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**ARAŞTIRMA MAKALESİ** **RESEARCH ARTICLE**

# Serological and Molecular Detection of Single and Mixed Infections Symptomatology Associated with Potato Viruses Infecting Commercial Potato in Punjab Province, Pakistan

Pakistan'ın Pencap Eyaletinde Ticari Patatesleri Etkileyen Patates Virüsleriyle İlişkili Tek ve Karışık Enfeksiyonların Semptomatolojisinin Serolojik ve Moleküler Tespiti

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## Abstract

Potato is an important source of food in developing countries, and potato viruses have been a major constraint on sustainable production of the potato crop in Sialkot, Punjab Province, Pakistan. During February 2021, a survey of nine commercial potato fields was conducted to determine the incidence of PVY, PVX, and PLRV, respectively. The results of serological and RT-PCR assays indicated that PVX was not detected in any of the fields. PVY and PLRV were dominant in all fields with infection levels up to 63.15% and 45.83% respectively. The incidence of co-infection of potato with PVY and PLRV was 8.3%. The RT-PCR was the most reliable method for the detection and identification of viruses. The coat protein sequences of PVY-$SKT_1$, PVY-$SKT_2$, PLRV-$SKT_1$ and PLRV-$SKT_2$ were deposited in the Genbank database and assigned the accession numbers MW881191, MW881192 MW881193, and MW881194. The BLASTn search indicated PVY sequence shared the greatest similar score, at 99% similarity, with PVY isolates from China (HM036202.1) and Kashmir (KY851109.1). The PLRV sequence was most closely related to PLRV-Pakistan (MF276872.1) (99.8%) and China (KR051180.1) (99%) isolates. The results of co-inoculation experiments corroborated previous reports that infection of potato by multiple viruses dramatically increased disease severity (50-70%), compared to single virus infections (45-65%). The ELISA and RT-PCR results were confirmatory in that virus was detected in plants by serological and molecular methods, respectively. The prevalence of single and mixed PVY and PLRV infections in commercial potato fields underscores the need for routine virus indexing to foster routine planting of virus-free seed tubers.

**Keywords:** Molecular detection, Potato leafroll virus, Potato virus Y, Plant virus co-infection, Serology

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## Öz

Patates, gelişmekte olan ülkelerde önemli bir gıda kaynağıdır ve patates virüsleri, Pakistan'ın Pencap Eyaleti, Sialkot bölgesindeki patates üretiminin sürdürülebilirliğini ciddi şekilde kısıtlamaktadır. Şubat 2021'de, PVY, PVX ve PLRV virüslerinin görülme sıklığını belirlemek amacıyla dokuz ticari patates tarlasında bir tarama yapılmıştır. Serolojik ve RT-PCR analiz sonuçları, PVX'in hiçbir tarlada tespit edilmediğini göstermiştir. PVY ve PLRV'nin ise tüm tarlalarda baskın olduğu ve enfeksiyon oranlarının sırasıyla %63,15 ve %45,83'e ulaştığı tespit edilmiştir. PVY ve PLRV'nin birlikte enfekte ettiği patateslerin oranı %8,3 olarak belirlenmiştir. RT-PCR, virüslerin tespiti ve tanımlanmasında en güvenilir yöntem olarak öne çıkmıştır. PVY-SKT1, PVY-SKT2, PLRV-SKT1 ve PLRV-SKT2'ye ait dış kılıf proteini dizileri GenBank veri tabanına kaydedilmiş ve sırasıyla MW881191, MW881192, MW881193 ve MW881194 erişim numaraları atanmıştır. BLASTn analizi, PVY dizisinin Çin'den (HM036202.1) ve Keşmir'den (KY851109.1) izolatlarla %99 oranında benzerlik gösterdiğini ortaya koymuştur. PLRV dizisinin ise Pakistan'dan (MF276872.1) %99,8 ve Çin'den (KR051180.1) %99 oranında benzerlik gösterdiği belirlenmiştir. Ortak enfeksiyon deneylerinin sonuçları, önceki raporlarla uyumlu olarak, birden fazla virüsün patatese bulaşmasının, tek bir virüsle enfeksiyona kıyasla hastalık şiddetini (%50-70) önemli ölçüde artırdığını doğrulamıştır (tek virüs enfeksiyonlarında %45-65). ELISA ve RT-PCR sonuçları, bitkilerde virüslerin serolojik ve moleküler yöntemlerle tespit edildiğini doğrulamıştır. Ticari patates tarlalarındaki tekli ve karışık PVY ve PLRV enfeksiyonlarının yaygınlığı, virüssüz tohum yumrularının düzenli olarak dikimini teşvik etmek için rutin virüs indeksleme uygulamalarının önemini vurgulamaktadır.


**Anahtar Kelimeler:** Moleküler tespit, Patates yaprak kıvırcıklığı virüsü, Patates virüsü Y, Bitki virüsü ko-enfeksiyonu, Seroloji

## 1. Introduction

Potato (*Solanum tubersoum*) is an important crop in terms of human consumption and nutrition, ranks third after wheat and rice (Devaux, et al., 2014). Countries in tropical and sub-tropical climates account for half of the production of the world's potato crop, which is affected by many abiotic and biotic factors. Among the biotic factors, viruses are of among the most important based on distribution, incidence, and economic loss. More than 50 viruses and two viroids have been reported to infect potatoes. However, the viruses that pose the greatest threat to the potato crop (Kreuze et al., 2020; Harahagazwe et al., 2018) are *Potato virus Y* (PVY) (genus, *Potyvirus*; family, *Potyvirdae*) and *Potato leafroll virus* (PLRV) (genus, *Polerovirus*; Luteoviridae), causing up to 80% yield loss. *Potato virus X* (PVX) (genus, *Potexvirus*; family Alfaflexiviridae) is less prevalent than PVY and PLRV and causes minor (10-25%) tuber infections (Mughal et al., 1981). These viruses may occur as a single or mixed infection of potato plants, and co-infection often results in greater disease severity, due to synergistic effects (Garcia-Cano et al., 2006). A survey was conducted to quantify the incidence of RNA viruses infecting potatoes in Pakistan. The results suggest the prevalence of multiple viruses in all surveyed areas with PVS, PVX, and PVY exhibiting infection levels of upto 50%. Co-infections were detected, with highest incidence recorded at 15.5% for PVX and PVS (Hameed et al., 2014).

Potato is vegetatively propagated, making it vulnerable to plant virus spread when seed tubers are infected at time of planting. Further, the rate of seedling emergence is reduced in virus-infected seed tubers and infected potato plants that emerge and survive often exhibit reduced vigor and yield (Wright and Bishop 1981; Kumar et al., 2017). The availability of virus-free potato seed tubers can be a barrier to sustainable potato production (Wales et al., 2008), making sensitive, accurate detection of potato viruses crucial for the production of virus-free seed tubers. When resources are limited ELISA (enzyme-linked immunosorbent assay) is a reliable detection method for the detection of potato viruses (Önder and Korkmaz, 2008; Wrobel, 2014; Ilbagi and Geyik, 2014). The most optimal virus detection assays offer sensitive, specific detection and rapid turn-around time. Reverse transcription polymerase chain reaction (RT-PCR) is more than 100 times more sensitive compared to ELISA (Dietgen, 2002). The objective of this study was to examine the prevalence of the potato viruses PLRV, PVX, and PVY in commercial potato fields in Sialkot, Punjab province, Pakistan.

## 2. Materials and Methods

### *2.1. Sample collection*

During 2021, surveys were conducted in commercial potato fields of Pasrur, Sambrial and Daska, Sialkot, Punjab, Pakistan, to estimate the incidence of the three viruses of interest in potato cultivars 'Cardinal' and 'Desire'. Leaf samples (n=190) were collected randomly from 21 symptomatic and asymptomatic plants in nine fields, three fields from each location. Leaves were collected from the top, middle, and lower portions of plants and placed into plastic bags. Samples were stored on dry ice during transportation and stored at -80 °C freezer.

### *2.2. Serological detection*

Potato leaf samples were tested for the presence of the three potato viruses of interest using a double-antibody sandwich enzyme-linked immune sorbent assay (DAS-ELISA) on a polystyrene microtiter plate, as previously described (Clark and Adam, 1977). The ELISA kit for PVX, PVY, and PLRV were purchased from Bioreba (Reinach, Switzerland). Leaf samples were ground in sample buffer provided and the assays were run, according to the manufacturer's instructions. Briefly, to detect virus in the sap preparations, the purified, respective virus-specific polyclonal antibodies were diluted 1000x in carbonate buffer (pH 9.6) and used to coat each well of the microtiter plate (200ul/well), with incubation for 24 hours at 4 °C. Following three washes with PBS-Tween, the diluted leaf sap was applied to each well (200ul/well), incubated for 24 hours at 4 °C, and washed three times with PBS-tween. A 200ul volume of the alkaline phosphatase-conjugated virus antibody was transferred to each well followed by incubation for 3 hours at 37 °C. After the final washes, plates were incubated with p-nitrophenyl phosphate substrate buffer for 30min at 25 °C in dark. The absorbance values were recorded using a spectrophotometer, at an optical density (O.D.) of 405nm. Mean $OD_{405}$ values of healthy samples and extraction buffers were averaged their standard deviation was calculated and multiplied by 3 (Clark and Adam, 1977). Values equal to, nearly equal to, and greater than the standard deviation were considered positive for virus presence. The

percent disease incidence was calculated based on a previously published, standard method (Abbas et al., 2012). Percent incidence was calculated as,

$$\text{Percentage of virus} - \text{positive samples} = \frac{\text{DAS}-\text{ELISA virus}-\text{positive potato samples}}{\text{total number samples}} \times 100 \quad \text{(Eq. 1)}$$

### *2.3. Greenhouse inoculation experiment*

Virus-free, presumed healthy potato plants were grown from tubers planted in pots containing sand, field soil, and leaf composite later infected at 35 days after planting. Test plants were either aphid-inoculated or mechanically inoculated against PVY and PLRV. Because PVX was not detected in potato plants in the field, the virus was not included in the greenhouse experiments. For aphid-inoculation experiments, symptomatic potato leaves were detached from 8-9 week old source plants (14-16 leaf stage). To maintain the turgidity in leaves for 2-3 hours, individual leaf petioles were inserted into 1.5ml plastic tubes containing water and sealed with paraffin (Shrestha et al., 2014). Non-viruliferous, apterous green peach aphids *Myzus periscae* (Sulzer) were collected from potato plants maintained in the National Agriculture Research Centre, Islamabad, Pakistan and starved for 2 hours. This aphid species was selected for the experiments because it is among the most efficient aphid vectors of the plant viruses of interest in Pakistan (Veerbek et al., 2010). Aphids were transferred to the underside of detached leaves from selected potato source plants and allowed a 2min acquisition-access period. Ten aphids per plant were transferred to the abaxial surface of upper leaves of each test plants using a fine bristle camel's hair brush, confined to plants by a mesh cage (3-cm diameter x 1.5cm depth). The aphids were allowed a 2 hours inoculation-access period (IAP) after which they were removed from the plants.

Mechanical inoculation was carried out by first macerating leaves from source plants with a sterilized pestle and mortar in 50mM potassium phosphate buffer (pH 7.0) at a ratio of 10%w/v tissue to buffer (1g tissue/10ml buffer). The potato leaves were inoculated by rubbing sap on carborundum powder-treated leaves. Inoculation experiment was repeated three times and plants were maintained in a greenhouse under controlled conditions, 25°C day and 21°C night temperature, 50-70% relative humidity, and 16-hour natural daylight. Plants were observed periodically for symptom development for 1-4 weeks post-inoculation.

### *2.4. Total RNA isolation*

In RNA isolation procedure 100mg of ELISA confirmed leaf tissues were homogenized in 1ml Tri-Reagent. The mixture was transferred to a centrifuge tube and 200ul of chloroform was added and centrifuged at 12000rpm for 15min. The aqueous phase was transferred to a fresh microfuge tube with 500ul isopropanol. The RNA pellets were obtained after centrifugation at 12000rpm. The supernatant was discarded, and the pellet was washed three times with 70% ethanol by gently inverting the tubes. The air-dried RNA pellet was dissolved in diethyl pyrocarbonate (DPEC) treated water.

### *2.5. Reverse transcriptase-polymerase chain reaction*

The cDNA was synthesized from total RNA by using 10pmol of PVY and PLRV-specific reverse primers with reverse transcriptase and incubated at 45°C for 60min. The reaction was terminated by incubation at 70°C for 5min. The PCR reaction was run in a total volume of 25ul containing 16.25ul of double-distilled water, 2.5ul of 10x PCR buffer, 2ul dNTPs, 1ul each primer ((PLRV-CPF ATGAGTACGGTCGTGGTTAA, PLRV-CPR CTATTTGGGGTTTTGCAAAG) (PVY-CPF ACGTCCAAAATGAGAATGCC, PVY-CPR TGGTGTTCGTGATGTGACCT)), 2ul of cDNA, and 0.25ul Taq enzyme. The cycling parameters for PVY were for an initial denaturation of 94°C for 3min followed by 35 cycles of 94°C for the 30sec, 51°C for 30sec, 72°C for 30sec, with a final extension at 72°C for 5min. The PLRV cDNA PCR amplification parameters were for one cycle of 94°C for 3min, followed by 35 cycles of 94°C for 45sec, 58°C for 45sec, 72°C for 45sec, with a final extension at 72°C for 5min. PCR amplified product was visualized on a 1% agarose gel stained with ethidium bromide. A 1kbp molecular marker (Waltham, Massachusetts, United States) was co-electrophoresed with amplicons as a size (mass) reference. The amplicons were eluted from gel using the Gene JET gel extraction kit (Waltham, Massachusetts, United States).

### *2.6. DNA sequencing and phylogenetic analysis*

Following the successful amplification of target region of the genome. The amplified products were prepared and outsourced to macrogen Korea to obtain DNA sequencing. The obtained raw sequencing data were subsequently utilized for phylogentic analysis. To infer the evolutionary relationship among the sequences, phylogenetic analysis was conducted using the Clustal W version. The clustal W algorithms aligns multiple sequencing. The aligned sequences were used to construct a phylogenetic tree. Providing insights into the genetic relatedness and evolutionary divergence of the studied DNA fragments.

## 3. Results and Discussion

In this study 190 symptomatic and non-symptomatic potato leaves were collected from nine potato fields located in Sialkot, Punjab Province and subjected to ELISA to detect the presence of PLRV, PVY, and PVX. Virus-specific PVX, PVY, and PLRV antisera were used for virus detection by DAS-ELISA. Based on ELISA results for the 190 samples tested, PVY and PLRV were detected in all of the areas surveyed in this study. The most prevalent virus was PVY, with an incidence of 63.15% in both cultivars, followed by PLRV at 45.83%, respectively. Also, mixed infections of PVY with PLRV were detected at a frequency of 8.3%. However, PVX was not detected in any potato plants among all of the locations sampled. Of the 190 samples tested, 16% were negative for all three viruses (*Figure 1*). Potato plant samples identified as positive for PLRV by ELISA testing exhibited upward rolling of leaf margins, foliar mottling, and stunting of plants. Potato plants found to be infected with PVY developed symptoms consisting of foliar mosaic, necrosis, and vein-clearing, stunting of plants, and reduced tuber size. Plants found to be co-infected with PVY and PLRV exhibited interveinal chlorosis, overall reduced size of the plant, and prominent upward leaf rolling. In some of the latter field-infected plants symptoms were extremely severe, and consisted of overall stunting, leaf distortion, and necrotic blotches.

***Figure. 1*** **A survey of potato fields to assess the incidence of PLRV, PVX, and PVY in the district Sialkot**

Inoculation of virus-free or otherwise 'healthy controls', the potato plants maintained, in greenhouse developed symptoms reminiscent of field-collected plants found to be infected with PVY or PLRV by ELISA testing. The symptoms on test plants developed ten days post-inoculation and were indistinguishable from those observed in the field-infected potato plants reported previously in Pakistan (Abbas et al., 2014) and Turkey (Yardımcı et al., 2015). In greenhouse experiments, virus infection of aphid-and sap-inoculated test plants were verified by RT-PCR and confirmatory DNA sequencing of cloned amplicons (Genbank Accession no. OK188143 and OK188144).

Yield loss was estimated by counting the number of tubers in 'healthy controls' and virus infected potato plants (Rahman et al., 2010). In this study results showed that among three potato viruses studied here, PVY was the predominant virus infecting potato, at 63.15%, with PLRV being the second most prevalent, at 45.83%. High incidence of PVY and PLRV indicated that the environmental conditions were conducive to disease development, transmission, and susceptible cultivars, and use of virus-infected seed tubers.

Accurate detection of plant viruses is essential for selecting disease-free seed tubers for commercial planting. The coat protein gene is the most conserve and reliable region for virus detection for most plant viruses. In this study, PLRV-specific primers from coat protein gene amplified 627bp fragments, while the PVY-specific primers from coat protein gene amplified 790bp fragments by RT-PCR, with no non-specific amplification in any sample. The BLAST analyses indicated that the amplicons shared 99% and 99.8% similarity to PVY and PLRV, respectively. The PVY isolate shared it's best match (highest similarity score, coverage, and e-value) with the isolates from China (HM036202.1) and Kashmir (KY851109.1), at 99%, while PLRV was most closely related to isolates MF276872.1 and KR051180.1 reported previously from Sahiwal, Pakistan, and China, with a 99.8% similarity score. The isolates identified from the mixed infections of potato were most closely related to PVY (MW881192) and PLRV (MW881193) isolates from the United Kingdom and China.

Knowledge of phylogenic relationships among viral species and strains is useful for understanding differences in prevalence and distribution among geographical isolates and trace the source in plant materials derived by vegetative propagation (Mattews, 2002; Gibbs and Ohshima, 2010). To determine the relationships between Sialkot isolates of Pakistan and other isolates in NCBI database of respective viruses, the viral coat protein sequences were aligned using the ClustalW method (Thompson et al., 1994) and phylogenetic analysis was carried out according to Kumar et al. (2016). The phylogeny (neighbor-joining) phylogenetic tree grouped the Pakistan potato PLRV isolates with PLRV isolates reported from Heilongjiang, China and Pakistan (Accession no. KR051180.1 and MF276872.1), respectively (*Figure 2*), and one PVY isolate was most closely related to an isolate from Xinjiang, China (HM036202.1), while a second PVY isolate was most closely related to a PVY isolate from the United Kingdom (EU161658.1) (*Figure 3*).

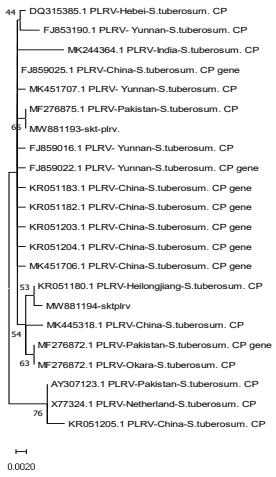


***Figure 2. Phylogenetic analysis of Potato leafroll virus detected from Sialkot district of Pakistan using nucleotide sequences from different isolates from NCBI. The phylogenetic tree was constructed using MEGA7 using the neighbor-joining method***

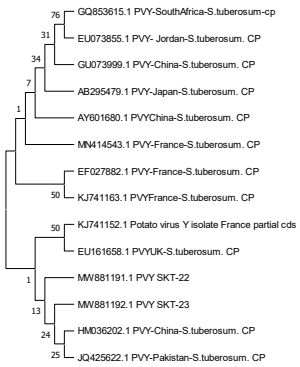


***Figure 3. Phylogenetic analysis of Potato virus Y detected from Sialkot district of Pakistan using nucleotide sequences from different isolates from NCBI. The phylogenetic tree was constructed using MEGA7 using the neighbor-joining method***

Previous studies in Pakistan have shown that PVY, PVS and PVX were most prevalent in the Punjab province of Pakistan (Hameed et al., 2014). However, in this study, PVX was not detected in any of the commercially grown potato fields in Pasrur, Sambrial and Daska, Sialkot in the Punjab province. This result suggests that dominance of a particular virus has changed over the time. The possible reason for this could be resistant cultivar of potato against PVX and disease management. However, PVY and PLRV were major pathogens threatening potato production worldwide (Ali et al., 2019). Based on this study, PVY and PLRV were the two most prevalent viruses infecting potato crops in Sialkot within this sampling time frame, with both viruses detected in cv. 'Cardinal' and cv. 'Desire' potato varieties. The observations from this study are consistent with previous reports that PVY and PLRV occur as single and mixed infections in most potato-growing regions of the world. Mixed virus infections in potato can result in substantial economic loss by reducing tuber size and quality compared to potato plants infected with only one virus infection (Wang et al., 2011). Results further underscore that management of potato-infecting RNA viruses is essential and relies on sensitive, accurate assays capable of detecting and identifying virus variants year-to-year to not only ensure that clean seed is available for planting, while also providing knowledge of possible shifts in viral strains or species. The production of virus free seed potato is therefore one of most important steps in reducing virus disease incidence in potato plantings, particularly early in the growing season, which can lead overall to consistently profitable, sustainable production of potato crops.

## 4. Conclusions

The results demonstrated the prevalence of individual virus and co-infection effects on two varieties 'Cardinal' and 'Desire'. The results revealed that disease severity associated with the mixed infection were higher than value from individual virus infection. Monitoring the phytosanitary status of potato crops in Pakistan with highly sensitive technique such as RT-PCR must be a short-term target. Thus, diagnosis of potato-infecting RNA viruses at all stages of the certification process of potato seed-tubers is important to support a sustainable potato industry.

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**Ethical Statement**

There is no need to obtain permission from the ethics committee for this study.

**Conflicts of Interest**

We declare that there is no conflict of interest between us as the article authors.

**Authorship Contribution Statement**

Concept: Yasmin, S.; Design: Yasmin, S.; Data Collection or Processing: Yasmin, S.; Statistical Analyses: Yasmin, S.; Literature Search: Yasmin, S.; Writing, Yasmin, S.; Review and Editing: Yasmin, S.; and Kayım, M.

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