruses and PVY strains. Lane M, molecular markers; lane 1, PVY^0; lane 2, PVY^N; lane 3, PLRV; lane 4, PLRV + PVY^N + PVY^0, and lane 5, PVY^N + PVY^0. A faint band of about 190 bp is produced by all isolates of PVY^N group.

PVY was detected in the uniplex, duplex or triplex RT-PCR (Fig. 1). The band intensity of both PLRV and PVY (Fig. 1, lanes 1 & 2) was high in the uniplex but slightly lower in the duplex (lane 3). In triplex, the band intensity of PLRV was significantly decreased (lane 4), therein indicating an interference with the other primers. However, irrespective of the intensity, the specific amplification of each virus took place as indicated by the absence of bands in the negative control (lane 5).

In a second experiment, involving another set of PVY and PLRV primers, a lower intensity of PLRV band was again observed in the mixed amplification (data not shown). However, use of increased amount of PLRV antisense primer, at the cDNA stage (Singh *et al,* 2000), substantially increased the yield of PLRV fragment (Fig. 2, lane 4). Additional adjustment of the PLRV and PVY antisense primer for the cDNA preparation were needed to achieve bands of equal intensity of both viruses.

In systems, where the cDNA is prepared using a common antisense primer (Nie & Singh, 2002 a,b) the quality of cDNA is generally uniform and the amplified products reflect the true template concentrations. This was demonstrated when two strains of PVY were used (Fig. 2, lanes 1 & 2), where PVY^0 strain was of lower concentration (lane 1) and the PVY^N type strain was in high concentration (lane 2). This relationship was not affected by the mixed amplification (lane 4 & 5). According to the band position (lane 2) this isolate yielded an amplified product of 443 bp, which indicated that this strain could be a member of the PVY^N group. Additional biological and molecular characterization would be needed for the specific placing of this isolate in the PVY^N group. However, it is clear that the primers and the competitive form of multiplex RT-PCR, where a common antisense primer for cDNA with multiple sense primers is used, can provide differentiation of various isolates and strains of a virus.

This study has demonstrated the feasibility of the detection of mixed infection by more than one virus simultaneously in a single sample by multiplex RT-PCR. Similarly, the closely related strains, where molecular variability is limited to a short stretch of the genome segment, can be differentiated using the competitive multiplex RT-PCR (Nie & Singh, 2002 b) as shown with the limited isolates of PVY in this study.

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