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Simultaneous detection of potato viruses A and M using *CP* gene specific primers in an optimized duplex RT-PCR

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Abstract
A duplex reverse transcription PCR (RT-PCR) assay was developed for simultaneous detection of a potyvirus (*Potato virus A*; PVA) and a carlavirus (*Potato virus M*; PVM) infecting potato crop. Six coat protein (*CP*) gene specific primer pairs (three each for PVA and PVM, respectively) were designed for both the viruses. Among these, primers PVACPF3/ PVACPR3 and PVMCPF2/PVMCPR2 successfully co-amplified the *CP* genes of both viruses at an annealing temperature of 54 °C. Primer PVACPF3/ PVACPR3 produced an amplicon of 560 bp for PVA and primer PVMCPF2/PVMCPR2 produced an amplicon of 408 bp for PVM in the same reaction. The present study has demonstrated for the first time that *CP* gene specific primers can be used to detect PVA and PVM simultaneously in an optimized RT-PCR. These primer pairs and a duplex RT-PCR optimized in the present study offers a reliable diagnostic tool for simultaneous detection of PVA and PVM in infected plant leaves and quarantine programmes.

Keywords: Potato, PVA, PVM, *CP* gene, Primers, Duplex-RT PCR.

Introduction
Potato, a tuber crop is the third most important source of staple food, after rice and wheat, for an ever-increasing population in the world (Bhaskar *et al*., 2009) [1]. This crop is infected by many pathogens, including bacteria, fungi and viruses, causing significant reduction in yield and quality of the tubers. Among these pathogens, viruses sometimes may pose serious threat since they alone can cause up to 50 % reduction in tuber yield (Pushkarnath, 1976, Sun and Yang, 2004) [24, 33]. About forty viruses and two viroids have been reported to infect potato crop (Jeffries *et al*., 2005) [12]. Etiological observations would suggest that mixed viral infection in potato is very frequent (Kerlan and Moury, 2008) [14]. The most common viruses known to infect potato crop are *Potato virus A* (PVA, genus *Potyvirus*), *Potato virus M* (PVM, genus *Carlavirus*), *Potato virus X* (PVX, genus *Potexvirus*), *Potato virus Y* (PVY, genus *Potyvirus*) and *Potato leafroll virus* (PLRV, genus *Polerovirus*) (Singh, 1999) [32]. Among the viruses, PVA and PVM are of economical concern since they significantly reduce the tuber yield. PVM has been reported to reduce the tuber yield by 15 to 45 % (Jeffries, 1998) [13]. The reduction could be 100 %, in some potato growing regions of the world, if the virus incidence is high due to early or severe infection.

PVA and PVM infects the potato crop either singly or simultaneously (coinfection). When infected singly, both viruses show very mild foliar symptoms of curling, vein deepening and leaf bronzing that are difficult to characterize. However, upon coinfection severe foliar symptoms appear that can be easily characterized as mosaic, mottling, crinkling and rolling of leaves (Dolby and Jones 1987, Rose, 1983) [6]. Coinfection of viruses in the host may result in either additive or antagonistic or synergistic interactions. Hameed *et al*., (2014) [9] detected a significant increase in PVM titer compared to PVA titer upon coinfection in potato and suggested that a synergistic interaction exist between PVA and PVM. However, (Rentería-Canett *et al*., 2011) [25] demonstrated that a synergistic interaction establish as a result of increased nucleic acid concentration of participating viruses in an equal proportion during coinfection. Since, PVM titer seen to increase compared to PVA titer upon coinfection in Hameed *et al*., (2014) [9] study an antagonistic interaction between these viruses could be hypothesized.

Disease diagnostic and management strategies increasingly require fast and accurate methods for the detection of multiple pathogenic microorganisms from complex samples. Easiness, sensitivity and reliability are the pre-requisite for any diagnostic method employed in the detection of pathogens. Keeping this in mind different serological, immunodiagnostics and molecular diagnostics methods have been developed for the detection of pathogens.

In this context, a molecular diagnostic method based on polymerase chain reaction (PCR) has been shown to be fast, efficient and specific for the detection of pathogens, including viruses. The detection efficiency of PCR-based method is 128-fold greater than that of an immunodiagnostic based enzyme linked immunosorbent assay (ELISA) (Meunier *et al*., 2003) [16]. Until 1990s most studies based on PCR detection of viruses involved *in vitro* amplification of deoxyribonucleic acids (DNAs) extracted from virus infected plants. Technics have been developed to amplify viral ribonucleic acids (RNAs), extracted from infected plants, by reverse transcribing it into its complementary DNA (cDNA) through the use of reverse transcriptase. The process is called reverse transcription PCR (RT-PCR). Several RT-PCR protocols have been developed to detect individual viruses from dormant potato tubers (Singh and Singh 1996, 1998) [29, 31, 34]. Detection of several viruses individually by RT-PCR is expensive and time consuming. Therefore, detection of two or more viruses in one PCR assay (duplex or multiplex, respectively) would be more economical and time saving. Duplex and multiplex PCR assays have been developed for the detection of various viruses including those infect potato crop (Nie and Sing 2000; 2001; 2002; Jeevalatha *et al*., 2013) [11, 19, 20, 21].

The success of duplex and multiplex PCR mainly depends on specificity of primers used in the assay. Schubert *et al*., (2007) [27] tried to differentiate the PVY variants by RT-PCR using different primer combinations published previously (Boonham *et al*., 2002; Glais *et al*., 2005; Moravee *et al*., 2003, Nei and Singh 2002; 2003; Singh and Singh 1996; Weidemann and Maiss 1996; Weilguny and Singh 1998) [2, 7, 17, 22, 29, 31, 32, 35, 36]. However, the authors reported that most of these primers did not satisfactorily differentiate all the isolates of PVY. Moreover, the primers lacked specificity for new variants of PVY in their study. In another study, a common oligo (dT) primer has been developed for the reverse transcription of RNAs from several potato viruses (Nei and Singh, 2000). The oligo (dT) primer facilitated the use of multiplex RT-PCR (m-RT-PCR) for simultaneous detection of several potato viruses. Similarly, random primers have also been used to amplify RNAs from various potato viruses and viroids (Nei and Singh, 2001). However, coat protein (*CP*) gene specific primers are known to be more sensitive than any other primers; since, they can detect the virus in micro plants and dormant potato tubers harvested from infected plants (Jeevalatha *et al*., 2013) [11]. This is because, *CP* gene is the most conserved gene in geminiviruses. The *CP* gene specific primers have been used previously for the detection of viruses including begomovirus (Brown *et al*., 2001) [3] and geminivirus (Jeevalatha *et al*., 2013) [11] in potato. Considering the sensitivity of *CP* gene, different primer pairs specific to this gene were designed and used for simultaneous detection of PVA and PVM in a duplex PCR optimized in the present study.

Material and Methods

Total RNAs from PVA and PVM infected potato leaves were extracted using a Spectrum™ plant total RNA kit (Sigma-Aldrich, India), following the Manufacturer's instructions. Quantification of total RNAs were done by Nanodrop 2000 spectrophotometer (Thermo Scientific, India). The samples with an acceptable purity (260/230 ratio≥2.0) were used for the preparation of cDNAs. First strand cDNA was prepared using a Revert Aid First Strand cDNA Synthesis Kit (Fermentas, India) and amplified by PCR, using random primers as anchored and arbitrary primers.

CP gene specific primers for PVA and PVM were designed from sequences available in National Centre for Biotechnology Information (NCBI) database using a free online tool, Primer3Plus (Untergasser *et al*., 2007) [34]. Specificity of the primers were analysed by BLAST and best combination of primer pairs were selected for PCR (Table 1). Annealing temperatures of selected primer pairs (three each for PVA and PVM) were optimized in a uniplex gradient PCR. PCR mixture of 20 µl volume contained 2.0 µl of cDNA, 2.0 µl of 10x PCR buffer, 1.6 µl of 2mM dNTPs, 0.5 µl each of 10 µM forward and reverse primers, 0.5 µl of 1.5 U *Taq* DNA polymerase and 12.9 µl of sterile double distilled water. The temperature profile of PCR cycle was pre-incubation at 94 °C for 2 min followed by 40 cycle of melting at 94 °C for 45 s, annealing at 52 °C, 54 °C and 56 °C for 30 s and extension at 72 °C for 45 s followed by final extension at 72 °C for 7 min. PCR was performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, India). The amplified products were detected by resolving 20 µl of each PCR product mixed with 4 µl of 6X gel loading dye on 1.5 % agarose gel.

A duplex PCR was performed with different concentrations (150–750 nM) of the *CP* gene specific primers to optimize the primer concentration. The primer pairs, PVACPF3/PVACPR3 and PVMCPF2/PVMCPR2 were selected for duplex PCR analysis based on their ability to detect viruses in a uniplex PCR. The optimized duplex-PCR mix included 1.0 µl of cDNA, 2.5 µl of 10× PCR buffer, 2.0 µl of 2 mM dNTPs, 2.0 µl each of 10 µM forward and reverse primers, 1.5 µl of 1.5 U *Taq* DNA polymerase and sterile double distilled water to make up the volume of 20 µl. PCR cycle conditions were the same as used in uniplex PCR except that the annealing temperature was 54 °C. The amplified products were detected by resolving 20 µl of each PCR product mixed with 4 µl of 6X gel loading dye on 1.5 % agarose gel.

Results and Discussion

A uniplex gradient PCR results revealed that all the six primer pairs could amplify *CP* genes in both viruses. Primers PVACPF1/ PVACPR1, PVACPF2/ PVACPR2 and PVACPF3/ PVACPR3 produced the amplicons of 541 bp, 571 bp and 560 bp, respectively, from infected leaves (Fig. 1a). Similarly, primers PVMCPF1/ PVMCPR1, PVMCPF2/ PVMCPR2 and PVMCPF3/ PVMCPR3 could produce the amplicons of 416 bp, 408 bp and 429 bp, respectively, from infected leaves (Fig. 1b). Both negative (cDNA from non-infected, healthy plant) and negative (PCR without cDNA) controls respectively did not produce any amplicons in the PCR.

In a duplex RT-PCR, primers PVACPF3/ PVACPR3 and PVMCPF2/PVMCPR2 successfully co-amplified the *CP* genes of both viruses at an annealing temperature of 54 °C for 30 s (Fig. 2). Primer PVACPF3/ PVACPR3 produced an amplicon of 560 bp for PVA and primer PVMCPF2/PVMCPR2 produced an amplicon of 408 bp for PVM in the same reaction. Same amplicons (560 bp for PVA and 408 bp for PVM) were produced in individual RT-PCRs performed with respective primers. A duplex RT-PCR confirmed the co-amplification of *CP* genes of both viruses with selected primers. A negative control (cDNA from non-infected, healthy plant) did not produce any amplicon in the PCR.

This is the first report of simultaneous detection of PVA and PVM using *CP* gene specific primers in a duplex RT-PCR.

Primers PVACPF3/ PVACPR3 and PVMCPF2/PVMCPR2 designed in the present study could be successfully used for simultaneous detection of PVA and PVM. PVA has been reported to co-infect the potato crop with PVX and PVS but not with PVM in a previous study (Dedic, 1975) [5]. However, in a recent study Hameed *et al*., (2014) [9] detected the coinfection of PVA and PVM along with other potato viruses but they used a serological method, ELISA.

Both PVA and PVM are transmitted through aphids and spread in non-persistent manner in the host (Halterman *et al*., 2012) [8]. When infected singly, PVA shows very mild foliar symptoms of curling, vein deepening and leaf bronzing that are difficult to characterize. Sometimes the crop even remain symptomless. As a consequence, growers who saves the tubers, from such symptomless crop with latent PVA infection, for planting in the next growing season may bear huge losses. PVA has been reported to cause yield losses of up to 40% in potato (Dedic, 1975) [5]. Upon coinfection of PVA and PVM in potato, severe foliar symptoms appear that can be easily characterized as mosaic, mottling, crinkling and rolling of leaves (Dolby and Jones 1987; Rose, 1983) [6, 27]. However, these symptoms are always attributed only to high PVA incidence; since, this virus is commonly found to infect potato crop worldwide. Consequently, growers use systemic insecticides to kill its vector but could not get good control of the disease. Nauen and Denholm (2005) [18] reported that virus acquisition by its vector and spread in the healthy plants take only seconds and systemic insecticides are ineffective in its control. In this context, we speculate that the very less time is taken by vector for virus acquisition and spread in the neighboring healthy plants compared to the time taken by systemic insecticides to translocate from one plant part to another. However, once the vector population is under control, with the help of systemic insecticides, it is difficult for PVA to spread and produce severe foliar symptoms. This suggest that either PVA transmits through other means besides vector or another virus with different mode of transmission is present and producing the severe foliar symptoms.

PVA has already been reported to co-infect with other potato viruses including PVM (Hameed *et al*., 2014) [9]. Interestingly, besides through aphids PVM has also been reported to transmit through mechanical injury (Halterman *et al*., 2012) [8]. As described by Dolby and Jones (1987) [6] and Rose (1983) [26], the coinfection of PVA and PVM produces severe foliar symptoms that can be easily characterize. Alike PVA, single infection of PVM has also been reported to reduce the tuber yield by 45 % (Jeffries, 1998) [13]. When co-infected, both PVA and PVM may cause huge yield losses in potato. Hence, it is obvious to develop a diagnostic tool for simultaneous detection of PVA and PVM in potato crop.

PCR can be used for the detection of both DNA and RNA viruses. Since, most plant viruses are RNA viruses, amplifying an RNA instead of DNA from the infected plant would increase the chances of their accurate detection. The RNA-dependent DNA polymerase (reverse transcriptase) is used to amplify an RNA molecule in a variant of PCR called RT-PCR. RT-PCR offers an easy, sensitive and reliable approach for the diagnosis of viruses in plants (Meunier *et al*., 2003) [16]. Possibility of simultaneous detection of two or more viruses in a single RT-PCR has speed-up the process of diagnosis (James *et al*., 2006, Park *et al*., 2005) [10, 23]. Hence, detection of viruses through RT-PCR is not only efficient but also faster. Considering these and several other advantages of RT-PCR over the other virus diagnostic methods, a duplex RT-PCR for simultaneous detection of potato viruses A and M has been optimized in the present study. In RT-PCR oligo (dT) is generally used to prepare cDNA from mRNA. However, in the present study random primers were used for this purpose. Oligo (dT) can only be used if the viral RNAs contain polyadenylated tail. This means, in order to obtain cDNAs from viruses the mRNAs should be intact in terms of size and shape and their numbers must be known. In contrast, random primers can be used for the reverse transcription of all RNAs, irrespective of their size, shape and numbers. Random primers for the preparation of first strand cDNAs from viral RNAs have also been used previously (De Haan *et al*., 1989, Shen *et al*., 1993, Nie and Singh, 2001) [4, 20, 28].

Different primer pairs have been developed for the detection of potato viruses through amplification of their cDNAs (Boonham *et al*., 2002; Glais *et al*., 2005; Moravee *et al*., 2003; Nei and Singh 2002; 2003; Singh and Singh 1996; Weidemann and Maiss 1996; Weilguny and Singh 1998) [2, 7, 17, 22, 29, 31, 32, 35, 36]. However, most of these primers lacked specificity for new variants of some potato viruses and hence could not satisfactorily differentiate all the isolates, such as in case of PVY (Schubert *et al*., 2007) [27]. Since, *CP* gene is the most conserved gene in geminiviruses the primers specific to this gene can detect most plant viruses not only from infected potato leaves but also from dormant tubers harvested from infected plants (Jeevalatha *et al*., 2013) [11]. This indicate that the *CP* gene specific primers are more sensitive than any other primers. Therefore, in the present study different primer pairs specific to *CP* genes of PVA and PVM were designed and used for further amplification of cDNAs of these viruses in a duplex PCR. Indeed, the *CP* gene specific primers PVACPF3/ PVACPR3 and PVMCPF2/PVMCPR2, designed in the present study, successfully amplified the cDNAs of PVA and PVM. The *CP* gene specific primers have also been used previously for the detection of RNA viruses infecting the potato crop (Jeevalatha *et al*., 2013, Brown *et al*., 2001) [3, 11].

Determination of an annealing temperature for primers is a crucial parameter in PCR. Even though the primers designed are specific and more efficient, the successful amplification of target templates depends primarily on annealing temperature. In the present study *CP* gene specific primers successfully co-amplified the *CP* genes of PVA and PVM at an annealing temperature of 54 °C in a duplex RT-PCR. (Singh *et al*., 2000) [30] and (Majumder *et al*., 2008) [15] also reported the significance of annealing temperature during optimization of RT-PCR for the detection of viruses.

Table 1: List of primers designed in the present study to amplify *CP* genes of *potato virus A* and *potato virus M*

Target Viruses	Name of primer	Sequence of the primers	Expected amplicon size (bp)
PVA	PVACPF2	5'-GACACTACCAATGCTCAAAG-3'	571
	PVACPR2	5'-CTCTTCTGAAGGTGTGACAT-3'	
	PVACPF3	5'-TGCTCAAAGGTAAGAGTGTC-3'	560
	PVACPR3	5'-CTCTTCTGAAGGTGTGACAT-3'	
	PVACPF1	5'-CGTTAACCTAGATCACTTGC-3'	541
	PVACPR1	5'-CTCTTCTGAAGGTGTGACAT-3'	
PVM	PVMCPF3	5'-CCACATCTGAGGATATGATG-3'	429
	PVMCPR3	5'-GCGTATTGTGAGCTACCTT-3'	
	PVMCPF1	5'-GCATATATGTGAACCTGGAG-3'	416
	PVMCPR1	5'-GTCTTTGTGCGTATTGTGAG-3'	
	PVMCPF2	5'-GCATATATGTGAACCTGGAG-3'	408
	PVMCPR2	5'-GCGTATTGTGAGCTACCTT-3'	

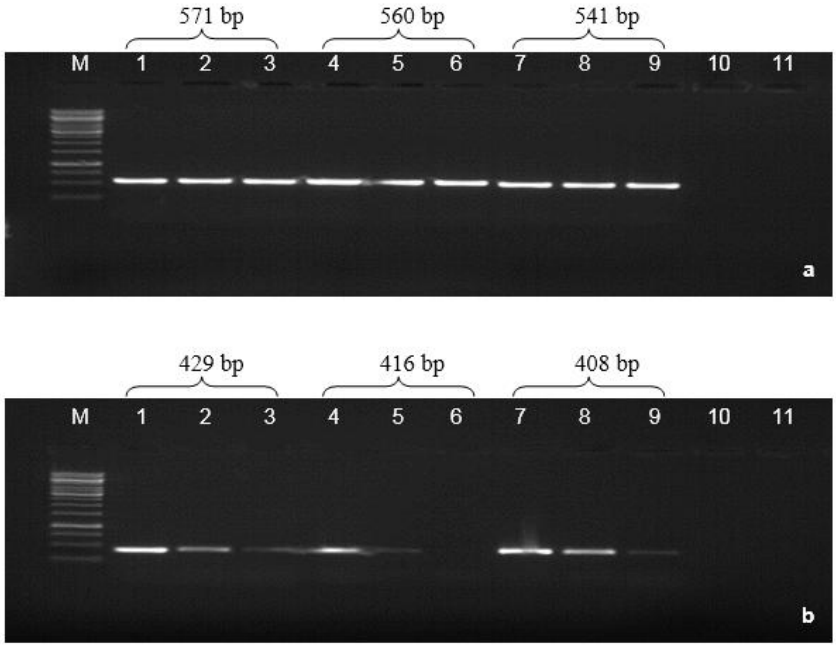

Fig 1: A uniplex gradient PCR of *CP* genes from potato viruses PVA (a) and PVM (b) amplified with six primer pairs designed in the present study. Primers PVACPF2/ PVACPR2 PVACPF3/ PVACPR3 and PVACPF1/ PVACPR1, produced the amplicons of 571 bp, 560 bp and 541 bp, (Lane 1, 2, 3, 4, 5, 6, 7, 8, 9 DNA bands from infected leaves) respectively in PVA. Whereas, primers PVMCPF3/ PVMCPR3, PVMCPF1/ PVMCPR1 and PVMCPF2/ PVMCPR2 produced the amplicons of 429 bp, 416 bp and 408 bp, (Lane 1, 2, 3, 4, 5, 6, 7, 8, 9 DNA bands from infected leaves) respectively in PVM. Both negative (Lane 10) and negative (Lane 11) controls, respectively did not produce any amplicons in the PCR. M: 1 kb DNA ladder.

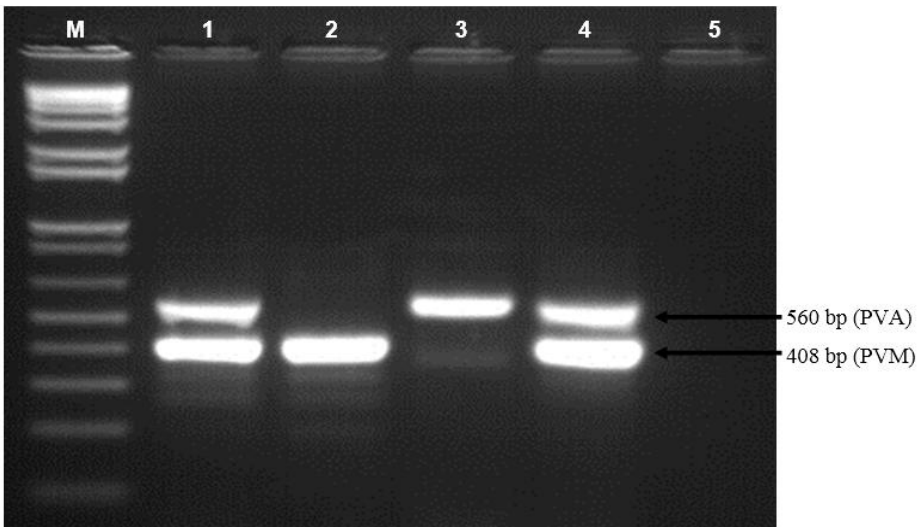

Fig 2: A duplex RT-PCR of *CP* genes from potato viruses PVA and PVM amplified with two primer pairs selected in the present study. Primer PVACPF3/ PVACPR3 produced an amplicon of 560 bp for PVA and primer PVMCPF2/PVMCPR2 produced an amplicon of 408 bp for PVM in (Lane 1; DNA bands from infected leaves). amplicons (408 bp for PVM; Lane 2; DNA bands from infected leaves. amplicons 560 bp for PVA; Lane 3; DNA bands from infected leaves); A duplex RT-PCR amplicon of 408 bp for PVM and 560 bp for PVA in (Lane 4; DNA bands from infected leaves) were produced with respective primers. A negative control (Lane 5) did not produce any amplicon. M: 1 kb DNA ladder.

Conclusions

The present study has demonstrated for the first time that *CP* gene specific primers can be used to detect PVA and PVM simultaneously in an optimized RT-PCR. The symptoms appear due to coinfection of PVA and PVM are often confused with the presence of only PVA. This is because, PVA is a common virus infecting potato crop worldwide. Despite of spraying systemic insecticides to control the vectors transmitting PVA, the growers bears huge losses in tuber yield. This happens because PVM, which also transmits mechanically and co-infects with PVA remains undetected. Primers PVACPF3/ PVACPR3 and PVMCPF2/PVMCPR2 designed to use in a duplex RT-PCR optimized in the present study offers a reliable diagnostic tool for the simultaneous detection of PVA and PVM.

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