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Full Length Research Paper

ELISA-based detection of major potato viruses in tissue culture produced potato germplasm

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Potato leaves samples of different potato plantlets of three varieties (Cardinal, Desiree, Diamant) produced through tissue culture, were collected from incubation room, glass houses, tunnels (Faisalabad and Murree) and field and tested against major potato viruses through ELISA. 537 out of 6581 samples were infected with different potato viruses. Maximum incidence was of PVX (4.51%) whereas 1.21% of PVY, 0.41% of PLRV, 1.52% of PVS, 0.39% of PVM and incidence of PVA was 0.11% in all tissue cultured potato plantlets. Cardinal variety was infected with PVX (3.21%), PVY (1.27%), PLRV (0.63%), PVS (1.99%); PVM (0.63%) and PVA (0.23%) and Desiree potato variety was infected with PVX (5.90%), PVY (1.52%), PLRV (0.38%), PVS (1.35%); PVM (0.42%) and PVA (0.08%) where as Diamant was infected with PVX (4.30%), PVY (0.80%), PLRV (0.20%), PVS (1.20%) and PVM (0.10%). No virus infection was observed in the samples of all three varieties collected from incubation room. Plantlets collected from glass house, Cardinal variety was infected with PLRV (1.25%), PVS (0.42%) and PVM (0.42%) and plantlets of Desiree infected with PVX (0.75%), PVY (0.75%), PLRV (0.93%), PVS (0.37%) and PVM (0.56%) where as plantlets of Diamant were infected with PVX (1.71%), PVY (0.86%), PLRV (1.14%), PVS (0.57%) and PVM (0.29%). The samples collected from tunnels, Cardinal plantlets were infected with PVX (10.32%), PVY (7.74%), PLRV (2.58%), PVS (12.90%) and Desiree plantlets were infected with PVX (13.92), PVY (4.40) andd PVS (5.86) and plantlets of Diamant were infected with (8.89), PVY (5.93) and PVS (8.89%). The samples collected from Murree tunnels, Cardinal was infected with PVX (2.44%), PLRV (0.28%), PVS (0.70%), PVM (0.42%) and PVA (0.14%) where as samples of Desiree and Diamant were infected with only PVX (4.51%, 3.65% respectively). The samples collected from field, Cardinal variety was infected with PVX (25.00%), PVY (20.00%), PVS (15.00%), PVM (7.50%), PVA (3.75%) and plantlets of Desiree were infected with PVX (44%), PVY (26.67%), PLRV (5.33%), PVS (18.67%), PVM (9.33), PVA (2.67%) and plantlets of Diamant were infected with PVX (34.78%), PVY (8.70%) and PVS (21.74%). These samples were collected from the suspected plantlets and ELISA test was carried out to identify the infecting virus in order to produce healthy potato plantlets for further multiplication of disease free seed potato.

Key words: Potato viruses, symptomatology, ELISA, germplasm, screening

INTRODUCTION

Potato *(Solanum tuberosum* L.) species are host to the largest number of viruses. At least 37 viruses naturally infect cultivated potatoes (Beemster and de Bokx, 1987; Salazar 1996; Jeffries, 1998). Some of these viruses,notably potato leaf roll (PLRV), potato virus A (PVA), potato virus Y (PVY), potato virus V (PVV), potato virus M (PVM), potato virus X (PVX), potato virus S (PVS), potato mop top virus (PMTV) and potato aucuba mosaic virus (PAMV) occur worldwide in potato crop; others are important only in some geographical areas (Brunt 2001, Mughal, 1990). Degeneration due to virus diseases has been reported from 1 to 17 % in 1st year & 37 to 56 % in subsequent years (Jagidar et al., 1982). According to report (Mughal & Khalid. 1985) more than 50 % yield losses occur in potato due to viral diseases whereas individually, PVX causes yield loses from 10 to 25%, PVY & PLRV up to 70%, PVA 40%, PVS 20%, PVM 30% (Mughal et al., 1988). If disease free material is available, it can be utilized to generate basic seed

which could be multiplied several times and obtain good quality seed. It is well-known that it is possible to develop virus free potato plants through in-vitro meristem culture (Faccioli 2001). It is, however essential to ensure that the supplied in vitro regenerated plants or microtubers are virus free for commercial potato cultivation. There are a number of systems available to detect viruses (Singh 1999). Many certification authorities prefer visual detection of pathogens on the potato plants or seed tubers. Relying on such visual detection of symptoms is, however not always conclusive. In many cases plants may carry a latent infection which does not produce detectable symptoms, as with certain strains of potato virus X (Banttari et al. 1993). Serological procedures form the most reliable method for detection and quantitative assay of viruses. In this context the direct double antibody sandwich enzyme-linked mmunosorbent assay (DAS-ELISA) is used far and wide for virus detection (Clark and Adams 1977). In the present investigation we have studied the presence or absence of the virus particles in in-vitro generated plants of three varieties namely Cardinal, Desiree & Diamant with six Potato Virus X (PVX), Potato Virus Y (PVY), Potato Leaf Roll Virus (PLRV), Potato Virus S (PVS), Potato Virus M (PVM) and Potato Virus A (PVA) using DAS-ELISA.

MATERIAL AND METHODS

These studies were carried out during cropping seasons of 2007 to 2009, in the Plant Virology Section, Ayub Agricultural Research Institute, Faisalabad.
Three cultivated potato (*Solanum tuberosum* L.) varieties namely Cardinal, Diamant and Desiree samples were collected.

COLLECTION OF PLANT SAMPLES

Leaf samples were collected from in-vitro raised plants of all varieties which were grown in the incubation room, glass house & tunnels (Murree & Faisalabad) where as field samples were collected from the plants showing virus-like symptoms as well as from symptomless ones from the field area.
Frequent inspections of plantlets in test tubes, seedlings in sand trays (incubation room), plants in pots (glass house), plantlets in tunnels & fields were carried out. 6581 potato leaves samples of three varieties, Desiree, Cardinal, Diamant (produced through tissue culture) were collected at random in polythene bags and stored at 4 ^{o}C until processed during 2007 to 2009.
65 samples of Cardinal, 48 of Desiree & 44 of Diamant from incubation room, 480 of Cardinal, 535 of Desiree & 350 of Diamant from glass house, 155 of Cardinal, 273 of Desiree & 135 of Diamant from tunnels, 80 of Cardinal, 75 of Desiree & 46 of Diamant from field area and 1432 of Cardinal, 1440 of Desiree & 1423 of Diamant from Murree tunnels were collected. In total 2212 samples of Cardinal, 2371 of Desiree and 1998 of Diamant were tested in laboratory of Plant Virology Section, PPRI, Faisalabad.

SOURCE OF ELISA KITS

Antiserum, antibody conjugated with alkaline phosphatase (IgG conjugate) and positive control for PVX, PVA, PLRV, PVS, PVM and PVX were purchased from Bioreba A.G. Christoph Merian Ring-7, CH-4153, Reinach BL1 Switzerland.

DAS-ELISA:

It was performed following the protocols described by Clark and Adams (1977). Microtitre plate wells (8 ∇ 12 flat-bottom wells of c. 400 µl/well) were coated with antiserum diluted in carbonate buffer (pH 9.6) according to the supplier's specifications. Plates were incubated for overnight at 4 ^{o}C. IgG used in was diluted in the carbonate buffer in the proportion of 1: 1000. Following incubation plant extracts were applied on these wells. The plant extracts were prepared in pestle and mortar (grind the potato leaves in extraction buffer at 1:10 ratio (1gm sample: 10 ml buffer. After adding the crude plant extract, plates were incubated for overnight at 4 ^{o}C. The virus was detected by the corresponding antibody conjugated with alkaline phosphate diluted in conjugate buffer (PBS-TPO, pH 7.4) according to the suppliers' specifications. Plates were incubated for overnight at 4 ^{o}C. Plates were washed with phosphate buffered saline (PBS, pH 7.4) at each stage. Prepare the fresh p-nitrophenyl phosphate substrate buffer and coated plates after addition of p-nitrophenyl phosphate tablets (1mg/ml) into buffer. Buffer and healthy samples were also charged as a control. Incubation was done at room temperature (25 ºC) for 30 minutes and reaction was visually observed for the development of yellow colour. The reaction was stopped by adding 50µl 3M NAOH to each well. A sample was considered positive if the yellow colour was observed as that of the control. The results were analyzed statistically and

Table 1: Viral incidence in potato varieties

Virus	Potato	Cardinal	Desiree	Diamant
PVX	4.51±0.55 A	3.21±0.34 A	5.90±0.68 A	4.30±0.37 A
PVY	1.21±0.35 BC	1.27±0.37 BC	1.52±0.37 B	0.80±0.19 BC
PLRV	0.41±0.10 BC	0.63±0.14 C	0.38±0.11 B	0.20±0.07 C
PVS	1.52±0.28 B	1.99±0.24 B	1.35±0.37 B	1.20±0.25 B
PVM	0.39±0.08 BC	0.63±0.21 C	0.42±0.11 B	0.10±0.05 C
PVA	0.11±0.02 C	0.23±0.05 C	0.08±0.04 B	0.00±0.00 C

Means sharing similar letters in a column are statistically non-significant (P>0.05).

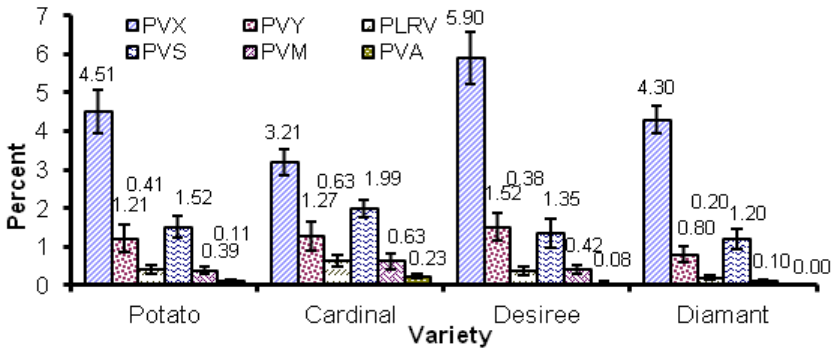

Figure 1: Graphical representation of viral diseases in different potato varieties

%age incidence was calculated as follows.

$$\% \text{ Incidence of virus} = \frac{\text{No. of infected samples}}{\text{Total no. of samples tested}} \times 100$$

RESULTS AND DISCUSSION

The main objective of the investigation was to identify the viruses infecting potato in order to produce healthy potato plantlets for further multiplication of virus free seed of three varieties viz. Cardinal, Desiree & Diamant. Maximum incidence of all tested viruses in all tissue cultured potato varieties was recorded in field. All these infected planlets were roughed out to produce virus free potato seed.

On the whole 537 samples out of 6581 were infected with different potato viruses. Maximum incidence was of PVX (4.51%) whereas 1.22% of PVY, 0.41% of PLRV, 1.52% of PVS, 0.40% of PVM and incidence of PVA was 0.11% in all tissue cultured potato varieties plantlets. Cardinal variety was infected with PV (0.08%) where as Diamant was infected with PVX (4.30%), PVY (0.80%), PLRV (0.20%), PVS (1.20%) and PVM (0.10%). Results given in Table-1 (3.21%), PVY (1.27%), PLRV (0.63%), PVS (1.99%); PVM (0.63%) and PVA (0.23%) and Desiree potato variety was infected with PVX (5.90%), PVY (1.52%), PLRV (0.38%), PVS (1.35%); PVM (0.42%) and PVANo virus infection was observed in the samples of Cardinal collected from incubation room where as samples collected from glass house were infected with PLRV (1.25%), PVS (0.42%) & PVM (0.42%), from tunnels infected with PVX (10.32%), PVY (7.74%), PLRV (2.58%), PVS (12.90%), from Murree tunnels infected with PVX (2.44%), PLRV (0.28%), PVS (0.70%), PVM (0.42%) & PVA (0.14%) and in field, Cardinal was infected with PVX (25.00%), PVY (20.00%), PVS (15.00%), PVM (7.50%), PVA (3.75%) Table-2. No virus infection was observed in the samples of Desiree collected from incubation room where as samples collected from glass house were

Table 2 : Viral incidences in Cardinal variety at Plant Virology Section

Virus	Incubation room	Glass house	Tunnels	Murree tunnels	Cardinal field
PVX	0.00±0.00	0.00±0.00 B	10.32±0.95 AB	2.44±0.20 A	25.00±8.63 A
PVY	0.00±0.00	0.00±0.00 B	7.74±0.58 B	0.00±0.00 C	20.00±4.36 AB
PLRV	0.00±0.00	1.25±0.43 A	2.58±0.60 C	0.28±0.07 BC	0.00±0.00 C
PVS	0.00±0.00	0.42±0.21 AB	12.90±0.64 A	0.70±0.09 B	15.00±1.65ABC
PVM	0.00±0.00	0.42±0.39 AB	0.00±0.00 C	0.42±0.14 BC	7.50±2.80 ABC
PVA	0.00±0.00	0.00±0.00 B	0.00±0.00 C	0.14±0.06 C	3.75±0.48 BC

Means sharing similar letters in a column are statistically non-significant (P>0.05).

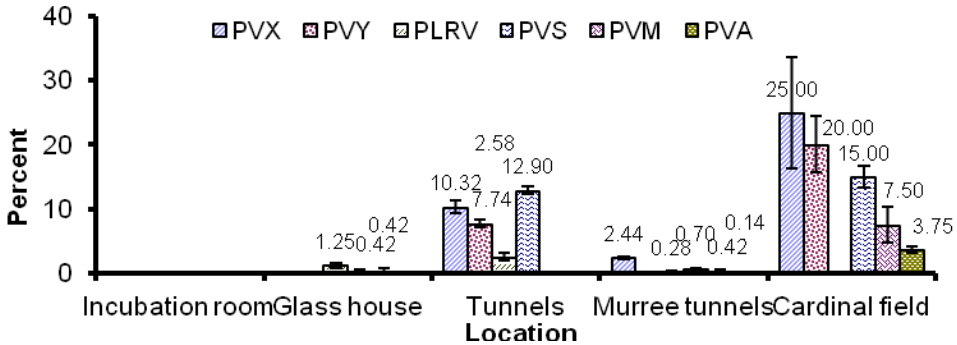

Figure 2. Graphical representation of viral diseases in Cardinal at different locations

Table 3: Viral incidences in Desiree variety at Plant Virology Section

Virus	Incubation room	Glass house	Tunnels	Murree tunnels	Desiree field
PVX	0.00±0.00	0.75±0.29 A	13.92±2.69 A	4.51±0.88 A	44.00±11.3 A
PVY	0.00±0.00	0.75±0.42 A	4.40±0.56 BC	0.00±0.00 B	26.67±4.65 AB
PLRV	0.00±0.00	0.93±0.27 A	0.00±0.00 C	0.00±0.00 B	5.33±1.09 B
PVS	0.00±0.00	0.37±0.17 A	5.86±0.85 B	0.00±0.00 B	18.67±2.13 B
PVM	0.00±0.00	0.56±0.33 A	0.00±0.00 C	0.00±0.00 B	9.33±2.03 B
PVA	0.00±0.00	0.00±0.00 A	0.00±0.00 C	0.00±0.00 B	2.67±1.41 B

Means sharing similar letters in a column are statistically non-significant (P>0.05).

infected with PVX (0.75%), PVY (0.75%), PLRV (0.93%), PVS (0.37%) and PVM (0.56%) samples from tunnels were infected with PVX (13.92%), PVY (4.40%) & PVS (5.86%) where as samples collected from Murree tunnels were infected with PVX (4.51%) and plantlets of Desiree from field were infected with PVX (44%), PVY (26.67%), PLRV (5.33%), PVS (18.67%), PVM (9.33), PVA (2.67%) Table-3.

No virus was observed in Diamant samples, collected from incubation room where as plantlets of Diamant from glass house were infected with PVX (1.71%), PVX (8.89%), PVY (5.93%) & PVS (8.89%), samples from Murree tunnels were infected with PVX (3.65%) and samples taken from field were infected with PVX (34.78%), PVY (8.70%) and PVS (21.74%). Results given in the Table-4.

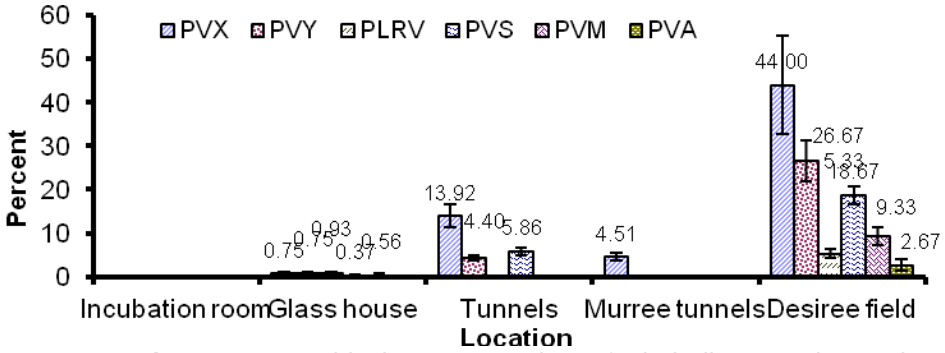

Figure 3: Graphical representation of viral diseases in Desiree at different locations

Table 4 : Viral incidences in Diamant Variety at Plant Virology Section

Virus	Incubation room	Glass house	Tunnels	Murree tunnels	Diamant field
PVX	0.00±0.00	1.71±0.52 A	8.89±3.87 A	3.65±1.18 A	34.78±10.3 A
PVY	0.00±0.00	0.86±0.02 AB	5.93±1.39 AB	0.00±0.00 B	8.70.14±5.57 AB
PLRV	0.00±0.00	1.14±0.21 AB	0.00±0.00 B	0.00±0.00 B	0.00±0.00 B
PVS	0.00±0.00	0.57±0.47 AB	8.89±0.91 A	0.00±0.00 B	21.74±8.08 AB
PVM	0.00±0.00	0.29±0.26 AB	0.00±0.00 B	0.00±0.00 B	0.00±0.00 B
PVA	0.00±0.00	0.00±0.00 B	0.00±0.00 B	0.00±0.00 B	0.00±0.00 B

Means sharing similar letters in a column are statistically non-significant (P>0.05).

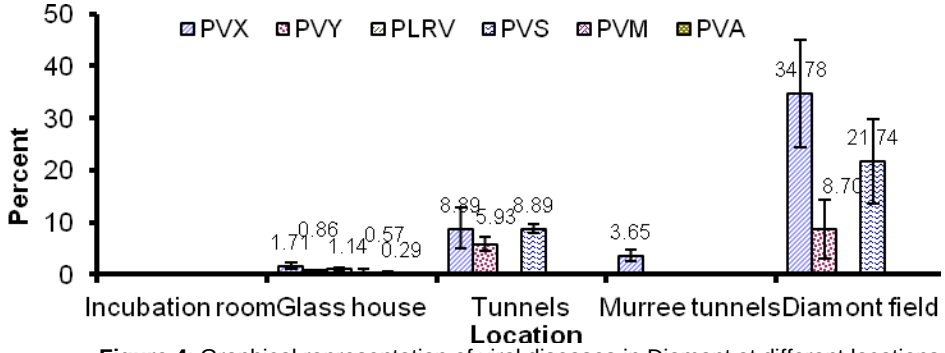

Figure 4: Graphical representation of viral diseases in Diamant at different locations

PVY (0.86%), PLRV (1.14%), PVS (0.57%) & PVM (0.29%), samples from tunnels were infected with

Potato Virus X (PVX):

The symptoms of plants infected with PVX showed mild leaf mosaic pattern, crinkling, thickening and wrinkled leaves. These symptoms appeared in all the three varieties from all locations except the samples (Cardinal, Desiree & Diamant) taken from Incubation room & the Cardinal samples collected from glass house are also free from PVX. The antiserum reacted strongly with all infected plants by the DAS-ELISA test. All the three varieties were more sensitive to PVX as compared to other viruses tested as Burhan et al. (2006) reported

prevalence of PVX in samples taken from Ayub Agricultural Research Institute Faisalabad. Koenig (1988) also observed similar results for the detection of potato viruses through serological tests. Maximum PVX incidence in all tissue cultured potato varieties was recorded in field as reported by Burhan et al., 2006.

Potato Virus Y (PVY):

Symptoms like leaf mottling, mosaic and necrosis were observed in infected plants. It was observed through DAS-ELISA test that antiserum reacted with the samples taken from all three varieties from all locations except the samples taken from incubation room and Cardinal samples taken from glass house. Burhan et al. (2006) reported same results in samples taken from Ayub Agricultural Research Institute Faisalabad. Ahmad et al. (2003) also detected PVY at different percentage on different varieties. Maximum PVY incidence in all tissue cultured potato varieties was recorded in field. Similar results were reported by Mughal and Khalid (1985), who detected eight Potatoviruses including PVY.

Potato Leaf Roll Virus (PLRV):

Viral symptoms like paleness and upward rolling of young leaves, especially at the base, stunted growth and yellowing of leaf margins were recorded from the samples taken from glass house (Cardinal, Desiree, Diamant), tunnels (Cardinal) and field (Desiree). Rodriguez and Jones (1978) also observed similar responses from field grown potato plants. The presence of PLRV was confirmed by DAS-ELISA test where antiserum reacted in the infected plant samples. In-vitro grown saplings of all three varieties in (test tubes & sand trays) incubation room, Desiree & Diamant from Tunnels, Cardinal & Diamant samples collected from field are free from PLRV. Maximum PLRV incidence in all tissue cultured potato varieties was recorded in field. Similar results were observed by Mughal et al., 1988.

Potato Virus S (PVS):

All the three varieties did not show any symptom. However, in the DAS-ELISA test all the infected plants showed positive reaction with antiserum except samples taken from incubation room & Diamant, Desiree samples collected from Murree tunnels. Similar findings were reported earlier by Schiessendoppler and Forchum (1990) using dot ELISA & Burhan et al. (2006) also found same results for PVS. Maximum PVS incidence in all tissue cultured potato varieties was recorded in field.

Potato Virus M (PVM):

Mildly mosaic patterns, crinkling and serious necrosis of leaves were observed in all three infected varieties as observed by Khalid et al., (2000) for PVM. In the DAS-ELISA test antiserum reacted only with the infected plants and as expected did not show any reaction with the samples taken from saplings grown in incubation room. Sample of all three varieties obtained from Faisalabad tunnels, samples of Desiree, Diamant from Murree tunnels & Diamant samples from field were also free from PVM. The result proved that PVM is also infectious to all varieties used. Faccioli and Colombarini (1996) observed similar results and detected the presence of PVM contents in potato tissue culture raised varieties. Maximum PVM incidence in all tissue cultured potato varieties was recorded in field as recorded by Khalid et al., 2000.

Potato Virus A (PVA):

Potato varieties Cardinal & Desiree samples were infected at all locations except samples taken from incubation room & Faisalabad tunnels. A mild mosaic, thick, wrinkled and marginal distorted leaves were observed as reported by Mughal et al, 1988, Mughal, S.M. 1990, Mughal, S.M. and Khalid, S. 1985. In the DAS-ELISA test antiserum reacted positively with the samples of Cardinal & Desiree taken from the field & samples of Cardinal taken from Murree tunnels. The above result indicated that Cardinal & Desiree are more sensitive to PVA than Diamant. Maximum PVA incidence in all tissue cultured potato varieties was recorded in field. Same results described by Mughal et al., 1988.

Pakistan is belonging to developing countries, where virus diseases are of major concern which lowers the yields of potato and requires the development of suitable, sensitive and reliable detection method (Salazar 1994, Mughal et al., 1988, Ahmad, I. 1998). Control of potato viruses is not easy & their spread is thus best be minimized by deterrent measures. As potato is a vegetatively propagated crop, infectivity increases through subsequent generations. The most effective approach to control viruses in potato includes the development of sensitive,

simple and economical detection method in any particular region.

Use of disease free seed is one of the durable and economical method for increasing potato production in Pakistan. Three approved varieties viz Cardinal, Desiree, Diamant that are commercially grown in extensive area, were maintained for true to type and disease free. The pre-basic seed is produced for further multiplication to basic & certified categories. These are pre-basic – I and Pre-basic – II, Basic – I and Basic – II, Certified – I and Certified – II. The approved varieties Cardinal, Desiree & Diamant were cleaned and multiplied through tissue culture. Therefore ELISA test was carried out to identify the healthy potato plantlets for further multiplication. Then huge quantity of seed potato produced and supplied to growers.

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