INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–060/2014/16–5–941–945
http://www.fspublishers.org

Full Length Article

Presence of New Strain of Potato Virus Y in Pakistan

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Abstract

Potato virus Y (PVY) occupied fifth position among the economically damaging plant viruses in the world and its increasing incidence achieved an evolving concern for potato (*Solanum tuberosum* L.) crop of Pakistan. Serological, biological and molecular tools were used to confirmed new strains of PVY in different potato growing countries. From Pakistan, only ordinary strain (PVY^{O}) was reported serologically and no nucleotide evidence was reported previously. During the year 2010-11 and 2011-12, 462 potato samples were collected form Islamabad, Rawalpindi, Sahiwal and Faisalabad and serological confirmation of PVY was performed through Double Antibody Sandwich (DAS) Enzyme Link Immunosorbent Assay (ELISA). According to ELISA results, the percentage incidence of PVY was increased 9%, 10%, 17% and 22% in Sahiwal, Islamabad, Faisalabad and Rawalpindi respectively in second year (2011-12) as compared to first year (2010-11). The biological confirmation of PVY was performed on Samsun (*Nicotiana tabacum* cv. Samsun NN), Chenopodium (*Chenopodium album*) and white burley (*Nicotiana rustica)* and molecular detection of PVY was done by observing 795 bp coat protein (CP) gene fragment through Polymerase Chain Reaction (PCR) assay with the help of CP gene specific sense and antisense primers. Phylogenetic tree (sequence analysis) clearly indicated four main and different groups of PVY strains while nucleotide sequence of Pakistani isolates fall in PVY^{NTN}-$PVY^{N/NTN}$ group with minimum genetic diversity and maximum homology and PVY^{C} group showed maximum genetic diversity.

Keyword: ELISA; PCR; CP gene; Nucleotide evidence; Phylogenetic analysis

Introduction

The rank of cultivated potato is fourth in production and it is a staple food which provides fifteen times more yield and produced more calories than wheat, maize and rice. A good quantity of vitamins, protein and carbohydrates are produced by potato tubers (McGillivray, 1953). During 2011, Potato crop produced 3726.5 thousand tons on 127.7 thousand hectares area which is comparatively low rather than other potato growing countries due to different biotic and a biotic factors. Fungus, bacteria (Ashraf *et al.,* 2012), virus (Abbas and Hameed, 2012), nematode along with different pests are much responsible in yield reduction and can be easily transferred to next generation through vegetative material. In Pakistan, Mughal *et al.* (1986) reported potato virus A, S, M, X, Y, potato leaf roll virus and potato mop top virus in autumn, spring and summer potato crop. Among them, the increasing trend of PVY is becoming a threat for potato in main potato growing areas by causing 80% yield losses and no molecular evidence of PVY was reported from Pakistan (Abbas *et al.,* 2012). Aphid borne virus was represented as Y component which was responsible for darkening of green color with veins banding to tobacco plants and also considered as origin of PVY belonging to *Potyvirus* (Smith, 1931). In early study, PVY was known as potato virus 20, potato virus C, potato acropetal necrosis virus, potato leaf drop streak virus, potato severe mosaic virus, potato stipple streak virus, potato streak virus, potato veinal necrosis virus, solanum virus 2, tobacco veinal banding virus and tobacco veinal necrosis virus but nucleotide sequence techniques were used rather than the host response to acronyms and differentiate among the different strains of PVY (Singh *et al*., 2008).

This virus is single stranded positive-sense RNA with 10 kb genome and having VPg protein at 5` end and poly-A at 3` end (Shukla *et al*., 1994) while a short Open Reading Frame (ORF) of PVY was reported within the previous large ORF (Chung *et al.,* 2008). PVY infects a wide host range including *Solanaceae* family members (Shukla *et al*., 1994) and causes yield losses up to 80%. PVY^{O} (ordinary) PVY^{C} (common) PVY^{N} (necrotic) PVY^{Z} and PVY^{E} are five strains group of potato isolates while new recombinant genotype/strains ($PVY^{N/NTN}$, PVY^{NTN}, PVY^{NWI} and $PVY^{NTN-NWI}$) have been evolved from the genomic recombination in PVY (Ali *et al*., 2010). PVY^{NTN} and PVY^{NWI} were first found in Hungary and Poland respectively but within a short period of time they have become common in potato fields (Ali *et al*., 2007). It is a need of time to have a complete and comprehensive study

of PVY disease on serological, biological and molecular level (nucleotide evidence) for understanding this disease.

Materials and Methods

Serological Confirmation

During the year 2010-2011 and 2011-2012, 462 symptomatic potato plants suspected to PVY were collected randomly from main potato growing areas of Rawalpindi, Islamabad, Sahiwal and Faisalabad. Top, middle and bottom leaves were plucked from the susceptible potato plants and transferred in appropriate labeled polythene bags. Immunoglobulin (IgG), alkaline phosphate enzyme (ALKP) conjugated IgG (BIOREBA kit) and p'nitrophenyl phosphate (PNP) tablets were used for successful identification of PVY. Coating buffer was used for diluting of IgGs (1:500) while diluted IgGs were loaded (100 µL) and incubation of plates was done for 3h at 37°C. Extraction buffer was used for homogenizing the suspected potato leaves with the help of ice chilled autoclave pestle and mortal and sap of infected samples (100 µL) were loaded. The incubation of loaded plates were performed over night (4°C) and conjugate buffer diluted (1:500) ALKP conjugated IgG were loaded (100 µL/well) in plates. Plates were kept at room temperature for 3 h and plates were washed between each step with phosphate buffer saline with tween (PBST). PNP tablets were diluted in freshly prepared substrate buffer (0.75 mg/mL) and loaded (150 µL/well) in each plate.

Yellow color development was visually assessed after an hour as mild (+), moderate (++), strong (+++) and no reaction (-) while optical density (OD) of yellow color was measured at 405 nm in an ELISA reader (EPSON LX-300). Mean OD_{405} values of healthy samples and extraction buffer were averaged, their standard deviation was calculated and multiplied by 3 (Clark and Adams, 1977). Any value above and equal/nearly equal the standard deviation was considered as positive while only ELISA positive potato plants were included and percentage (%) incidence of PVY was calculated by following formula (Abbas *et al.,* 2012).

$$\% \text{ Incidence PVY} = \frac{\text{DAS-ELISA confirmed potato samples}}{\text{Total potato samples tested}} \times 100$$

Infectivity Assay and Total RNA Extraction

Susceptible host indicator Chenopodium (*Chenopodium album*), Samsun (*Nicotiana tabacum* cv. Samsun NN) and white burley (*Nicotiana rustica)* were shifted in glasshouse (30 ± 2°C) while indicator host plants were injured with carborandum powder and inoculums were prepared by crushing ELISA confirmed leaf in phosphate buffer. The inoculums were used for transmission of PVY while symptoms on mechanically inoculated plants were observed weekly and further confirmed serologically. Leaf tissues (100 mg) were crushed in TRI Reagent (1 mL) and chloroform (200 µL) was added in mixture. The aqueous phase was separated with centrifugation (12,000 rpm) and transferred to a fresh tube with isopropanol (500 µL). The total RNA pellets were obtained with centrifugation (12,000 rpm) and were washed thrice with ethanol (75%). Tubes were centrifuged (7,500 rpm) and total RNA was dissolved in diethyl pyrocarbonate (DEPC) treated water after removing the ethanol.

Polymerase Chain Reaction Assay

The CP gene specific sense and antisense primers were designed from previously reported strains of PVY which are available at National Centre for Biotechnological Information (NCBI). The downloaded CP gene sequences were used for sense and antisense gene specific primers and the antisense primer was used in cDNA synthesis along with total RNA in the presence of Revert Aid reverse transcriptase at 42°C for 60 min while the reaction was terminated at 70°C for 5 min. The final PCR reaction mixture (50 µL) was comprised of template (cDNA), Taq reaction buffer (1X), $MgCl_2$ (3 mM), Taq enzyme (2.5U), mix dNTPs (0.3 mM) and each primer (10 pmol). The PCR conditions were at 94°C/3 min followed by 30 cycles of 30 sec/94°C, 30 sec/51°C, 1min/72°C and the final extension was done at 7min/72°C (PERKIN ELMER PCR System 2400). CP fragments were visualized on agarose gel using 1 kb DNA ladder and amplified products were eluted by using Gene JET^{TM} Gel Extraction Fermentas Kit.

Sequencing and Sequence Analysis of CP Gene

The purified PCR products were sequenced from Macrogen Ibc (Korea) in sense and antisense direction and the final sequence was submitted on NCBI. The evolutionary history of 26 sequences of different strains and Pakistani isolates was inferred using the Neighbor-Joining method on the JTT matrix-based model with the help of Molecular Evolutionary Genetics Analysis (MEGA).

Results

A total of 462 (231 each year) potato leaf samples showing stunting, systemic vein clearing, mosaic, mottling, curling, shortening of leaves, dark green and vein-banding were collected from Islamabad (140), Rawalpindi (100), Faisalabad (130) and Sahiwal (92) and serologically confirmed through DAS-ELISA. The presence of yellow color indicates the presence and white color confirms the absence of PVY, while OD_{405nm} value was measured to determine the concentration of PVY in infected potato samples (Fig 2). During the year 2010-11, maximum disease incidence was observed in Sahiwal 39% (mean OD_{405nm} 0.475) followed by Faisalabad 26% (mean

Table 1: PVY incidence percentage during the year 2010-2011 and 2011-2012

Location	Year				
	2010-2011		2011-2012		
	Potato Plants Infected/ Examined	Percentage (%) Incidence	Potato Plants Infected/ Examined	Percentage (%) Incidence	Percentage (%) Increase/Decrease
Islamabad	14/70	20	21/70	30	10
Rawalpindi	6/50	12	17/50	34	22
Faisalabad	17/65	26	28/65	43	17
Sahiwal	18/46	39	22/46	48	9
Mean	14/58	24	22/58	39	15

Fig. 1: Biological confirmation of PVY on susceptible indicator Samsun (A) and white barley (B) plants

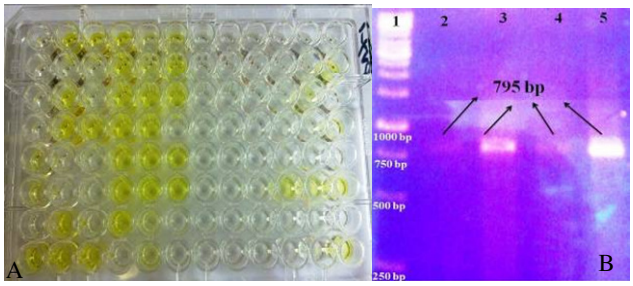

Fig. 2: Serological (A) and molecular (B) detection of PVY infecting potato crop

OD_{405nm} 0.382), Islamabad 20% (mean OD_{405nm} 1.421) and Rawalpindi 12% (mean $OD_{405\ nm}$ 0.552).

During second year, Islamabad showed minimum 30% (mean OD_{405nm} 0.619) and Sahiwal showed maximum 48% (mean OD_{405nm} 0.690) disease incidence, while the disease incidence in Rawalpindi and Faisalabad was 34% (mean OD_{405nm} 1.176) and 43% (mean OD_{405nm} 0.730) respectively (Table 1). Maximum increasing incidence of PVY disease was found in Rawalpindi 22%, Faisalabad 17%, Islamabad 10% and Sahiwal 9% in second year as compared to first year while the maximum concentration of virus was found in Faisalabad. The biological confirmation of PVY was done on chenopodium, Samsun and white burley by observing local lesions, necrotic whitish lesion and mottling, respectively (Fig 1).

Specific forward and reverse primers amplified 795 bp of CP fragment through PCR assay and no non specific amplification was found in any sample (Fig 2). The final sequences of Pakistani isolates FHM (JQ425622) and PK (JQ518266) were found 795 nucleotides along with 265 amino acids and multiple sequence alignment (Fig 3) was determined with Clustal W (Version 2.1). In 795 nucleotide sequence, Thymine/uracil (T/U) and cytosine (C) were same (21.8 and 20.3), while adenine (A) and Guanine (G) were 34.6% and 23.4% and 34.2% and 23.8% in FHM and PK respectively. In 265 amino acid sequence, Alanine (GCU, GCC, GCA and GCG) was found maximum in FHM (7.92%) and PK (7.54%) while Tryptophan was lowest with same quantity 1.132% in both isolates. The sequences of Pakistani isolates (JQ425622) and JQ518266), PVY^{O} (AY792597.1, AY061994.1 and JN635310.1), PVY^{C} (AF012026.1 and AF012027.1), PVY^{N} (EF027891.1, AB025415.1, AB025417.1, EF027859.1, EF027861.1, EF027886.1, AY319647.1 and GQ853665.1) PVY^{NTN} (AJ133454.1, EF027869.1, EF027879.1, EF027901.1, EF027897.1, EF027863.1, EU161658.1, EF027881.1 and EF027898.1), $PVY^{N/NTN}$ (AB295479.1, HQ631374.1 and AB295477.1) and $PVY^{N\text{-}Wi}$ (GQ853667.1) strains were further analyzed and a phylogenetic tree with four different groups PVY^{NTN}-$PVY^{N/NTN}$, PVY^{N}-$PVY^{N\text{-}WI}$, PVY^{O} and PVY^{C} was obtained (Fig 4). Both Pakistani isolates of PVY fall in first group with 12 isolates of PVY form four different countries of the world and showed minimum range of genetic diversity with first group (0.0038 to 0.0031) followed by second (0.026 to 0.043), third (0.089 to 0.108) and fourth (0.092) group respectively.

Discussion

Potato growers introduced high yielding foreign potato varieties in Pakistan which have significantly increased the yield of potato crop along with different new pathogens which are dominant in potato areas (Mughal *et al.,* 1986). During last two decades, the percentage incidence of PVY disease was 6.81% in Sahiwal (Ahmad *et al*., 1995) and it increased 39% and 48% in 2010-11 and 2011-12, respectively. The increasing incidence of PVY has become the major threat in Pakistan and only PVY^{O} strain was confirmed serologically and no nucleotide evidence was reported previously (Abbas *et al.,* 2012). Symptomolgy is not a reliable criteria for detection of potato viruses because symptom development is due to different biotic as well as a biotic factors but it can play a vital role for disease diagnosis (Batool *et al*., 2011). A large number of potato samples were certified serologically through ELISA techniques, because these techniques are less expensive and more samples can be certified with in short period of time but ELISA is unable to detect low concentration of virus during early stage of infection (Betancourt *et al*., 2008).

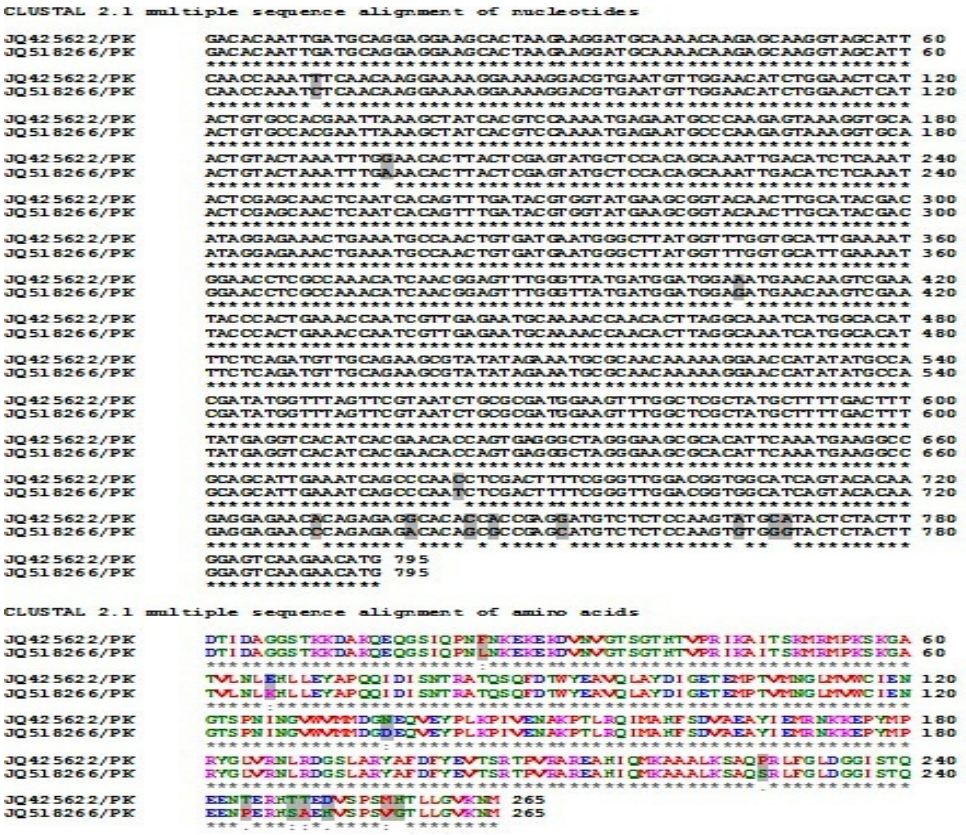

Fig. 3: Multiple sequence alignment of PVY coat protein gene from Pakistani isolates

New specific, sensitive and more reliable molecular tools (PCR) were introduced and CP gene specific sense and antisense primers successfully amplified, while few symptomless and ELISA negative plants also produced a required band through PCR. The rapid evolution of PVY and the continuous emergence of the recombinant PVY strains, however, make it necessary to update these assays at nucleotide level (Ali *et al.,* 2010). Nucleotide evidence is relatively quick, reliable and straight forward procedure to confirm PVY strains. The amino acids sequence of Pakistani isolates showed maximum genetic similarity 99.97% with PVY^{NTN}-$PVY^{N/NTN}$ group and minimum 99% with PVY^{O} group (Fig 3) while higher percentage nucleotide identity is due to high conserve sequence of CP gene in the genome of PVY (Rodriguez *et al*., 2009).

Nucleotide evidence of Korean isolates of PVY was 99% genetically similar (Choi *et al.,* 2005) and Mexican isolates exhibiting only 1% genetic diversity (Rodriguez *et al.,* 2009) while Egyptian PVY^{NTN} CP gene isolates showed maximum homology of nucleotides 99.5% with previously reported isolates of PVY (Amer *et al*., 2004). Rodriguez *et al*. (2009) reported new necrotic strain of PVY in Mexican potato on the basics of biological and molecular (sequence) analysis and observed that necrotic strain reported form different countries shared same nucleotide sequence and fell in same group of phylogenetic tree. On

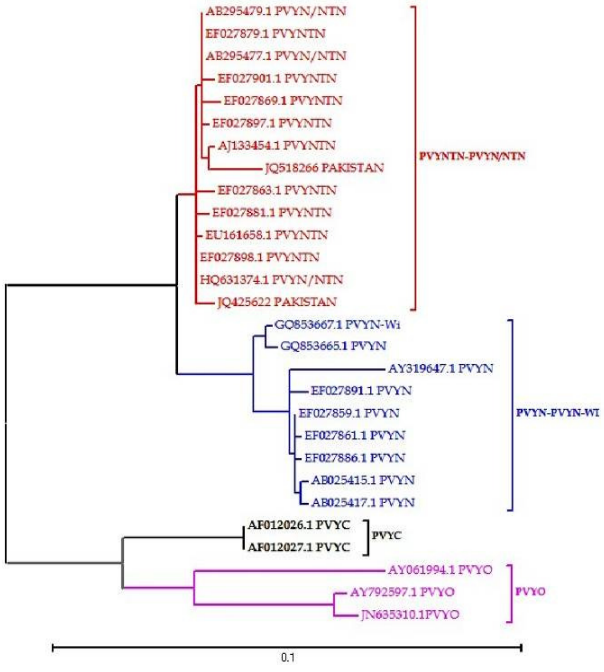

Fig. 4: A phylogenetic tree calculated from the twenty eight amino acid sequences of different strains of coat proteins of PVY

the basics of biological, molecular and nucleotide evidence, the necrotic strain of PVY has been reported in Germany (Lindner and Billenkamp, 2005), Washington and Oregon (Crosslin *et al*., 2006), North America, Belgium (Rolot, 2007), Czech Republic (Dedic *et al*., 2007), France (Rolland *et al*., 2007), Netherlands and Europe (Van der Vlugt *et al*., 2007), South Africa (Visser and Bellstedt, 2009), Canada (Nie, 2010), Poland (Yin *et al.*, 2012) and now in Pakistan. Ghosh *et al.* (2002) used nucleotide sequence of CP gene from and Indian isolate (PVY^{O}) and developed CP gene mediated resistance in tobacco plant while solanaceous hosts showed stability in transgenic virus resistance (Reavy *et al.*, 1997). Pathogen derived resistance in potato plant, tolerant or resistant potato varieties, weeds and insect vector control, meristem culture and treatment with chemotherapy and thermotherapy are more effective to control PVY diseases in potato crop and may improved the potato grower's income.

This is the first report of new strain of PVY in Pakistani potato with two PVY isolates belonging to PVY^{NTN}-$PVY^{N/NTN}$ group along with biological, serological and nucleotide evidence.

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(Received 16 January 2013; Accepted 13 January 2014)