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UNIPLEX AND DUPLEX RT-PCR PROTOCOLS FOR DETECTION OF PVY AND PLRV IN APHIDS FROM POTATO FIELDS

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ABSTRACT: ***Myzus persicae*** **is the principal vector of economically important potato viruses, especially** ***Potato virus Y*** **and** ***Potato leaf roll virus*****. Uniplex and duplex reverse transcription polymerase chain reaction (RT-PCR) assays were standardized and validated to detect PVY and PLRV in field collected populations of** ***M. persicae*** **using primers specific to coat protein. Results revealed that an annealing temperature of 62°C was found optimum for amplification of both viruses. Uniplex assay developed in this study could detect PVY in** ***M. persicae*** **samples of Modipuram, Jalandhar, Solan, Patna and Shillong. Duplex assay could successfully detect both viruses in** ***M. persicae*** **samples of Kodaikanal and Shillong. PCR products of PVY and PLRV were direct sequenced and got desired nucleotides of 380 and 492 bp, respectively which further confirmed the robustness of the protocols. Therefore, these protocols could be helpful to detect PVY and PLRV in** ***M. persicae*** **for healthy potato seed production.**

KEYWORDS: Coat protein, COI, *Myzus persicae*, RT-PCR, PVY, PLRV

INTRODUCTION

In India, potato (*Solanum tuberosum* L) is considered as one of the most economically important vegetable crop with an annual production of 45 million tonnes (Anonymous 2014-15). But the potential yield of potato is often limited by vector borne viral diseases that cause huge yield loss (Sun and Yang, 2004). Among these viruses, *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV) are the most important potato viruses worldwide (Radcliffe, 2002) including India. PVY also cause serious damage in potato (10-100% yield losses) and other solanaceous crops. PLRV is one of the most significant viruses that cause yield loss up to 60-75% (Paul Khurana, 1999). However, these viruses are transmitted to healthy plants horizontally under field conditions by aphid vectors. It is reported that more than 60% of virus transmission is by aphid vectors (Radcliffe, 2002). PLRV and PVY are transmitted efficiently in persistent and non-persistence manner, respectively by *Myzus persicae* (Sulzer). The incidence of PVY and PLRV correlates positively with aphid population buildup. Due to unfavourable environmental conditions on their primary host plants, aphids migrate to adjacent vegetable crops including potato, where they may acquire the virus from an infected plant and subsequently transmit it to a healthy plant. In Potato, the management of PLRV and PVY is indirectly related to monitoring and management of aphid vectors. Effective management of these viral diseases entails early detection and breeding for resistance (Sridhar *et al.,* 2016). The uniplex RT-PCR was used to detect viruses from single aphids (Lopez-Moya *et al.*, 1992; Singh *et al.*, 1995). RT-PCR based

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diagnostic technique has been demonstrated as efficient and reliable for detection of potato viruses (Singh and Nie, 2003) which provides both narrow and broad specificity for detection of RNA viruses (Singh, 1998). RT-PCR for detection of PLRV in aphids was developed in India (Raigond *et al.*, 2014) but not for PVY. Duplex RT-PCR protocols for detection of two viruses in potato leaves at a time have been developed which saves time and cost of the assay (Singh *et al.*, 1996; Verma *et al.*, 2003; Raigond *et al.*, 2013). However, such duplex RT-PCR protocol for simultaneous detection of PVY and PLRV in *M. persicae* is lacking. Keeping this in view, a uniplex and duplex RT-PCR protocols were formulated to detect the PVY and PLRV in field collected *M. persicae.*

MATERIALS AND METHODS

Collection of aphids and total RNA isolation

The adults and nymphs of *M. persicae* were collected from nine potato growing areas of India (Shillong, Modipuram, Shimla, Jalandhar, Patna, Kodaikanal, Sikkim, Nagaland and Arunachal Pradesh). These aphids were preserved in 70% ethanol and stored at room temperature till further use. Total RNA was isolated with the help of Axygen Mini Total RNA extraction kit (Axygen, USA) by following user protocol and it was stored at -80°C for further use. The primer sequences specific to coat protein of viruses were designed and synthesized (Geno Biosciences), and mitochondrial COI region of *M. persicae* was also included in the study as internal control (**Table 1**). All the laboratory work was carried out in the Division of Plant Protection, ICAR-Central Potato Research Institute, Shimla.

Reverse transcription

Total RNA was used for cDNA synthesis using random primers with Revert Aid™ cDNA synthesis kit from Fermentas Life Sciences. The reverse transcription mixture i.e., 4.0 ml of 5X buffer, 2.0 ml of 10 mM each dNTP mix, 1.0 ml of 20 U/ml RNase inhibitor, 1.0 ml of 0.2 mg/ml random primer, 1.0 ml of 200 U/ml RT enzyme, 6.0 ml of template RNA and 5.0 ml of RNase free water, were added to provide a final volume of 20 ml. All the reactions were carried in ice chilled condition to avoid premature cDNA formation and to minimize the risk of RNA degradation. The reaction mixture was incubated at 25°C for 05 min, 42°C for 59 min, 75°C for 10 min.

Uniplex and Duplex assays

Uniplex RT-PCR reaction for PVY was carried out at two annealing temperatures i.e. 60°C and 62°C in a thin walled 0.2 ml PCR tubes (Gene Amp PCR 9700, Applied Biosystems, USA). The reaction mixture of 20 µl containing 2.5 µl of 10x Taq buffer A, 1.5µl of

Table 1. Details of primer sets and size were used in the study.

Primer Name	Nucleotide Sequence 5'-3'	Amplicon size (bp)	Target virus/pest
PVY-FCP	ACGTGGTATGAGGCAGTGCGGA	380	PVY
PVY-FCP	ATGTGCGCTTCCCTAGCCCTCA		
PLRV-FCP	CTAACAGAGTTCAGCCAGTGGTTA	492	PLRV
PLRV-RCP	CGGTATCTGAAGATTTTCCATTTC		
MPCoI-1F	TCGGGTATAATTGGATCATCA	579	*M. persicae*
MPCoI-4R	TCCTCCTGCTGGGTCAAA		

2 mM dNTP mix, 1.0 µl of 10 pM forward and reverse primers each, 2 µl of cDNA template, 1.0 ml of 1.0 U/µl of Taq DNA polymerase and 11 ml sterile nano pure water. PCR reaction was carried out to amplify coat protein gene of PLRV, PVY and COI region of *M. persicae* along with water control as follows; initial denaturation at 94°C for 2 min, 94°C for 30 sec, annealing temperature at 62°C for 45 sec, followed by extension at 72°C for 35 cycles for 1 min along with final extension step at 72°C for 5 min. The amplified products were resolved in 1.0% agarose gel, stained with ethidium bromide (10 µg/ml) and visualized in gel documentation system.

Duplex RT-PCR reaction was attempted for simultaneous detection of PLRV and PVY in which the same primer sets were used for detection of viruses. PCR conditions were applied as that of uniplex and they were found promising results. Therefore, annealing temperature of 62°C and same PCR conditions were applied for screening the field collected aphid samples.

Gel elution and Cycle sequencing

The amplified products were gel eluted with the help of gel elution kit by following manufacturer's protocol. The eluted DNA was subjected to cycle sequencing in a total volume 20 µl mix containing 3 µl of RR (reaction mixture), 3 µl of 5X sequencing buffer, 2 ml sterile nano pure water,4 µl (0.8 pmol) of PLRV/PVY/ MP primers and 8 µl of eluted DNA. The cycle sequencing was performed with following conditions i.e., 96°C/10 sec, 50°C/5 sec, 60°C/4 min for 25 cycles in Gene Amp PCR 9700 system (Applied Biosystems, USA), later the product was purified. The cycle sequenced product was purified, denatured at 95°C for 2 min and it was chilled immediately on ice. The denatured product was sequenced with the help of 3500 Genetic Analyzer, Applied Biosystems.

RESULTS AND DISCUSSION

Uniplex and duplex RT-PCR protocols for detection of PVY and PLRV in field collected populations of *M. persicae* and their validation was done in this study to supplement virus diagnostics for the management of these viruses in potato. Besides, aphid populations were also confirmed their identity by both morphological and molecular approaches. The mitochondrial COI gene of *M. persicae* was amplified (579 bp) using specific primers (**Fig. 1**) and confirmed it's identity by direct sequencing and BLAST analysis.

Uniplex RT-PCR protocol

The uniplex protocol was standardized for the detection of PVY in field collected *M. persicae* populations. Among two annealing temperature i.e. 60°C and 62°C tried for amplification of PVY coat protein gene in *M. persicae*, 62°C was observed as optimum for uniplex RT-PCR protocol. Annealing temperature plays a crucial role for specific amplification of target viral coat protein in PCR reactions (Majumder *et al.*, 2008, Jeevalatha *et al*., 2012, Raigond *et al*., 2013, 2014). The results revealed that PVY was detected in aphids of Shillong and Modipuram (380 bp) (**Fig. 1**). This protocol was further validated by processing field collected aphids from various locations. It was observed that *M. persicae* from Modipuram, Jalandhar, Patna, Solan and Shillong were viruliferous as they were found positive for PVY. However, *M. persicae* from Shimla, Sikkim, Nagaland and Arunachal Pradesh were not carrying PVY (**Fig. 2**). These results are similar with the findings of Raigond *et al.* (2013) and Levy *et al.* (1994) that amplification of PVY in aphid's optimum at 62°C.

The uniplex protocol could detect PLRV in aphid populations of Kodaikanal and Shillong, while other aphid samples were not carrying virus (**Fig. 3**). The results of the

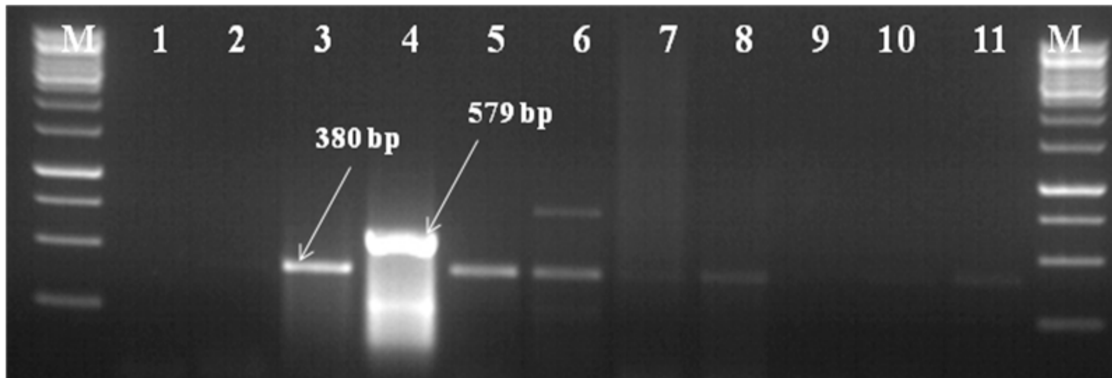

Fig. 1. Detection of PVY in field collected M. persicae from different locations. M-1 kb ladder, Lane1-water control, 2-healthy control, 3-Positive control (PVY), 4-Internal control (M. persicae), 5- Shillong, 6- Modipuram, 7- Jalandhar, Lane 8, 9, 10 & 11 are from Shimla, Sikkim, Nagaland and Arunachal Pradesh.

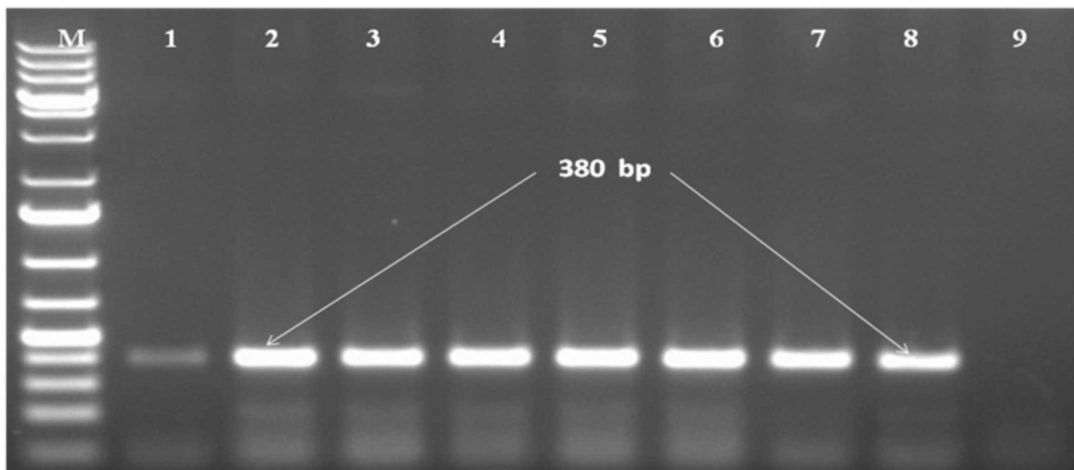

Fig. 2. Detection of PVY in aphids. M-1 kb ladder, Lane1-Pune, 2-Positive control PVY, 3-Solan, 4-Patna, 5-Jalandhar, 6&7-Shillong, 8- Modipuram, 9- water control.

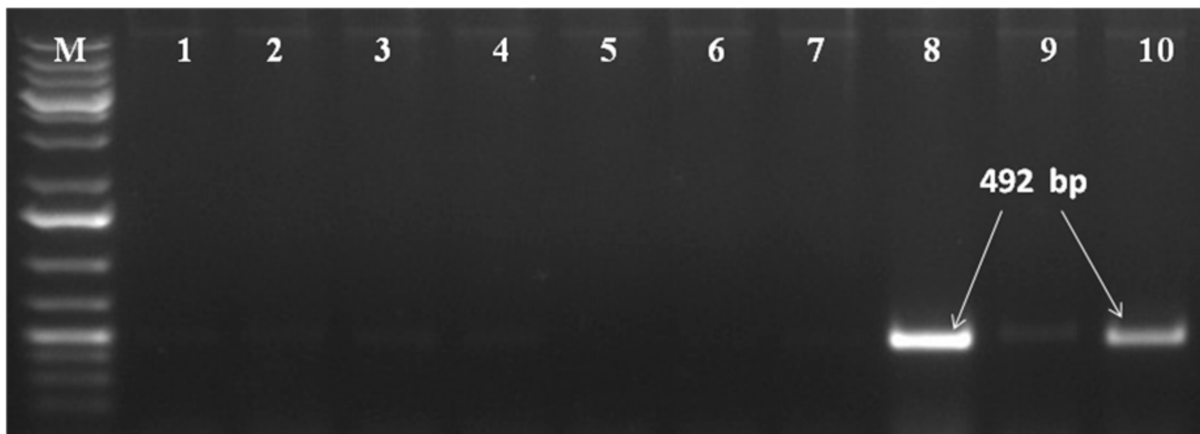

Fig. 3. Detection of PLRV in aphids. M-1 kb ladder, Lane1-water control (PLRV), 2-Shillong, 3-Shimla, 4-Jalandhar, 5-Modipuram, 6-Patna, 7- Pune, 8-Kodaikanal, 9 & 10-Shillong.

study are similar with the findings of Singh *et al.* (1995) and He *et al.* (2006) who reported the RT-PCR based protocol for detection of PLRV and PVY in aphids.

Duplex RT-PCR protocol

Annealing temperature play a crucial role in successful amplification and detection of target virus in aphid vectors. Therefore, two annealing temperatures (60°C and 62°C) were tried for duplex RT PCR detection of PVY and PLRV simultaneously in a single reaction and annealing temperature of 62°C was found ideal. The Compatibility of two primers is important in success of the reaction in detection of PLRV and PVY (Nie and Singh, 2000, 2001). Duplex reverse transcription coupled with PCR assay provides rapid and sensitive to overcome some of the problems associated with ELISA (Singh *et al.*, 1996).

The duplex RT-PCR results revealed that the aphids from Kodaikanal and Shillong carried both PVY and PLRV (**Fig. 4**) but in other locations only PVY was detected confirming the viruliferous nature of the aphids. These findings were similar with Singh *et al.* (1996) in detecting PVY and PLRV in aphids using duplex protocol, and Singh *et al*. (2000) also reported that higher concentration of primer can compensate the lower concentration of given target or template. Majumder *et al*. (2008) reported that two fold increase in primer concentration increased detection efficiency in duplex PCR for simultaneous detection of PLRV and PVY.

The reliability of the assay was improved by including the COI gene of *M. persicae* as internal control. Mitochondrial COI gene is well conserved and it is a robust marker for species identification, determining phylogenetic relationships and genetic variability, geographical studies in various insect species (Galtier *et al.*, 2009; Prabhakar *et al.*, 2013). Co-amplification of cytochrome *c* oxidase subunit I act as an indicator of RNA quality. Ahouee *et al*. (2010) explained similar kind of results i.e. amplification of COI region as internal control in RT PCR based protocol for detection of PLRV in aphids.

The RT-PCR protocol was able to detect and amplify the PVY, PLRV and COI region of aphids with an expected amplicon of 380, 492 and 579 bp, respectively. The results were reconfirmed by sequencing the amplified fragments and BLAST analysis revealed that the coat protein gene of PVY and PLRV has a homology of 98% with reported PVY and

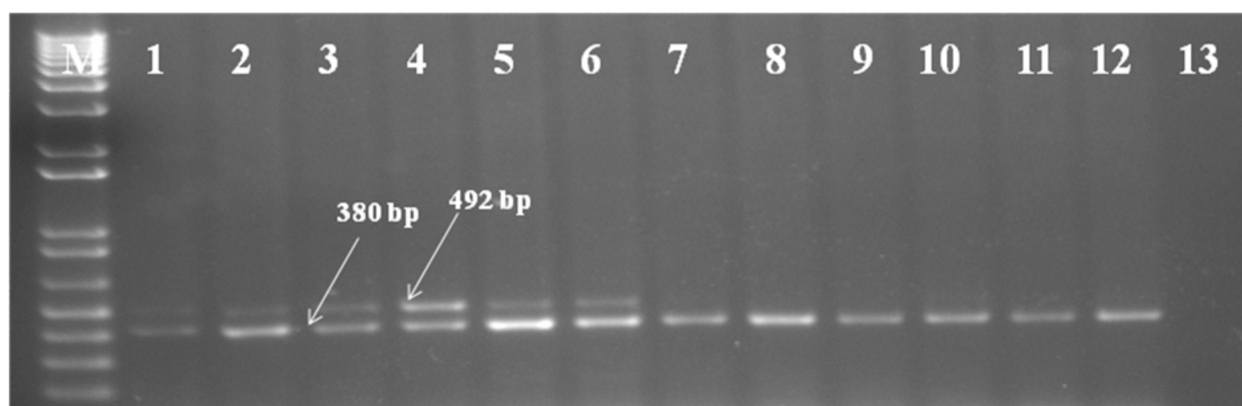

Fig. 4. Duplex RT-PCR detection of PVY and PLRV in aphid samples. M-1 kb ladder, 1-Positive control (PVY and PLRV), 2-Kodaikanal, 3-7 Shillong, 8-Pune, 9-Shimla, 10-Jalandhar,11- Patna, 12- Solan, 13-water control.

PLRV from different geographical locations, while COI was showing 99% homology with already reported COI region.This method has also been found to be highly efficient method of detection of PVY and PLRV (Ghosh and Bapat, 2006). Therefore, this protocol can be successfully employed for duplex detection of PVY and PLRV in populations of *M. persicae*.

CONCLUSION

The developed assays are robust and could detect PVY and PLRV in aphids collected from different geographical locations. This study helps in taking timely virus and vector management decisions for healthy seed potato production.

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