Monitoring variability responses of cultivated potato varieties infected with *Potato virus Y* pepper isolate

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ABSTRACT

The PVY isolate-infected pepper plants confirmed by DAS-ELISA and isolated on *Chenopodium amaranticolor* L. where gave chlorotic local lesions. Eleven potato cultivars (*Solanum tuberosum)* mechanically inoculated with PVY isolate under greenhouse condition were divided to three categories, resistant (Diamond, Execusa, Hermes and Valor), tolerant (Sisi, Lady Balfor, Nikola, Ditta and Lady Rosetta), susceptible (Anabel and Spunta) based on different external symptoms, the severity infection and virus concentration. Expressed protein was differed in leaves of potato cultivars. Since PVY infection unexpectedly increased in (Anabel, Sisi, Lady Balfor, Nicola and Execusa) or decrease of total protein in (Hermes and Spunta). The potato cultivars (healthy and infected) varied in number and density of protein bands. As well as specific bands with 72% polymorphism, commonly bands were percentage 24%. On the other hand, the unique band appeared only in infected plants of Valor cv. with MW 85 KDa. Total phenols and proline were increased in tolerant and resistant potato cultivars compared to susceptible cultivars and healthy ones. Scavenging enzymes (SOD, CAT, POX and PPO) were increased mostly in tolerant and resistant cultivars compared with susceptible cultivars and healthy ones. The total number of phenol oxidizing isozymes (POX and PPO) bands in infected plants of eleven cultivars was varied considerably by increasing, decreasing or no changes comparing with healthy plants. The presence of $\boldsymbol{Ry_{adg}}$ gene has been observed in Anabel, Diamond, Nicolas, Dita, Execusa, Hermes and Lady Rosetta. The nucleotide sequence of $\boldsymbol{Ry_{adg}}$ of cultivars Dita, Execusa and Lady Rosetta was aligned with other recommended PVY strains registered in GenBank. A phylogenetic tree of $\boldsymbol{Ry_{adg}}$ gene based on nucleotide sequence revealed 93% a moderate degree of similarity to Potato gene Y-1 isolated from *Solanum tuberosum* subsp. *andigenum*

Keywords: Disease severity, POX, PPO, CAT, SOD, Phenols, Proline, Isozymes, SDS-PAGE, potato cultivars, Ry_{adg}

INTRODUCTION

PVY is wide spread all over the world and causes economically important disease not only to solanaceous crops but also too many solanaceous weeds (Quintero-Ferrer and Karasev, 2013). *Potato virus Y* (PVY) is the most harmful virus infecting potato crops (Valkonen, 2007) and causes a mosaic leaf pattern, vein clearing; leaf necrosis and leaf drop symptoms, although the type and severity of symptoms will differ among potato cultivars (Nie *et al*., 2011).

ELISA enabled the highly sensitive detection and assaying of a number of morphologically different viruses in purified preparations unclarified extracts of herbaceous hosts (Clark and Adams, 1977; Rakib *et al*., 2011).

Attributed increase protein content partly due to proteinous nature of virus itself or decrease of total soluble proteins due to

viral infection has been reported by several workers (Taiwo and Akinjogunla, 2006).

Proline accumulation is a common metabolic response to both abiotic and biotic stress and when higher plants are exposed to stress; many plants accumulate high amounts of proline in tissues (Mazid *et al.*, 2011).

Many defense enzymes are involved in defense reaction against plant pathogens. These include oxidative Peroxidase (POX) and polyphenol oxidase (PPO), which catalyzes the formation of lignin and other oxidative phenols that contribute, to the formation of defense barriers for reinforcing the cell structure. SODs, POXs and CATs are the most common mechanism for detoxifying ROS synthesized during stress response (Mittler, 2002; Kumar *et al*., 2009) very few reports are available for antioxidative enzymes activity in plants subjected to biotic stresses especially, viral infection.

Plant can synthesize and accumulate phenolic compounds in response to stress (Mamdouh *et al*., 2002).The antioxidant activity of phenolic compounds can play an important role in neutralizing ROS (Zheng and Wang, 2001; Kumar *et al*., 2010).

Genes triggering hypersensitive response and field immunity against PVY are present in several potato species. There are also *Ry* genes conferring ER to PVY in *S.tuberosum* sub sp. *andigena* (Ry_{adg}) and *S. stoloniferum* (Ry_{sto}). Already these loci have been introduced into several commercial cultivars by conventional breeding (Mahfouze, 2009).

The present study aimed to evaluation responses of potato cultivars to (PVY-EG) based on disease severity and phytochemical constituents.

MATERIALS AND METHODS

Source of Potato Cultivars

Forty tubers of each potato cultivar (Execusa, Valor, Lady Rosetta, Diamond, Anabel, Sisi, Hermes, Lady Balfor, Ditta, Spunta and Nikola) were obtained from (Inventory project and the fight against brown rot in potatoes) belong to agriculture researches center (ARC), Dokki, Giza. Potato cultivars were checked before cultivation and inoculation for potential infection with PVY, PVX and PLRV tested by DAS-ELISA technique using specific polyclonal antibodies (Clark and Adam, 1977).

Source of Virus Isolate

Potato virus Y potyvirus was isolated from naturally infected pepper plants**.** The isolated PVY was confirmed by local lesions assay on *Ch. amaranticolor* and serologically by DAS-ELISA. Twenty tubers from each cultivar which gave –ve results DAS ELISA with PVY, PVX and PLRV were planted in plastic bags (diameter 30 cm^2) containing sterilized soil (one tuber per bag) in a virology greenhouse, Microbiology Dept., Agric. Fac., Ain Shams Univ. and with practical agriculture recommendation.

PVY isolate was mechanically inoculated on ten plants (2 wreaks old) as well as ten plants without inoculation as a healthy of each cultivar and maintained under a greenhouse. After 3 weeks post inoculation the development external symptoms was recorded and infected plants were confirmed by DAS-ELISA.

Disease Severity

The disease severity of infected potato plants was determined using the following formula according to Yang *et al*. (1996).

$$DS(\%) = \frac{\Sigma(\text{disease grade} \times \text{No. of plants in each grade})}{(\text{total No . of plants} \times \text{highest disease grade})} \times 100$$

Virus concentration was determined using PVY specific polyclonal antibodies by DAS-ELISA according to Clark and Adam (1977).

Extraction and Determination of Phenolic Compounds

Extraction and determination of phenolic compounds in leaves were carried out according to method described by Daniel and George (1972) using spectrophotometer (Unico2000) at the wave length 725 nm.

Quantitative Determination of Free Proline

Free proline content was determined according to the method described by Bates *et al*. (1973) at 520 nm using UV-spectrophotometer (Unico 2000).

Determination of Total Proteins

This was made according to the method of Lowery *et al*. (1951) using casein as a standard protein determination of total protein in leaves by spectrophotometer (Unico2000) at the wave length 750 nm. The protein pattern was determined by sodium dedocyl sulphate polyacrelamyde gel electrophoresis (SDS-PAGE) according to Laemmli (1970), modified by Studier (1973).

Determination of Enzymes

The plant materials used for estimation of enzymes were 2 g of the terminal buds were homogenized with 10 ml of phosphate buffer pH 6.8 (0.1 M), then centrifuge at 2^{o}C for 20 min at 20000 rpm in a refrigerated centrifuge. The clear supernatant (containing the enzymes) was taken as the enzymes source (MuKherjee and Choudhuri, 1983).

Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol using a method described by Marklund and Marklund (1974) at 325 nm wave length using UV- spectrophotometer (Labomed, inc.23).

Catalase (CAT)

Catalase activity was determined by measuring the rate change of H_2O_2 absorbance with a UV- spectrophotometer (Labomed, inc.23) at 250 nm wave length. according to the method of Chengwei *et al*. (2000).

Peroxidase (POX)

Peroxidase activity was assayed using the rate of increase in absorbance as pyrogallol was determined by UV-spectrophotometer (Labomed, inc.23) at 470 nm (Bergmeyer, 1974).

Polyphenol Oxidase (PPO)

Polyphenol oxidase activity of polyphenol oxidase enzyme was determined according to the method adopted by Matta and Dimond (1963). The absorbance was measured at 495 nm wave length. by UV-spectrophotometer (Labomed, inc.23)**.**

Isozymes Electrophoresis

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among tested cultivars using two isozyme systems, peroxidase (POX) and polyphenol oxidase (PPO) according to Stegemann *et al*. (1985).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from infected *D. metel* L. leaves with PVY-isolate according to the instruction manual of High

Pure RNA tissue kit (Version 1, 2000) from Roche diagnostics GmbH, Germany.

Set primer pairs of the forward and reverse for ***Ry$_{adg}$*** Gene was designed from the only accession AJ300266 that is available in NCBI GeneBank database. The ***Ry$_{adg}$*** gene is confers disease resistance of PVY in *S. tuberosun ssp. andigena* (Vidal *et al.,* 2002). The designed primer (manufactured by Bioron) of ***Ry$_{adg}$*** gene with length of 20 bp (table 1).

Table 1. sequence of *Ry$_{adg}$* gene primer

Primer for *Ry* gene	**Sequences of direct (d) and reverse (r) primer (5´…3´)**		**size (bp)**
Ry $_{adg}$-2	**d**	CTCCGGTGAATCAGGGATTA	**242**
	r	TTGTGTCTAATTGCCGTGGA	

PCR reaction was carried out in a DNA thermocycler (Biometra, biomedizinische Analytic GmbH). performed with the cycling program of denaturation (one cycle) 94°C for 4 min, annealing 35 cycles for 1 min, 94°C, 1 min at 72°C and with final extension of 5 min at 72°C.

Agarose Gel Electrophoresis

PCR amplified products were analyzed using 1.2% agarose gel electrophoresis in 1x TBE buffer staining with ethedium bromide. The amplified RNA bands were visualized under UV light and the size of the fragment were estimated based on a RNA ladder of 100 to 2000 bp (manufactured by Bioron).

Sequence Alignments and Phylogenetic Analyses

Multiple alignments of sequences were performed using DNAMAN software (Wisconsin, Madison, USA) and clustalw (Ver.1.74) program (Thompson *et al.,* 1994). Phylogenetic relationships were evaluated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) through DNAMAN software and Neighbour Joining (NJ) implemented through MEGA 4.0 software, and bootstrap analysis (1000 replicates) was performed to assess the reliability of the constructed phylogenetic tree.

RESULTS

The virus infectivity of isolate was confirmed biologically, that gave chlorotic lesions on *Ch. amarannticolor* (Fig. 1) as well as gave (+ve) results with PVY polyclonal antibodies by DAS-ELISA.

Figure 1. *Ch. amaranticolor* as indicator host to PVY isolate showing similarity of local lesions

Forty tubers of each potato cultivars, local tuber seeds (Execusa, Valor, Lady Rosetta, Diamond, Anabel, Sisi, Hermes, Lady Balfor, Ditta, Spunta and Nikola) were tested to the extent of its infection or virus

free from PVY, PVX and PLRV using specific antibodies by applying the DAS-ELISA assay.

Reaction of Tested Potato Cultivars with PVY-EG isolate

Eleven potato cultivars inoculated with (PVY-EG) isolate and grown under a greenhouse (28±2°C) showed variation external symptoms after 30 days, post-inoculation. The symptoms were recorded in table (2) and Fig. (2).

All potato cultivars showed systemic symptoms but it was different. These symptoms were confirmed by DAS-ELISA against specific polyclonal antibodies for PVY isolate. Based on external symptoms, the severity of the infection, and the virus concentration, potato cultivars were differed in the degree of sensitivity to (PVY-EG) and divided to three categories:

(1) PVY resistant cultivars, Diamond, Execusa, Hermes, and Valor which showed mild mosaic, mosaic and veinal necrosis with severity up to 25%. (2) PVY tolerant cultivars, Sisi, Lady Balfor, Nikola, Ditta and Lady Rosetta showed vein clearing, mild mosaic, mosaic, necrosis and top necrosis and severity were 25 to 50%. (3) PVY susceptible cultivars, Anabel and Spunta cv. produced vein clearing, severe mosaic, leaf deformation, crinkling veinal necrosis, necrosis and top necrosis with severity > 50%.

Variation of phytochemical constituents

PVY infected potatoes cv. were varied in the protein content compared with healthy ones as a result of (PVY) infection, where the infection led to cultivars Anabel, Sisi, Lady Balfor, Nicola and Execusa showed a significant increase in soluble protein content. Whereas, non-significant increase observed in cultivar Diamond. Significant decreased in both Hermes and Spunta cultivars, while non-significant decreased recorded in cultivars Ditta, Lady Rosetta and Valor (Table 3).

Table 2. Susceptibility of eleven different potato cultivars to infection with PVY Egyptian isolate

Cultivar	Symptoms	*ELISA reading	Disease Severity%	Type of resistance
Anabel	VC, LD, VN, N	0.183	53.3	Susceptible
Sisi	VC, N, TN	0.145	33.3	Tolerant
Lady Balfor	M, N	0.141	40	Tolerant
Diamond	mM,VN	0.144	20	Resistant
Nicola	VC, N	0.195	43.3	Tolerant
Ditta	VC, N	0.223	46.6	Tolerant
Execusa	mM, VN	0.122	23.3	Resistant
Hermes	VC, N	0.160	21.6	Resistant
Lady Rosetta	VC, mM, N	0.151	36.6	Tolerant
Valor	M, VN	0.157	23.3	Resistant
Spunta	VC, SM, C, N, TN	0.196	71.6	Susceptible

VC= Vein clearing, **mM**= Mild mosaic, **SM**= Severe mosaic, **M**= Mosaic, **LD**= Leaf deformation, **C**= crinkling, **N**= Necrosis, **VN**= Veinal necrosis, **TN**= Top necrosis,

***Optical density at 405 nm, **Negative control**= 0.042, **Positive control**= 0.275

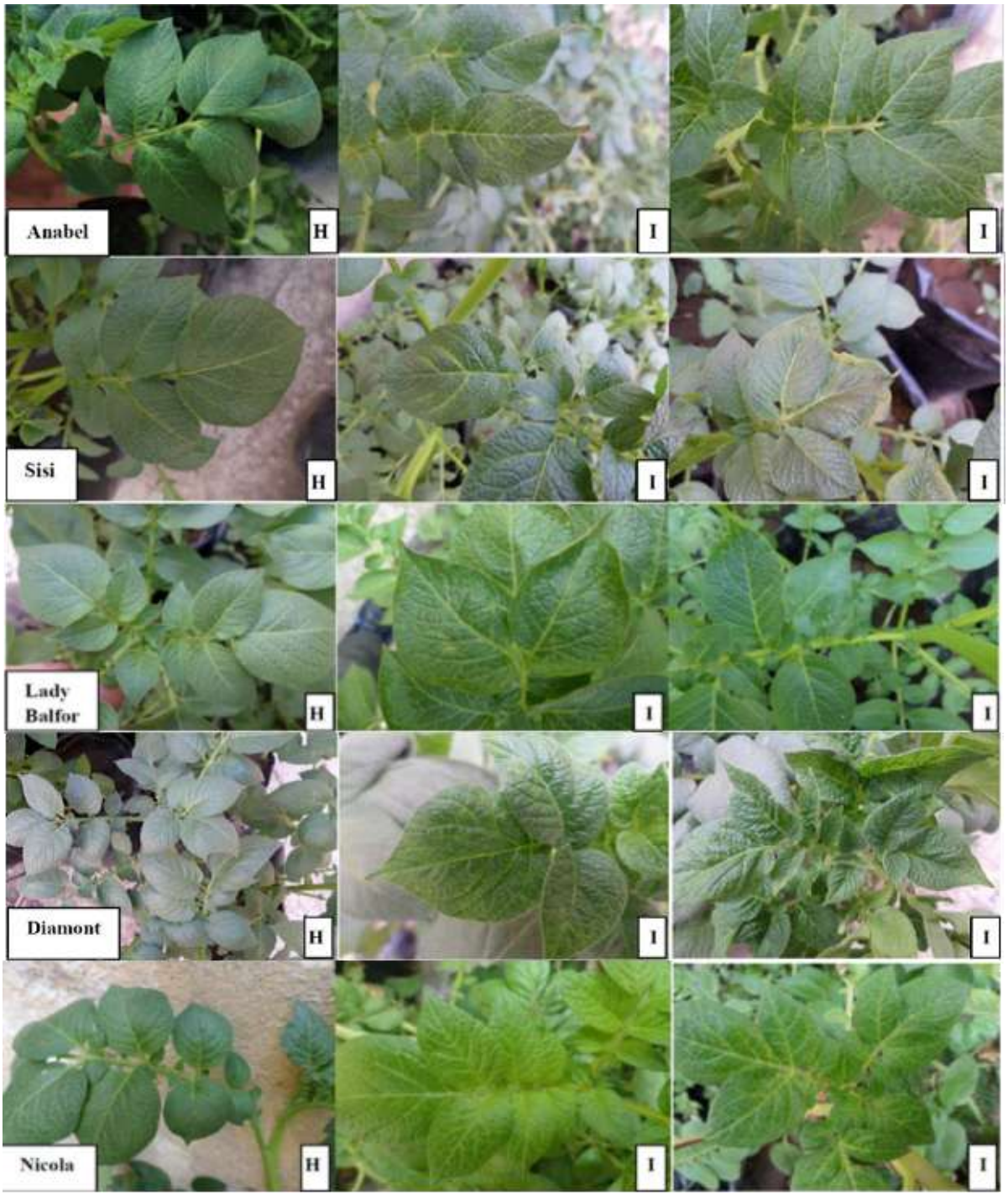

Figure 2. Tested potato cultivars inoculated with PVY Egyptian isolate were showing different external symptoms at greenhouse conditions

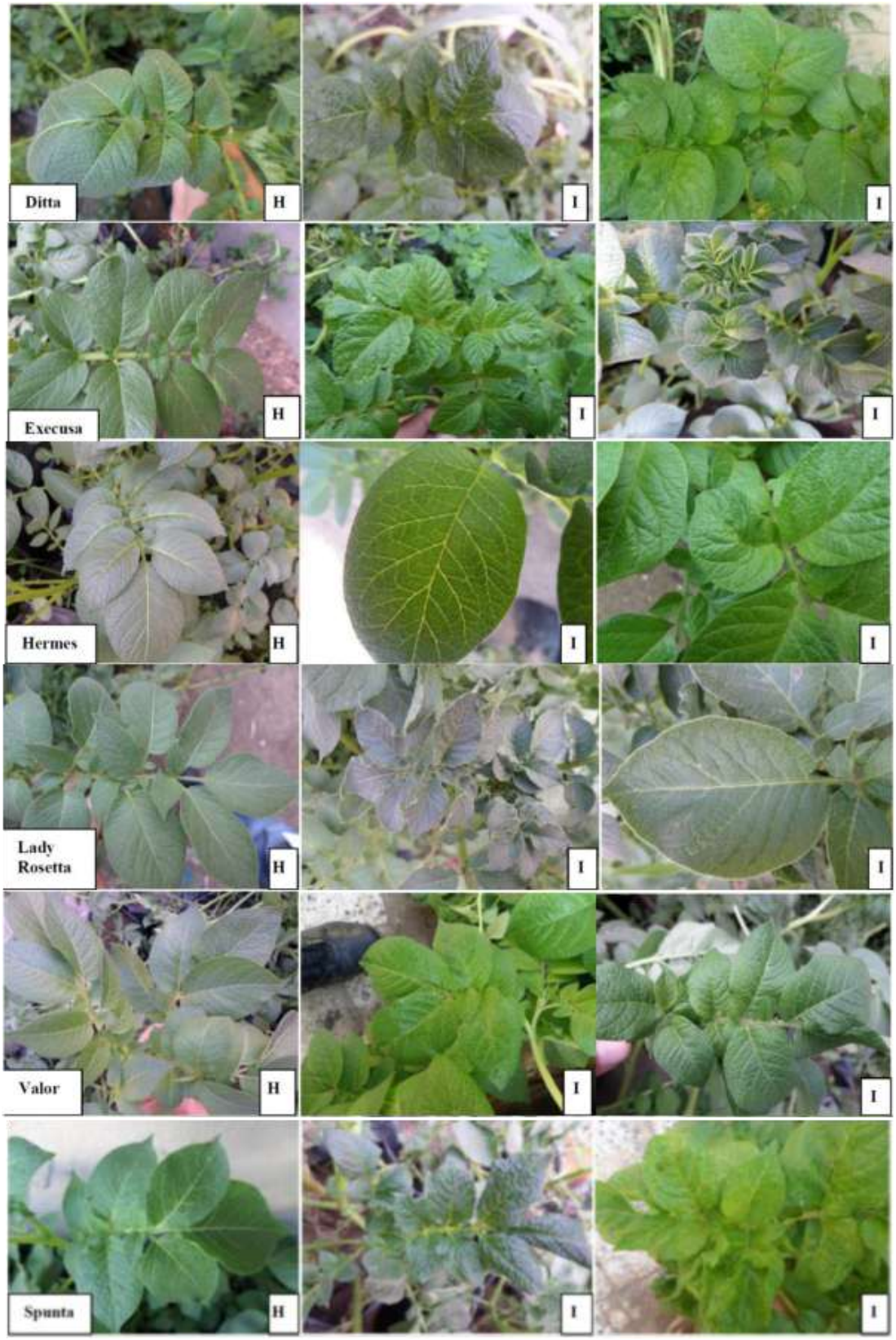

Figure 2. Continue

The difference in the content of bioactive components, i.e. total proline and total phenols due to PVY infection where proline content mostly increasing, where cultivars Anabel, Sisi, Lady Balfor, Diamond, Lady Rosetta and Valor showed significantly increased, compared with healthy ones, while cultivars Nicola, Ditta, Hermes and Spunta showed a non-significant increase in the proline content. As well as, the results showed non-significant decrease observed only in cultivar Execusa (Table 3).

Also, table (3) showed PVY resistant and tolerant cultivars, Lady Balfor, Ditta, Hermes, Lady Rosetta and Valor showed a significant increase in total phenols, compared with healthy ones, whereas, non-significant increase observed in both Lady Balfor and Diamond cultivars. While PVY susceptible cultivars Anabel, Spunta and Sisi showed a significant decrease.

Table 3. Effect of PVY infection on proteins, phenols, proline (mg/g dry weight) of shoots of potato cultivars by tow way analysis of variance (ANOVA)

Cultivars			**Protein (mg/g dry weight)**	**Proline (mg/g dry weight)**	**Phenol (mg/g dry weight)**
PVY resistant cvs.	Diamond	H	11.3±0.3	0.3±0.004	0.068±0.001
		I	11.8±0.3 **NS**	0.65±0.03 **S**	0.068±0.001 **NS**
	Execusa	H	9.0±0.2	0.14±0.01	0.078±0.000
		I	10.3±0.2 **S**	0.12±0.01 **NS**	0.075±0.001 **NS**
	Hermes	H	11.6±0.1	0.25±0.01	0.102±0.001
		I	10.5±0.2 **S**	0.31±0.009 **NS**	0.115±0.001 **S**
	Valor	H	11.4±0.5	0.15±0.004	0.068±0.000
		I	11.1±0.6 **NS**	0.3±0.009 **S**	0.078±0.001 **S**
PVY tolerant cvs.	Sisi	H	9.8±0.05	0.54±0.08	0.106±0.001
		I	11.8±0.3 **S**	0.87±0.01 **S**	0.094±0.001 **S**
	Lady Balfor	H	10.3±0.2	0.25±0.04	0.063±0.000
		I	12.2±0.4 **S**	0.42±0.01 **S**	0.087±0.000 **S**
	Nicola	H	11.7±0.3	0.31±0.006	0.009±0.000
		I	12.9±0.5 **S**	0.33±0.008 **NS**	0.014±0.000 **NS**
	Ditta	H	11.2±0.3	0.33±0.008	0.074±0.001
		I	10.7±0.3 **NS**	0.40±0.007 **NS**	0.084±0.001 **S**
	Lady Rosetta	H	11.6±0.3	0.54±0.07	0.062±0.001
		I	11.1±0.4 **NS**	0.8±0.007 **S**	0.080±0.000 **S**
PVY susceptible cvs.	Anabel	H	9.5±0.15	1.6±0.16	0.083±0.000
		I	13.5±0.11 **S**	2.15±0.02 **S**	0.069±0.001 **S**
	Spunta	H	10.9±0.1	0.09±0.003	0.090±0.001
		I	9.9±0.6 **S**	0.13±0.014 **NS**	0.072±0.001 **S**

Each value is the mean of 4 replicates

±= standard error of means, **H**= Healthy plants, **I**= Infected plants, **NS**= Non significant, **S**= Significant at $P<0.05$

Enzyme activities were differed among healthy and infected potato cultivars**,** table (4) revealed that, in the superoxide dismutase enzyme (SOD), cultivars Lady Balfor and Diamond showed significantly increased of enzymatic activity due to (PVY) infection, compared with healthy ones. While cultivars Anabel, Sisi, Ditta, Execusa, Hermes, Valor and Spunta was the increase in enzymatic activity were not significant, while both Nicola and Lady Rosetta cultivars did not show any changes enzymatic activity of superoxide dismutase.

Table 4. Effect of PVY infection on oxidative enzymes (superoxide dismutase, catalase, peroxidase and polyphenol oxidase) of shoots of Potato cultivars by tow way analysis of variance (ANOVA).

Cultivar			**Polyphenol oxidase**	**Peroxidase**	**Catalase**	**Superoxide Dismutase**
PVY resistant cv.	Diamond	H	0.37±0.03	2.0±0.25	6.25±1.25	2.5±0.5
		I	0.37±0.03 **NS**	2.5±0.25 **NS**	6.25±1.25 **NS**	5.5±0.5 **S**
	Execusa	H	0.3±0.07	2.75±0.25	16.25±1.25	6.5±0.5
		I	0.57±0.07 **S**	2.5±0.25 **NS**	18.75±2.2 **NS**	7.0±0.5 **NS**
	Hermes	H	0.37±0.09	1.25±0.25	8.75±0.5	5.0±0.5
		I	0.57±0.12 **S**	0.75±0.00 **S**	21.25±1.25 **S**	6±0.00 **NS**
	Valor	H	0.1±0.06	1.5±0.00	16.25±1.25	2.5±0.5
		I	0.3±0.06 **S**	3.5±0.25 **S**	7.5±0.00 **S**	3.0±0.00 **NS**
PVY tolerant cvs.	Sisi	H	0.43±0.03	2.0±0.25	12.5±1.25	2.0±0.25
		I	0.77±0.03 **S**	0.83±0.08 **S**	7.5±2.16 **S**	2.5±0.5 **NS**
	Lady Balfor	H	0.27±0.03	1.5±0.00	3.75±0.00	5.0±0.5
		I	0.1±0.00 **S**	0.75±0.00 **S**	12.5±1.25 **S**	8.0±0.5 **S**
	Nicola	H	0.4±0.03	1.0±0.25	10±1.25	5.5±0.5
		I	0.3±0.06 **NS**	2.75±0.25 **S**	10±1.25 **NS**	5.5±0.5 **NS**
	Ditta	H	0.37±0.03	4.25±0.25	16.25±1.25	2.5±0.5
		I	0.3±0.03 **NS**	4.5±0.25 **NS**	21.25±1.25 **S**	3.5±0.5 **NS**
	Lady Rosetta	H	0.73±0.03	1.5±0.00	12.5±1.25	3.5±0.5
		I	0.67±0.07 **NS**	2.25±0.00 **S**	16.25±1.25 **S**	3.5±0.5 **NS**
PVY susceptible cvs.	Anabel	H	0.37±0.03	3.0±0.43	5.0±1.25	5.5±0.5
		I	0.27±0.03 **NS**	3.25±0.3 **NS**	8.75±1.25 **S**	6.5±0.5 **NS**
	Spunta	H	0.67±0.03	1.0±0.25	3.75±0.00	7.0±0.5
		I	1 ± 0.06 **S**	2.5±0.25 **S**	10±1.25 **S**	7.5±0.9 **NS**

Each value is the mean of 4 replicates

±= standard error of means, **H**= Healthy plants, **I**= Infected plants, **NS**= Non significant, **S**= Significant at $P<0.05$

The results revealed also that, in catalase (CAT) activity of cultivars Anabel, Lady Balfor, Ditta, Hermes, Valor and Spunta showed a significant increase in enzymatic activity as a result of (PVY) infection, compared with healthy plants. While increased in Execusa cv. enzymatic activity was not significant. Both Diamond and Nicola did not show any changes in enzymatic activity of (CAT) enzyme in infected plants comparing with healthy ones. On the contrary, both Sisi and Valor cultivars showed significant decrease in enzymatic activity of infected plants (Table 4).

The difference in the enzymatic activity of peroxidase enzyme (POX) as a result of (PVY) infection, led to a significant increase in the peroxidase activity in cultivars Nicola, Lady Rosetta, Valor and Spunta,. Whereas, Anabel, Diamond and Ditta cultivars showed non-significant increase in the peroxidase activity, compared with healthy plants. On the contrary, cultivar Sisi, Lady Balfor and Hermes showed significant decrease in (POX) activity of infected plants, while, non-significant decrease in enzymatic activity of infected plants of Execusa cultivar was observed.

On the other hand, table (4) revealed that, at cultivars Sisi, Execusa, Hermes, Valor and Spunta, significant increase in the of polyphenol oxidase activities were recorded in response to (PVY) infection, compared with healthy ones. In the contrast, Lady Balfor cultivar only showed significant decrease in (PPO) activity of infected plants, while, non-significant decrease in enzymatic activity of infected plants of cultivars Anabel, Nicola, Ditta, and Lady Rosetta was observed. Whereas Diamond cultivar did not show any changes in the enzymatic activity of polyphenol oxidase.

Biochemical markers

Protein analysis

SDS-PAGE revealed a total number of 25 bands with different molecular weights (MWs) ranged from about 8 to 250 KDa as shown in Fig. (3) and table (5). The potato cultivars (healthy and infected) varied in number and density of protein bands. Among such protein bands, 18 specific bands were polymorphic with 72% polymorphism which varied in PVY infected or healthy ones. While 6 protein bands were commonly (monomorphic) with MWs (100, 75, 50, 35, 15 and 10) KDa with percentage 24% related to potato plants. On the other hand, the unique band appeared only in infected plants of Valor cv. with MW (85) KDa. The total protein bands of eleven healthy potato cultivars were varied in number, whereas Ditta revealed the highest total number with 18 bands followed by Valor and Sisi with 17 and 16 bands respectively, each of Anabel, Diamond, Hermes and Lady Rosetta showed similar number with 15 bands, Spunta and Lady Balfor displayed 14 and 13 bands, respectively. While Execusa displayed the lowest number with 12 bands were shown. It is interest to note that, the total number of protein bands in the PVY infected plants were increased comparing with the healthy plants. For instance, they were increased six bands in Sisi, five bands in Hermes, three bands in Lady Balfor, Execusa, Lady Rosetta and Valor. Moreever, each of the two remainder pairs displayed similar number of increasing total bands, whereas Anabel and Nicola displayed two increasing bands and Diamond and Spunta showed similar number of increasing band with one band.

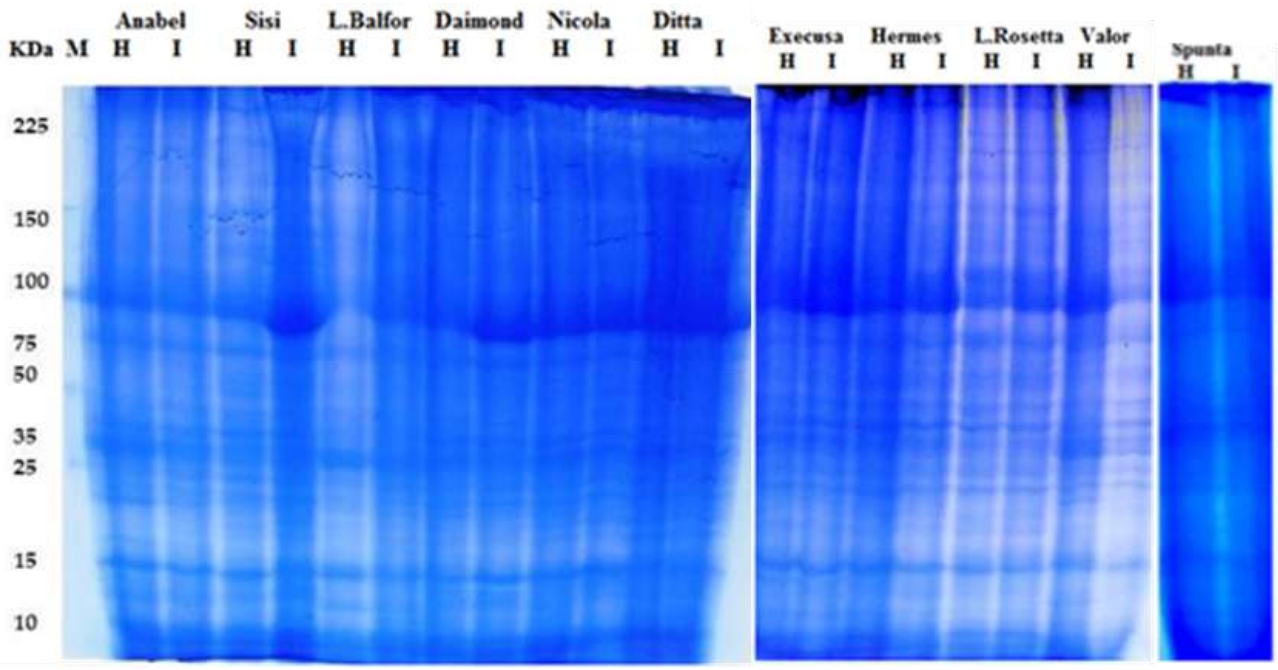

Figure 3. SDS-PAGE (12%) of total protein fraction for healthy and PVY infected leaves of eleven potato cultivars

Oxidative Isozyme Profile

Peroxidase (POX) isozymes

Peroxidase isozyme (POX) analysis displayed a total of 29 bands at different RF values varying from 0.52 to 0.92 (Fig. 4). It is interest to note that the total number of peroxidase bands in infected plants of eleven cultivars was varied considerably by increasing, decreasing or no changes comparing with healthy plants as shown in table (5). For instant, the PVY infected plants were increased three bands in Diamond and one band in each of Execusa, Hermes, Valor and Spunta. While they were decreased in both of Lady Balfor and Nicola by one band. While Anabel, Sisi, Ditta and Lady Rosetta displayed the same number of total bands.

Polyphenol oxidase isozymes

Polyphenol oxidase isozyme (PPO) analysis displayed a total of 29 bands at different RF values varying from 0.46 to 0.96. It is interest to note that the total number of polyphenol oxidase bands in infected plants of eleven cultivars were varied considerably by increasing, decreasing or no changes comparing with a healthy plants as shown in Fig. (5) and table (5). For instant, the PVY infected plants were increased one band in Anabel, Diamond, Execusa, Lady Rosetta and Valor. While were decreased in both of Ditta and Sisi by two and one band, respectively. While Lady Balfor, Nicola, Hermes and displayed the same number of total bands.

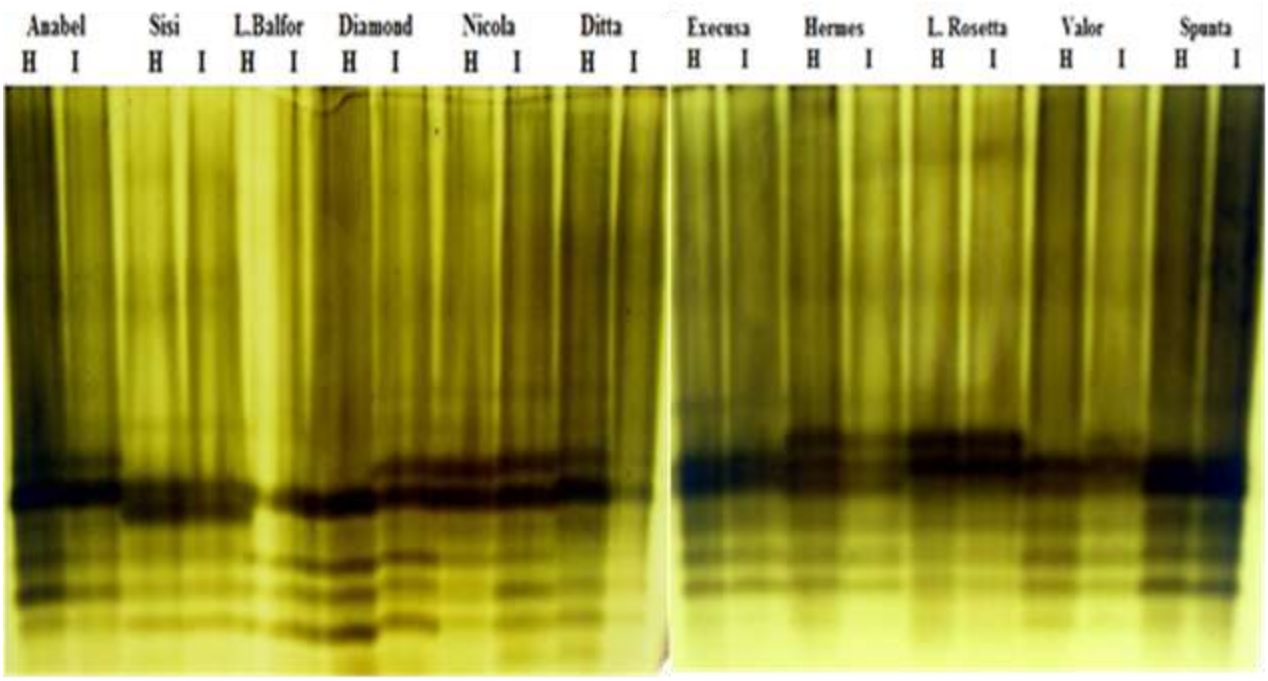

Figure 4. Peroxidase (POX) isozymes profiles of eleven potato cultivars

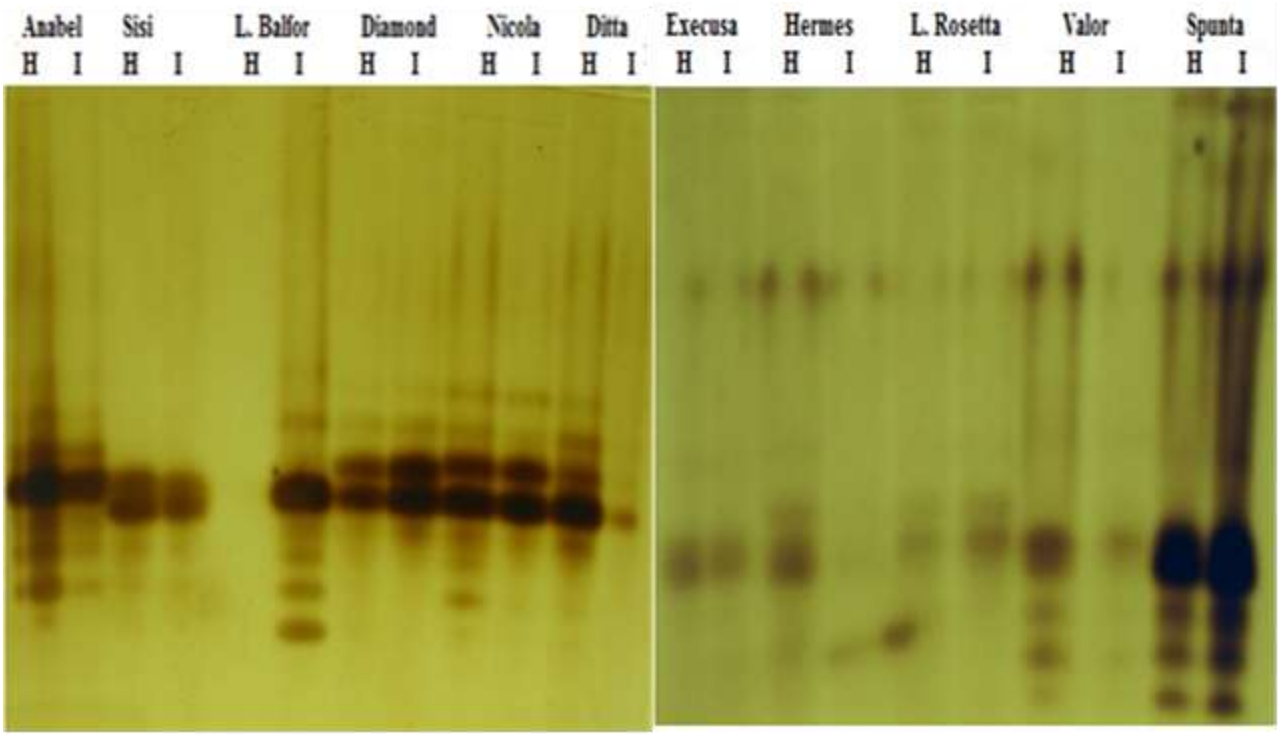

Figure 5. Polyphenol oxidase (PPO) isozymes profiles of eleven potato cultivars

Molecular characters of *Ry$_{adg}$* gene

The total RNAs prepared from 11 cultivars were reverse transcribed using ***Ry$_{adg}$*** antisense primer (5′-TTGTGTCTAATTGCCGTGGA-3′) primer. The ***Ry$_{adg}$***-cDNA was amplified from RNAs

extracted from 11 cultivars leaves using one step PCR-technique. The resulting complementary DNA (1 µl of cDNA) was mixed with PCR reaction mixture, taq DNA polymerase and (sense and antisense) primers directly. RT-PCR was used to amplify a fragment of about 229 bp corresponding to the ***Ry$_{adg}$*** in 7 cultivars only (Execusa, Lady Rosetta, Diamond, Anabel, Hermes, Ditta, and Nikola) (Fig. 6). On the other hand, 4 cultivras gave –ve results indicating the absence of ***Ry$_{adg}$*** gene in these cultivars (i.e. Valor, Sisi, Lady Balfor and Spunta) (Fig. 6).

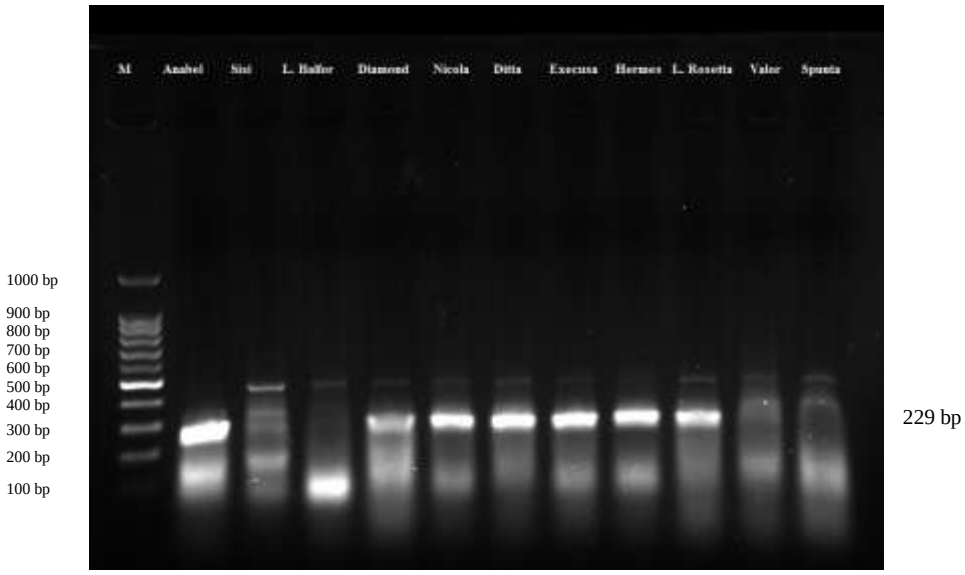

Figure 6. 1% Agarose gel electrophoresis showing *Ry$_{adg}$* RT-PCR product amplified from total RNA extracted from 11 potato cultivars using sense and antisense primers

The partial nucleotide sequence of the PCR-amplified fragment the ***Ry$_{adg}$*** was done to determine the relationship with other recommended ***Ry$_{adg}$*** registered in GenBank. The sequencing was done from the forward direction using Macrogen 3730XL6-1518-009, Korea. Nucleotides were found to be 229 bp corresponding to ***Ry$_{adg}$***. The predict numbers of amino acids were produced from translation of partial ***Ryadj*** gene nucleotide sequence were 75 amino acids starting with Leucine. The partial nucleotide sequence and amino acids sequence of ***Ry$_{adg}$*** gene were aligned with two genes registered in GenBank which are: Potato gene Y-1 isolated from *Solanum tuberosum* subsp. *andigenum* (Accession no. AJ300266), and TMV resistance protein N-like isolated from *Solanum tuberosum* (Accession no. XM_006352385) (Figs. 7 & 8).

A phylogenetic tree of ***Ry$_{adg}$*** gene based on the nucleotide sequence and the amino acids sequence revealed 92% and 89%, respectively a moderate degree of similarity to Potato gene Y-1 isolated from *Solanum tuberosum* subsp. *andigenum* (Accession no. AJ300266) (Fig. 9).

Table 5. Expressed proteins of potato cultivars under PVY infection

Potato Cultivars / Expressed Protein	Anabel		Sisi		Lady Balfor		Diamond		Nicola		Dilta		Execusa		Hermes		Lady Rosetta		Valor		Spunta	
	H	I	H	I	H	I	H	I	H	I	H	I	H	I	H	I	H	I	H	I	H	I
Protein pattern	15	17	16	22	13	16	15	16	15	17	18	18	12	15	15	21	15	18	17	20	14	15
POX-isozymes	5	5	5	5	5	4	4	7	8	7	7	7	5	6	5	6	6	6	4	5	4	5
PPO-isozymes	4	5	4	3	3	3	4	5	7	7	6	4	1	2	4	4	3	4	4	5	4	4

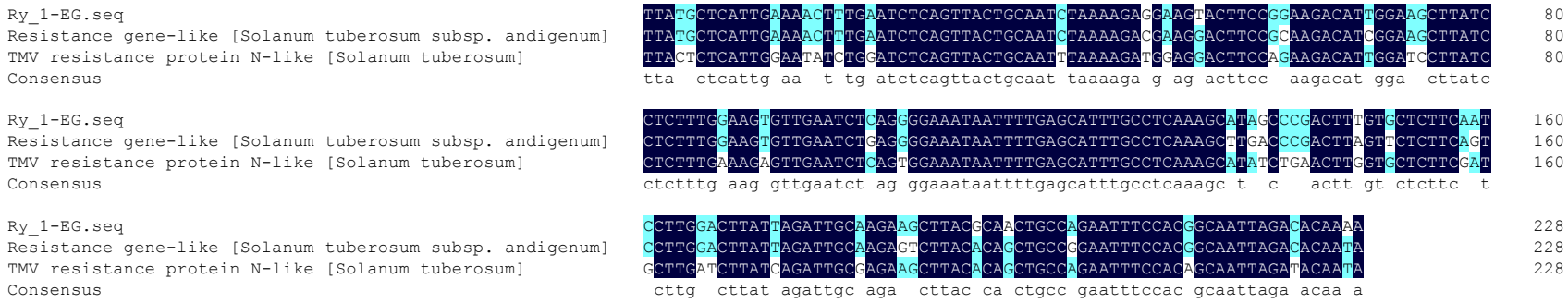

Figure 7. Multiple sequence alignment of the partial nucleotide sequence of the *Ry*$_{adg}$ gene with the corresponding sequence of two genes available in GenBank

Figure 8. Multiple sequence alignment of the partial amino acids sequence of the *Ry*$_{adg}$ gene with the corresponding sequence of two genes available in GenBank

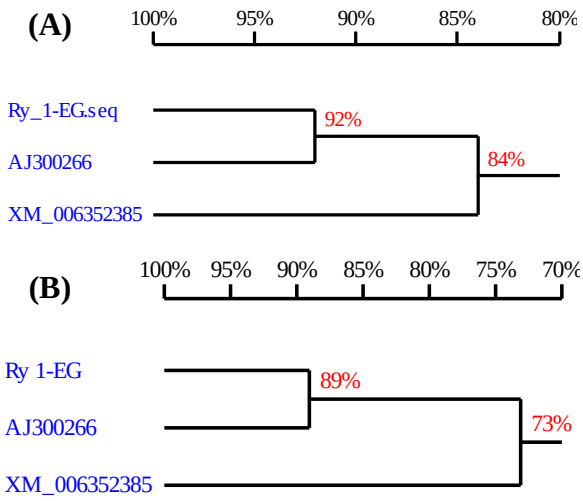

Figure 9. Phylogenetic tree of Ry_{adg} gene based on the nucleotide sequence (A) and amino acids sequence (B) displaying the percentage of sequence homology between the Ry_{adg} gene and the two other genes published in GenBank.

DISCUSSION

Potato virus Y spread in Egypt (Mahfouze *et al.*, 2004) and all over the world (Glais *et al.*, 2002; Quintero-Ferrer and Karasev, 2013). It causes economically considerable losses in several solanaceous crops (De Bokx and Huttinga, 1981).

The PVY Egyptian isolate-infected pepper plants, which gave +ve results with polyclonal antibody by DAS-ELISA was isolated on healthy *Chenopodium amaranticolor* L. where gave chlorotic local lesions (De Bokx *et al.*, 1975; Mahfouze *et al.*, 2004).

The potato cultivars were inoculated with PVY-EG (pepper-Fayoum) showed different susceptibility based on external symptoms and ELISA test. Spunta and Anabel cultivars were susceptible. Top necrosis symptoms produced in Spunta and Anabel cultivars constitute a hypersensitive reaction (Mahfouze *et al.*, 2004). Sisi, Lady Balfor, Nikola, Ditta and Lady Rosetta cultivars were tolerant. Vein clearing and veinal necrosis in these cultivars connstitute a moderate sensitive reaction (Singh *et al.*, 1994).

Total protein levels were differed in potato cultivars. Since virus infection unexpectedly increased the incorporation of amino acids into proteins, which was consistent with the reduced amino acid accumulation. It is probably that virus infection increased protein accumulation via the incorporation of amino acids rather than via the carbohydrates translocation. This cannot confirm the explanation of Prasad *et al.* (1985) in which they attributed increase protein content partly due to proteinous nature of virus itself. A decrease of total soluble proteins due to viral infection has been reported by (Taiwo and Akinjogunla, 2006). Plant acclimation to stress is associated with profound changes in their proteome and metabolome. Since proteins are directly involved in plant response to biotic stresses, proteomics studies can significantly contribute to unravel the possible relationships between protein abundance and plant pathogen interactions (Moshe *et al.*, 2012).

Scavenging enzymes (Superoxide dismutases, Catalases, peroxidases and polyphenol oxidase) were increased mostly in tolerant and resistant cultivars compared to susceptible cultivars and healthy ones. Since many defense enzymes are involved in defense reaction against plant pathogens. These include oxidative peroxidase (PO) and polyphenol oxidase (PPO), which catalyze the formation of lignin and other oxidative phenols that contribute to the formation of (defense barriers for reinforcing the cell structure (Avdiushko *et al.*, 1993). The induction of antioxidant enzymes, such as SODs, POXs and CATs is the most common mechanism for detoxifying ROS synthesized during stress response in order to protect cellular membranes and organelles from the

damaging effects of ROS (reactive oxygen species) (Mittler, 2002; Lee, 2007; Kumar *et al.*, 2009). Virus infection appears to stimulate peroxidase activity in all hosts in which necrotic or chlorotic symptoms are induced, the degree of stimulation correlating with severity of symptoms (Wood, 1990).

Total phenols were increased in tolerant (Sisi, Lady Balfor, Nikola, Ditta and Lady Rosetta) and resistant (Diamond, Execusa, Hermes and Valor) potato cultivars compared to susceptible cultivars (Spunta and Anabel) and healthy ones. These results in agreement with (Raj *et al*., 2005; Treutter, 2006; Kumar *et al*., 2010), they reported that phenols play an important role in host pathogen interaction, disease development and defense reaction of infected plants. Hence, the increased quantity of phenolics in the infected parts of pumpkins presumably appears to contribute towards the resistance against viral infection. Phenolic compounds including flavonoids are structurally diverse group of plant secondary products that can play a variety of roles in plant defense against pathogens, such as phytoanticipins, phytoalexins, structural barriers and activators for plant defense genes (Junqueria *et al*., 2004; Treutter 2006). The antioxidant activity of phenolic compounds can play an important role in neutralizing ROS (Zheng and Wang, 2001).

Also, proline was increased in tolerant (Sisi, Lady Balfor, Nikola, Ditta and Lady Rosetta) and resistant (Diamond, Execusa, Hermes and Valor) potato cultivars compared to susceptible cultivars (Spunta and Anabel) and healthy ones. Since increased levels of proline in plants correlate with enhanced stress tolerance (Ashraf and Foolad, 2007; Mazid *et al.*, 2011). Proline and other amino acids are the most common compatible solutes that occur in a wide variety of plants. They provide protections against stress by maintaining redox homeostasis, scavenging free radicals (Chen and Dickman, 2005).

The current study suggested that PVY-EG infection in hypersensitive host induced oxidative stress which enforces the host to produce high level of reactive oxygen species (ROS) where ROS play a central role in plant pathogen defense.

The SDS-PAGE protein profiles of total proteins extracted from leaves (healthy and infected) of eleven potato cultivars showed differences in band patterns. These results were similar to (Chaudhry *et al*., 1994; Mahfouze *et al*., 2004). Van Loon and Van Kammen (1970); Van Loon and Van Strien (1999) showed that viral infection of tobacco induced the accumulation of a distinct set of proteins, called pathogenesis-related proteins (PR proteins).

The highest POX activity appeared in infected plants of Diamond (three bands), the lowest POX activity showed in Execusa, Hermes, Valor and Spunta (one band). While both of Lady Balfor and Nicola decreased by one band comparing with healthy ones, whenever the other cultivars were equally in number of bands. Otherwise, the PPO activity appeared in infected plants of Anabel, Diamond, Execusa, Lady Rosetta and Valor (one band), while both of Ditta and Sisi decreased by two and one respectively, whenever the Lady Balfor, Nicola, Hermes and Spunta were equally in number of bands. These results were in an agreement with Neog *et al*. (2004) who mentioned that enzymes control biochemical reactions, and their synthesis are under the control of specific gene, any changes in the activity of an enzyme would reflect the pattern of gene expressions and corresponding metabolic events in the cell. Hence, enzymes can be used as tool to study the induced responses of plants showing disease symptoms at biochemical level. Differences in the isozymes binding pattern

are due to variation in the amino acids content of the molecule, which in turn is dependent on the sequence of nucleotides in DNA. Different bands obtained indicate different electrophoretic mobilities of the isozymes, which are coded by different alleles or separate genetic loci (Chittoor *et al*., 1998). Ali *et al*. (2006) suggested that the increased POX activity following virus infection was a reflection of physiological changes associated with, but not responsible for, induced resistance whereas up-regulated peroxidase might be responsible for growth reductions and malformations in virus infected plants. Also, Sofy *et al*. (2013) found that the polyphenol oxidase (PPO) activity was varied among eight wild potato species inoculated with PSTVd-EG and the control. On the other hand the PPO isozymes profiles showed differences in banding patterns compared with healthy plants ones.

The presence of the gene has been observed in Anabel, Diamond, Nicolas, Dita, Execusa, Hermes and Lady Rosetta, while the gene absent in Sisi, Lady Balfor, Valor and Spunta. The nucleotide sequence of the PCR-amplified fragment for the $\boldsymbol{Ry_{adg}}$ of cultivars Dita, Execusa and Lady Rosetta was done to determine the relationship with other recommended PVY strains registered in GenBank. A phylogenetic tree of $\boldsymbol{Ry_{adg}}$ gene based on the nucleotide sequence and the amino acids sequence revealed 92% and 89%, respectively a moderate degree of similarity to Potato gene Y-1 isolated from *Solanum tuberosum* subsp. *andigenum* (Accession no. AJ300266) (Mahfouze, 2009).

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