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DETECTION OF POTATO VIRUS Y (PVY) BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION USING SPECIFIC PRIMERS

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ABSTRACT

Potato virus Y (PVY) is considered as one of the most threatening viruses in commercial potato production in Sri Lanka. In the global scale, the losses due to PVY infection have been rated between 40-70% of total production. Therefore, a highly sensitive, reliable and early detection method is important for rapid multiplication programmes of healthy potato plants and seed tubers, as well as for quarantine and breeding purposes. Potato samples with PVY-like symptoms were collected from Badulla, Sita Eliya, Nuwara Eliya, Bandarawela, Hali-Ela, Welimada and Beragala in the Uva province of Sri Lanka in early 2010. The preliminary screening for PVY was done by Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS ELISA). The PVY positive samples identified by ELISA were then subjected to molecular diagnosis by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The total RNA was extracted by 'Size fractionated silica extraction method' for RT-PCR. An amplicon of 480 bp specific for this primer pair was obtained when optimized conditions for the detection of PVY were used with specific primers P_3 and P_4. The optimum PCR conditions were determined as 5 µl of 5 x PCR buffer, 1.5 µl of 25 mM $Mgcl_2$, 1.0 µl of 10 mM dNTP mixture, 1.0 µl of each 10 mM primer pairs, 0.5 units of Taq polymerase, and 3 µl of cDNA template in 25 µl reaction volume with annealing temperature 57°C for 30 seconds.

KEYWORDS: Potato Virus Y, RNA, Reverse Transcription Polymerase Chain Reaction (RT-PCR)

INTRODUCTION

Potato (*Solanum tuberosum*), a tuberous crop from the family Solanaceae is the world's fourth-largest food crop, following rice, wheat and maize (http://en.wikipedia.org/wiki/Potato). Sri Lanka has successfully cultivated potato since 1812, and the districts of Badulla, Nuwara Eliya, Jaffna and Puttalam are the most desirable places for the crop to grow. However, 99,622 Mt. of potato were imported to Sri Lanka in 2009 (Anon, 2009). The most commonly cultivated potato varieties in the country are 'Granola' and 'Arnova'. Although the annual maximum seed potato requirement of the country is 22,000 tons, approximately 6,000–7,000 tons of seed potatoes are imported every year as the seed potato production is kept at a low phase due to many reasons, including the susceptibility to pests and diseases (Nugaliyadde *et al.*, 2005).

Potato leaf roll virus (PLRV), potato virus X (PVX), potato virus Y (PVY), Potato virus M (PVM) and potato virus S (PVS) are the most prevalent viral diseases in Sri Lanka and among these PVY is the second most harmful virus disease for potato in the country (http://www.agridept.gov.lk/Production/of/virus/free/in-vitro/plants/of/potato). Shortening of the stem internodes, severe malformation of the upper leaves and stunting of potato plants appear as common problems in potato cultivation in Sri Lanka due to viral infections.

The PVY is a member of the largest plant virus family Potyviridae affecting potato, tobacco, tomato and pepper as well as wild plant species, especially those in the Solanaceae family. In potato, PVY causes a severe disease called mosaic or rugose mosaic. Symptoms are variable depending on the viral strain, host cultivar, climatic conditions, and the type of infection, irrespective whether it is a primary or secondary infection. Symptoms in the aerial parts of the infected plants consist of a mild to severe mottle, often associated with distortion of the leaves. Yellowing and necrosis frequently occur in the lower leaves. Symptoms also include collapse and fall of intermediate leaves, which remain clinging to the stem. Secondary infected potato plants are dwarfed and brittle, with crinkled and puckered leaves. Symptoms in foliage and growth reduction are more severe when PVY occurs in combination with other viruses, especially PVX or potato virus A (PVA).

The PVY has a single positive-sense genomic RNA ~ 10 kb long and forms flexuous filamentous non-enveloped, rod shape virus. The genomic RNA contains a unique ORF encoding a polyprotein, which is processed into functional virus proteins by virus encoded proteases. The PVY is naturally transmitted by aphids in a non-persistent manner (El-Araby *et al.*, 2009).

The PVY can be identified by serological and molecular detection techniques such as ELISA, nucleic acid hybridization, Polymerase Chain Reaction (PCR), etc. The serological method ELISA is used worldwide including Sri Lanka for the detection of many plant viruses and PVY (http://www.agridept.gov.lk/institutes_sub_more.php/id=219&mMenu=Horticulture&Agriculture/Research/Station-/Sita-Eliya/ARS). But no viral genome-based PVY detection methods are employed in Sri Lanka. Therefore, it is important to develop a nucleic acid-based detection system, which is highly reliable for PVY identification in Sri Lanka. The objective of this study is to develop and optimize conditions for a Reverse Transcription Polymerase Chain Reaction (RT-PCR) system conditions for detection of PVY.

MATERIALS AND METHODS

The study was conducted at the Plant Virus Indexing Centre (PVIC), Gabadawatta, Homagama, Sri Lanka.

Collection of potato samples

Leaves and sprouted tubers showing PVY-like symptoms were collected from fields grown at Sita Eliya, Nuwara Eliya, Bandarawela, Hali-Ela, Welimada and Beragala districts in the Uva province in August 2010. Twenty four samples were collected and the potato plants sampled were stunted with mosaic leaves and yellowing symptoms. Collected samples were immediately used or preserved at -80°C.

Screening of plant material for PVY

The presence of the virus in collected samples was confirmed by polyclonal PVY specific antibodies (IgG: AS-0137, IgG-AP: AS-0137, DSMZ, Germany) in Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA).

Optimization of PCR system conditions for detection of PVY

The serologically (ELISA) positive samples from the tested samples were used for the optimization of PCR conditions.

Extraction of RNA

Total RNA was extracted from PVY-infected plants and healthy plants using 'Size fractionated silica extraction method' (Gunasinghe *et al.,* 2009).

cDNA synthesis and Reverse transcription

All extracted RNA (PVY) samples were used for cDNA synthesis with PVY antisense primer (P_4) (Table 1). The reverse transcription reaction mixture contained 4 µl of 5×reverse transcriptase buffer (M-MuLV), 2 µL of dNTPs (10mM), 1µL antisense primer, 0.1 µL of RNAsin ribonuclease inhibitor (Promega), 0.25 µL of M-MuLV reverse transcriptase (Promega), 4.65 µL of deionized water and 8 µL of RNA template. The first strand synthesis was carried out at 37°C for 60 min followed by inactivation at 72°C for 15 min. The primer annealing temperatures were determined theoretically using software (http://www.sabina.anzlovar.com).

Amplification of cDNA by polymerase chain reaction (PCR)

The synthesized PVY cDNA were amplified with virus specific primer pair specific for the coat protein gene of PVY (Shalaby *et al.*, 2002; Singh and Singh, 1995; Table 1). The PCR reactions were carried out separately with different annealing temperatures, starting from 49°C up to 60°C. The PCR mixture consisted of 5 µL of 5x PCR buffer, 1.5 µL of $MgCl_2$ (25 mM), 1.0

μL of dNTPs (10mM), 1.0 μL each of 10 mM sense (P_3) and antisense (P_4) primer, 0.5 units of *Taq* polymerase (5u/μL) (Promega), 3 μL of cDNA template and deionized water to a total volume of 25 μL. The thermocycler conditions were initial denaturation 94°C for 1min, followed by 35 cycles, denaturation at 94°C for 1min, annealing at T_a°C for 30 sec, extension 72°C for 40 sec with final extension at 72°C for 10 min.

Table 1. Specific primers for detection of PVY, and the size of the amplified product

Virus	*Primer*		*Primer sequences (5'-3')*	*Product size*
PVY	PVY 3S	P_3	ACGTCCAAAATGAGAATGCC	480 bp
	PVY-A4	P_4	TGGTGTTCGTGATGTGACCT	

Analysis of PCR product

The PCR products were separated by gel electrophoresis on a 1% agarose gel in TAE buffer (40mM Tris acetate, 1mM EDTA, pH 8.0) containing 1 μL of ethidium bromide (0.05 μg/mL) (Sambrook *et al.*, 1989). The 2000 bp DNA ladder (Gene Script Corp) was used as the size marker to determine the amplified 480 bp products specific for this primer pair.

Confirmation of protocol

Potato samples with PVY symptoms collected from farmer fields were tested with above procedure to determine the reproducibility of the optimized conditions. This experiment was repeated to determine the consistency of the results.

RESULTS AND DISCUSSION

The detection of the occurrence of PVY in potato samples by DAS-ELISA

Twelve samples from the 24 samples tested by DAS-ELISA were positive for PVY (Table 2). Sprouted tubers from Bandarawela (S_{21}) recorded the highest positive results.

Table 2. Symptoms of test samples collected from potato cultivations and absorbance values at 405nm

Sample No.	*Sample location*	*Plant No.*	*Yellowing of leaf vein*	*Yellowing full leaf*	*Leaf Mosaic*	*Stunted plant*	*Absorbance*	*Disease status*
S_1	Bandarawela	35	Y	-	-	-	0.285	+
S_2	Bandarawela	33	-	Y	-	-	-0.033	*
S_3	Bandarawela	01	Y	-	-	-	0.215	+
S_4	Bandarawela	08	-	Y	-	-	-0.007	*
S_5	Bandarawela	11	-	-	Y	-	-0.010	*
S_6	Bandarawela	16	Y	-	-	-	-0.017	*
S_7	Nuwara Eliya	20	-	-	Y	-	-0.007	*
S_8	Nuwara Eliya	21	-	Y	-	-	-0.001	*
S_9	Nuwara Eliya	24	-	-	Y	-	-0.023	*
S_{10}	Nuwara Eliya	28	-	Y	-	-	-0.019	*
S_{11}	Nuwara Eliya	32	-	-	Y	-	-0.021	*
S_{12}	Welimada	19	Y	-	Y	-	-0.044	*
S_{13}	Nanu Oya	48	Y	Y	-	Y	2.508	+
S_{14}	Sita Eliya	63	Y	-	Y	-	2.243	+
S_{15}	Sita Eliya	64	Y	-	Y	Y	2.250	+
S_{16}	Sita Eliya	57	Y	-	Y	-	2.246	+
S_{17}	Sita Eliya	54	Y	-	Y	-	2.347	+
S_{18}	Sita Eliya	52	Y	-	Y	-	2.363	+
S_{19}	Sita Eliya	56	Y	Y	Y	Y	2.406	+
S_{20}	Sita Eliya	25	Y	-	Y	-	0.115	+
S_{21}	Bandarawela tubers	30	-	-	-	-	2.687	+
S_{22}	Bandarawela tubers	26	-	-	-	-	-0.042	+
S_{23}	Bandarawela tubers	07	-	-	-	-	0.164	+
S_{24}	Welimada	15	Y	-	-	-	-0.059	*
S_{25}	Kit positive control 1	P 1					0.390	+
S_{26}	Kit positive control 2	P 2					0.458	+
S_{27}	Nanu Oya (Healthy)	17	-	-	-	-	0.018	*
S_{28}	Kit negative control	H 1					0.016	*

- Absence of Symptoms, Y presence of symptoms, + Diseased, * Healthy

Optimization of Reverse transcription polymerase chain reaction (RT-PCR) system conditions

Product amplification at different annealing temperatures: The product amplification was not observed at annealing temperatures of 49°C, 50°C, 56°C, 58°C, 59°C and 60°C. But annealing temperatures of 52°C and 54°C produced light color bands (480 bp) with lower quality. Clear bright bands were obtained in products amplified at annealing temperature of 57°C (Figure 1).

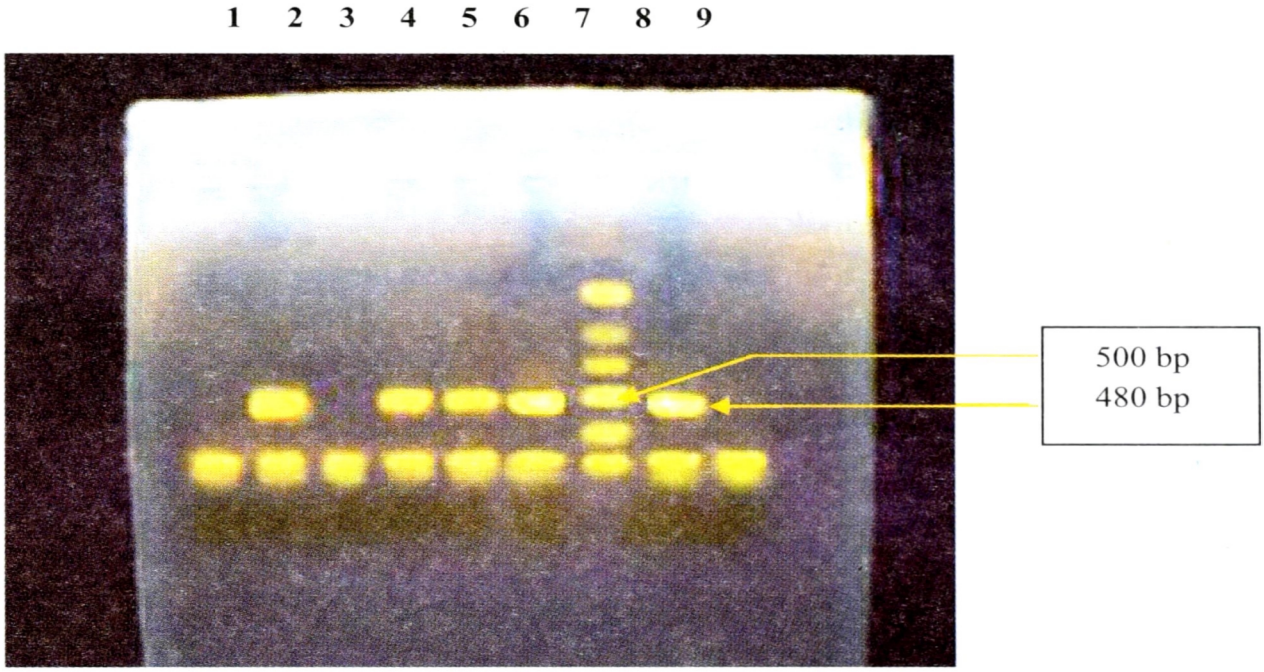

Figure 1. Agarose gel electrophoresis of RT-PCR using primer pair P_3 and P_4 at annealing temperature 57°C. PVY-infected plants (Lane 1, 2, 3, 4, 5, 6), Molecular size marker (2000bp DNA Ladder Gene Script Corp) (Lane 7), Positive control (Lane 8), Negative control (Lane 9).

Product amplification at different magnesium chloride concentrations: The prepared cDNA was amplified with a range of $MgCl_2$ concentrations in the PCR mix. Positive results were obtained for PVY (480 bp), when samples were amplified at 57°C and 1.0, 1.25, 1.50, 1.75 µL of $MgCl_2$ concentrations. The most significant band was observed at 1.5 µL $MgCl_2$ concentration (Plate 2). No amplification was given for 0.75 µL and 2.00 µL (Figure 2).

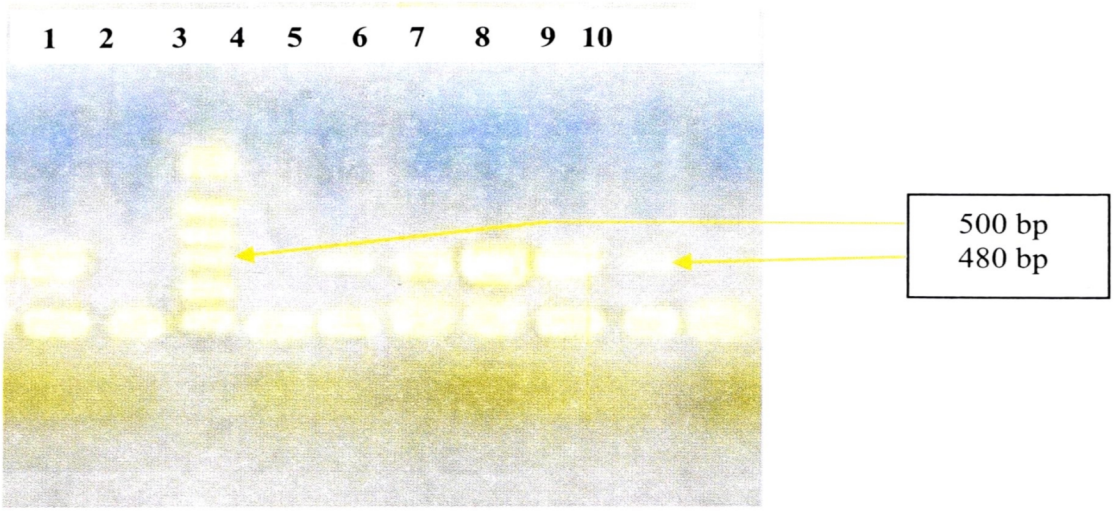

Figure 2. Amplified products of PVY at annealing temperature 57°C using different magnesium chloride concentrations of 25mM. Positive control (Lane 1), Negative control (Lane 2), Molecular size marker (2000bp DNA Ladder, Gene Script Corp) (Lane 3), $MgCl_2$ concentrations of: 0.75 µL (Lane 4), 1.0 µL (Lane 5), 1.25 µL (Lane 6), 1.50 µL (Lane 7), 1.75 µL (Lane 8), 1.85 µL (Lane 9), 2.00 µL (Lane 10).

Product amplification at different dNTPs concentrations: Positive results were given for PVY (480 bp), when purified virus samples were amplified at 57°C with 0.75, 1.0 and 1.5 µL of dNTPs concentrations. The most effective band was obtained at 1.0 µL of dNTPs concentration (Figure 3). The sample amplified with 0.5 and 2.0 µL of dNTPs gave negative results.

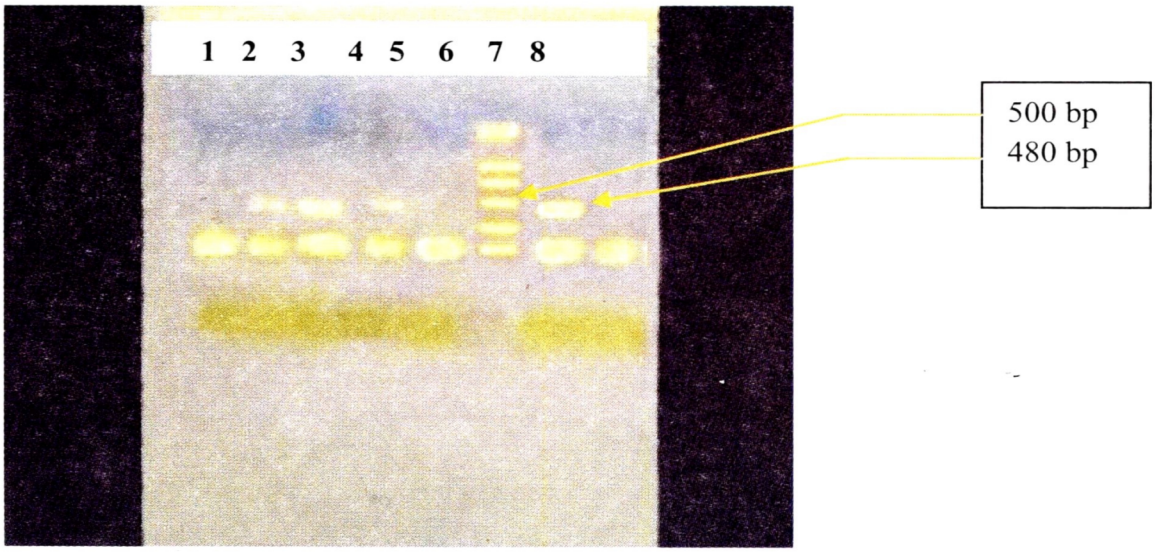

Figure 3. Amplified products of PVY at annealing temperature 57°C using different dNTPs concentrations. dNTP concentrations 0.5 µL (Lane 1), 0.75 µL (Lane 2), 1.00 µL (Lane 3), 1.50 µL (Lane 4), 2.00 µL (Lane 5), Molecular size marker (2000bp DNA Ladder, Gene Script Corp) (Lane 6), Positive control (Lane 7), Negative control (Lane 8).

Detection of PVY in symptomatic plants using optimized conditions

The diseased potato samples collected from farmer fields amplified a band of 480 bp with RT-PCR, at optimized annealing temperature 57°C and optimized PCR system conditions (Table 3).

Table 3. Results of Product amplification, non amplification and reproducibility test of PVY.

Sample Location	*Plant No:*	*Amplified / Not Amplified*								
		Annealing temperature								
		52°C			*54°C*			*57°C*		
		1	*2*	*R*	*1*	*2*	*R*	*1*	*2*	*R*
Sita Eliya	S_8	-	-	x	-	+	+	++	++	+
Sita Eliya	S_6	-	-	x	*	+	+	++	+	x
Sita Eliya	S_3	-	-	x	-	*	x	-	*	-
Bandarawela	B_8	+	*	+	-	-	-	++	++	++
Bandarawela	B_{15}	-	-	x	-	-	-	*	++	++
Bandarawela	B_{17}	-	-	x	-	-	-	++	+	++
Bandarawela	B_3	-	*	-	-	-	-	++	++	++
Bandarawela tubers	Bt_{21}	-	-	x	-	-	x	++	++	++
Bandarawela tubers	Bt_{23}	-	-	-	-	-	x	+	++	++
Sita Eliya	S_{59}	-	-	x	-	-	-	++	++	++
Nanu Oya	S_{48}	-	-	-	-	*	x	++	++	++
Sita Eliya	S_{56}	-	-	x	-	-	x	+	*	+
Sita Eliya	S_{52}	-	*	x	-	-	-	++	++	++
Nanu Oya	S_{25}	-	-	-	-	*	x	++	*	++
Sita Eliya tubers	S_{26}	-	-	x	-	*	-	++	++	++
Sita Eliya tubers	S_{27}	-	*	x	-	*	x	+	+	+
Sita Eliya	S_{54}	-	-	x	-	*	x	++	++	++
Sita Eliya	S_{57}	-	*	x	-	*	x	++	*	-
Sita Eliya	S_{64}	-	-	x	-	*	x	++	++	+
Sita Eliya	S_{63}	-	*	x	-	-	x	+	*	-

++ : Product amplified
\- : Product not amplified
* : Sample not tested
x : Reproducibility test not done
\+ : light band
1 : Replicate 1
2 : Replicate 2
R : Reproducibility test

The most common method for the detection of plant viruses is ELISA, because it is rapid and inexpensive. However, the serological methods have drawbacks of limited availability of antisera and questions regarding the specificity of antisera produced from preparations containing virus mixtures. Raising antisera is also time-consuming. In contrast, a diagnostic technique using RT-PCR can be rapidly implemented in independent laboratories. The common methods used to detect PYV in Sri Lanka are based on biological properties such as nature of symptoms and biochemical properties such as ELISA serology and the authors found no previous work reporting nucleic acid-based diagnosis for PVY in Sri Lanka.

A comparison of three conventional RNA extraction methods for the detection of plant virus/viroid RNAs by RT-PCR with same set of specific primers, were carried out by Sipahioglu *et al.* (2007). The same Silica capture RNA extraction method was used in this study, too. Most of the methods used in the present study was based on the published information by previous workers, but only few of already established systems' information could be used, such as annealing temperature 55°C for 1 min (Shalaby *et al.*, 2002), 54°C for 1 min (Ghosh and Bapat, 2005) , 57°C for 1 min (Sipahioglu *et al.*, 2007) and 55°C for 1 min (El-Araby *et al.*, 2009). In this study annealing temperature was the optimized 57°C for 30 sec in primer condition using extracted purified and viral RNA from field samples.

A large number of PVY isolates have been tested in different countries, collected from different locations. For example, 119 samples were tested in Moscow (Lorenzen *et al.*, 2006) 193 samples were tested in USA (Piche *et al.*, 2004). In the present study, the sample number was limited to 30, which were positive for PVY in ELISA, due to various limitations.

When reproducibility testing was conducted, the previously amplified viruses were not present in some samples. Rosner and Muslenin (2005) revealed that virus detection in the field grown plant can be influenced by two levels of interferences that exist between the templates of two closely related PVY isolates in a linked PCR-transcription amplification. It seems that there is a mutual interference between the two virus templates during the PCR amplification process. This could be due to the increase in the concentration of one virus, in the reaction mixture suppressing the amplification of the other virus isolate.

The results obtained in this study demonstrate the successful use of RT-PCR to detect plant viruses in infected leaf tissues of potato and indicated its feasibility as a rapid laboratory assay for detecting PVY. This optimized protocol can be used in indexing in order to establish virus free potato cultivation, thus helping to increase production, reduce the imports and save foreign exchange.

CONCLUSIONS

Potato Virus Y can be detected with specific primers (PVY 3S–ACGTCCAAAATGAGAAT GCC and PVY A4-TGGTGTTCGTGATG TGACCT). Amplified products of PVY were obtained with RT-PCR, at optimized annealing temperature 57°C for 30 sec, using specific primers. The optimized PCR conditions are 5 µL of 5 x PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 1.0 µL of 10 mM dNTPs mixture, 1.0 µL of each 10 mM primer pairs, 0.5 units of Taq polymerase and 3 µL of cDNA template.

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REFERENCES

Anon. 2009. Estimated Extent and Production of Potato: Agriculture and Environment Statistics Division, Department of Census and Statistics, Sri Lanka. Available from: (http://www.statistics. gov.lk/agriculture/seasonalcrops/PotatoNationalandsubNational.html (Accessed 18 July 2010).

El-Araby, W.S., I.A. Ibrahim, A.A. Hemeida, A. Mahmoud, A.M. Soliman, A.K. El-Attar and H.M. Mazyad. 2009. Biological, Serological and Molecular Diagnosis of Three Major Potato Virus in Egypt. International Journal of Virology 5 (2): 77-88.

Ghosh, S.B. and V.A. Bapat, 2005. Development of RT-PCR based method for detection of *potato virus Y* in tobacco and potato. Indian Journal of Biotechnology 5: 232-235.

Gunasinghe, W.A.D.S.K., E.M. Dassanayake and N.M. Ubeysekara. 2009. Successful Detection of RNA viruses by Reverse Transcription Polymerase Chain Reaction. Annals of the Sri Lanka Department of Agriculture 11: 57-62.

http://en.wikipedia.org/wiki/Potato, (Accessed 18 July 2010).

http://www.agridept.gov.lk/institutes_sub_more.php/id=219&mMenu=Horticulture&Agriculture/Research/Station-/Sita-Eliya/ARS. (Accessed 10 June 2010).

http://www.agridept.gov.lk/Production/of/virus/ free/in-vitro/plants/of/potato, (Accessed 18 July 2010).

htpp://www.sabina.anzlovar.com. (Accessed 20 February 2010).

Lorenzen, J.H., L. M.Piche, N.C. Gudmestad, T. Meacham and P. Shiel. 2006. A multiplex PCR assay to characterize *Potato virus Y* isolates and identify strain mixtures. Plant Diseases. 90:935-940.

Nugaliyadde, M.M., H.D.M. De Silva, R. Perera, D. Ariyaratna and U.R. Sangakkara. 2005. Annals of the Sri Lanka Department of Agriculture. 7: 199-208.

Piche, L. M., R.P. Singh, X. Nie and N.C. Gudmestad. 2004. Diversity among *Potato virus Y* isolates obtained from potatoes grown in the United States. Phytopathology. 94: 1368-1375.

Rosner, A. and L. Muslenin. 2005. Interference in PCR: Transcription amplifications of mixed PVY isolates. Plant Protection Science. 41:125–131.

Sambrook, J., E.F. Fritch and T. Maniatis. 1989. Molecular cloning: A. 2nd ed. Cold Spring Harbour laboratory press: Cold Spring Harbour.

Singh, M., and R.P. Singh. 1995. Digoxigenin-labelled cDNA probes for the detection of *potato virus Y* in dormant potato tubers. Journal of Virological Methods. 52: 133-143.

Shalaby, A.A., M.K. Nakhla, A.M. Soliman, H.M. Mazyad, A. Hadidi and D.P. Maxwell. 2002. Development of a highly sensitive multiplex reverse transcription-polymerase chain reaction (m-RT-PCR) method for detection of three potato viruses in a single reaction and nested PCR. Arab J. Biotech. 5 (2): 275-286.

Sipahioglu, H.M., M. Ocak and M. Usta. 2007. Comparison of three conventional extraction methods for the detection of plant virus/ viroid RNAs from heat dried high-phenolic host leaves. Asian Journal of Plant Science. 6 (1): 102-107.