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ROLE OF REACTIVE OXYGEN SPECIES AND ANTI-OXIDANTS IN HYPERSENSITIVE LOCAL VIRUS-INFECTED PLANTS

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ABSTRACT

Oxidative stress in compatible virus-host plant interactions was studied in virus-inoculated *Nicotiana glutinosa* plants. Leaves virus-infected plants showed highly increase in lipid peroxidation of polyunsaturated fatty acids indicating an advanced disintegration of membranes as elucidate by scanning electron microscope (SEM). A chlorotic (chlorophyll reduction) appeared 2-4 days after inoculation followed by necrotic was observed on inoculated leaves. Radical intermediates formed during lipid peroxidation co-oxidize both photosynthesis pigments and phenolic molecules, thus it might account for virus-induced yellowing and brownish symptoms.

Furthermore, in local lesion infected plants change of enzyme activities involved in the detoxification of reactive oxygen species (catalase (CAT), peroxidase (POD) and polyphenoloxidase (PPO)) was observed. After infection catalase activity was declined, while peroxidase and polyphenoloxidase activities were increased. SDS-PAGE reveals five high density proteins pattern and new proteins detected in infected leaves, may function as radical scavengers and catalyze the formation of H_2O_2. Thus it can be presumed that the enhancement of peroxidases contributes to the oxidative stress in plant and virus interactions. Moreover, infected leaves showed elevation in phenol, salicylic, gallic, T-cinnamic acid and p-hydroxy benzoic acids, besides, appearance of Kaempherol and hydroquinone as compared with healthy plants. These increases in antioxidant enzymes, phenolic compounds and high level of malonic dialdehyde (MDA) may inhibit virus infection. In addition, phenolic compounds led to increase the ability of the plant to scavenge reactive oxygen species may hinder virus replication.

INTRODUCTION

Production of activated oxygen species (O_2^- superoxide anion, OH hydroxyl radical & H_2O_2 hydrogen peroxide) is one of the biochemical changes possibly occurring when plants are subjected to harmful stress. The chloroplasts and mitochondria of plant cells are important intracellular generator of activated oxygen species can seriously disrupt normal metabolism through oxidative damage of lipids (Fridovich, 1986; Wise, and Naylor, 1987), protein (Davie, 1987; Halliwell, and Harvey 1995) and nucleic acids (Fridovich, 1986; Imlay and Linn 1988). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage (Wise, and Naylor, 1987; Young and Jung 1999). Plants have evolved specific protective mechanisms, involving antioxidant molecules and enzymes that can help prevent the cellular damage caused by free radicals and defend themselves against oxidants.

Polyphenols play a vital role in the growth and propagation of plants and protect plant tissue from damage (Gutmann and Feucht, 1994). They neutralize free radicals and thus protect biologically vital molecules from

oxidation. Polyphenols are a part of a complex immunity system, which can be acquired in the tissues under stress (Feucht, 1994).

Individuals infected with a virus often have damaged or deficient antioxidant systems. This can result in an excess of free radicals (molecules that compromise the integrity of cell walls and leave them vulnerable to viruses). The plants cannot, contrary to animals, synthesize antibodies but they can produce numerous substances phytoalexins. Those are secondary metabolites, which inhibit and kill pathogenic organisms (Bennett and Wallsgrove, 1994). In addition, polyphenols protect plants against insects and herbivorous mammals (Harborne, 1995). The response to fungus diseases and bacterial diseases in grapevine has been well investigated (Robert *et al.*, 2001). The species of the genus Vitis, which contain larger amounts of polyphenols (e.g.Vitis rotundifolia) are more resistant to infections caused by downy mildew (Plasmopara viticola Berl. & de Toni) and when infected they produce larger amounts of polyphenols than varieties which are more susceptible (Dai *et al.*,1994). A positive correlation between the content of catechin in grape and resistance to grey mould (Botrytis cinerea Pers.) has been discovered (Goetz *et al.*, 1999).

However, Kaur *et al.*,1989; Kaur *et al.*1991;Baruah and Chowfla,1994 suggest that there are high contents of polyphenols present in healthy plants.High content of secondary metabolites in healthy plants protect the plants from infection. On the contrary, Kumar (1991), Sharma and Chowfla (1991) as well as Suresh *et al.* (1991) state that there are higher amounts of total polyphenols in virus infected plants. By way of oxidation of indole-3-acetic acid upregulated peroxidases might also be responsible for growth reductions and malformations in virus-infected plants (Riedle-Bauer 2000).

The present study was conducted to elucidate the correlation between the antioxidants system (antioxidant enzymes peroxidase, polyphenoloxidase, catalase), beside total phenols, leaves soluble protein, and the photosynthesis pigments change with formation local lesion. Also, lipid peroxidation malonic dialdehyde (MDA) and the scanning of *N.glutinosa* leaf infected, beside the changes in protein pattern in both healthy and infected leaves. In addition, the influence of virus on the content of polyphenolic compounds in plants by HPLC analysis after plant infected with virus was also studied. To demonstrate the role of reactive oxygen species in this damage and the mechanism of how could the plants stopping or decrease this harmful, to established a system whereby the resistance to ToMV can be measured and interpret the defense mechanism.

MATERIALS AND METHODS

Host plant and Virus inoculation

The host plant used was *Nicotiana glutinosa* L, which react with *Tomato mosaic tobamovirus* (ToMV) local lesions. The plants were grown under greenhouse conditions. When the plants had 5-6 full expanded leaves, they were trimmed to 2 leaves, the third and fourth from the soil line. These two leaves were inoculated mechanically with ToMV (diluted with distilled water to 10^{-2}). The inoculated plants were kept in greenhouse at 28±2 °C for

7 days. The host plants and ToMV was obtained from Virology Lab. Fac.Agric.Ain Shams Univ.

Scanning Electron Microscope (SEM)

For electron microscopy rectangular pieces, ranging in size from 4 to 25 mm^2 of the experimental and control tissues were excised from fully expanded leaves submerged in cold glutaraldehyde (2.5%) for 24 hours at 4°C,then post-fixed in 1% osmeium tetraoxide for 1 hour at room temperature (Harley and Fergusen 1990). The specimens were then dehydrated with path through ascending concentration of acetone. The samples were dried till critical point and finally sputter coated with gold. The examination, measurements and photographing were done through Jeol Scanning Electron Microscope (JSM-T330A) equipped with image recorded and processing system (Semafore), Central Lab., Fac. Agric., Ain Shams Univ.

Biochemical and Physiological analysis

Lipid peroxidation as Malonic dialdehyde (MDA) was measured as described by Heath and Packer (1968). Protein determination was accomplished according to Bradford (1976) using bovine serum albumin as standard. Enzyme extraction and assay (Peroxidase and polyphenoloxidase) activity was determined according to the methods described by Sadasivam and Manickam (1992). Catalase assay was performed according to the method reported by Sinha, (1971). Chlorophyll a, b, total Chlorophyll and Carotenes were estimated according to the method mentioned Jayaraman (1985). Total polyphenols were assayed as mentioned by Malik and Singh (1980).

HPLC analysis

Phenolic extracts intended for the HPLC analysis were prepared according to the method applied by Candela et al., (1995). Phenolic compounds analysis was conducted with HPLC apparatus Hewlett-Packard HP (model 1100) Liquid Chromatograph, with a reverse-phase hypersil C-18 (5 µm packing, 4.6x250 mm) column. The HPLC connected to UV/VIS detector. HPLC grade solvent used as follow: solvent A, 0.5% acetic acid in bidistilled water at pH 2.6, solvent B, 0.5% acetic acid in 99.5% acetonitrile. Gradient development was linear starting with A and ending with B during 50 min. at flow rate 0.3 ml/min, with detector wave length 254 nm, and injection volume 5 µl. in the Lab of General Organization for Agricultural Equalization Fund (G.O.A.E.F).

SDS-PAGE

Sodium dodocyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% (w/v) slab gels containing SDS (Laemmli, 1970) as modified by Studier (1973). The gels were cross-linked with 0.3% (w/v) N.N-methylene bisacrylamide at pH 8.3 and stacking gels were made 5.0% (w/v) polyacrylamide at pH 6.8. Samples (50 µl) were denaturated by heating at 100 °C for10 min in 1% SDS containing 2-mercaptoethanol. Molecular weight of the protein was estimated from a low molecular weight standard (M.W. range from 14 to 96 kDa Pharmacia Montreal).

Statistical analysis.

Statistical analysis were estimated using student's t-test ($p<0.05$).

RESULTS

Morphological of local lesion

The virus, ToMV was inoculated on indicator host, *N.glutinosa*. Data showed necrotic local lesions appeared after 2-4 days. It is circular necrotic local lesion (1-2 mm diameter) appeared which are then turned into red-brownish spots with necrotic center and chlorotic margin surrounded by yellow hallo (Fig. 1).

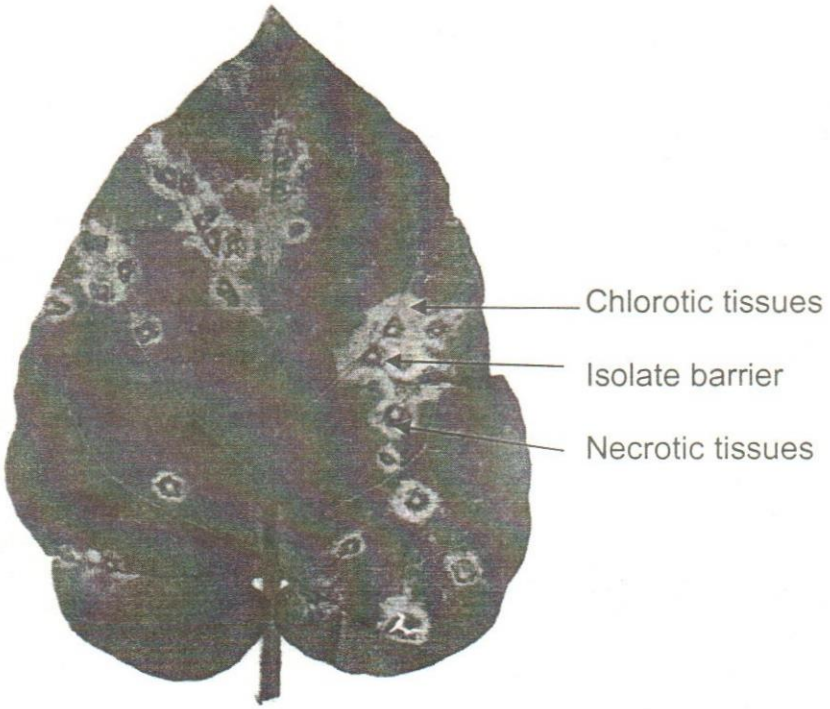

Fig. (1): Infected leaf of *N. glutinosa* inoculated with *Tomato mosaic tobamovirus* (ToMV).

Scanning Electron Microscope Examination (SEM)

SEM was done to study the effect of virus infection on indicator host epidermal tissue cells of ToMV infected *N.glutinosa* leaves and it was illustrated in Fig. (2). Scanning electron microscopy (SEM) was carried out on local lesion with healthy tissue specimens. The blade of infected leaf appears slight downward lesions shows clear bulging from the upper and lower sides. The hairs appear disrupted from the upper epidermal cells. It was shown that the epidermal cells of healthy tissues were turgid and normal in shape with wavy walls. Many simple, multicellular and glandular hairs appeared. The stomata were numerous and appeared closed or slightly opened. The epidermal cells of the infected tissues (local lesion) appeared shrinking with

clearly broken, deformed hairs and widely opened stomata. The epidemal cells surrounded by local lesions were broken and deformed beside formed isolated layer between healthy and infected tissues (Fig. 2).

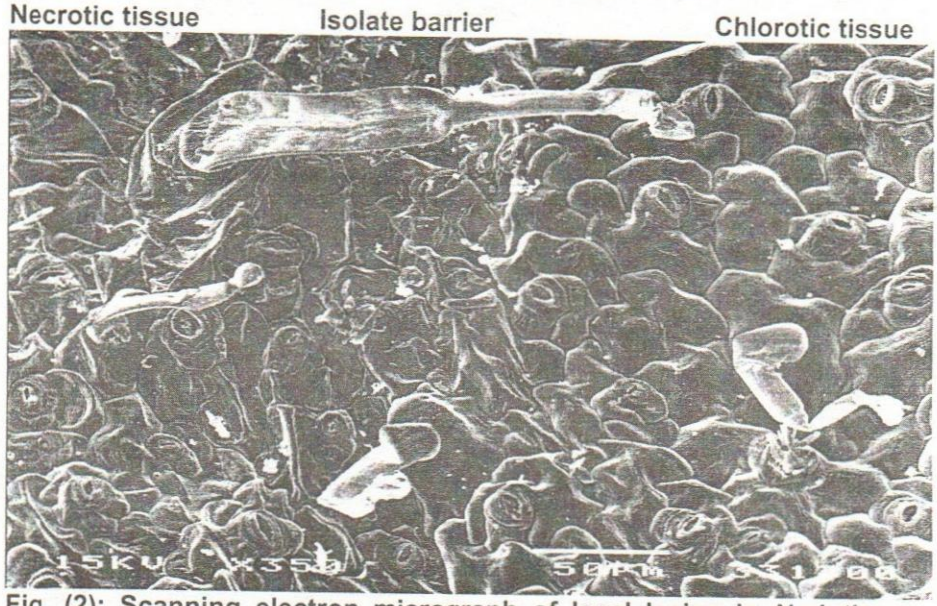

Fig. (2): Scanning electron micrograph of local lesion in *N.glutinosa* inoculated with *Tomato mosaic tobamovirus* (ToMV).

Biochemical and Physiological analysis

Chlorophyll a, b and Carotenes.

Data in Table (1) revealed that chlorophyll a,b and total chlorophyll were decreased while carotenes content were increased in infected leaves compared with healthy leaves.

Table (1): Biochemical alteration in chlorophyll & carotenes as affected by ToMV virus inoculated in leaves of *N. glutinosa*.

Parameter	Total chlorophyll (mg/100 g fw)	Chlorophyll a	Chlorophyll b	Carotenes (g/100 g fw)
Healthy leaves	150.94 ± 3.82	85.57 ± 2.5	65.37 ±1.98	3.15 ± 0.42
Infected leaves	89.23 ± 2.95	45.95 ± 1.87	43.28 ±1.36	8.16 ± 0.90
Alteration %	40.89 ↓	46.31 ↓	33.80 ↓	259.10 ↑

All data were express as mean ± standard error. fw = fresh weight.

Despite healthy plant has larger amount of Chlorophyll a than b but chlorophyll a:b ratio was decreased to be equalized after infection. Moreover, the color becomes orange of carotene and yellow green of chlorophyll b. The carotenes pigments were increased to reach about three fold in infected leaves corresponding to healthy ones.

Lipid peroxidation

Lipid peroxidation is increased after infection with virus and the value obtained gives the highest value of MDA in infected leaves compared to healthy ones. The MDA level in infected leaves rose significantly to be eight fold of MDA in healthy plant leaves (Table 2).

Table (2): Effect of infection with virus ToMV on lipid peroxidation and antioxidants system in leaves of *Nicotiana glutinosa*.

Parameter	Healthy leaves	Infected leaves with virus ToMV	Changing Ratio Healthy:Infected
Malonic dialdehyde MDA (nmol/g fw)	72.58 ±5.26	580.65 ±9.38	1:8
Peroxidase activity (unit/g fw)	418.50 ±8.25	604.80 ±10.12	1:1.5
Polyphenoloxidase (unit/g fw)	51.40 ±3.20	68.30 ±2.88	1:1.3
Catalase activity (unit/g fw)	306.81 ±7.54	103.52 ±5.26	3:1
Soluble protein (mg/g fw)	38.82 ±2.32	12.75 ±1.93	3:1
Total phenols (mg/g fw)	8.916 ±1.22	8.749 ±0.95	1:1

All data were expressed as mean ± standard error.

Antioxidant enzymes and soluble protein

Data in Table (2) revealed that the activity of antioxidant enzymes (peroxidase and polyphenoloxidase was increased in infected leaves about one and half due to virus infection. Meanwhile, catalase activity was lowered. Also, leaves soluble protein was decreased to be 1/3 in infected leaves.

Antioxidant compounds, total polyphenols (mg/g fw)

The content of total polyphenols is slightly decreased in infected compared to healthy leaves.

HPLC analysis of phenolic antoxidant compounds.

HPLC analysis for phenolic compounds revealed the presence of about 18 phenolic compounds in healthy leaves compared to 20 compounds in infected leaves. Standard phenolic compounds were presented in Fig (3). The distributions and types between infected and healthy leaves in phenolic compounds was found and detected by HPLC analysis as it is shown in Table (3).

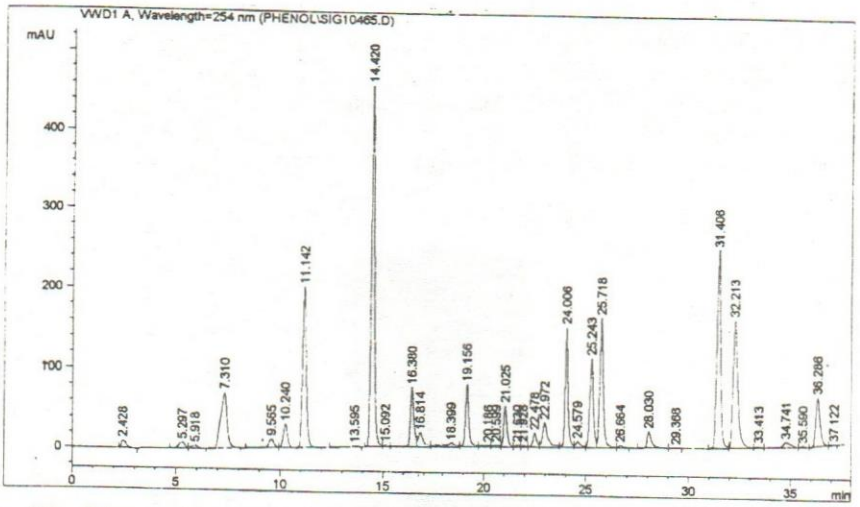

Fig. (3): Chromatogram of standard phenolic compounds achieved by reverse-phase HPLC. and its Retention time (Rt).

Pyrogallic acid 5.29, Hydroquinone 5.91, Gallic acid 7.31, Resorcinol 9.56, Protocatechuic acid 11.14, p-hydroxy benzoic acid 14.42, Chlorogenic acid 16.38, Catechin 16.81, Phenol 18.39, Vanillin 19.16, p-Coumaric acid 21.02, Ferulic acid 22.48, Salicylic acid 22.97, Rutin 24.01, o-Coumaric acid 25.24, Coumarin 25.71, Myricetin 28.03, Cinnamic acid 31.40, Quercetin 32.21, Kaempherol 36.28.

Table (3). HPLC analysis (%area) of extractable phenolic compounds for *N. glutinosa* in healthy and inoculated leaves with virus (ToMV).

Phenolic name	Healthy (%area)	Infected (%area)	Retention time (Rt)
Unknown	----	0.75	3.26
Pyrogallic acid	----	----	5.29
Hydroquinone	----	1.93	5.91
Gallic acid	1.48	12.30	7.31
Resorcinol	50.85	44.10	9.56
Protocatechuic acid	0.22	---	11.14
p-hydroxy benzoic acid	4.07	4.98	14.42
unknown	2.81	4.38	15.09
unknown	11.14	----	15.49
Chlorogenic acid	6.08	----	16.38
Catechin	0.71	----	16.81
Phenol	2.40	3.52	18.39
Vanillin	1.02	---	19.16
p-Coumaric acid	6.72	2.00	21.02
unknown	----	0.77	21.63
Ferulic acid	1.24	1.17	22.48
Salicylic acid	1.40	1.94	22.97
Rutin	2.78	2.78	24.01
o-Coumaric acid	----	----	25.24
Coumarin	----	----	25.71
Myricetin	----	----	28.03
Cinnamic acid	0.68	2.09	31.40
Quercetin	1.18	----	32.21
Unknown	----	3.06	34.74
Unknown	----	1.33	34.99
Kaempherol	----	6.73	36.28
Unknown	----	2.08	37.12
Unknown	----	0.87	38.17
Unknown	----	2.02	39.25
Unknown	----	0.66	39.70
Unknown	4.36	----	40.24
Unknown	0.86	----	40.65

Protein elctrophoresis (SDS-PAGE)

Related protein due to virus infection was extracted from infected tissues, separated by SDS-PAGE analysis and illustrated in Fig. (4). The obtained data in Fig. (4) show the proteins pattern from infected tissues which reveal presence of 5 high density proteins pattern in infected leaves compared to healthy tissues with molecular weight 90, 85, 70, 40 and 14 kDa.

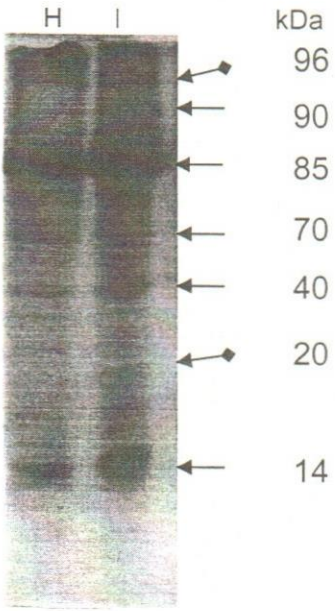

Fig. (4): SDS-PAGE for Healthy (H) and Infected (I) leaves of *N.glutinosa* inoculated with ToMV show new proteins (↔) and five high density proteins (←—).

DISCUSSION

Data obtained in Table (1) & Fig. (1) illustrate presence of chlorotic (chlorophyll reduction) appeared after 2-4 days of inoculation followed by necrotic on inoculated leaves. Biochemical analysis reveals lowering in chlorophyll a, b and total chlorophyll. Chlorophyll lowering was noticed more in a than b and the chlorophyll ratio a:b was decreased in infected leaves. Although in healthy plant chlorophyll a was more than b in general, but this ratio was decreased to be equal after infection, which mean that virus infection led to destroy chlorophyll a at the expense of b. The decrease in chlorophyll a/b ratio is considered to be a symptom of oxidative stress condition. This decrease in the ratio after virus infection might be due to the generation of reactive oxygen species (ROS) causing damage to chlorophyll a. That is mean the plant failed to capture the light and so photosynthesis will decrease or stopped. The decreasing in chlorophyll and the increasing in carotenes interpret the color change after infection. Now, the question, is the virus destroy chlorophyll a in its process and why? The color becomes

orange & yellow green of carotenes and chlorophyll b. This may be due to the virus try to stop photosynthesis which gives the plant resistance power, as phenolic compounds, antioxidant substances beside antioxidant enzymes which support the plant and give it the power to overcome the virus infection. On the other hand, the highest levels of MDA (Table 2) in infected leaves (580.65) corresponding to (72.58 nmol/g fw) in healthy leaves besides, decreasing in soluble protein, reveal rapid increase in free radical formation of reactive oxygen species which mean that, radical intermediates formed during lipid peroxidation co-oxidize both pigment and phenolic molecules, thus it might account for virus-induced yellowing and red-brownish symptoms.

These results are coincide with Hernandez *et al.*, (2004 & 2006) they reported that in infected plants, a decrease in the efficiency of excitation energy capture by PSII (Fv'/Fm') was observed, which was accompanied by a decrease in non-photochemical quenching (NPQ). p-Hydroxy-mercury benzoic acid (pHMB)-insensitive ascorbate peroxidase (APX) activity (class III peroxidase) was detected in both chloroplast and soluble fractions. According to these results, as a consequence of plum pox virus (PPV) infection, an oxidative stress, indicated by an increase in lipid peroxidation and in protein oxidation, was produced only in the leaves from the susceptible cultivar of peach, which was monitored by the diaminobenzidine (DAB) peroxidase-coupled H_2O_2 probe. PPV infection produced an alteration in chloroplast ultrastructure, giving rise to dilated thylakoid membranes. They suggest that long-term PPV infection produces an oxidative stress, and that an antioxidative metabolism imbalance may be related to the progress of plum pox virus (PPV) infection and symptoms in peach plants. The loss of PSII, indicative of activated oxygen species production, and the decrease in the levels of antioxidant enzymes in chloroplasts from susceptible plants could be responsible for the chlorosis symptoms observed.

Antioxidant enzymes peroxidase, polyphenol oxidase increased while catalase was decreased. High activity of peroxidase and polyphenol oxidase may be responsible for red-brownish color in infected leaves. In addition, protein electrophoresis for related protein virus infection extracted from healthy and infected tissues and subjected to SDS-PAGE analysis (Fig. 4) showed differences in density in protein pattern between infected and healthy leaves. It was found 5 high density proteins pattern in infected than healthy tissues with molecular weight 90, 85, 70, 40 and 14 kDa. beside two new proteins. This protein may be explained as defended proteins in infected leaves to stop virus infection in defence mechanism. Furthermore, lowering of catalase activity which means high level of H_2O_2 in infected cell led to trigger enhancement of peroxidase activity to remove this toxic metabolite, consequently, this higher concentration resulted in cell death due to imbalance in metabolism. Also, decreasing catalase activity may be attributed to high level of O_2^- and $^{\cdot}OH$ which affect on catalase active site. However, the new proteins and the high density proteins (5 patterns) may be function on removing H_2O_2 and produce more antioxidant substances like (salicylic acid in present study) to protect cell from metabolism imbalance.

A decrease in catalase was observed under virus infection may be responsible for virus inhibition, because catalase catalyze decoposation of

H_2O_2 which result from dismute of O_2^- by SOD so, the plant need to save high level from H_2O_2 to destroy the invaded virus and to oxidize phenolic compounds to make the complex polymers to isolate the virus in infected area. While peroxidase and polyphenoloxidase need co-factor to oxidize H_2O_2 and therefore POD & PPO are raised and CAT is lowered. The other interpretation for catalase lowering may be due to that some virus product block catalase gene expression in the host plant.

This data are in accordance with Hernandez *et al.*, (2006) who reported that, catalase decreased in the soluble fractions from both susceptible and resistant infected peach cultivars after plum pox virus (PPV) infection. However, Yourk and Marchall (2003) reported that phenolic compounds serve as substrate of oxidative enzymes to yield o-quinone which eventually polymerize in non-enzyme catalyzed reactions, resulting in brown pigments. On the other hand, Vandenabeele *et al.* (2004) reported that, in plants, hydrogen peroxide (H_2O_2) plays a major signaling role in triggering both a defense response and cell death. Increased cellular H_2O_2 levels and subsequent redox imbalances are managed at the production and scavenging levels. Because catalases are the major H_2O_2 scavengers that remove the bulk of cellular H_2O_2, therefore altering their levels allows in plant, a modulation of H_2O_2 concentrations. Also, Clarke, *et al.*, (2002) reported that catalase activity in leaves of *Phaseolus vulgaris* L. var. was declined after infection with a non-lesion-forming isolate of *White clover mosaic potexvirus* (WCIMV). They added that, plants treated with salicylic acid and jasmonic acid prior to WCIMV inoculation showed elevated catalase and peroxidase activity, and these treatments inhibited virus replication with enzyme activities remaining near control levels. They propose that a decline in free radical scavenging capacity may be required before a rapid increase in virus replication can take place. Treatments increasing the ability of the plant to scavenge reactive oxygen species may hinder virus replication.

Also, it was found that virus infection led to induce protein synthesis resistance to pathogens and the production of PR proteins (new host proteins, named pathogenesis related) in tobacco and other plants, can be induced by salicylic or acetylsalicylic acid (asprin), even in the absence of pathogenic organisms (Pennazio *et al.*, (1987). It has been claimed that salicylic acid act as the internal signal that triggers general resistance in plants and induces the expression of messenger RNAs, which presumably direct the synthesis of the PR proteins (Moffat, 1992). Yalpani *et al.*, (1991) showed an increase in endogenous salicylic acid level in tobacco mosaic virus, which caused a hypersensitive response with systemic induction of PR proteins. It is possible that salicylic acid is an endogenous messenger that activates important elements of host resistance to pathogens (Raskin,1992). Salicylic acid is thought to be involved in induced resistance but may not be the long distance signal (Raskin,1994).

Total polyphenols (mg/g fw) content is increased in healthy compared to infected leaves, in addition, the total polyphenols and types were changed. Consequently, it could be concluded that viral infections do not affect only on the synthesis of polyphenols but also have effects on the distribution type of polyphenols in an infected leaves (Table 3,& Fig. 5).

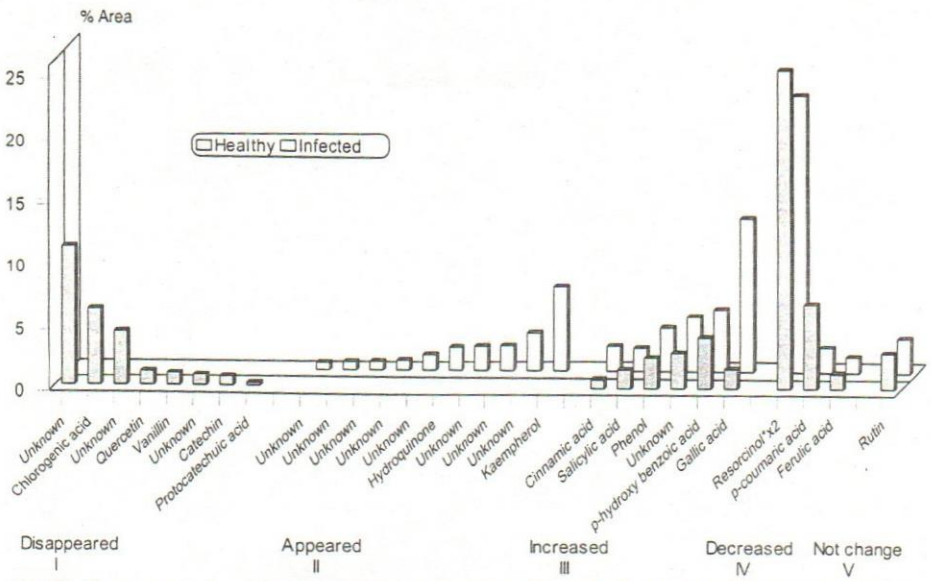

Fig.(5): Groups of phenolic compounds analyzed by HPLC in healthy and infected leaves of *N.glutinosa* inculated with *Tomato mosaic tobamovirus* (TOMV).

Effect of infection with virus on extractable polyphenols of *N.glutinosa*.

To establish the true influence of viruses on polyphenols it should be return to SEM (Fig. 2) and MDA (Table, 2) which revealed that the high content of MDA was reflected in the apparent damage in plasma membrane of infected leaves as cleared in SEM micrograph. It is obviously cleared that plant after infection with virus suffers from stress condition during infection period and plant goes to stop the invasion and the pathogen. However, it could be concluded that the profile of antioxidant system and phenolic compounds tended to change due to viral infections. Some phenolic compounds do not increase but others show increase and these might be responsible for the resistance in infected plant. In the present study, after infection with virus, phenolic compounds could be classified into five main groups, 1st, phenolic which disappeared, 2nd, appeared, 3rd increased, 4th decreased, 5th not changed (Fig.5). In addition, the present work illustrates the ability of plant to activate the defense reactions which determined its resistance. Certain phenolic compounds play a role in plant defense system as appeared in increasing the salicylic acid and chatechin in infected plant compared with the healthy control. Results of phenolic analysis revealed that gallic acids play a principal role in protecting the plant from pathogen as seen from its highest increasing from 1.48 to 12.30. The infected leaves, compared to healthy ones, exhibited more gallic acid compounds, and less in the control. Gallic acid differences between the healthy and infected leaves was observed in high content of gallic acid as analyzed by HPLC since it increased from 1.4 in control to 12.30% in infected plant, which may be defined as gallic acid indicator for virus infection (GAVI).

The results are in accordance with Candela *et al.* (1995) who revealed that soluble phenolic compounds have different resistance to infection, which appeared in qualitative and quantitative variation after inoculation and the most pronounced inhibitory effect was produced by T-cinamic acid, followed by p-hydroxy benzoic, vanillic and salicylic acids, in their study on soluble phenolic acids in *Capsicum annuum* stems infected with *Phytophthora capsici*. Furthermore, after infection the pathogen induces changes in the host plant, some of the changes in the infection zone suggest that phenolic compounds may be the first line of defence (Matern & Kneusel, 1988), either as toxic products (free phenolic acids or their oxidative products, Quinones) or as impedance in the individual tissues (Bhullar *et al.*,1972).

Index of vanilline:

With the index of vanilline the differences between the healthy and infected leaves was observed (in the content of low-molecular phenolic substances as analyzed by HPLC). The infected leaves, compared to healthy ones, exhibited more low-molecular phenolic compounds, and less in the control. This index was found in the present study where the chlorogenic and qurcetin with high molecular weight are disappeared, which mean that those phenolic compound are hydrolyzed to lower molcular weight

There was very significant variation between the phenolic acids analyzed. Caffeic acid was not detected, probably owing to its rapid transformation into ferulic acid (by methylation). Ferulic acid which forms covalent cross-links between wall polymers and restrict wall extensibility and digestiblity (Fry, 1987), disappeared in the infected tissues. This acid is also one of the precursors of lignin formation (Harborne, 1980) and might form vanillic acid by b-oxidation of its side chain. Vanillic acid is formed from vanillin by oxidation is consider as benzoic acid and T-cinnamic acid, the precursor of the other phenolic acids, they cause sever inhibition of fungal growth (Candela *et al.*, 1995). Certain phenolic compounds play a role in plant-pathogen interactions. The phenolic protocatechic acid has been cited as responsible for the resistance of pigmented onions to fungus *Colletotrichum circinans*, and chlorogenic acid is thought to be involved in the resistance of potato tubers to *Streptomyces scabies* (Dickinson & Lucas, 1987). Moreover, phenolic compounds have been linked with the resistance of *Eucalyptus marginata* to *Phytophthora cinnamomi* (Cahill *et al.*, 1993).

The current study suggest that virus infection in hypersensitive host induce oxidative stress which enforce the host to produce high levels from reactive oxygen species (ROS) that have high oxidative power activity to oxidize and attack any oxidisable thing in infected cell especially unsaturated fatty acids in plasma membrane for cell and included organelles, beside phenolic compounds in the cell. Once, ROS oxidize phenolic compound result in complex polymer in infected area (isolate barrier) that stop virus to transfer or move and infect the surrounding cells. In addition, destroying membranes of organelles i.e. lysozome cause flow out of its contents from hydrolytic enzymes which hydrolyze the bio-molecules in the cell thus, finally lead to cell death in infected area and make local lesion (red-brownish dead spot). Thus, increased cellular H_2O_2 levels and subsequent redox imbalances may be responsible for cell death in plants.

Conclusions

The examination of viruses influence on leaves show that viral infections have different effects on the plant than the infections caused by other pathogenic plants which induce production of antioxidant substances i.e. polyphenols. Pennazio and Roggero (1998) stated that there had not been a case of a plant acquiring resistance against systemic virus infection. Antioxidants are produced in viral infections only when the infected tissue becomes hypersensitive. Hypersensitivity does not disturb replication but it causes the decay of the tissue around the infection and thus limits the expansion into other cells. The infected tissue produces salicylic acid which induces the accumulation of lipid prooxidants through free radicals of salicylic acid. These oxidants induce the expression of defense genes (Anderson *et al.*,1998). It is also possible that in the place of infection oxidation conditions occur due to the reduction antioxidative enzymes activities. This triggers the accumulation of free radicals and the peroxidation of lipids that leads to the tissue decay. When the production of oxidized derivatives is enlarged the genes which code antioxidative enzymes are induced to limit the decay of the tissue around the necrosis (Fodor *et al.*, 1997).

Finally, there is a marvelous design might be need more study to discover the whole defense mechanism in virus-infection.

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دور شقوق الأكسيجين النشطة ومضادات الأكسدة فى النباتات عالية الحساسية للإصابة الفيروسية والمكونة للبقع الموضعية المحددة

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صممت هذه الدراسة لمعرفة الدور الذى تلعبه الشقوق الحرة ومضادات الأكسدة فى الحد من الإصابة الفيروسية للنبات . فتم اختيار نبات *N.glutinosa* (عائل حساس لفيروس تبرقش الطماطم ToMV) حيث أحدثت إصابة للنبات بفيروس تبرقش الطماطم ToMV.

أوضحت النتائج أن الإصابة بهذا الفيروس قد أدت إلى حدوث بقع حمراء بنية يحيط بها هالة صفراء وانحصرت الإصابة فى بقع موضعية محددة Local lesions .

تم دراسة التغيرات البيوكيميائية والمورفولوجية والفسيولوجية فى الأوراق المصابة والسليمة . أظهرت النتائج حدوث نقص فى الكلوروفيل الكلى وكلوروفيل أ ، ب بينما زادت نسبة الكاروتينات فى الأوراق المصابة بالفيرس. وكان نقص كلوروفيل أ أكثر من ب . كما حدثت زيادة فى نشاط إنزيمات الأكسدة والإختزال البيرأكسيديز والبولى فينول أكسيديز بينما انخفض نشاط إنزيم الكتاليز فى الأوراق المصابة.

حدثت زيادة معنوية فى مستوى المالونالدهيد MDA فى النباتات المصابة مما يشير إلى حدوث أكسدة عالية للأحماض الدهنية الغير مشبعة فى الأغشية الخلوية وكذلك حدوث أكسدة للبروتينات والتى ظهر انخفاض تركيزها . كما بين تحليل الفينولات بجها زالكروماتوجرافى السائل HPLC حدوث تغيرات كمية ونوعية فى أنواع الفينولات فى الأوراق المصابة مقارنة بالسليمة ، فحدثت زيادة فى أحماض السينامك والجاليك والسالسيليك وبارا-هيدروكسي بنزويك والفينول بينما وجدت فينولات فى الأوراق المصابة لم تظهر فى الأوراق السليمة مثل الهيدروكينون والكامفيرول وكذلك اختفت فينولات كلوروجينيك ، والكاتيكين والكورسيتين والفانيلين والبروتوكاتشيك فى الأوراق المصابة مقارنة بالسليمة ويمكن تفسير تلك التغيرات على أساس أن تلك الفينولات التى ظهرت نتيجة الإصابة ذات أدوار متخصصة فى الحماية ووقف انتشار الإصابة الفيروسية وتكوين مواد راتنجية نتيجة الأكسدة وبالتالى إحداث جدر عازلة تحد من انتشار الإصابة الفيرسية وتحديدها فى بقع موضعية محددة Local lesions.

كما أظهر نتائج تحليل البروتين SDS-PAGE وجود بروتينات مستحثة نتيجة الإصابة و يمكن عزوها إلى زيادة قدرة تلك البروتينات فى الحماية من الشقوق الحرة والناتجة عن الإصابة الفيروسية .

وتوصى الدراسة إلى إمكانية استخدام المواد الفينولية فى رش النباتات للحد من الإصابة الفيروسية وذلك عن طريق زيادة قدرة النباتات من مضادات الأكسدة كخط دفاع أول ضد الإصابة ولزيادة الحماية .