Australian Journal of Crop Science AJCS
AJCS 9(5):438-444 (2015) ISSN:1835-2707

A simple diagnostic technique to detect potato viruses at post-harvest conditions

Mohamed S. Al-Saikhan

Agriculture of Arid Land Department, College of Agriculture and Food Sciences, King Faisal University, 400 Al-Hofuf, Al-Hassa 31981, Saudi Arabia

***Corresponding author: msaikhan@kfu.edu.sa**

Abstract

Potato (*Solanum tuberosum* L.) is one of the major vegetable crops. Viral diseases are the major limiting factor for successful cultivation of potato. In the present study, a reverse transcription polymerase chain reaction (RT-PCR) based protocol for PVX detection was developed and was compared to an enzyme linked immuneosorbent assay (ELISA) based PVX detection methods. Three potato cultivars (Lady Rosetta, Spunta and Hermes) were stored for 1 and 3 months at 4 ºC (95% RH) Detection of potato leaf roll virus (PLRV), potato virus Y (PVY) and potato virus X (PVX) were carried out on potato leaves and tubers before and after storage using RT-PCR method with specific primers designed to detect these viruses. Spunta and Lady Rosetta have moderate and high resistances, respectively, while Hermes loses in chemical quality progressively during storage at 9°C, and similar has virtually no resistance to LTS at 4°C. To insure good quality of seed potato, changes in sugars and ascorbate content were investigated in different genotypes and storage periods. The different parameters of LTS resistance or susceptibility in these cultivars were indicated by changes in sugars (total soluble and total reducing) content and ascorbate concentration in relation to tuber respiratory profiles during wound healing (9 °C), LTS (4°C) and reconditioning (16 °C). Tuber ascorbate content began to decrease during storage period and continued to decline progressively through storage stages, consistent with susceptibility of potato genotypes to LTS. RT-PCR might represent fast, practical, and sensitive alternatives for the detection of PLRV, PVY and PVX from green and/or tuber tissues.

Keywords*:* Potato, Storage, Potato viruses, ELISA, RT-PCR.
Abbreviations: AsA_ ascorbic acid; LTS_ low temperature sweetening**;** PLRV_ potato leaf roll virus; PVY_ potato virus Y, PVX_ potato virus X, RT-PCR_ reverse transcription polymerase chain reaction.

Introduction

Potato (*Solanum tuberosum*, L.) is a staple crop in parts of the world with temperate climates. The potato crop is the fourth major world food crop after corn, rice and wheat, in economic importance, and is used for human consumption, animal feed, and as a source of starch and alcohol. Potato covers a production area of about 19.3 million ha worldwide that yielded 368 million Ton (FAO, 2012). In Saudi Arabia, it covers a production area of about 16 thousand ha that yielded 405.7 thousand ton according to 26th statistical book, Ministry of Agriculture, Saudi Arabia. Production of the potato in the developing world has been accelerated in recent years than any other major food crop. Storage of potato is very important because fresh potatoes are available only for a few months in a year. Thus, it is required to control sprout growth and extend shelf life of potatoes. The critical factors involved in properly storing potatoes are temperature, humidity, CO_2 and air movement (Harbenburg et al., 1986). Losses through storage are mainly caused by evaporation of water from the tubers, respiration, sprouting, spread of diseases, changes in the chemical composition and physical properties of the tubers (Eltawil et al., 2006). The ability to store potato tubers long-term (> 6 months) can increase grower' returns by 30 – 60% (NASS, 2012). Cold storage is high energy consuming that also makes it costly. Storage at 10-12 °C is less energy consuming and prevents undesirable accumilation of reducing sugars (Mahto and Das, 2015). Potatoes for processing purpose are stored at this temperature, but requires application of sprout suppressants. Gamma irradiation is a safe and effective method of sprout inhibitation that allows potatoes to be stored at high temperature (Mahto and Das, 2014). Like most vegetatively propagated crops, potato suffers from wide range of diseases such as fungal, bacterial, viral and nematode diseases. Potato virus Y (PVY-poty viruses), potato virus X (PVX-potex virus) and potato leafroll virus (PLRV-luteo virus) are the most common viruses affecting potato crop (Singh, 1999). Most countries have enacted legislations that set reduce tolerances for the spread of the viruses (Gudmestad, 1991). The transmission of viral diseases through seed potato has led to progressive decline in potato yield (Bhat et al., 2010). Most potato viruses can be detected by ELISA, which is the most popular technique that has been used in seed potato certification systems and in quarantine laboratories (Petrunak et al., 1991). However, new advanced techniques were developed using nucleic acid hybridization (Agindotan & Perry, 2008), multiplex RT-PCR (Bostan & Elibuyuk, 2010), real-time PCR (Agindotan & Perry, 2008), macroarrays (Agindotan et al., 2007) and microarrays (Nicolaisen, 2011). Therefore, this study aimed to detect viruses in potato tissues by one step RT-PCR as a practical and alternative diagnostic technique to ELISA test. Changes in sugars and ascorbic acid contents in response to genotype and storage temperature and duration were also investigated.

Results and Discussion

Diagnostic technique of potato viruses using ELISA test

During field survey, the ELISA test as most rapid diagnostic technique using specific antibodies for PLRV, PVY and PVX

Table 1. The sequences of the specific primers used to detect the coat protein genes of potato viruses PLRV, PVY and PVX.

Viruses	Primer's Name	Nucleotide Sequences	Size (bp)	Reference
PLRV	PLRVCPv*Eco*RI	5`-AATAGAATTCTAATGAGTACGGTCGTGGTTARAGG-3`	650	Shalaby et al. (2002)
	PLRVCPc*Nco*I	5`-AAAACCATGGCTATYTGGGGTTYTGCARAGCTAC-3`		
PVY	PVYCPv*Bam*HI	5'-TCAAGGATCCGCAAATGACACAATTGATGCAGG-3'	825	Shalaby et al. (2002)
	PVYCPc*Eco*RI	5'-AGAGAGAATTCATCACATGTTCTTGACTCC-3'		
PVX	PVXCPv*Eco*RI	5`-GATAGAATTCAGATGACTACACCAGCCAACACC-3'	750	Shalaby et al. (2002)
	PVXCPc*Nco*I	5`-TACGCGTCGGTTCCATGGACGTAGTTATGGTGG-3`		

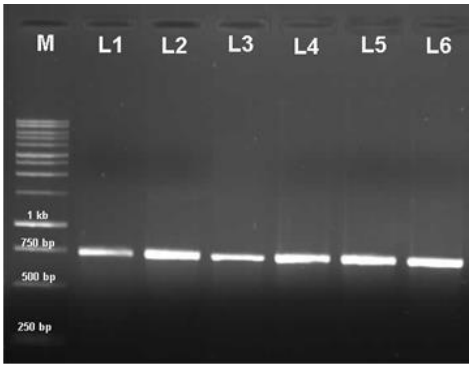

Fig 1. Agarose gel electrophoresis of RT-PCR products of potato viruses using primers for CP genes. M: 1 Kb DNA Ladder; from L_1 to L_3: leaves of three samples infected with PVX (750 bp); and from L_4 to L_6: sprouts of the same three samples infected with PVX (750 bp). [(L_1 & L_4 for Lady rosetta), (L_2 & L_5 for Spunta) and (L_3 & L_6 for Hermes) cultivars].

was conducted on 9 samples of leaves, tubers and growing sprouts. ELISA readings at wavelength 405 nm and the results of the ELISA test are presented in Table (2). The results were in semi conformity with field symptomatic visually infected samples of the three potato cultivars. No positive sample was found with PVY or PLRV antibodies against these viruses. In case of tubers, 8 samples were positive with PVX antibody against this virus and were spread on over 88.9% of the Hermes cultivar, while in other varieties its spread was restricted to less than 33.3 and 22.2% for Spunta and Lady rosetta cultivar, respectively. On the other hand, PLRV and PVY were equally disappeared in the whole samples of the three potato cultivars. In sprouts, the pattern of PVX was occupied the whole tissue, with a considerable concentration of PVX on the borders and in the vascular tissue. For PVX detection samples, ELISA had a significantly higher detection percentage than PLRV and PVY (Table 2). For samples collected from sprouts, the three tested viruses were equally sensitive in detection and they had equal percentage as tubers samples. Statistical analysis of all potato viruses detection showed that ELISA as serological method had a significant differences among the three tested tissues. The use of sprouts for viruses detection proved better than other organs.

Diagnostic technique of potato viruses using one step RT-PCR test

RT-PCR amplification of viral RNAs was carried out on the total RNAs isolated from infected plants using specific primers designed to amplify the coat protein genes (CP) genes of PLRV, PVY and PVX viruses with an expected amplicon size of 650, 825 and 750 bp, respectively. It was found to be highly efficient method of detection as shown in Fig. (1). The PVX CP gene amplification in case of all samples of three cultivars (leaves and tuber sprouts) of naturally infected potato plants was found to be very high level. Electrophoresis analysis of RT-PCR products where single amplified fragments of 750 bp for PVX-CP was obtained for three potato samples (leaves and tuber sprouts), while no fragments were amplified from the RNAs samples treated with PLRV or PVY primers (Fig. 1). PVX CP gene amplification from all field samples was achieved and the bands were clearly visible including those of visually infected samples which detected by ELISA test (Fig. 1). Samples of sprouts which were grown and harvested repeatedly for first generation in growth chamber those sprouts started showing PVX specific disease symptoms of leaves curling and yellowing. For field survey, the results showed that ELISA might be used successfully for the detection of three major potato viruses PLRV, PVY and PVX and that each virus has a different and somewhat specific pattern. Localization of each of the three viruses in potato green tissues matched the pattern described in the literature which was mainly detected using electron microscopy (Kogovsek et al., 2011). To determine the level of virus infection by ELISA in imported potato seed, it is recommended to incubate the tubers for 2 – 3 weeks until sprouts appear (Fox *et al*., 2005). The ELISA technique was less effective than one step RT-PCR for detection of three viruses (PLRV, PVY and PVX) in potato sprouts. It might be used interchangeably providing simple, cost effective and easy use. Therefore, a possible alternative would be to test directly from the tuber tissue. Testing for PVX from eyes, stem ends or sprouts by ELISA and one step RT-PCR would give the same result due to the homogenous distribution of PVX in all plant tissues. However, using tuber tissues for PVX testing by ELISA or one step RT-PCR is less efficient than the sprouts.

Effect of short term storage on quality of potato tubers and existence of PVX virus as affected by low temperature sweetening (LTS)

Total soluble and reducing sugars

Changes in total soluble sugars (TSS) and total reducing sugar (TRS) concentrations during wound healing, LTS and reconditioning at different temperatures and a statistical comparison of sugar concentrations among cultivars following each phase of storage are presented in Table 3.

Table 2. The number of positive, negative samples and percentage of infection of collected samples of potato leaves and tubers of three cultivars "Lady rosetta, Spunta and Hermes" from Harad, NADEC co., Saudi Arabia.

Potato varieties	Potato Viruses	No. of tested samples	No. of positive (+) samples	No. of negative (-) samples	Percentage of infection (%)
			Leaves		
	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	2.0 ^{A}	7.0	22.2
			Sprout		
Lady Rosetta	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	2.0 ^{A}	7.0	22.2
			Tuber		
	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	2.0 ^{A}	7.0	22.2
			Leaves		
	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	5.0 ^{A}	4.0	55.6
			Sprout		
Spunta	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	3.0 ^{A}	6.0	33.3
			Tuber		
	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	3.0 ^{A}	6.0	33.3
			Leaves		
	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	9.0 ^{A}	0.0	100.0
			Sprout		
Hermes	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	8.0 ^{A}	1.0	88.9
			Tuber		
	PLRV	9	0.0 ^{B}	9.0	0.0
	PVY	9	0.0 ^{B}	9.0	0.0
	PVX	9	8.0 ^{A}	1.0	88.9

- Values presented are mean of three replicates; Values in the same group in the same column with different uppercase alphabetical letters superscripts in case of No. of positive (+) samples are significantly different at ($p < 0.05$).

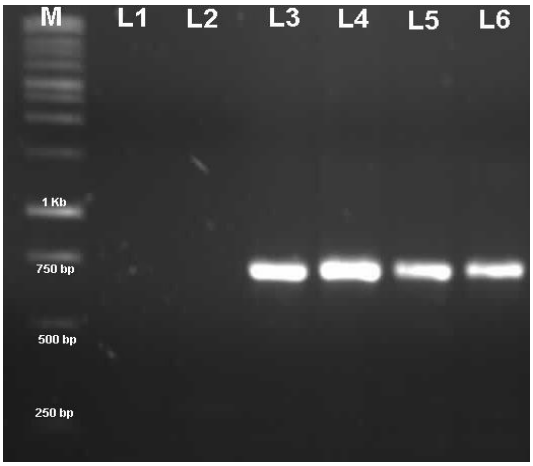

Fig 2. Agarose gel electrophoresis of RT-PCR products of potato PVX virus using primers for CP genes of leaves from re-grown sprouts tubers of stored potato tubers up to 3 month. M: 1 Kb DNA Ladder; L_1 for Lady rosetta cv. after 3 month; L_2 for Spunta CV. after 3 month; L_3 for Hermes CV. after 3 month. L_4 for Lady rosetta CV. after 1 month; L_5 for Spunta CV. after 1 month; L_6 for Hermes CV. after 1 month.

Table 3. Effect of short-term storage at 75% RH on sugar contents (mg/g DW) and ascorbate (mg/100g FW) of collected samples of potato leaves and tubers of three cultivars "Lady rosetta, Spunta and Hermes" from Harad, NADEC Co., Saudi Arabia.

Storage stages	Potato varieties		
	Lady rosetta	Spunta	Hermes
	Total soluble sugars (mg/g dray weight)		
Control (at zero time)	0.32 ± 0.01 [a]	0.42 ± 0.02 [a]	0.50 ± 0.05 [b]
1st Stage (WH after 12 day / 9◦C)	0.34 ± 0.02 [a]	0.43 ± 0.03 [a]	0.54 ± 0.03 [ab]
2nd Stage (LTS after 69 day / 4◦C)	0.34 ± 0.01 [a]	0.44 ± 0.01 [a]	0.56 ± 0.02 [ab]
3rd Stage (REC after 90 day / 16◦C)	0.35 ± 0.03 [a]	0.46 ± 0.02 [a]	0.59 ± 0.04 [a]
L.S.D. $_{(0.05)}$	n.s. 0.039	n.s. 0.037	0.066
	Total reducing sugars (mg/g dray weight)		
Control (at zero time)	0.17 ± 0.01 [b]	0.24 ± 0.02 [b]	0.31 ± 0.03 [c]
1st Stage (WH after 12 day / 9◦C)	0.17 ± 0.01 [b]	0.25 ± 0.01 [b]	0.34 ± 0.02 [c]
2nd Stage (LTS after 69 day / 4◦C)	0.18 ± 0.00 [ab]	0.27 ± 0.02 [b]	0.40 ± 0.01 [b]
3rd Stage (REC after 90 day / 16◦C)	0.20 ± 0.02 [a]	0.30 ± 0.01 [a]	0.45 ± 0.03 [a]
L.S.D. $_{(0.05)}$	n.s. 0.023	** 0.029	*** 0.045
	Ascorbate (mg/100g fresh weight)		
Control (at zero time)	32.07 ± 1.92 [a]	27.33 ± 2.46 [a]	24.37 ± 1.46 [a]
1st Stage (WH after 12 day / 9◦C)	31.21 ± 0.94 [ab]	25.61 ± 1.28 [ab]	21.18 ± 1.73 [b]
2nd Stage (LTS after 69 day / 4◦C)	30.08 ± 2.41 [ab]	23.25 ± 0.70 [b]	14.26 ± 0.43 [c]
3rd Stage (REC after 90 day / 16◦C)	28.52 ± 0.57 [b]	20.07 ± 1.40 [c]	10.06 ± 0.10 [d]
L.S.D. $_{(0.05)}$	n.s. 3.08	** 2.99	*** 2.30

Values presented are mean of three replicates; WH : Wound Hailing (1st Stage after 12 day/9◦C); LTS: (2nd Stage after 69 day/4◦C); REC: (3rd Stage after 90 day/16◦C). Values in the same column with the same uppercase alphabetical letters superscripts are non-significantly different at ($p < 0.05$).
L.S.D. $_{(0.005)}$: Lowest significance differences at ($P < 0.05$); n.s.: non-significant differences between treatments; **: moderate significant differences between treatments; ***: Highly significant differences between treatments.

While Spunta and Hermes tubers retained quality at low temperature, their mechanisms of LTS resistance were varied significantly. Total soluble sugars concentration in Hermes tubers increased by 18.0% while RS highly increased with 45.16% after 90 days of LTS storage. In contrast, TSS and TRS concentrations in Lady rosetta tubers increased relatively slowly to become 9.38 and 17.65%, respectively, after 90 days of LTS storage. When held continually at 9 ˚C, TSS content in tubers of three cultivars remained relatively constant, the trends in LTS of these cultivars were highly consistent. There were no significant differences between the two sweetening cultivars (Spunta and Hermes) in their TSS concentrations after 57 days at 4 ˚C (Table 3), however, TRS accumulated more gradually in Hermes cv. than in Spunta tubers. Despite these differences in the kinetics of sweetening, an increase in TSS and TRS at 9.52% and 25%, respectively, was obtained over the 90-day storage period at LST in 'Spunta' tubers. Control of three cultivars potato tubers stored constantly at 9˚C for 90 days exhibited no change in TSS levels and a relatively minor increase in RS compared with that induced by 4◦C (LTS). Following 57 days of LTS, storage temperature was rapidly increased to 16˚C for 3 weeks of reconditioning to ascertain the capacity for recovery of TSS and TRS in each cultivar. The TSS and TRS concentrations before and after reconditioning are statistically compared and presented in table 3. Since TRS concentrations were relatively low increase in Spunta and Hermes tubers after 57 days at 4◦C (Table 3), reconditioning would not normally be necessary. Lady rosetta tubers exhibited the most complete LTS resistance, maintaining low concentrations of both TSS and TRS at 4˚C storage. At low temperatures, TSS synthesis in potatoes is under substrate level control confirming previous findings by Sowokinos et al. (2000) and Daniel et al. (2014) on 'Premier Russet' tubers. Moderate LTS resistance in 'Spunta' tubers was characterized by substantial accumulation of TSS and low accumulation of TRS during 57 days of storage at 4˚C. Consistent with our findings, total activity of acid invertase correlated closely with TRS content in cold-stored tubers and the relative contribution of an endogenous inhibitor was highly genotype-dependent (Zrenner et al., 1996). A strong correlation between basal activities (inhibitor present) and LTS susceptibilities in 'Spunta' and 'Hermes' tubers is consistent with the results of Pressey (1969) in which low inhibitor activity was associated with TRS accumulation during LTS. In contrast, invertase activity in 'Hermes' tubers in the presence and/or absence of inhibitor was extremely low over the 57 days of LTS at 4˚C and throughout the 12 days of storage at 9˚C. Similar trends were described by Matsuuro-Endo et al. (2004) in the sweetening resistant clone 'Inca-no-mezame'.

Loss of ascorbate during short term storage

Total ascorbate content in tubers at zero time (at harvesting) varied by genotype (Table 3). Lady rosetta tubers had the highest concentration (32.07 mg 100 g^{-1} FW), followed by Spunta (27.33 mg 100 g^{-1} FW) and Hermes (24.37 mg 100 g^{-1} FW). Total AsA concentrations were decreased most rapidly in all cultivars over the first stage of storage (WH) at 9˚C (75% RH), followed by moderate decrement in the rate of loss through next 69 days of storage (stage 2 and 3). Hence, the decline was best described at second or third short term storage stages by the rates of loss over the initial 12 days of storage. It was recorded 20.07 and 10.06 mg total AsA 100 g^{-1} FW in case of Spunta and Hermes cultivar, respectively with a significant difference at $P < 0.05$ (LSD values = 2.99 and 2.30, respectively). Also, the decline was reached up to 28.52 mg/100^{-1} FW from over the entire 69 days of the 2nd and 3rd stages of storage for Lady rosetta cultivar which recorded the highest AsA concentrations at the end of storage, with a significant difference at $P < 0.05$ (LSD value = 3.08). Wound-induced AsA synthesis was also affected by tuber age (storage period). Finally, tubers stored for 90 days at 4˚C had 11.07, 26.56 and 58.8% less AsA than freshly tubers at zero day in case of Lady rosetta, Spunta and Hermes cultivars, respectively. The AsA content is affected by genotype and storage temperature and period. Previous report by Jacob *et al.* (2013) stated the effect of low oxygen on the initial rapid loss of AsA from freshly harvested tubers

which indicates that AsA loss during storage is tied to oxidative metabolism. Wounding of 12 days old stored tubers stimulated substantial AsA biosynthesis in tubers. Ascorbate concentration increased 2.68, 6.29 and 3.19% for Lady roseta, Spunta and Hermes, respectively within 12 days of wounding, correlating with increased expression of genes in transcript levels increased within 24 h in response to wounding. So, the wound-induced increases in GGP1 and GPP transcripts are particularly relevant as potential rate limiting steps in AsA biosynthesis (Ioannidi et al., 2009). The high per capita consumption of potatoes makes them a significant source of dietary vitamin C (FAO, 2008). Chu et al. (2002) estimated that AsA from potatoes accounts for about 13.3% of their total anti-oxidant activity. However, as demonstrated herein, the AsA content of tubers varies substantially during storage (Table 3), and temperature stress.

Effect on existence of PVX virus using one step RT-PCR

The one-step RT-PCR was repeated twice on the stored tubers of selected three cultivars as affected by short term storage at low temperature sweetening, after one and three months of storage. PVX CP gene amplification from all samples stored after one month was achieved and the bands were clearly visible (Fig. 2). On the other hand, there was one positive sample with PVX after three month of storage which belongs to Hermes cultivar, while other two samples of Lady rosseta and Spunta cultivars are given a negative reaction with PVX-CP primers after three months of storage (Fig. 2).
This investigation focused on developing alternative detection method which is cost effective and maintains an efficient or acceptable level of detection of potato viruses in two situations (1) testing viruses in potato seed whether imported or locally produced and (2) surveying for viruses in green tissue samples collected from potato fields. A good quality of seed potato is an essential factor for production of high yields. Certification schemes require that the total incidence of potato viruses in potato seed lots does not exceed 1- 5%, with some variations between countries or states. This may be expected since our results showed that PVX is not evenly distributed in the tuber and thus the efficiency of detection using eye tissue would be approximately half of that of sprouts. Therefore, the results of PVX detection in the eyes by ELISA might be doubled to compensate for this reduced detection due to uneven distribution of the virus in tuber tissue, for example if PVX was detected in 1.5% of the samples, then the real percentage of infection would be around 3%. In the present study, a reverse transcription polymerase chain reaction (RT-PCR) based protocol for PVX detection was developed and was compared to an enzyme linked immunosorbent assay (ELISA) based PVX detection methods. As it had been reported earlier by Singh (1998) and Ghosh and Bapat (2006) that RT-PCR based protocol is more efficient and sensitive in detecting PVY from field samples. It was found that RT-PCR with a pair of CP gene specific primers could detect the presence of very small amount of infection in field samples which not detected by ELISA. This method will also help to correctly identify resistant plants in the population so that they could be separated for further multiplication. Samples from eyes or stem ends of imported seed potato might be collected and used for serological assay (ELISA); however, proper correction factors should be calculated locally with respect to the recommended tests using sprouts.

Materials and Methods

Plant materials

Three potato cultivars (Lady Rosetta, Spunta and Hermes) were used as genetic materials in this study. According to ECPD (2014), These cultivars are classified to virus infestation as resistance, moderately susceptible and susceptible, respectively. Tuber seeds were randomly selected from visually virus infected spots of growing area in the fall and stored at 4 ºC (95% RH) until planting date. Tubers of nine selected plants of each cultivar were harvested 169–176 days after planting (DAP), and sorted for post-harvest studies. Potatoes with equal size from each cultivar were washed, dried and packed separately in low density polyethylene (LDPE) bags (37.12 microns; 21.5 × 15 cm). Each bag contained 3 tubers was considered as one replicate for each treatment. Leaves of collected potato samples were subjected directly to ELISA tests and RT-PCR for detection of potato viruses PLRV, PVY and PVX. Meantime, pieces of tubers (each bearing one eye) were planted in pots under growth chamber conditions (20 – 25 ºC) and irrigated as described by Driskill et al. (2007). Ten days after planting, the young leaves of the developed sprouts, were also sampled for virus detection.

Potato viruses detection

Potato viruses (PLRV, PVY and PVX) were detected in fresh potato leaves, young leaves of the developed sprouts and in tubers at the end of storage period (after 3 months) for each cultivar.

ELISA

ELISA tests for the disclosure of potato viruses (PLRV, PVY and PVX) in the collected potato samples (leaves and tubers) were conducted as described by ELISA kits (Bioreba AG, Switzerland). Leaves of symptomatic potato plants were collected, labeled and placed in plastic bags. A sample consisted of 18 leaves (two leaves per plant, one from the upper and another from the lower part of the plant) was taken from nine plants in the same field grown with chosen cultivars. Tubers were taken from the same nine labeled plants. The collected samples were kept in an ice box within the same afternoon and stored overnight in a refrigerator till assayed for viruses next day. The double antibody sandwich ELISA (DAS-ELISA) method was used for virus detection as described by Clark and Adams (1977). The samples were tested for occurrence of the three mainly remarkable potato viruses (PVX, PVY and PLRV) using ELISA kits from Bioreba AG, Switzerland.

Primers for PLRV, PVY and PVX

Pair of specific primers for PLRV coat protein gene (PLRV-CP), PVY coat protein gene (PVY-CP) and PVX coat protein gene (PVX-CP) was used for the CP genes (Shalaby et al., 2002). The forward primers and the complementary primers for the three potato viruses were used for the detection of coat protein genes (Table 1).

One step RT-PCR

One step RT-PCR was done using platinum quantitative RT-PCR thermo script one step system (Invitrogen Company,

USA). The samples (leaves and tubers) which gave positive reactions in ELISA tests at the beginning of the experiment and the one step RT-PCR was repeated twice on the stored tubers (after one and three months of storage). Total RNA extracted from infected potato plants using RNeasy® plant mini kit obtained from Qiagen was used as molding for one-tube RT-PCR amplification reactions. RT-PCR mixture prepared by combining 12.5 µL of 2× thermo script reaction mix, 5 µL of total RNA, 1 µL of 10 µM of each primer (forward and reverse primers; Table 1), 0.5 µL of thermo script *Taq* enzyme mix and the reaction was completed to 25 µL with double distilled water. Reverse transcription reaction ongoing with incubation at 50 °C for 30 min, followed by denaturation at 95 °C for 5 min. PCR amplification was performed by 35 cycles in a thermal cycle starting with denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and extension at 72 °C for 1 min with final extension at 72 °C for 10 min. Five microliters aliquots of RT-PCR products were analyzed on 1% agarose gels in 0.5× Tris/Borate/EDTA (TBE) buffer.

Short-term storage study

At harvest time in 2013, tubers were randomly collected from each visually symptomatic infected potato plots of three selected cultivars. Short term storage studies were conducted to profile the low temperature storage (lab refrigerator). Changes in sugar content (total sugars and total reducing sugars) and ascorbate level during low temperature sweetening (LTS) and reconditioning of cultivars (90 day storage) were measured directly following harvest. After sorting, tubers from each of the three cultivars were wound-healed for 12 days at 9 °C /75% RH during which time basal respiration rates were established (1st Stage call "wound-healing"). The storage temperature was then rapidly (15 min) dropped to 4°C/75% RH for a 57 day during LTS period (2nd Stage call "low temperature sweetening"), followed by 3 weeks of reconditioning at 16 °C/75% RH (3rd Stage call "reconditioning"). Tubers from visually healthy plants of all cultivars were maintained at 9°C/75% RH over the 90 day storage period to serve as negative control, and another group of tubers from visually healthy plants of all cultivars were maintained at all storage steps as mention above over the 90 day storage period to serve as positive control. Three replicates of each treatment "Stage" and three tubers per each replicate. Sprouting over the 90 days storage period was inhibited by treating tubers with 0.75 mmol kg^{-1} of 3-nonen-2-one as needed (Knowles and Knowles, 2012). Tubers were stored for 90 days, then re-growing tubers of three cultivars to get sprouts after 10 days with length 3 cm and later examined for PVX infection using one step RT-PCR as previously described.

Carbohydrate analysis

Determination of total soluble sugar

The amount of total soluble sugars was determined by phenol sulphuric acid reagent method (Dubois et al., 1951), using 500 mg dry weight of tuber tissues. The optical density of the formed yellow orange color thus developed was measuring at 490 nm using a spectrophotometer against the blank sample; standard curve was prepared by using known concentration of glucose. The quantity of sugar was expressed as mg/g fresh weight of stored potato tuber tissues.

Determination of reducing sugar

The amount of reducing sugar was determined according to the method of Miller (1972) using 500 mg dry weight of tuber tissues. The developed color absorbance was measured using spectrophotometer at 515 nm. The quantity of reducing sugar was expressed as mg/g fresh weight of stored potato tubers tissues.

Ascorbate analysis

One hundred milligrams of lyophilized tuber tissues was extracted with 1.0 mL of 6% (w/v) trichloroacetic acid and centrifuged at 11,000 × *g* for 6 min (4 °C). Reduced, oxidized and total ascorbic acid were analyzed as described by Gillespie and Ainsworth (2007) using a microplate reader (Power Wavex, Bio-Tek Instruments, Inc., USA). The Fe^{2+} generated forms a colored complex (wave length 525 nm). The total AsA (reduced + oxidized) was obtained by reduction of dehydroascorbate in one aliquot of extract with dithiothreitol prior to the assay. Quantification of AsA was achieved by linear regression from a standard curve of 0.05 – 1.0 mM authentic ascorbic acid.

Statistics analysis

Data were analyzed using analysis of variance (ANOVA) and the means were compared by the least significant differences (LSD) at $P \leq 0.05$ described by Snedecor & Cochran (1980). All data were subjected to ANOVA with time, temperature and cultivar as independent variables.

Conclusion

The study showed that RT-PCR with a pair of CP gene specific primers could detect the presence of very small amount of infection in field samples which not detected by ELISA. RT-PCR might represent fast, practical, and sensitive alternatives for the detection of PLRV, PVY and PVX from green and/or tuber tissues. Changes in the quality of potato tubers in terms of reducing sugars and ascorbate contents during storage are closely related to cultivar and storage period.

References

Agindotan BO, Patrick JS, Philip HB (2007) Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan_ realtime RT-PCR. J Virol Methods. 142: 1-9.

Agindotan BO, Perry KL (2008) Macroarray detection of eleven potato-infecting viruses and potato spindle tuber viroid. Plant Dis. 92: 730-740.

Bhat JA, Bhat ZA, Dar WA, Rashid R (2010) Economically significant bacterial and viral diseases of potato and their management. Int J Curr Res. 2: 73-77.

Bostan H, Elibuyuk IO (2010) A simple and rapid nucleic acid preparation method for reverse transcription polymerase chain reaction (RT- PCR) in dormant potato tubers. Afr J Biotechnol. 9: 1982-1986.

Bulley SM, Wright M, Rommens C, Yan H, Rassam M, Lin-Wang K, Andre C, Brewster D, Karunairentnam S, Allan AC, Laing W (2012) Enhancing ascorbate in fruits and tubers through over-expression of the L-galactose pathway gene GDP-L-galactose phosphorylase. Plant Biotechnol. 10: 390–397.

Chu Y, Sun J, Wu X, Liu R (2002) Antioxidant and antiproliferative activities of common vegetables. J Agr Food Chem. 50: 6910–9616.

Clark MF, Adams AN (1977) Characteristics of the microplate method of the enzyme linked immunosorbent assay for the detection of plant viruses. J Gen Virol. 34: 475-483.

Daniel HZ, Knowles LO, Knowles NR (2014) Tuber respiratory profiles during low temperature sweetening (LTS) and reconditioning of LTS-resistant and susceptible potato (*Solanum tuberosum* L.) cultivars. Postharvest Biol Tec. 92: 128–138.

Driskill EP, Knowles LO, Knowles NR (2007) Temperature-induced changes in potato processing quality during storage are modulated by tuber maturity. Am J Potato Res. 84: 367–383.

Dubios MK, Gilles JK, Robers PA, Smith F (1951) Calorimetric determination of sugar and related substance. Anal Chem. 26: 351-356.

ECPD (2014) European Cultivated Potato Database; is an online database of potato varieties. Available in http://www.europotato.org/menu.php.

Eltawil M A, Samuel DVK, Singhal OP (2006) Potato storage technology and store design aspects. Agr Eng Int CIGR. 8 (11): 1-18

FAO (2008) International Year of the Potato 2008; New Light on a Hidden Treasure, an End-of-year Review. FAO, Rome, Italy.

Fox A, Evans F, Browning I (2005) Direct tuber testing for potato Y poty virus by real-time RT-PCR and ELISA: reliable options for post-harvest testing. Bulletin OEPP. 35: 93-97.

Ghosh SB, Bapat VA (2006) Development of RT-PCR based method for detection of potato virus Y in tobacco and potato. Ind J Biotechnol. 5: 232-235.

Gillespie KM, Ainsworth EA (2007) Measurement of reduced, oxidized and total ascorbate content in plants. Nat Protoc. 2: 871–874.

Gudmestad NC (1991) A historical perspective to pathogen testing in seed potato certification. Am J Potato Res. 68 (2): 99-102.

Harbenburg RE, Watada AE, Wang CY (1986) The commercial storage of fruits, vegetables and florist and nursery stocks. USDA. 66-68.

Ioannidi E, Kalamaki MS, Engineer C, Pateraki I, Alexandrou D, Mellidou I, Giovannonni J, Kanellis AK (2009) Expression profiling of ascorbic acid-related genes during tomato fruit development and ripening and in response to stress conditions. J Exp Bot. 60: 663–678.

Jacob MB, Mohan Kumar GN, Knowles LO, Dhingra A, Knowles NR (2013) Changes in ascorbate and associated gene expression during development and storage of potato tubers (*Solanum tuberosum* L.). Postharvest Biol Tec. 78: 76–91.

Kogovsek P, Kladnik A, Mlakar J, TusekZnidaric M, Dermastia M, Ravnikar M, Pompe-Novak M (2011) Distribution of Potato Virus Y in Potato Plant Organs, Tissues, and Cells. Am Phytopathol Soc. 101: 1292-1300.

Knowles LO, Knowles NR (2012) Toxicity and metabolism of exogenous, unsaturated carbonyls in potato (*Solanum tuberosum* L.) tubers. J Agr Food Chem. 60: 11173–11181.

Mahto R, Das M (2014) effect of gamma irradiation on the physico-mechanical and chemical properties of potato (*Solanum tuberosum* L.), cv. 'Kufri sindhuri', in nono-refregerated storage conditions. Postharvest Biol Tec. 92:37-45.

Mahto R, Das M (2015) effect of $^{\gamma}$ irradiation on the physico-mechanical and chemical properties of potato (*Solanum tuberosum* L.), cv. 'Kufri chandramukhi', and 'Kufri jyoti', during storage at 12 °C. Radiat Phys Chem. 107:12-18.

Matsuuro-Endo C, Kobayashi A, Noda T, Takigawa S, Yamauchi H, Mori M (2004) Changes in sugar content and activity of vacuolar acid invertase during low-temperature storage of potato tubers from six Japanese cultivars. J Plant Res. 117: 131–137.

Miller GL (1972) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem. 31: 426-428.

NASS- National Agricultural Statistics Service (2012) September. Potatoes 2011 Summary,USDA. : 1949-1514.

Nicolaisen M (2011) An oligonucleotide-based microarray for detection of plant RNA viruses. J Virol Methods. 173: 137-143.

Petrunak DM, Gildow FE, Christ BJ (1991) Incidence and distribution of six viruses infecting potatoes in Pennsylvania. Plant Dis. 75: 644.

Pressey R (1969) Role of invertase in the accumulation of sugars in cold-stored potatoes. Am J Potato Res. 46: 291–297.

Shalaby AA, Nakhla MK, Soliman AM, Mazyad HM, Hadidi A, Maxwell DP (2002) Development of a highly sensitive multiplex reverses transcription-polymerase chain reaction (m-RTPCR) method for detection of three potato viruses in a single reaction and nested PCR. Arab J Biotechnol. 5 (2): 275-286.

Singh RP (1998) Reverse transcription polymerase chain reaction for the detection of viruses from plants and aphids. J Virol Methods. 74:125-138.

Singh RP (1999) Development of the molecular methods for potato virus and viroid detection and prevention. Genome. 42:592-604.

Snedecor GW Cochran WG (1980) Statistical Methods. 7th Edn. Iowa State Univ. Press, Iowa, USA.

Sowokinos JR, Shock CC, Stieber TD, Eldredge EP (2000) Compositional and enzymatic changes associated with the sugar-end defect in Russet Burbank potatoes. Am J Potato Res. 77: 47–56.

Zrenner R, Schuler K, Sonnewald U (1996) Soluble acid invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers. Planta. 198: 246– 252.