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Full Length Research Paper

A simple and rapid nucleic acid preparation method for reverse transcription polymerase chain reaction (RT-PCR) in dormant potato tubers

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In order to shorten and facilitate the preparation of nucleic acid (without using tuber slicer, santurugation, vacuum devices and nanocalorimeter (NCM)) for reverse transcription polymerase chain reaction (RT-PCR), pieces of tuber were placed directly into eppendorf tubes containing 30 µl of detergent (0.5% Triton XL-80 N) buffers, vortexed for 3 min and then 2.5 µl of this aliquots was used in RT-PCR. Alternatively, evaluation of the efficiency of different buffers (sodium sulphite buffer, 0.1 M HCl, 0.1 M NaOH, 0.1 M Tris, 0.1 M EDTA, 0.1 M phosphate buffer, 0.1 M LiCl, 0.1 M KCl, 1 M NaCl and water (H_2O)) was carried out. The strongest band was obtained from sodium chloride (NaCl), potassium chloride (KCl), sodium sulphite buffer (SSB), phosphate buffer and lithium chloride (LiCl), respectively. To determine the minimum concentration and delectability of potato leafroll virus (PLRV) and potato virus S (PVS) that can be assessed by RT-PCR, the sap obtained from tubers known to be infected with PLRV and PVS were diluted (1:1, 1:2, 1: 4, 1:8, 1:16 1:32 and 1:64) and tested in RT-PCR. PLRV and PVS bands were observed up to 1:8 in NaCl and phosphate buffer, 1:4 in KCl, SSB and LiCl. However, except NaCl, no difference between the intensity of band obtained from other buffers was observed. On the other hand, increasing the cut surfaces of tuber pieces increased the dilution rate of nucleic acid. To determine the efficiency of the NaCl buffer, when a total of 60 dormant tubers (60 days after the harvest) were tested, PLRV and PVS were detected at the rate of 57/60 and 54/60, respectively.

Key words: Nucleic acid preparation, reverse transcription polymerase chain reaction, dormant tubers, potato leafroll virus, potato virus S.

INTRODUCTION

The vegetative propagation of potato (*Solano tuberous* L.) presents ample opportunity for the accumulation and multiplication of viral diseases (Spiegel and Martin, 1993). Under field conditions, potato often becomes infected with several viruses during a growing season (McDonald, 1984). Although there are more than 30 viral pathogens that can affect potato worldwide (Salazar, 1996), potato leafroll virus (PLRV, genus *Polerovirus*), potato virus X (PVX, genus *Potexvirus*), potato virus Y (PVY, genus *Potyviruses*), and potato virus S (PVS, genus *Carlavirus*) are the most common viruses economically affecting potato crops either singly or in combination (Singh, 1999). The first requirement to reduce extensive yield losses due to viral diseases in potato crops is the use of certified virus-free tuber for planting. However, routine diagnosis of potato viruses from dormant seed tubers in post-harvest potato indexing requires reliable, inexpensive and rapid procedures.

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Abbreviations: PLRV, Potato leafroll virus; **PVX,** potato virus X; **PVY,** potato virus Y; **PVS,** potato virus S; **ELISA,** enzyme-linked immunosorbent assay; **NCM,** nanocalorimeter; **dNTPs,** deoxyribonucleotide triphosphates; **SSB,** sodium sulphite buffer; **d_2H_2O,** distilled water; **EDTA,** ethylenediaminetetraacetic acid.

Molecular diagnosis, especially polymerase chain reaction (PCR)-based diagnostic tests have demonstrated reliability and efficiency for the detection of potato viruses in dormant tubers and aphid vectors (Singh et al., 1995; Singh and Singh, 1996, 1997, 1998; Singh and Nie, 2003). However, successful application of these methods for pathogen detection requires efficient recovery of target nucleic acids with minimal PCR inhibitors such as polyphenolic and polysaccharide compounds from infected plants (Singh et al., 2002). Furthermore, detection of several individual viruses from dormant tubers by reverse transcription polymerase chain reaction (RT-PCR) is expensive and time consuming. In order to reduce labor and equipment costs for test, a duplex RT-PCR (d-RT-PCR) (Singh et al., 2000) and a multiplex RT-PCR (m-PCR) protocols were developed for the detection of five viruses and one viroid in potato tubers and simplified by using an oligo (dT) in the synthesis of cDNA for potato viruses (PLRV, PVA, PVS, PVX and PVY) (Nie and Singh, 2000). This was used for the differentiation of geographical strains and isolates of PVY (Nie and Singh, 2002; Singh and Nie, 2003; Lorenzen et al., 2006) and used for the routine detection of PLRV, PVS and PVX from dormant tubers in certification programme (Bostan and Peker, 2009).

Nucleic acid extraction from plant tissues in large-scale surveys is the most laborious and time-consuming step in RT-PCR. Therefore, the preparation of sample RNA should be rapid, inexpensive and suitable for use in routine diagnostic testing. In order to alleviate former problems, reduce the use of organic solvent in nucleic acid extraction, minimize the dependence on well-equipped laboratories and facilitate the sample storage at room temperature and long distance transportation, various RNA extraction protocols were described (Rowhani et al., 1995; La Notte et al., 1997; Weidemann and Buchta, 1998; Singh, 1999; Fattouch et al., 2001; Dovas and Katis, 2003; Singh et al., 2004; Garg et al., 2008; Li et al., 2008). But, many of these methods are laborious, time-consuming, and expensive and still require full equipped laboratories.

The main purpose of this study was to simplify the preparation of nucleic acids from potato leaves and dormant tubers of PVS and PLRV for RT-PCR. For this purpose, the efficiency of different buffers without tuber slicer, centrifugation, vacuum devices and nitrocelluse membrane were tested.

MATERIALS AND METHODS

Plant material and viruses

Potato tubers (cv. Granola) known to be infected with both PVS and PLRV were obtained from our previous studies and multiplied in greenhouse. The presence of viruses in tubers was confirmed by enzyme-linked immunosorbent assay (ELISA) and RT-PCR (Bostan and Haliloglu, 2004; Bostan et al., 2006; Bostan and Peker, 2009). The leaves and tubers obtained from these plants were used in the tests.

Phenol methods for nucleic acid extraction

As a control, nucleic acid was extracted from individual potato tubers using the Na_2SO_3 procedure (phenol method) as described by Singh et al. (2002). The RNA extraction buffer at pH 7.4 had the following composition: 0.1 M Tris HCl, 2.5 mM $MgCl_2$, 0.65% Na_2SO_3 and 6 U of RNase-free DNase I. Three hundred microlitre of extraction buffer was combined with six drops (150 - 180 µl) of sap, collected from Tuber Slicer (Electrowerk, Behcke, Hannover, Germany). The mixtures were vortexed for 10 - 15 s and then incubated at 37°C for 10 min. For removing protein, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and then the samples were centrifuged (12 000 g, 15 min, 4°C). The top aqueous phase was transferred to fresh tubes and precipitated with 1 volume of isopropenol in the presence of 0.1 vol. of 3 M sodium acetate (-20°C overnight). The precipitated nucleic acid was collected by centrifugation (12 000×g, 15 min, 4°C), washed with 70% cold ethanol. The pellet was dried under vacuum and dissolved in distilled water (100 µl for tuber samples and in 1000 µl for leaf samples).

Comparison of buffers for RT-PCR

For the preparation of nucleic acid from potato leaves and tubers, the efficiency of the following buffers containing 0.5% Triton XL-80 N (Sigma-Aldrich Canada Ltd., Oakville, Ont.) were evaluated: (1) Sodium sulphite buffer (SSB) at pH 7.4 consisting of 0.1 M Tris-HCl and 0.65% Na_2SO_3 (Singh et al., 2002); (2) 0.1 M HCl; (3) 0.1 M NaOH; (4) 0.1 M Tris (pH 7.0); (5) 0.1 M EDTA (pH 7.0); (6) 0.1 M phosphate buffer (pH 7.0); (7) 0.1 M LiCl (pH 7.0); (8) 0.1 M KCl (pH 7.0); (9) 0.1 M NaCl (pH 7.0) and (10) sterile distilled water (d_2H_2O). Seven drops of sap were obtained from potato leaves and tubers tissue by passing the tissue through a tissue slicer/grinder (Electrowerk, Behncke and Co., Hannover, Germany) in a micro centrifuge tube containing 300 µl extracting buffers as described above and vortexed for 3 min. In order to obtain nucleic acid, individual nanocalorimeter (NCM) spots (BA-S 85, pore size 0.45 µm, Schleicher and Schuell, Keene, NH) were cut-out using a paper hole-punch, immersed directly into extracts, and then left separately in plates for each buffer. After spots were dried overnight at room temperature, they were transferred into micro centrifuge tubes containing 30 µl of distilled water and vortexed for 5 min. The eluted solution was used for RT-PCR. The concentration of PVS and PLRV in individual NCM spots prepared from leaf and dormant tuber samples was determined by diluting the eluted liquid and testing by RT-PCR. The dilutions were: 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64.

Obtaining nucleic acid from tubers without using vacuum, santurufugation, tuber slicer and NCM

In an attempt to shorten protocol and facilitate the preparation of nucleic acid (without using tuber slicer, santurugation and NCM) for RT-PCR, pieces of tuber (two pieces of tubers was about 5 mm^3) were placed directly into eppendorf tubes containing 30 µl of elution buffers, vortexed for 3 min and then 2.5 µl of this aliquots was used in RT-PCR.

Table 1. The primer pairs used for PVS and PLRV in RT-PCR.

Virus	Sequence	Polarity	Fragment
PVS	5'-TGGCGAACACCGAGCAAATG-3'	Sense	187 bp
	5'-ATGATCGAGTCCAAGGGCACTG-3'	Antisense	
PLRV	5'- CGCGCTAACAGAGTTCAGCC-3'	Sense	336 bp
	5'-GCAATGGGGGTCCAACTCAT-3'	Antisense	

RT-PCR

For reverse transcription (RT), 2.5 µl of total RNA was incubated at 65°C for 5 min, and then kept on ice for 5 min to denature the RNA. The RT mixture of 7.5 µl was added to provide a final concentration of 20 ng/µl of reverse primer specific to PLRV, 20 ng of oligo dT primer (Roshe), 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM dithiothreitol (DDT), 2.5 mM $MgCl_2$, 1.5 mM of each dNTPs (Promega), 200 U RNasin (Promega, Madison, WI) and 20 U Moleney Murine Leukemia virus-reverse transcriptase (MMLV-RT) (Invitrogen). Samples were incubated for 1 h at 42°C for RT and incubated subsequently at 95°C for 3 min to terminate the RT reaction.

PCR was carried out using 2 µl aliquots of the cDNA mixture in 23 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.2 µM of each of antisense and sense primers, and 0.625 U of *Taq* polymerase (Sigma). Samples were amplified in 35 cycles. Annealing temperature was 57°C (30 s), denaturation (92°C, 1 min), primer extension (72°C, 90 s), and a final extension (72°C, 12 min). 10 µl of amplified products were separated by electrophoresis in a 2.0% agarose gel containing 0.5 µg/ml ethidium bromides and photographed. Positive control for the viruses under study was included in each experiment. In order to determine the size of amplified products in the gel, as a marker, a low mass ladder (invitrogen) was used. The antisense and sense primers used for PLRV (Singh et al., 1995) and PVS (Matousek et al., 2000) are given in Table 1.

RESULTS AND DISCUSSION

Comparison of the efficiency of buffers for RT-PCR

In preliminary experiments, to determine which buffers were suitable for virus release, the nucleic acid eluted in 30 µl of water from individual cut-out spots prepared (without centrifugation and vacuum devices) from leaf and dormant tuber samples (the sap obtained from tuber slicer but not centrifuged) using each buffers was analyzed by RT-PCR and it was observed to be the bands seen on UV for each virus in all buffers (Figure 1). The band intensity obtained from both leaf and tuber samples in NaCl, KCl, SSB, phosphate buffer and LiCl were better than d_2H_2O, HCl, Tris, NaOH and EDTA (Figure 1). Therefore, except NaCl, KCl, SSB, phosphate buffer and LiCl, the remaining buffers were not used in subsequent applications. However, the band intensity obtained from leaf and tubers for PLRV and PVS in NaCl, KCl, SSB, phosphate buffer and LiCl were similar. When the results were evaluated according to the viruses, PVS gave a stronger band than PLRV in leaf samples but PLRV gave a stronger band than PVS in tubers (Figure 1).

To determine the minimum concentration and delectability of PVS and PLRV that can be assessed by RT-PCR, the nucleic acid eluted from NCM were tested at 1:1, 1:2, 1: 4, 1:8, 1:16 1:32 and 1:64 dilutions, and 2.5 µl aliquot from each diluted preparation was used for cDNA preparation. PLRV from leaf samples was detected up to the dilution of 1:16 in LiCl and KCl; 1:32 in phosphate buffer, NaCl and SSB buffers (data is not shown). Dilution rate for PVS from leaf samples were 1:32 in LiCl, phosphate buffer and KCl; 1:64 in NaCl and SSB buffers. When the extract obtained from tubers was diluted with sap obtained from uninfected tubers, PLRV bands were observed up to 1:4 in KCl, SSB and LiCl; 1:8 in NaCl and phosphate buffer. On the other hand, dilution rate for PVS from tuber samples were 1:4 in KCl, SSB and LiCl; 1:8 in NaCl and phosphate buffer (Table 2). However, except for NaCl, no difference between the intensity of band obtained from other buffers was observed.

In order to shorten protocol and to facilitate the preparation of nucleic acid (without using tuber slicer) for RT-PCR, small pieces of tubers (about 5 mm^3) were placed directly into eppendorf tubes containing 30 µl of detergent buffers, vortexed for 2 - 3 min and then 2.5 µl of aliquots was used in RT-PCR. In this application, the band for PLRV and PVS was obtained from all buffers up to the 1:8 and 1:4 dilutions. No distinct difference in the band intensity between the buffers were observed. However, the band intensity obtained from PLRV was stronger than PVS. On the other hand, increasing the cut surfaces of tuber pieces (increasing the number of tuber pieces) increased the dilution rate twofold for both PLRV and PVS.

To determine the efficiency of the NaCl buffer, when a total of 60 dormant tubers (60 days after the harvest) were tested, PLRV and PVS were detected at the rate of 57/60 and 54/60, respectively.

In preliminary studies, several simple RNA release protocols were reported. But, most of the studies did not used non-ionic detergents, and have involved a multitude of reagents. Furthermore, the use of phenol methods requires well-equipped laboratories (Rowhani et al., 1995, Thomson and Dietzgen, 1995, Olmos et al., 1996, Singh et al., 1996; Fattouch et al., 2001; Dovas and Katis, 2003). However, non-ionic detergent (Triton X series) has been used as a RNA release agent (Olmos et al., 1996,

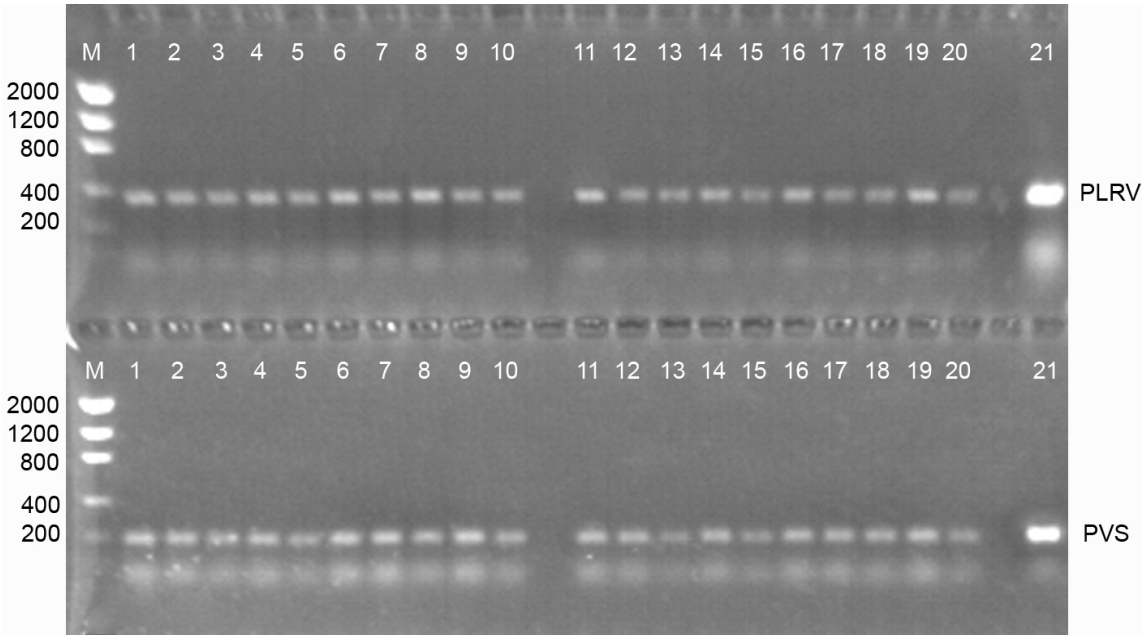


Figure 1. Evaluation of eluted material from NCM spots prepared from leaf and tuber samples by different buffers. RT-PCR products of PLRV from leaf samples (upper panel, left side); from tubers samples (upper panel, right side); RT-PCR products of PVS from leaf samples (lower panel, left side); from tubers samples (lower panel, right side); SSB, (lanes 1 and 11); HCl, (lanes 2 and 12); NaOH, (lanes 3 and 13); Tris, (lanes 4 and 14); EDTA, (lanes 5 and 15); phosphate buffer, (lanes 6 and 16); LiCl, (lanes 7 and 17); KCl, (lanes 8 and 18); NaCl, (lanes 9,19); d_2H_2O (lanes 10 and 20); pozitive control (lane 21); M, molecular size marker.

Table 2. Dilution rate of extract obtained from leaf and tuber samples using different buffers for PLRV and PVS from potato leaves and tubers.

Buffers	Tissue	PLRV	PVS
SSB + XL-80N	Leaf	1:32	1:64
	Tuber	1:4	1:4
NaCl,+ XL-80N	Leaf	1:32	1:64
	Tuber	1:8	1:8
KCl + XL-80N	Leaf	1:16	1:32
	Tuber	1:4	1:4
Phosphate buffer + XL-80N	Leaf	1:32	1:32
	Tuber	1:8	1:8
LiCl + XL-80N	leaf	1:4	1:32
	Tuber	1:4	1:4

Singh, 1999, Fattouch et al., 2001 and La Notte et al., 1997; Singh et al., 2002; Singh et al., 2004). PLRV could be detected by one-step procedure using detergent method from leaf, stem, tuber and single aphids, and it was determined that sterile distilled water and cDNA reaction mix were suitable for viral RNA releasing agents (Singh, 1999).

In our study, the obtained bands from tuber sap up to 1:4 dilution from PLRV and PVS from buffers showed that the viral nucleic acids released from tubers was enough to detect these viruses in RT-PCR. To detect PLRV-specific bands up to 1:8 dilutions on UV is particularly important. PLRV is a member of luteovirus, which occurs in low concentration in its hosts (Martin, 1995). Alternatively, increasing the cut surfaces of tuber pieces increased the dilution rate up to 1:16 for PLRV and 1:32 for PVS in all buffers. The observation shows that the detergent buffers could be used for the preparation of

viral nucleic acid from potato tubers for RT-PCR. Furthermore, nucleic acid was obtained directly from tubers in 1-2 min without any devices such as centrifugation, vacuum devices, tuber slicer and even NCM. The buffers used for preparation of nucleic acid do not pose any risk to human health and environment, and the buffers can be prepared easily in any laboratory. As a result, this modified protocol is simple, cheap, rapid, and suitable for the routine screening of PLRV and PVS from tuber-seed pieces in a large number of samples throughout the year.

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