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Biosynthesis of novel phytochemicals in tobacco plant infected with *tobacco mosaic tobamovirus* (TMV)

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Abstract

The main purpose of this work was to study the effect of TMV infection on physiology of active gradient photochemicals and protein expression in infected tobacco plants. Impact of *Tobacco mosaic tobamovirus* (TMV) on active gradient photochemicals quantitative and qualitative was evaluated in *Nicotiana tobaccum* cv. white burly. First, the TMV samples were isolated from single local lesions of infected leaves of *N. glutinosa*. Then, the *N. tobaccum* cv. white burly plants were inoculated with TMV. The infected plants showed severe systemic mosaic symptoms and reduction of leave size. We used *Datura metel* as a diagnostic tool-plant (indicator) for mosaic virus because of its vast exhibitory ability to show the symptoms incited by viruses. It was confirmed that these symptoms were due to the effect of TMV virus, comparing with Datura plant (as control). Analysis of TMV infected leaves by GC-mass detected biosynthesis of novel photochemicals (2-cyclopenten-1-one, Furfural, Indene, Pyrrole, Benzonitrile, Guaiacol and Oxime, methoxy-phenyl) that could not be detected in healthy plants. Furthermore, a 56.17% decreased in nicotine content was observed in infected plants compared with healthy ones. Also, increase of soluble protein contents was observed in infected leaves in response to TMV infection, compared with healthy ones. Alterations in protein patterns were observed in *N. tabaccum* leaves in response to TMV infection using SDS PAGE. Several secondary bioactive compounds were also found to hold important functions in infected plants. For example, flavonoids could protect against free radicals generated during photosynthesis. Terpenoids may attract pollinators or seed dispersers, or inhibit competing plants. Alkaloids usually ward-off herbivore animals or insect attacks (phytoalexins).

Keywords: Tobacco plants, TMV, photochemical, GC-mass, SDS-PAGE, protein expression.

Introduction

Tobacco (*N. tabacum*) plant is a perennial erect glandular herb. It is basically native of America but it is now commercially cultivated throughout the world. Tobacco plants are rich in medicinal value. All parts of herb are gluey, sheltered with short viscid glandular hairs, which exude a yellow secretion that comprise alkaloid known as nicotine. *N. tabacum* has a uniquely high proportion of alkaloids i.e. nicotine (Kamal, 2014). Nicotine is an alkaloid, which only found in tobacco plants and accounts for 95% of its total alkaloid content (Hashimoto and Yamada, 1994). Nicotine is manufactured in the roots and transported in the xylem to the shoot (Alworth and Rapoport, 1965). The plant is susceptible to various bacterial, fungal and viral diseases (e.g., the tobacco mosaic virus) and is attacked by numerous species of worms, beetles and moths (Kamal, 2014). The TMV infection can slightly change quantitative and qualitative phenolic antioxidant compounds, total chlorophyll and soluble protein in infected plant. About twenty-two phenolic compounds were identified in leaves of *N. tobaccum* cv. while burley by HPLC. The N-Hydroxy acyl nornicotine has newly been discovered in fresh shoot plants (Dina and Sabah, 2008). However, (Ali et al., 2006) recommended that there are high contents of polyphenols in healthy plants. On the contrary, (Srivastava, 2002) stated that there are higher amounts of total polyphenols in virus-infected plants. The oxidation of indole-3-acetic acid up regulated peroxidases might also be responsible for growth reduction and malformations in virus-infected plants (Riodle-Bouer, 2000). Abbas, 2015 found that there is variability in quality and quantity of 32 compounds of essential oils among healthy and infected *O. basilicum* plants infected with Cucumber Mosaic Virus (CMV). Consequently, the objective of the present study was to evaluate the effect of virus infection on the most important group of secondary metabolites (active gradient photochemicals) by GC Mass analysis and protein pattern (SDS PAGE) on the selected plant *N. tabaccum* cv. White burley infected with *Tobacco mosaic virus*.

Results

Confirmation of TMV isolate and virus propagation

The data showed that TMV isolate has caused systemic symptoms on *N. tabaccum* cv. White burley (Fig. 1). The symptoms were more obvious in younger leaves, which were more newly developed from fully expanded older ones in the form of vein malformation (reduction of leaf blade) severe

mosaic, formation of green blisters, vein banding, malformation and dwarfing and deformation. Healthy leaves were symptomless. Necrotic local lesions were appeared on *D. metal* indicator host (Fig. 1). ELISA results showed positive and high values against polyclonal antibodies specific TMV while healthy plants exhibited negative results.

Active gradient photochemicals in tobacco leaves

The phytochemical composition of healthy and infected leaves is presented in Table 1 and Fig 2. The components are listed according to their elution on the column. Eleven compounds were recognized in healthy (31.50%), while 68.49% were new (not-identified) components. TMV infected leaves contained 9 components, whereas 17.73% were recognizable and 82.27% non-identified components by GC mass. The profile of healthy leaves were contained Nornicotine (1.35%), Myosmine (0.98), Anabasine (3.51), Isonicoteine (7.025), Anatabine (1.201), Nicotine (6.89), Lubimin(5.50), Phytuberin (3.07), Rishitin (1.10), Solavetivone (0.70), and Capsidiol (0.18). The profile of infected plants was differed, containing low quantity of Rishitin (0.61) compounds, 2-cyclopenten-1-one (1.05), Furfural (2.09), Indene (9.68), Pyrrole (0.63), Benzonitrile (0.53), Guaiacol (0.09) and Oxime, methoxy-phenyl (0.03).

Protein content

The polypeptides were more or less accumulated depending on the plant stress. The soluble protein contents also varied between TMV infected leaves and healthy ones. The highest protein content (35.5mg/100g dry weight leaves) was recorded in infected leave compared with healthy ones (31.75mg/100g dry weight leaves).

Protein patterns analysis

The SDS PAGE technique revealed diversity in number and density of ten Protein patterns among healthy and TMV infected *N. tabaccum* leaves (Fig. 2) and (Table 2). The virus infection revealed three novel bands with molecular weights of 90, 45 and 30 kDa out of ten bands. In addition, seven bands were recorded in both TMV infected and healthy *N. tabaccum* leave with molecular weights of 140, 75, 60, 50, 35, 25 and 14 Kda**.** Protein expression showed poorer concentration in healthy compared to infected *N. tabaccum* leave (Fig. 3).

Discussion

Higher plants have a broad range of mechanisms to protect themselves against various threats including physical, chemical and biological stresses, such as wounding, exposures to salinity, heavy metals, drought, cold, air pollutants and ultraviolet rays and pathogen attacks, like fungi, bacteria and viruses according to Agrios, 1997.

These stresses can stimulate the physiological and biochemical changes in plants, like physical strengthening of the cell wall through suberization, lignification and callose deposition (Callose is a plant polysaccharide). It is thought to be manufactured at the cell wall by callose synthases. It is formed in response to wounding, infection by pathogens, by producing phenolic compounds, phytoalexins and pathogenesis-related (PR) proteins, which subsequently inhibit various pathogen invasions (Bowles, 1990). Among these mechanisms, production and amassing of pathogenesis related proteins in plants is the reaction to invading pathogen and/or stress situation is very important. Phytoalexins are principally produced by healthy cells neighboring to the localized crashed and necrotic cells, while PR proteins accumulate locally in the infected and surrounding tissues and also in remote uninfected tissues. Production of PR proteins in the healthy parts of plants can inhibit the affected plants from further infection (Delaney, 1997). Van Loon and Van Kammen, 1970 stated that PR protein in the plants was firstly discovered and reported in tobacco plants infected with tobacco mosaic virus. Later, these proteins were found in many other plants.

Phytochemicals are essential to both human and animal as they have been associated with positive effect in health, including high blood pressure, coronary heart disease, cancer, diabetic, ulcer, muscular degradation, psychotic disease with multiple actions on human health. TMV isolate exhibited systemic symptoms on *N. tabaccum* cv. White burley in the form of severe mosaic, vein banding, and malformation (decreasing of leaf blade). The TMV infectivity has been defined by local lesion host with numerous local lesions that observed on *D. metal*.

These results were assured the infection of tobacco plants cv. White burley, while healthy plants showed negative results. Several researchers showed that, differential hosts and ELISA are the most easily and widely method for TMV detection and affirmation (El-Dougdoug, et al. 2007). Baker and Adkins (2000) noted that there are many symptoms such as different degrees of mosaic symptoms; chlorosis on leaves, stems and fruits; necrosis on several plant parts; and dwarfism on virus-infected pepper plants.

All various macroscopic and microscopic symptoms of diseases must initiate biochemical deviations induced directly or indirectly by the virus (Hull, 2009). Chlorophyll is the most important element in the photosynthetic system. Virus infection frequently involves the colour change in most plants, exhibiting less chlorophyll content in healthy plants or damaged to chlorophyll as a consequence of infection. The loss of chlorophyll in such cases has been related to the prevention of the formation of new plastid units after virus infection in addition to their destruction (Sharma and Sharma, 1981).

Several reports have shown the interactions between different plants and virus combinations. The Prior studies of (Palanisamy et al. 2009), showed that the level of total chlorophyll (*Chl*) and carotenoids (*Car*) in control and *Yellow vein mosaic virus* (YVMV) infected leaves were significantly reduced 64% and 62% in infected leaves, respectively. Another important finding was that *Zucchini yellow mosaic virus* (ZYMV) infected leaves of pumpkin showed severe symptoms as mosaic, green blisters, size reduction and deformation and the virus infection diminished the *Chla* (48%), *Chlb* (53%) and carotenoid contents (52%). Nevertheless proline contents of ZYMV infected plants were higher than controls (Radwan et al., 2007).

The photochemical composition of active gradients in healthy and infected leaves *N. tabaccum* L, cv. White burly was listed in order to elution on the column by Gc mass technique. Eleven compounds were identified in healthy plants with 31.40% and non-identified components with

68.49%, while TMV infected leave was containing nine identified components with 17.77% and non-identified components with 82.27% (using GC mass). The profile of healthy leaves was contained Nornicotine, Myosmine, Anabasine, Isonicoteine, Anatabine, Lubimin, Phytuberin, Rishitin, Solavetivone and Capsidiol. The profile of infected plants differed containing low quantity of Nicotine and Rishitin compounds, while, 2-cyclopenten-1-one, Furfural, Indene, Pyrrole, Benzonitrile, Guaiacol and Oxime, methoxy-phenyl were detected in infected leaf only. The study of (Abbas, 2015) stated that the essential oil were consisted of 32 compound and differed in quantity in healthy and infected with Cucumber mosaic virus (CMV) *O. basilicum* plant. The study of (Dina and Sabah, 2008) demonstrates that twenty two phenolic compounds were identified in the leaves of *N. tabaccum* cv. white burley and nineteen in healthy ones using HPLC and secondly the N-Hydroxy acyl nornicotine. The newly discovered compound was found in fresh shoot plants with higher amount in healthy compared with TMV infected plants but not in root tissues. They showed that the Nicotine was found in fresh tissues with low concentration of infected roots, stems and leaves but in high concentration in healthy ones. This outcome opposed to those that reporting Nornicotine is found in fresh healthy tissues of roots, stem and leaves but not recognized in TMV infected stem and leaves using TLC chromatography. The reduction of total soluble proteins that caused by viral infection has been reported by (Taiwo and Akinjogunla, 2006). A strong relationship has been found between acclimation of stressed plants and the 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 profusion and plant pathogen interactions (Moshe et al., 2012).

The current study found that SDS PAGE technique revealed diversity in number and density of ten Protein patterns among healthy and TMV infected *N. tabaccum* leave. Virus infection produced three novel bands with molecular weights of 90, 45 and 30 kDa out of ten visible bands. Whereas seven bands were recorded in both TMV-infected and healthy *N. tabaccum* leaves with different molecular weight. The previous studies confirmed the correlation between many host virus infection and the increasing demand of abnormal protein production which required for the rapid synthesis of virus particle and for this purpose there has been increase in diversion of assimilated carbon compounds towards protein synthesis eventually resulting in the decreased production of carbohydrate level in the leaves (Singh and Shuka, 2009). The reduction in the content of carbohydrate is due to increased respiration resulting from virus infection and the conversion of carbohydrate into amino acids used in protein synthesis of the viral coat. This reduction might also be due to the duration of virus infection (Muqit et al., 2007; Sinha and Srivastava, 2010). There are several studies suggesting that the virus multiplication inside the plant cell alters different biochemical constituents of plants and disrupt the physiological processes like photosynthesis, transpiration and respiration of the infected plants (Tajul et al. 2011). The reports also demonstrated that the determination of cellular constituents in virus-infected plant is very important to recognize the activities of the host cell, the nature and extent of damage caused by the virus. The results of Alam et al., 1996 noticed that the biochemical alterations are caused by different viruses only in leaves of tomato. The analysis of phyto-biochemical changes in virus infected plants by GC-mass revealed qualitative and quantitative changes due to virus infection, compared to healthy leaves. All the physiological changes of TMV infection are induced indirectly by the virus. The physiological effects due to TMV infection were (I) an increase in respiratory rate, (II) a decrease in rate of photosynthesis often associated with a decrease in protosynthetic pigments, chloroplasts and ribosomes (III) increasing the activity of certain enzymes, mainly polyoxidase and the accretion of oxidized polyphenols and (IV) a decreased or increased activity of plant growth regulators. In several virus diseases, the general pattern of metabolic change appears to resemble an accelerated aging process. For example, the mosaic virus infection of *Solanum khasianum* reduced the fruit content of solanonine (a medically useful alkaloid) to about one half (Rangarajus and Cheulu, 1973). Edeoga et al., 2005 investigated the presences of these chemical constituents with medicinal value in plant. The most interesting finding was that reduction in total medically useful alkaloids of leaves is a common feature of virus disease and the decrease in alkaloid contents may be due to reaction in plant total fresh weight specially leaves (Satton, et al. (1985). Elbeshehy et al. 2011 found an increase in endogenous salicylic acid level in *tobacco mosaic virus* which caused a hypersensitive response with systemic induction of PR proteins. Total polyphenols content was increased in healthy plant compared to infected leaves. In addition, the total polyphenols and types were changed. Consequently, it could be concluded that viral infections do not only affect the synthesis of polyphenols but also have effects on the distribution type of polyphenols in infected leaves. Some phenolic compounds do not increase but others show increase and these might be responsible for the resistance in the infected plant. Mofunanya and Nta, (2011) also reported marked decreases in alkaloids, flavonoid and tannins caused by *Telfariria* mosaic virus and increases in polyphenols, saponins and significant increases in glycosides and flavonoids and non-significant increase in tannins in infected samples. Uegaki et al. (1998) reported accumulation of stress compounds due to virus infection. The accumulation of these chemical constituent in inoculated plants suggest that their synthesis is stimulated by virus stress infection.

Nicotine is the main alkaloid in the interval tissue. It is produced in roots of *N. glauca, N. tabacum* and *N. glutinosa* which are transported to the aerial parts of plant, where it is demethylated to nornicotine (Qiumei, 2007). The nicotine has a high antioxidant compound activity, due to its ability to donate a hydrogen atom to a free radical, that terminating free radical reaction (Srivastava, 2002).

Van Loon (1985) demonstrated that in virus infected plants the development of symptoms is accompanied by the appearance of one or more new proteins. The occurrence of these new proteins is not pathogen specific, but determined by the type of reaction of the host plant, indicating that these proteins are of host origin. Virus multiplication in plants generally causes synthesis of abnormal proteins resulting in alteration of type and the amount of total proteins. Singh and Shukla (2010) reported that nitrogen and protein contents were higher in infected tissue of Carica

Table 1. Quantitative and qualitative phytochemical analytic in healthy and TMV infected *N, tabacum* cv. white burly by GC-Mass spectrometry.

No	Compound				Area %	
	RT	Name	M.Wt.	M.F.	H. P.	I.P.
1	6.12	Nornicotine	148	C9H12N2	1.35	-
2	6.20	Myosmine	146	C9H10N2	0.98	-
3	6.67	Anabasine	162	C10H14N2	3.51	-
4	6.77	Isonicoteine	156	C10H8N2	7.025	-
5	6.98	Anatabine	160	C10H12N2	1.201	-
6	33.01	Nicotine	162	C10H14N2	6.89	3.02
				C15H24O2	5.50	-
7	36.1	Lubimin	236			
8	36.01	Phytuberin	294	C17H26O4	3.07	-
9	48.2	Rishitin	222	C14H22O2	1.10	0.61
10	52.05	Solavetivone	218	C15H22O	0.70	-
11	54.02	Capsidiol	236	C15H24O2	0.18	-
12	21.01	2-cyclopenten-1-one	82	C5H6O	-	1.05
13	26.3	Furfural	96	C5H4O2	-	2.09
14	41.02	Indene	116	C9H8	-	9.68
15	46.01	Pyrrole	95	C5H5NO	-	0.63
16	47.20	Benzonitrile	103	C7H5N	-	0.53
17	48.98	Guaiacol	124	C7H8O2	-	0.09
18	51.03	Oxime,methoxy-phenyl	135	C8H9NO	-	0.03
19	53.15	Non- identified compounds	-	-	68.494	82.27

Molecure weight: M.wt. Molecular formula: M.F Health plant: H.P. Infected plant: I.P. Area %: the percentage of compound in healthy and infected plant. (-): Absences of compound.

TMV infected **Healthy** ***D. metal* L.L**

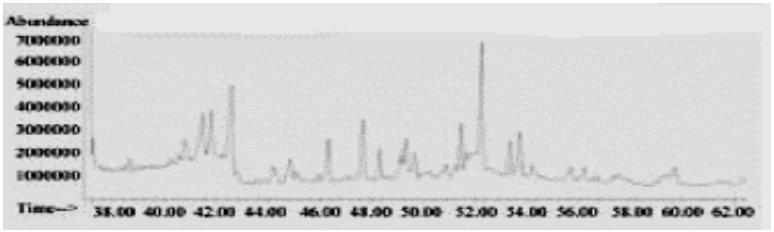
Fig 1. Photogram showing mosaic severe symptom on TMV infected leaves than healthy leaves of *N. tabaccum* cv. white burly leaves .local lesions on TMV inoculated *D.metal* **.**

Table 2. Density and molecular weight of Protein patterns analysis in healthy and TMV infected *N .tabaccum* cv. white burley by SDS PAGE technique

MW (Kda)	Protein patterns(bands)			
	Healty leaves		TMV infected leaves	
	Density	MW (Kda)	Density	MW (Kda)
140	++	140	++	140
90	.....	.....	++	90
75	++	75	+++	75
60	++	60	+++	60
50	++	50	+++	50
45	.....	.....	++	45
35	+++	35	++++	35
30	.....	.....	++	30
25	+	25	++	25
14	+++	14	++++	14
Total	7		10	

++++ = Very density band +++ =high density band ++ = density band
+ = Low density band--- = No detected band

(A)



(B)

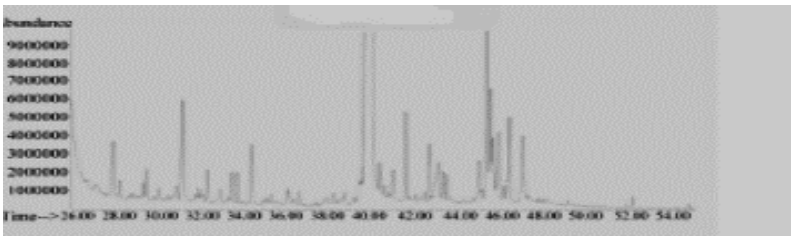


Fig 2. Main constancies of *N. tabaccum L*, *cv.* White burly healthy leaves GC/mass. (A) healthy and (B) TMV infected leaves.

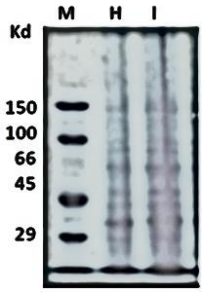


Fig 3. Gel electrogram of SDS-PAGE 12 % profile showing the changes in protein patterns of *N. tabaccum* cv. white burly leaves healthy (H) and infected with TMV (I) ones.

papaya L. infected by PRSV, when compared to healthy counterparts.

Materials and methods

Plant and virus materials

Seeds of *N. tabaccum* L, cv. White burly and *Datora metal* (Family of Solanaceae) were cultivated in a mixture sand and clay (1:2 v/v) in plastic pots (30 cm in diameter). The Isolate of *Tobacco mosaic tobamovius* (TMV) used in this study was prepared from single local lesions infected leaves of *N. glutinosa* L obtained from Virology lab. Fac. Agric. Ain Shams University.

The experiment

N. tabacum seedlings were transplanted in favorable conditions suitable for growth. The relative humidity was about 70%. The plants were kept at 100% water holding capacity. After 21 days of growth, plants with similar size were selected and divided into two groups. Each group consists of five replicates (a replicate is one pot containing three healthy plants). The first group, healthy plants sprayed with water and second group mechanically inoculated with TMV virus. These plants were kept under greenhouse condition. TMV infection was evaluated after three weeks of inoculation using healthy *D. metal* plants. They mechanically inoculated with sap of inoculated tobacco leaves. The percentage of infection and virus concentration were recorded as local lesions on *D. metal.* The DAS-ELISA was applied as described by (Clark and Adams, 1977) to measure TMV concentrations in inoculated plants using antibodies obtained from Virology lab. Fac. Agric. Ain Shams Univ.

Protein expression analyses: The leaves of *N. tabaccum* were collected for determined protein contents of infected and healthy leaves according to (Bradford, 1976).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The (SDS-PAGE) was carried out in 12% acrylamide slab gels following the system of (Laemmli, 1970). Separating gels composed of 0.75 M Tris – HCl pH 8.8, 10% SDS, 0.025% of N,N,N,N- tetramethylenediamine (TEMED) and 30% ammonium persulfate. Stacking gels contained 0.57M Tris-HCl pH 6.8, 10% SDS, 0.025% TEMED and 30% ammonium persulfate. Electrode buffer contained 0.025 M Tris, 0.192M glycine, 0.1% SDS and pH 8.3. Electrophoresis was carried out with a current of 25 mA and 130 volts per gel until the bromophenol blue marker reached the bottom of the gel after 3hrs. After electrophoresis, the Commassie Brilliant R250 staining method was used for protein bands and polypeptides.

Determination of photochemical:-Extraction of plant leaves

The youngest developed leaves of *N. tabaccum* from both healthy and TMV inoculated plants were collected after four weeks and air dried at room temperature from inoculation for phytochemical analysis. About 50 mg dried *Nicotiana* leaves were accurately weighted and introduced into a 15 ml centrifuge tube. A 2 ml of H_2SO_4 (0.1 M solution) was added and the mixture was kept in ultrasonic bath for 10 s. Then, the centrifuge tube was incubated in a water bath at 70°C for 10 min. 50 µl of extract were diluted in 1 ml with mobile phase and centrifuged for 6 min at 5000 rpm. Then, 50 µl of supernatant were diluted in 1 ml mobile phase.

The GC-MS analysis of the crude extracts were performed using Perkin Elmer system (GC clarus 600, USA) equipped with a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: Column Elite-5MS fused silica capillary column (30.0 m× 0.25 mm ID, 250 µmdf). Detection was operating in electron impact mode with ionization energy of 70 eV with helium (99.999%) as a carrier gas at a constant flow of 1mL/ min. The injection volume of 1 µL of sample was employed (split ratio of 10:1) injector temperature was at 240°C. The oven temperature was programmed at 60°C (isothermal for 2 min), with an increase of 10°C/min to 300°C/min for 6 min.

Conclusion

The present study shows that the *Tobacco mosaic tobamovirus* (TMV) make quantitative and qualitative changes in secondary metabolites of *Nicotiana tobaccum* cv. white burly, soluble protein expression contents and Alterations in protein patterns in *N. tabaccum* leave in response to TMV infection.

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