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SQUASH AND TISSUE PRINT PROTOCOLS FOR QUICK DETECTION OF *TOMATO LEAF CURL NEW DELHI VIRUS*-POTATO IN FRESH AND ETHANOL PRESERVED SINGLE WHITEFLY

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ABSTRACT: Squash and tissue-print PCR protocols were designed to detect *Tomato leaf curl New Delhi virus*-potato in *Bemisia tabaci* and *Trialeurodes vaporariorum*. Both whiteflies species were given an acquisition feeding of 24 hours separately on pure culture of this virus. Then, the individual whitefly tissues were directly printed on nitrocellulose membrane in tissue print method, and whiteflies were crushed in PBST buffer and spotted on membrane in squash print method. The uniplex PCR assay of eluted DNA revealed a sharp amplicon of 491 bp region of coat protein gene of ToLCNDV-potato. In simultaneous detection with mt *COI* gene as internal control, a sharp amplicon of 161, 300 and 491 bp for *T. vaporariorum*, *B. tabaci* and ToLCNDV-potato respectively, were observed in a single reaction. Gene sequencing results showed 99-100% similarity with reported sequences of ToLCNDV-potato. The protocols were validated for their sensitivity and reproducibility using ethanol preserved whiteflies collected from potato fields of Punjab, Uttar Pradesh, Madhya Pradesh and Maharashtra, and concurrent results were observed on repetition. This is the first report to detect ToLCNDV-potato in single whitefly along with internal control which will not only strengthen the virus diagnostics but also help to screen the field collected whiteflies for their viruliferous nature and to understand the epidemiology of virus vector which would guide us in taking timely vector management decisions.

KEYWORDS: Squash tissue print, ToLCNDV-potato, *Bemisia tabaci*, *T. vaporariorum*, detection

INTRODUCTION

The vector borne viruses are responsible for progressive degeneration of seed potatoes which result in poor yield (Thomas-Sharma *et al.,* 2016). Recently, whitefly transmissible geminivirus, *Tomato leaf curl New Delhi virus*-potato (ToLCNDV-potato) causing potato apical leaf curl disease has been posing serious threat to both healthy seed and ware potato production. This disease caused an yield reduction of 60.78% in K Khyati and even 100% infection in susceptible varieties due to horizontal transmission by whiteflies under Hisar climatic conditions (Lakra, 2010). It causes 40–75% infections in the potato cultivars grown in Indo-Gangetic plains of India (Venkatasalam *et al.,* 2005). Association of ToLCNDV with potato was first reported in 1999 in north India (Garg *et al.,* 2001) and cause was identified as a new strain of *Tomato leaf curl New Delhi virus* belonging to genus *Begomovirus* in the family *Geminiviridae* (Usharani *et al.,* 2004). ToLCNDV-potato is widely spreading in India at fast rate due to polyphagous nature, high multiplication rate and broader host range and quick adaptability of its whitefly vectors. Effective management of the viral diseases entails early detection and breeding for resistance (Bela-ong and Bajet, 2007). As of now, breeding for ToLCNDV-potato resistance in potato is in its infancy, therefore early and quick diagnosis, and elimination of

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the virus is one of the best options for the management of viruses. Various advanced diagnostic techniques including Print-capture PCR (PC-PCR) have been developed to detect viruses in plant and vectors with advent of molecular techniques. PC-PCR protocol was developed to detect TYLCV in tissue of plant as well as in whitefly by printing directly on nitrocellulose membranes (NCM) (Atzmon *et al.,* 1998). It was further improved up on for efficient amplification of geminivirus DNA genome from plant and whiteflies by printing tissues on Whatman 3MM paper (Navas-Castillo *et al.,* 1998). Recently, PC-PCR facilitated detection of *Groundnut bud necrosis virus* in thrips and potato leaves as well (Kaushal *et al.,* 2010), PVY virions in single viruliferous *Myzus persicae* (Gawande *et al.,* 2011), *Candidatus* Liberibacter solanacearum in single viruliferous potato psyllid (Teresani *et al.,* 2015). A robust, sensitive, reliable and well defined diagnostic technique including PC-PCR was developed to detect this virus from potato plants at CPRI (Jeevalatha *et al.,* 2013 and 2014). However, PC-PCR (squash and tissue print) protocols to detect ToLCNDV-potato in its whitefly vector are still lacking. The objectives of this study therefore were (i) to develop uniplex PC-PCR protocol, (ii) to develop protocol for simultaneous detection (along with internal controls) of ToLCNDV-potato in fresh and ethanol preserved single whitefly sample and (iii) to validate protocol for its sensitivity and reliability.

MATERIALS AND METHODS

Whitefly and virus cultures

The nucleus virus free field collected cultures of *B. tabaci* (**Fig. 1a**) and *T. vaporariorum* (**Fig. 1b**) were maintained on tomato and brinjal plants in insect rearing cages at ICAR-Central Potato Research Institute (CPRI), Shimla, India under controlled laboratory conditions. The identity of two whitefly species was confirmed using mitochondrial *COI* gene specific primers (**Table 1**). Preliminary studies were conducted to ensure whitefly cultures were free from virus (**Fig. 2**). Pure ToLCNDV-potato cultures (**Fig. 1c**) were obtained from virus culture facility maintained at CPRI, Shimla for conducting acquisition studies.

DNA isolation

Freshly emerged whitefly adults (both species) were released on to pure cultures of TOLNDV-potato in insect proof rearing cages and were allowed for an acquisition feeding period of 24 hours. Squash and tissue-print methods were used for isolation of total genomic DNA from these whiteflies individually. In squash print method,

Fig. 1a. Bemisia tabaci

Fig. 1b. T. vaporariorum

Fig. 1c. Pure culture of ToLCNDV-potato

Table 1. Primer sets, size and Genebank accession numbers used in the assay

S. No.	Primer code	Polarity	Nucleotide sequence	Amplicon size (bp)	GeneBank Reference sequences (accession #)
1	LCVCPF1	Sense	5′-AAAGTCATGTGTGTTAGTGATGTTACC-3′	491	AY286316
	LCVCPR1	Antisense	5′-TAGAAATAGATCCGGATTTTCAAAGTA-3′		
2	TVF2	Sense	5′-AGCGGCACGGGTTGAACTGTTTAT-3′	161	JQ995230
	TVR2	Antisense	5′-CCCAACGCCCGCATGTTTACAATA-3′		
3	BTF1	Sense	5′-TAGGAGTGAGGCTGGAAAGCTTGA-3′	300	KT851539
	BTR1	Antisense	5′-ATCCAGCAAACCAAAGTCCAAGCG-3′		

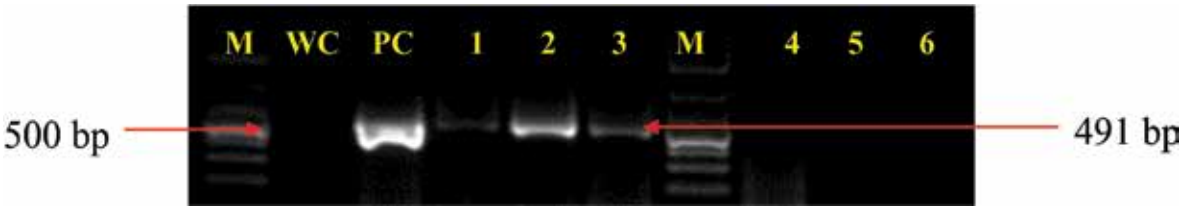

Fig 2. Detection of ToLCNDV-potato in T. vaporariorum. M-100 bp Marker; WC- Water control; PC- Positive control; Lane 1-3- T. vaporariorum acquiring ToLCNDV-potato; Lane 4-6- Healthy T. vaporariorum.

individual whiteflies were crushed in 15 µl of PBST buffer in a clean 1.5 ml eppendorf tube and spotted on NCM. Then, viral DNA was eluted from the NCM in 20 µl sterile distilled water by incubating at 95°C for 10 minutes and was quantified using Nano Drop (Thermoscientific, Leon-Rot, Germany) and also checked on the gel electrophoresis for its presence before proceeding for PCR amplification. In case of tissue print method, the individual whiteflies were directly printed on to NCM by pressing with sterile glass rod and incubated at 37°C for 30 minutes and proceeded further as above in squash print method to isolate total DNA.

Uniplex PCR assay

Total genomic DNA was used to perform polymerase chain reaction in a thermal cycler (Applied Biosystems 9700) using the cycling parameters; an initial denaturation step at 94°C for 5 minutes followed by 35 cycles at 94°C for 1 minute, annealing at 62°C for 1 minute, an initial extension step at 72°C for 1 minute and a final extension at 72°C for 10 minutes using the primers specific to coat protein of ToLCNDV-potato (**Table 1**). A total reaction volume of 20 µl having 1.0 µl of each primer (10 mM), 2.0 µl of 10X taq buffer, 1.0 µl of 2.0mM dNTP and 1.0 µl of 1.0 unit Taq DNA polymerase including 5 µl template DNA in 9 µl sterile double distilled water. The amplified products were resolved on 1.0% agarose gel, stained with ethidium bromide (10µg/ml) and visualized in a gel documentation system.

Simultaneous detection

PCR assay was performed for simultaneous detection of ToLCNDV-potato along with internal controls in a single reaction to enhance the reliability of the assay. Gradient PCR was performed to standardize annealing temperature (58, 60 and 62°C) for simultaneous detection of virus, *T. vaporariorum* and *B. tabaci*, and 60°C was found to be optimum annealing temperature (Data not shown). A total reaction volume of 20 µl having 0.5 µl of each primer (*B. tabaci*, *T. vaporariorum* and ToLCNDV-potato), 2.0 µl

of 10X taq buffer, 1.0 µl of 2.0mM dNTP, 0.5 µl of $MgCl_2$ and 1.0 µl of 1.0 unit Taq DNA polymerase including 5 µl template DNA in sterile double distilled water.

Validation of protocol

The protocol was validated using whiteflies (preserved in 70% ethanol) collected from potato fields of Jalandhar, Hoshiarpur, Ludhiana and Kapurthala locations of Punjab, Modipuram and Meerut of Uttar Pradesh, Gwalior of Madhya Pradesh and Pune of Maharashtra (**Table 2**).

Sequencing and analysis

The PCR amplicons were gel eluted using MinElute® gel extraction kit (Qiagen, Hilden, Germany) by following manufactures' protocol. The eluted products were further used for cycle sequencing in a total volume of 20 µl mix containing 3 µl of reaction mixture, 2 µl of sequencing buffer (Big dye® Applied Biosystem, UK), 4 µl (0.8 pmol) of selected primers and 5 µl of eluted DNA (Baswaraj *et al.*, 2014). The cycle sequenced product was purified, denatured at 95°C for 2 min and was chilled immediately on ice. The denatured product was sequenced in an ABI3500 genetic analyzer (Applied Biosystems). The sequences obtained were aligned, assembled and BLAST analysed for identity of ToLCNDV-potato, *B. tabaci* and *T. vaporariorum*.

Table 2. Field collected whitefly samples and detection of ToLCNDV-potato

Location and State	Sample No.	ToLCNDV-potato	Percent vectors
Jalandhar, Punjab	15	6/15	40.00
Hoshiarpur, Punjab	8	4/8	50.00
Ludhiana, Punjab	9	2/9	22.20
Kapurthala, Punjab	7	3/7	42.80
Modipuram, Uttar Pradesh	13	3/13	23.07
Karkhoda, Uttar Pradesh	11	5/11	45.45
Gwalior, Madhya Pradesh	12	2/12	16.66
Pune, Maharashtra	12	3/12	25.00
Total	87	28/87	32.18 (Mean)

RESULTS AND DISCUSSION

The viruliferous nature of whiteflies with respect to ToLCNDV-potato was determined using squash and tissue print PCR method individually and simultaneously along with internal control. Both the whiteflies were confirmed for their identity using mitochondrial *COI* gene specific primers. Sharp amplicons of 161 bp and 300 bp were observed for *T. vaporariorum* and *B. tabaci* respectively.

Squash and tissue print method for detection of ToLCNDV-potato

DNA isolation

Squash and tissue printed DNA from fresh and ethanol preserved individual whiteflies (both species) was ranging between 11 and 20 ng/µl. The quality of DNA was good without any smear and shear.

Uniplex PCR assay

Squash and tissue printed total DNA from fresh and ethanol preserved individual whiteflies (both species) was used for PCR amplification. The amplification of viral coat protein gene revealed a sharp and clear amplicon of 491 bp on gel electrophoresis (**Fig. 3**) without any non-specific bands.

Simultaneous detection assay

Simultaneous assay was performed to detect ToLCNDV-potato along with internal controls of *B. tabaci* and *T. vaporariorum* in a single reaction to save reagents and time (**Fig. 4**). This assay was standardized by keeping reaction at three different annealing temperatures i.e. 58, 60 and 62°C to find out optimum temperature for sharp amplification of target virus and both whiteflies in a single

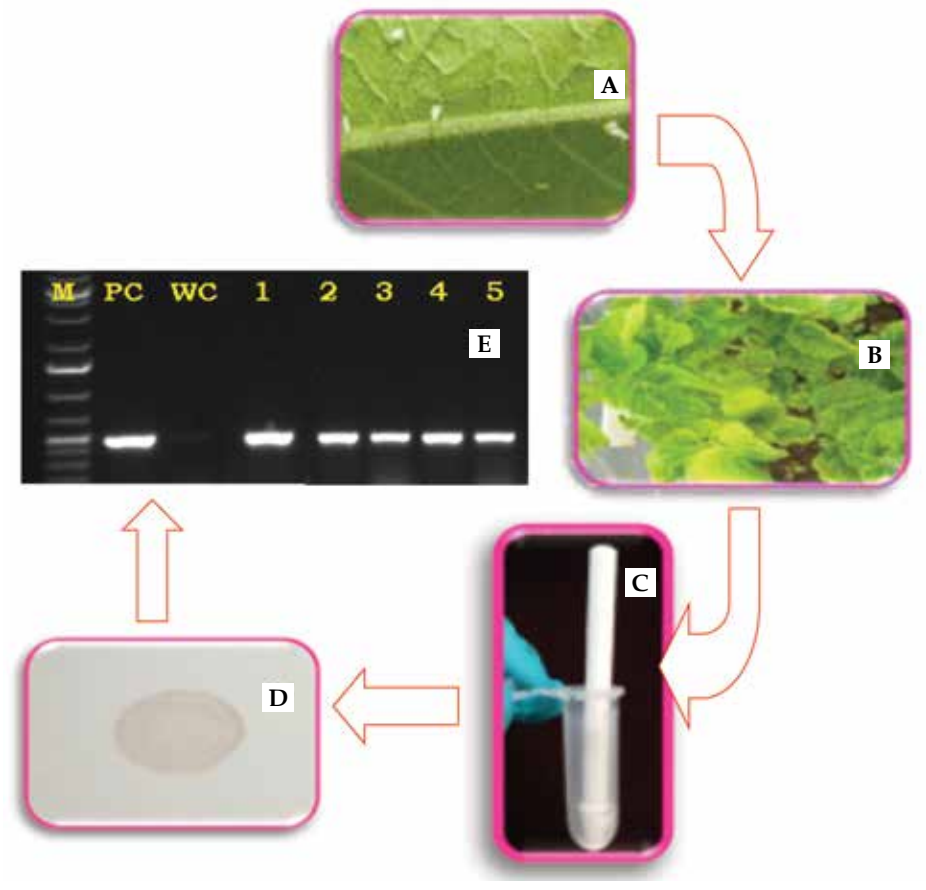

Fig 3. Pure culture of B. tabaci (A), Acquisition feeding on ToLCNDV-potato infected plant (B), Viral DNA isolation (C), Viral DNA spotting on NCM (D) and viral DNA detection (E)

reaction. Annealing temperature of 60°C was found to be optimum which was further used for validation of the assay. PCR results revealed that specific amplicons of virus, *B. tabaci* and *T. vaporariorum* were observed on gel electrophoresis. To further confirm and increase the precision of the assay, unidirectional sequencing of amplicons was done. It was found that *CP* gene sequence of ToLCNDV-potato and *COI* gene of both whitefly species showed 99-100% similarity with that of reported sequences in NCBI. Sometimes, the nucleic-acid based detection leads to false negative results due to presence of inhibitors in DNA preparation or human error (Jeevalatha *et al.*, 2013) which makes it almost difficult to conclude with certainty the viruliferous nature of vectors (Baswaraj *et al.*, 2014). Amplification of internal control indicates the reliability of the results as it was proved by He *et al.* (2006) that co-amplification of aphid mRNA from the cytochrome *c* oxidase subunit I serve as an indicator of DNA quality. Therefore, internal controls of *B. tabaci* (300 bp) and *T. vaporariorum* (161 bp) used in this study enhanced the reliability of the assay. Previously, *Citrus tristeza virus* was detected by quashing single aphid on to nylon membrane (Bertolini *et al.*, 2008; 2014). Wide variety of protocols were developed for efficient detection of potato viruses like PVY, PLRV, PVS, PVM, PVA and spindle tuber viroid in potato plant tissues as well as in single aphid vector but they demand

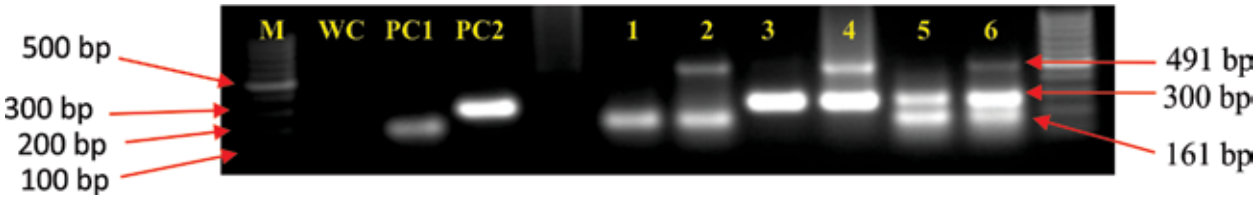

Fig. 4. Simultaneous detection of ToLCNDV-potato in B. tabaci and T. vaporariorum. M- 100 bp; WC-Water control; PC1-T. vaporariorum; PC2-B. tabaci; Lane 1-T. vaporariorum alone; 2-T. vaporariorum and ToLCNDV-potato; L3-B. tabaci alone; L4-B. tabaci and ToLCNDV-potato; L5-T. vaporariorum and B. tabaci; L6-T. vaporariorum, B. tabaci and ToLCNDV-potato.

a series of multiple sample preparation and nucleic acid extraction steps (incubation, washing etc.) which is time consuming, labour intensive and increase the cost of the assay (Singh *et al.*, 2000 & 1995; Nie and Singh, 2000). This assay guides us to detect ToLCNDV-potato in a mixed population of these two whitefly species with exact identity of the species at a time in a single reaction. This assay reduces incubation time by half (30 minutes instead of 60 minutes) and decreases use of DNA template by nearly 50 percent (5 µl as against 10 µl) as compared to previous protocols in detecting viruses such as PVY in single *M. persicae* (Gawande *et al.*, 2011), PVY° in aphid tissue (Oraby and El-Borollosy, 2013), ToLCNDV in plant tissues (Gawande *et al.*, 2007), *Groundnut bud necrosis virus* in lots of potato leaf and thrips tissues (Kaushal *et al.*, 2010). Consequently, this assay is not only time saving and cost effective but also detects virus in ethanol preserved individual whiteflies without compromising the sensitivity of the assay as whiteflies are small tiny insects weighing 30-40 µg that harbour very low titre of virions.

Validation of the assay

To validate the above standardized protocol, a total 87 whitefly samples (ethanol preserved) collected from eight different geographical locations of the country were processed (**Fig. 5**). This protocol could successfully detect virus in 28 viruliferous whiteflies. Virtually one third of *B. tabaci* samples were carrying virus (32.18%). Fifteen (38%), eight (33%) and two (16%) whiteflies were found to carry virus in Punjab, Uttar Pradesh and Madhya Pradesh respectively. The validation was repeated thrice for ToLCNDV-potato positive whitefly samples and protocol showed consistent results. Highest percent of viruliferous whiteflies were recorded in Hoshiarpur followed by Karkhoda and Kapurthala. Very less number of viruliferous whiteflies was reported in Gwalior (Madhya Pradesh) and Ludhiana (Punjab). These results indicates the robustness of protocol to detect ToLCNDV-potato in fresh and ethanol preserved whitefly samples and also viruliferous whiteflies that are responsible for progressive degeneration of seed and ware

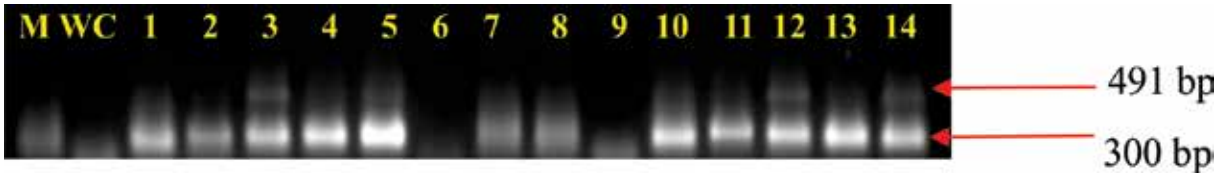

Fig 5. Validation of simultaneous detection of ToLCNDV-potato in B. tabaci. M- 100 bp; WC- Water control; Lane 1-14 ToLCNDV-potato (491 bp) in B. tabaci along with internal control (300 bp). Whitefly samples 3, 4, 12, 14 were found to be viruliferous.

potato production. Viruliferous nature of *B. tabaci* and horizontal transmission of virus under field conditions depend on prevalence of the virus and favourable environmental conditions for the vector. The indirect impact of whitefly vectors on potato yield under field conditions was reported by Lakra (2010) that ToLNDV-potato increased at a geometric rate with increase in whitefly population which inturn decreased yield drastically when same seed was used continuously.

CONCLUSIONS

This modified PC-PCR protocol is valuable tool by virtue of being time-saving, high throughput and also in detecting ToLCNDV-potato in fresh and ethanol preserved whitefly samples bypassing the DNA extraction step. Besides, it offers an edge over other PCR methods as it avoids risk of cross contamination (Nagata *et al.*, 2004), prints with long shelf life (Kaushal *et al.*, 2010) can be stored for up to 8 months before use without compromising sensitivity of assay (Navas-Castillo *et al.*, 1998), facilitate long distance transport (Gawande *et al.*, 2007) and deal with quarantine viruses without any risk (Bertolini *et al.*, 2008). It is the first report in detecting ToLCNDV-potato in fresh and ethanol preserved single *B. tabaci* and *T. vaporariorum* along with internal controls using squash and tissue print PCR directly. This assay would be useful to generate data on viruliferous nature of field collected whiteflies to and understand epidemiology and forecasting of virus disease and vector. It would also indirectly help in timely decision making for vector and virus disease management.

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