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IDENTIFICATION OF DISEASE FREE POTATO GERMPLASM AGAINST POTATO VIRUSES AND PCR AMPLIFICATION OF POTATO VIRUS X

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ABSTRACT

Potato virus caused 83% yield losses in potato crops of Pakistan. Potato virus X (PVX) is in the list of the top ten damaging plant viruses in the world. Thirty yhree varieties/advanced lines of potato were screened against potato virus A (PVA), potato leaf roll virus (PLRV), potato virus M (PVM), potato virus S (PVS) and PVX. CIP-72-Y-3 was infected with PVA, PVM, PVS and PVX while Sahiwal-3, 34-1-Y, Sahiwal-4, 34-2-Y, Sahiwal-5, Sahiwal-6 and CIP-72-Y-4 showed reaction against PVA, PVS and PVX. 34-Y-3 found positive against PVA and PVX while PVM and PVX were confirmed in 34-4-X and CIP-72-Y-1. Azado-3, 34-6-X and 36-2 were positive with PLRV and PVM. 36-1, 34-S-1, 34-S-3-H and 42-1(Rusal Invary) were infected with PLRV and PVX while PVS and PVX were detected in Sahiwal-2, Azado-2, 42-2 (Rusal Invary) and CIP-72-Y-2. Eleven, one, nine, two, eight and two varieties/advanced lines of potato were highly resistance, highly susceptible, moderately susceptible, resistance, susceptible, against PVX respectively. Coat protein (CP) gene specific primes successfully amplified a 750 bp fragment of PVX in infected samples.

Keyword: ELISA; PCR; coat protein gene, potato virus X,

INTRODUCTION

Potato (*Solanum tuberosum* L) is a staple food and vegetable crop. It is ranked fourth in production after rice, wheat and maize (Rauscher *et al*., 2006). It gives 12-15 times more yield per hectare. Its calories production per unit area is higher than wheat, maize and rice. The potato tuber is an excellent source of carbohydrates, protein and vitamins (MacGillivary, 1953). In Pakistan, potato is cultivated over an area of 127.7 thousand hectares and annual production is 3726.5 thousand tons (GOP, 2011). Propagated vegetative material transfers high number of pests and pathogens to the next generation and viruses are most important among them. Potato crop is naturally infected by 37 different plant viruses. Foreign potato varieties significantly increased the yield of potato crop in Pakistan but at the same time resulted the new viral problems like Potato PVX, PVY, PVS, PLRV, PVA and PVM have been reported in spring, summer and autumn potato crop of Pakistan and cause up to 83% yield losses (Mughal *et al.,* 1986). Most viruses can effectively be determined serologically by ELISA tests (Petrrunk *et al*., 1991). PVX is single stranded positive sense RNA genome having a cap structure at 5' end and poly (A) tail at 3' terminus (Sonenberg *et al.,* 1978). The 3' terminus encodes coat protein (CP) gene which is involved in various stages of infection cycle. The virus particles by weight contain 6% nucleic acid and approximately 94% protein (Tollin *et al.,*1967).

MATERIALS AND METHODS

A total thirty three varieties/advanced lines of potato of different origin/source were tested against five viruses by using Double Antibody Sandwich (DAS) ELISA as described by Abbas *et al.,* (2012) and plants were selected randomly for screening. Solutions required for DAS ELISA were prepared by following materials and methods.

Antibody coating buffer: Sodium carbonate (Na_2CO_3) 0.59 g, Sodium bicarbonate ($NaHCO_3$) 2.93 g and Sodium azide (NaN_3) 0.2g was dissolved into double distilled water (DDW) (500 mil) and the pH of the solution was adjusted to 9.6. Buffer was stored at 4°C after making volume 1L with DDW.

Phosphate buffer saline (PBS): Sodium chloride (NaCl) 8 g, Potassium phosphate (K_2PO_4) 0.2 g, Sodium phosphate (Na_2PO_4) 1.15 g, Potassium chloride (KCl) 0.2 g, Tween-20 (Polyoxy ethylene sorbitan monolayrate) 0.2 ml/L and NaN_3 2 g were dissolved in 1000 ml of DDW. It was stored at room temperature and used in the preparation of extraction and washing buffer.

Virus (antigen) extraction buffer: Sodium Sulfite 1.3 g, NaN_3 0.2 g, Polyvinyl pyrrolidone (PVP. MW 40000) 20 g, Tween-20 0.2 ml/L and final volume was made 1L by adding PBS. The pH was adjusted 9.8 and stored at 4°C.

Washing buffer: IL of final volume was made with PBS by adding 1ml Tween-20. This buffer was stored at room temperature after adjusting the pH 7.4

Conjugate buffer: 20 g PVP was added and final volume made 1L with PBS. The pH was maintained 7.4 and stored at 4°C.

Substrate buffer: Diethanol amine 97 ml, NaN_3 0.2 g, Magnasium chloride ($MgCl_2$) was added and final volume was made 1000 ml with DDW after adjusting pH 9.6. This buffer was stored at 4°C. The pH of each buffer was adjusted with 1N sodium hydroxide (NaOH) and diluted hydrochloric acid (HCL).

Leaf samples from 30 varieties/ advanced lines were collected for testing through DAS-ELISA against PVA, PVM, PVS and PVX. The 60 wells of ELISA plate was coated with Monoclonal Immunoglobulin (IgG) of PVA, PVM, PVS, PVX and PVY (BIOREBA AG kit). IgG was diluted (1:200) in coating buffer and 100 µl was loaded in each well and the coated plate was incubated at 37°C for 4h. Leaf samples were homogenized (1mg/ml) in autoclave ice chilled pestle and mortal and 100µl was added to each well along with negative and positive control. Antigen loaded plates were incubated over night at 4°C. Enzyme alkaline phosphatase (ALKP) conjugated IgG (BIOREBA AG kit) of PVA, PVM, PVS, PVX and PVY diluted at 1:200 and 100µl was loaded in each well. The plates were incubated for 4h at 37°C in a moist chamber. After each step three washing with 3 min interval was done with washing buffer and plates were dried at paper towel. 150µl of substrate buffer containing p-nitro phenyl phosphate (1 mg/ml) was added to each well and plates were incubated at room temperature in dark for 45 min. The reaction was visually observed for the development of yellow color and also read in ELISA reader at 405 nm. The reaction was stopped by adding 50µl of NaOH (3M) to each well. A sample is considered as virus infected when the absorbance of 405 nm was at least thrice of healthy control and also shows yellow color. Development of yellow color indicated the presence of a virus and OD_{405nm} value shows concentration of virus in the plant.

Total RNA Isolation: 100 mg Leaf tissue was crushed in ice chilled auto-clave pestle and mortar with liquid nitrogen and TRI Reagent 118 (1ml/1mg) was added. The homogenate was stored on ice for 5 min and it was transferred into new 2ml micro-centrifuge tubes containing 0.2 ml chloroform. Tubes were shaken vigorously for 15 second stored on ice for 10 min. Centrifugation at 12,000 rpm for 15 min was done and the aqueous phase was transferred to a fresh tube containing 0.5 ml isopropanol. Samples were stored on ice for 10 minutes and centrifuged at 12,000 rpm for 8 minutes. RNA pellet was washed with 1ml ethanol (75%) and centrifuged at 7,500 rpm for 5 min. All centrifugation was done at 4°C. The ethanol was removed and 20 µl of Diethylpyrocarbonate (DEPC) treated water was added after drying the pellet at remove temperature.

CP gene specific Primers: Two CP gene specific primers sense (GACCTCGAGAT GTCAGCACCAGCTAG) and anti sense GGCGAATTCTTATGGTGGTGGTAGAGTG) were used for molecular conformation of PVX in DAS-ELISA confirmed germplasm (Nosheen, 2011). Reverse primer was used in cDNA synthesis while both primers were used in PCR amplification of CPG.

Polymerase Chain Reaction (PCR): cDNA was synthesized using Revert Aid Reverse Transcriptase with total RNA and CP gene specific antisense primer.

PCR Reactions: A 50µl PCR reaction mixture contained 2µl of template (cDNA), 5µl of 10x Taq eaction buffer, 1.5 mM $MgCl_2$, 0.2mM dNTPs (dATP, dCTP, dGTP, dTTP), 0.25 unit of Taq polymerase and 0.2 µM of each primer.

PCR conditions: Initial melting for 3 min at 94°C followed by 35 cycles of 94°C/30 sec, 51°C/30 sec, 72°C/1 min with a 72°C/10 min final extension.

Gel Electrophoresis: PCR amplified fragments were analyzed by electrophoresis on 1% agarose gel in TBE buffer (89mMTris, 89mMboric acid, 2mM EDTA) stained with ethidium bromide and visualized under UV illumination light. CP gene fragment size was determined using the 1kb DNA Ladder.

RESULTS

A total thirty three varieties/advanced lines were tested against PVA, PLRV, PVM, PVS and PVX. CIP-72-Y-3 was infected with PVA, PVM, PVS and PVX while Sahiwal-3, 34-1-Y, Sahiwal-4, 34-2-Y, Sahiwal-5, Sahiwal-6 and CIP-72-Y-4 showed their reaction against PVA, PVS and PVX and 34-Y-3 was positive with PVA and PVX only. PVM and PVX were detected in 34-4-X and CIP-72-Y-1 while PLRV and PVM in Azado-3, 34-6-X, 36-2 and 36-1, 34-S-1, 34-S-3-H and 42-1 (Rusal Invary) were infected with PLRV and PVX. Sahiwal-2, Azado-2, 42-2

(Rusal Invary) and CIP-72-Y-2 confirmed PVS and PVX in DAS-ELISA. Sahiwal-1, Azado-1, 34-Y-X, 34-S-2, 42-Y-1, 42-Y-2, CIP-72-Y-5, 72-X-1 showed no reaction against any virus (Fig.1 and Table I).

Potato germplasm field screening to PVX: Among thirty three lines/varieties of potato only twenty two showed infection with PVX. Sahiwal-1, Azado-1, 34-S-2, 42-Y-1, 42-Y-2, CIP-72-Y-5, 72-X-1, 34-5-X, Azado-3, 34-6-X and 36-2 found to be highly resistant against PVX and only CIP-72-Y-3 was highly susceptible while 34-4-X and CIP-72-Y-1 were resistant and 34-3-Y and Cardinal were found to be moderately resistant. Sahiwal-334-1-Y, Sahiwal-4, 34-2-Y, Sahiwal-5, Sahiwal-6 and CIP-72-Y-4, Mirrato and Desire were moderately susceptible while susceptible against PVX (Table 2, 3). 36-1, 34-S-1, 34-S-3-H, 42-1(Rusal Invary), 42-2 Sahiwal-2, Azado-2, CIP-72-Y-2 and CIP-72-Y-3 were highly susceptible.

PCR amplification of Potato virus X: Specific sense and antisense primers of CP gene successfully amplified 750bp fragment of PVX in PCR. ELISA negative samples were also included in PCR and 42-Y-1 which was ELISA negative and highly resistance against PVX produced a dim band in PCR (Fig. 2).

Table 1. Serological confirmation of potato viruses in potato germplasm.

Virus confirmed in DAS-ELISA	Potato germplasm
PVA, PVM, PVS and PVX	CIP-72-Y-3
PVA, PVS and PVX	Sahiwal-3, 34-1-Y, Sahiwal-4, 34-2-Y, Sahiwal-5, Sahiwal-6, CIP-72-Y-4 and Mirrato
PVA and PVX	34-3-Y
PVM and PVX	34-4-X and CIP-72-Y-1
PLRV and PVM	Azado-3, 34-6-X and 36-2
PLRV and PVX	36-1, 34-S-1, 34-S-3-H, 42-1(Rusal Invary) and Cardinal
PVS and PVX	Sahiwal-2, Azado-2, 42-2 (Rusal Invary), CIP-72-Y-2 and Desire
Negative	Sahiwal-1, Azado-1, 34-S-2, 42-Y-1 and 42-Y-2 CIP-72-Y-5, 72-X-1 and 34-5-X

Table 2. Disease rating scale for PVX (Mughal & Khan, 2001).

Disease reaction (PVX)	Severity index disease	
No visible symptoms	0	HR
Mild mottling on the upper leaves.	1	R
Inter venial mosaic symptoms on more than one leaf.	2	MR
Mosaic symptoms on all leaves.	3	MS
Distinct mosaic symptoms on all leaves.	4	S
All above symptoms and small number of small sized tubers	5	HS

Table 3. Potato germplasm field screening to PVX

Resistance Level	No. of genotypes	Disease severity	Varieties/lines
HR	11	0	Sahiwal-1, Azado-1, 34-S-2, 42-Y-1, 42-Y-2, CIP-72-Y-5, 72-X-1, 34-5-X, Azado-3, 34-6-X and 36-2
R	2	1	34-4-X and CIP-72-Y-1
MR	2	2	34-3-Y and Cardinal
MS	9	3	Sahiwal-3, 34-1-Y, Sahiwal-4, 34-2-Y, Sahiwal-5, Sahiwal-6, CIP-72-Y-4, Mirrato and Desire
S	8	4	36-1, 34-S-1, 34-S-3-H, 42-1(Rusal Invary), Sahiwal-2, Azado-2, 42-2 (Rusal Invary), CIP-72-Y-2 and
HS	1	5	CIP-72-Y-3

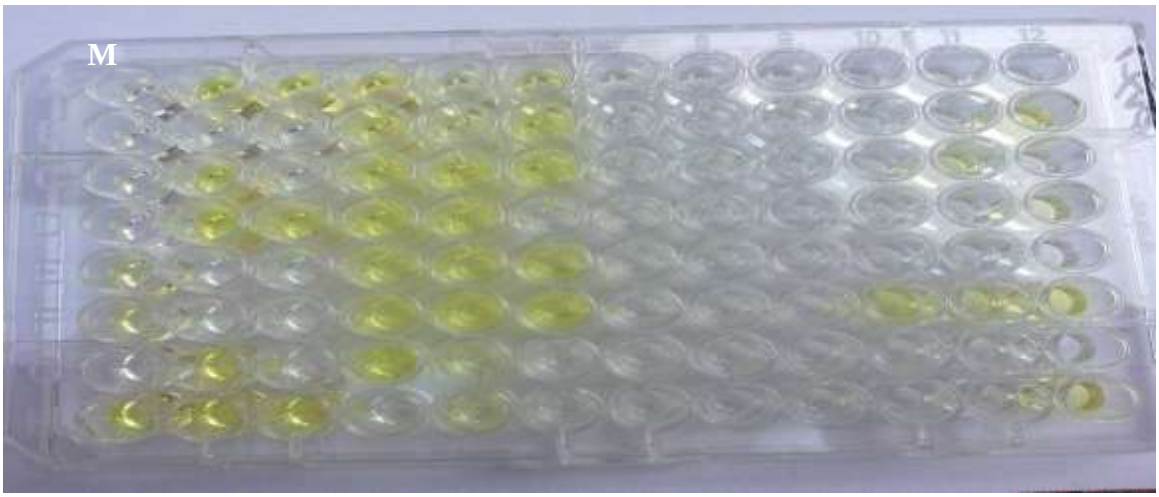

Fig 1. Yellow and white color in ELISA plate indicate the presence or absence of virus respectively.

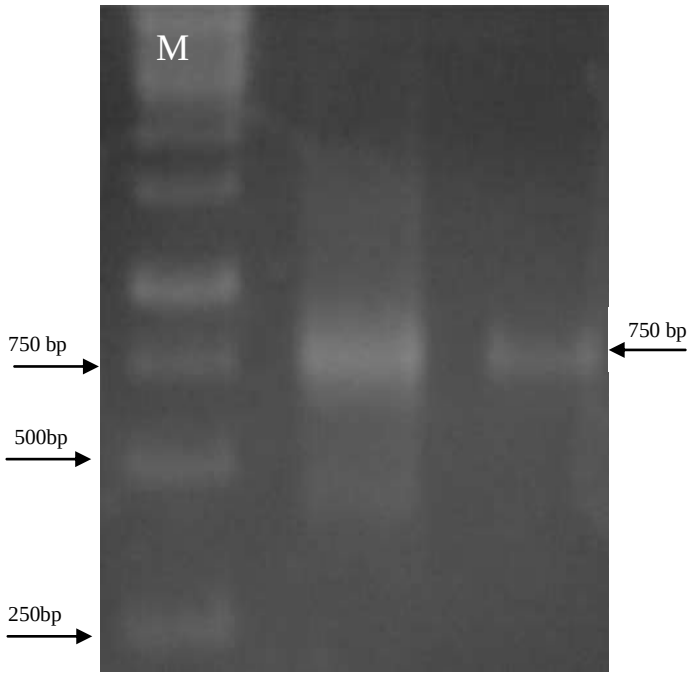

Fig 2. PCR amplification of PVX coat protein gene.

DISCUSSION

Symptomlogy is not a reliable method for the confirmation of viruses in field plants but is an initial step for disease diagnosis (Batool *et al*., 2011). A large number of potato samples examined in certification laboratories through ELISA because these tests are more reliable for screening of potato viruses. ELISA confirmed the potato viruses in twenty eight varieties/advanced lines and only five were disease free. Certification of germplasm requires a sufficient sensitive detection method to detect one infected sample among pooled healthy samples. Few virions (theoretically one) of PVX present at the initial stage of host plant increase to several billion copies of within a few days or weeks (Betancourt *et al*., 2008) and virus-free plants can infected in the open field by aphid and mechanically through PVX infected plants. A detection procedure for PVX must be rapid, sensitive, specific, reliable, easy to use and cost-effective. PCR technology offers further sensitivity and specificity to detect PVX. Nadeem *et al*. (2012) also screened the potato lines/varieties and they also found highly infection of PVX. In the present study, CP gene specific primers were used for amplification of PVX through PCR and successfully

amplified 0.75kb cDNA fragment of CP gene through PCR in few ELISA negative samples because ELISA tests are unable to detect virus in low concentration.

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