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First report on Potato Spindle Tuber Viroid (PSTVd) from field grown infected Potato plants (*Solanum tuberosum*) in Pakistan

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ABSTRACT

The degradation of RNA is main hindrance for dealing with fine quality RNA for its downstream processing. Mostly RNAs are degraded as tissues are taken from fields, therefore the protocol for PSTVd RNA isolation was optimized and improved for obtaining Pure and high quality RNA having an increased and nondegradable life from infected plants of Potatoes. The present study was aimed to confirm the presence and identification of potato spindle tuber viroid (PSTVd) on potato plants in Pakistan. RNA was extracted and purified using optimized Trizol reagent and RNeasy Miniplant Kit. The RNA obtained was converted into cDNA on the same day and amplified. The identification of PSTVd was carried out by Sequencing and BLAST. The advanced optimized Rapid Trizol reagent method was comparatively found best and reproducible as compared to the second method. The Sequencing and BLAST confirmed the presence of Pakistani PSTVd (Accession # MK.303578) in infected potato plants.

INTRODUCTION

Potato spindle tuber viroids was first identified small circular single stranded RNA viroid causing infection in many crops, weeds, ornamental plants, etc. This is a representative member of *Pospiviroidae family (*Bostan *et al*., 2004). Its genome is consisting of 359 nucleotides forming a rod-like conformation, having five distinct domains. All domains have been reported with different functions *(*Candresse *et al*., 2010). It was general perception that only pathogenic Domains (P) are involved for inducing infection in plants (Atsushi *et al.,* 2013) Viruses and Viroids are responsible for 40-50 billion of field losses every year effecting stored and cultivated crops equally, proving themselves one of the major hurdle in effective food production and supply (Owens, 2009). The short-lived vegetables and herbaceous annual crops, e.g. Tomato, Potato and Capsicum etc., grown by true seeds, display maximum losses due to such infections (EFSA, 2011). There is a tremendous increase in number of plant viroids identified in last three decades, thus creating attention of global community to protect their fields and farms from exotic and imported pathogens (Anna, 2013). Two decades earlier, it was a general opinion that any organism without a protein coat could not replicate itself, even with the help of host cell protein. However, discovery of PSTVd proved it wrong. The PSTVd was the first viroid identified, as physical entity, native to Australia and posing serious threat to world potato economy. Approximately 32 plant families have displayed sensitivity to viroids (Kovalskaya and Hammond, 2014).

The complicated plant pathogen and host interaction process is the key behind the development of disease signs and symptoms. The degree and type of infection depend on systemic transfer efficiency. PSTVd exhibits mild to severe symptoms in plants. Diagnoses of PSTVd depend on symptoms, appearing on leaves or stems of infected plants like Chlorosis, stunted growth in potatoes and purpling of leaves (Flores, 2009). By the time potato has become an important cash crop for both Pakistani farmers and consumers. It is the fourth most significant crop by volume and yield of production and gives highest returns to field growers (Barbara *et al*., 2012). Almost 86% of potato area and production in Pakistan is achieved from Punjab. Pakistan is self-sufficient in potatoes for country consumption. This is a tasty, nutritive and highly digestible vegetable with 75 % water contents. The average yield of potato is 30-50 tons/ha which it depends upon the location, variety and cultural practices (FAO, 2016). Although a lot of work has been performed on isolation of RNA from infected plants tissues, but this is first report of Isolation of PSTVd RNA from infected plants in Pakistan. The extraction of RNA is always a big

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problem and challenging in university laboratories. Moreover, it is not always possible to follow published protocols because of expensive and complicated apparatus discussed in research papers. The fast efficient protocol for isolation of PSTVd was optimized in the present work and found very short and effective protocol completing in less than three to four hours as compared to RNAeasy Miniplant Kit. Another plus point of the discussed method is that extracted RNA can be stored for longer periods without converting into cDNA. For completion of experiment required RNA was converted into c DNA.

MATERIALS AND METHODS

Selection and collection of infected plants

Plants were collected on the basis of symptoms discussed in the work of Owen, 2009. The fresh leaves were analyzed with yellowing and spindle like formation in Potato. The leaves were plucked, covered in Aluminum foil and immediately shifted to liquid Nitrogen small container to save RNA from degradation. Infected plants were collected from different geographical areas in Pakistan. Some basic signs & symptoms were also studied as morphological markers to identify PSTVd in infected plants.

RNA extraction with RNeasy plant mini kit

Total RNA was extracted with the modified protocol of Kolonko *et al*., 2006, using RNeasy Plant Mini Kit. The frozen leaves were ground and poured into falcon tube, added 400µl of ribozol reagent was added into it and mixed properly. These tubes were incubated at room temperature at 34^{o}C for 30 minutes. The tubes were put on ice and after fifteen minutes, the chilled tubes were centrifuged at 15000rpm at 15^{o}C for 5 minutes. Two clear layers were formed and the supernatant layer was transferred to fresh tubes, each containing 100µl of chloroform. The tubes were vortexed for a minute in fume hood and incubated at room temperature for 60 min. The incubated samples were centrifuged again for 15 min at 10000rpm. The clear aqueous phase was transferred to new tubes, each containing and 200ul of isopropanol. The mixing of samples was done by inverting tubes many times and then incubating again for third time. These tubes were centrifuged for 10 min at 12000 rpm. The supernatant was removed and 200µl of 75% Ethyl alcohol was added and RNAase free water was used in order to wash pellet. The final samples in tubes were spun at 7500 rpm for 10 min at 4^{o}C. The clear RNA Pellet was saved and dried for 5 min. Concentration of RNA was measured by spectrophotometry at 260nm ^{TM}Nanodrop 2000/2000c. The extracted RNA was preserved at -80^{o}C.

RNA extraction with modified Trizol protocol

The infected frozen plant tissues were ground in 500 µl of Trizol reagent and then 100 µl of chloroform reagent was added. The total sample was vortexed for 20 seconds and incubated at room temperature for almost 30 minutes. Then it was centrifuged at 13000rpm for 5-10 minutes. Two phases were formed, lower red interphase and colourless aqueous phase. The upper phase was collected and 250µl of cold Isopropanol was added at room temperature. This was again centrifuged at 13000rpm 4^{o}C for 20 min. The pellet was collected and 500 µl of 75% ethanol was added and centrifuge for three min at 750rpm. The remaining liquid was discarded and pellet was dried and suspended in 500 µl of Trizol reagent.

cDNA Synthesis

For completion of experiment Extracted RNA was converted in to cDNA on the same day using GScript First strand synthesis Kit (Cat No MB 305-0050) according to manufacturer‘s Instructions. Extracted RNA solution was added into eppendorf with following kit’s components i.e. Oligo (dT) 1µl, dNTPs 1µl, 13µl of Nuclease free water and mixed properly. Then it was centrifuged at 13500 rpm for 5 min and shifted to pre warmed water bath for almost 3-5 min at 65^{o}C. As incubation was completed, the whole mixture was spun shortly and shifted on ice. The available 5x is strand buffer (4µl) was added, soon after buffer’s addition the Gscript Rtase and DTT (0.1M) were added as 1µl Respectively (Owens & Baumstark, 2007).

Agarose gel run

The quality of cDNA was checked on 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. The quantity of cDNAs was also noted with spectrophotometry. All samples were found between 1-2 nm. The Quantification of cDNA was performed with ^{TM}Nanodrop 2000/2000c Thermo Fisher Scientific V1.0. The large peaks at260/280nm were obtained for purified sample. The Visualization of bands were done in gel documentation system and was photographed and fine quality bands were seen and scored (Fig A) (Gozmanova *et al*., 2003)

Sequencing

The presence of PSTVd was confirmed by

BLAST and sequencing. The obtained FASTA sequence showed 100% similarity with other reported accessions # (U.23058.1 and A.F459007.1) in NCBI BLAST results (Fig B-1). The amplified sequences were inserted into a vector according to protocol of Vachev *et al*., 2010 and sent for sequencing to Bio Basic Company.

Amplification of PSTVd

The PSTVd cDNA was further amplified with the primers designed by Vachev *et al*., 2014) and sent for sequencing. The amplified sequence also confirmed the presence of this deadly pathogen in the infected samples.

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RESULTS

The two methods of RNA isolation, RNA Extraction with RNeasy Plant Mini Kit and RNA Extraction with Modified Trizol Protocol from PSTVd were analyzed comparatively. It was noted that the second modified methods was found best and easy to use then the former. (Fig.1). This modified Trizol method was also found efficient in time saving and quality of RNA. The RNA was stored in 2µl of Trizol reagent for future use. This property of optimized Trizol reagent also found efficient over RNeasy Plant Mini Kit method. The prior addition of b-Mercaptoethanol (1-2 µl per ml of Trizol reagent) also gave best quality RNA. The amplified sequence also gave positive, compact and distinct bands for PSTVd (B).

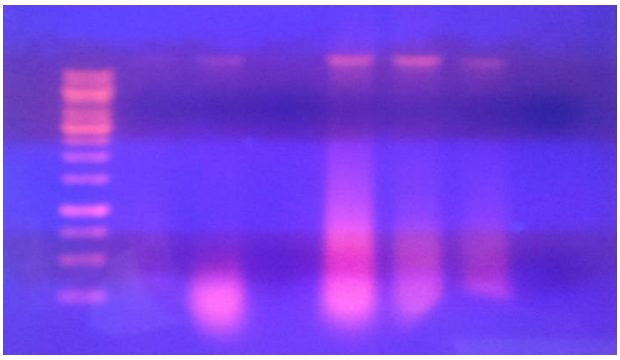

Fig.1: RNA from infected samples: 1st well: 100 bp ladder, 2nd 3rd well: RNA with RNeasy Plant Mini Kit; 4th well Modified Trizol reagent. 5th and 6th well; stored RNA (Trizol reagent)

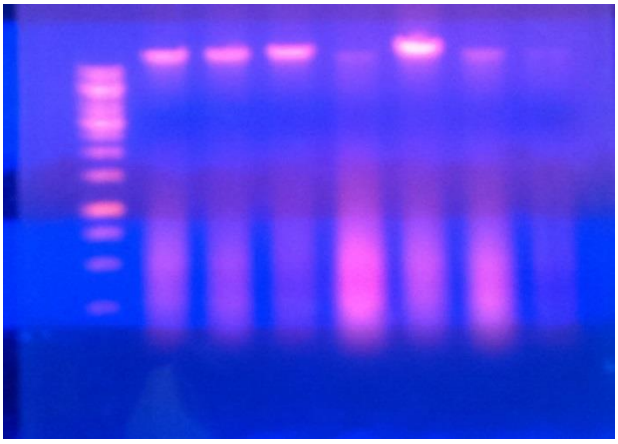

Fig. 2: Sharp bands of amplified PSTVd sequence with Modified Trizol methods

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Potato spindle tuber viroid (PSTVd) strain RG 1, complete genome	664	664	100%	0.0	100%	U23058.1
Potato spindle tuber viroid isolate M3/M1, complete genome	658	658	100%	0.0	99%	AF459007.1
Potato spindle tuber viroid isolate M1, complete genome	658	658	100%	0.0	99%	AF459005.1
Potato spindle tuber viroid isolate M12/M1, complete genome	658	658	100%	0.0	99%	AF458986.1
Potato spindle tuber viroid isolate AS2, complete genome	656	656	100%	0.0	99%	AY518940.1
Potato spindle tuber viroid isolate PSTVd-H112, complete genome	652	652	100%	0.0	99%	DQ308558.1
Potato spindle tuber viroid isolate AS1, complete genome	652	652	100%	0.0	99%	AY518939.1
Potato spindle tuber viroid isolate M2/M1, complete genome	652	652	100%	0.0	99%	AF459006.1
Potato spindle tuber viroid isolate M34/M1, complete genome	652	652	100%	0.0	99%	AF458993.1
Potato spindle tuber viroid isolate M33/M1, complete genome	652	652	100%	0.0	99%	AF458992.1
Potato spindle tuber viroid isolate M23/M1, complete genome	652	652	100%	0.0	99%	AF458990.1

Fig. 2a: NCBI blast results of PSTVd FASTA showing 100% and 99% similarity with other reported sequence.

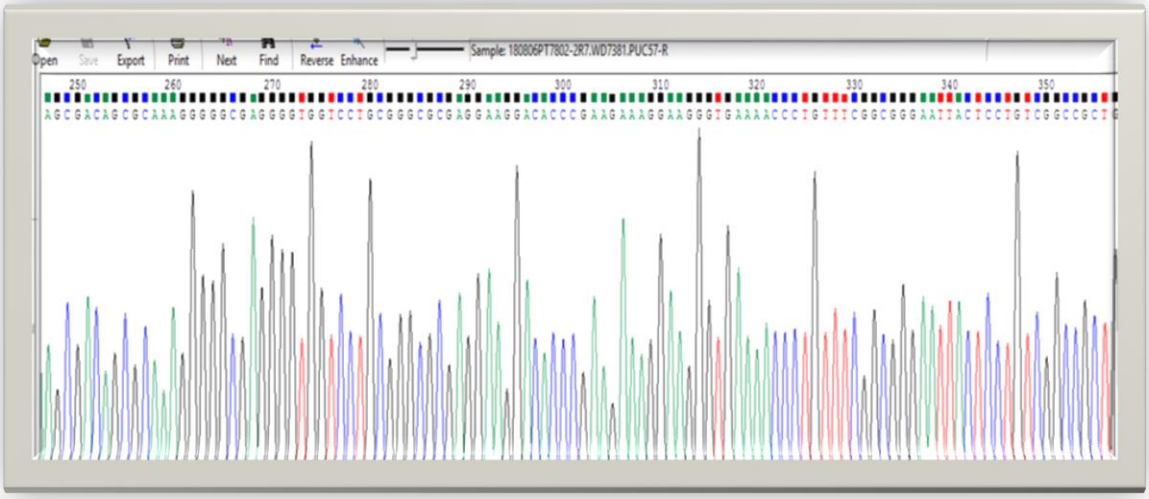

Fig. 2b: Sequencing results for PSTVd amplified samples inserted into vector pUC57-R

DISCUSSION

The extraction of PSTVd has been reported earlier by many researchers in different countries (Constable *et al*., 1996; Boonham *et al*., 2004; Singh *et al*., 2006; Di Serio, F. 2007;Qiu *et al*., 2016) but the present PSTVd isolation is first report of presence of PSTVd in Pakistan. The already reported methods of PSTVd RNA isolation were also found best but those all need highly equipped Laboratories, which is not possible in University Laboratories of progressing and low income countries in Pakistan. This presented work is reported as the modification of different researchers' work (Ding, Itaya, 2007; Guner *et al*., 2012) The present research is easy, simple and economic time saving isolation of PSTVd RNA from infected plants. The modification of extraction buffer by adding b-Mercaptoethanol in trizol reagent improved the isolation as compared to RNeasy Plant Mini Kit protocol (Mackie, 2015). It was worthy to note that isolated PSTVd RNA when amplified and sequenced showed complete match with NCBI BLAST sequences (Vachev *et al*., 2014). The obtained sequence was deposited in Gene bank (Accession # MK.303578) and Blastn searches at NCBI website demonstrated that this PSTVd isolate shared 100% similarity with Accession # U.23058.1 in *Solanum tuberosum*. These results have confirmed the presence of PSTVd in infected potatoes plants in Pakistan. This research could be served as a basic solid reference for planning and designing more explanatory research in viroids functions and structures for this pathogen control in Pakistan (PARC, 2010). The attack of pathogens always effected and slowed down the economy of agricultural countries like Pakistan. It is need of time to diagnose and identify actual cause of plant diseases (Hammond, 2001). The infection symptoms of virus and viroids often confused with each other therefore proper extraction and confirmation by sequencing is vital step for identification of viroids (Vachev *et al*., 2014).

In present work the infection symptoms were clearly observed and distinguished as morphological markers to identify PSTVd in infected plants according to literature. Three main signs and symptoms of PSTVd disease i.e yellowing, purpling of leaves with stunted stem growth were focused during present study (Mascia, 2009). It was also noted during infection plants collection that high grade temperature and light tropical regions of Pakistan) also favoured presence of PSTVd. The previous reports of PSTVd instability were also taken into accounts (Varadarajan, 2003; Mumford, 2004; Ward *et al*., 2010; Vachev *et al*., 2014; Mertelik *et al*., 2010 and shelf and storing condition of PSTVd RNA and cDNA were also improved during present study. The knowledge and findings about PSTVd has been discussed a lot during last decades in European countries.

However more need to be done, Like how this pathogen overtakes host plant's machinery to overtake advantage. It is also necessary determine source and route of PSTVd entry into crops. The present work would open up new avenues and eras for presence of PSTVd (Pathogen) and disease management.

CONCLUSION

The presented study has proved as an outstanding and efficient method for extraction of PSTVd RNA, its cDNA synthesis, amplification and storage. The Isolation of PSTVd from infected plants is a new addition to existing research.

Conflict of interest's statement

Authors declare that there is no conflict of interest for publishing this study.

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