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DIFFERENT DETECTION METHODS OF TOMATO MOSAIC VIRUS (TOMV) AND INDUCING RESISTANCE ON BELL PEPPER BY SOME PLANT EXTRACTS

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ABSTRACT: Tomato mosaic virus (ToMV) was isolated from naturally infected bell pepper plants in different areas at Menoufia Governorate, Egypt. The isolates were characterized by its reaction in indicator host (*Nicotiana glutinosa* L.) and systemic host (*Capsicum annum* L cv. Balady), serological reaction (DAS-ELISA, TIBA and DIBA) and electron microscope (EM). In addition to estimate the effect of four plants leaf extracts, guava (*Psidium guajava*), solanum (*Solanum nigrum*), spinach(*Spinacea oleracea*) and thyme (*Thymus vulgaris*) with three concentrations (2.5-5-10 % of stock ethanolic crude extract) on ToMV infection. Results showed that all plant extracts were efficient in reducing the local lesion formation by the virus on *Nicotiana glutinosa*. The extract of *Solanum nigrum* gave the highest inhibition percentage at all concentrations. Percentage of infection and disease severity (%) of bell pepper plants (*Capsicum annum* L cv. Balady) were measured in response to systemic induced resistance (SIR) to ToMV infection. Treating of all inducers on infected bean leaves were effective in reducing infection percentage and disease severity compared to infected control. Extract of *Solanum nigrum* L. was the greatest efficient against ToMV as a factor of resistance induction. It reduced percentage of infection by (20 %) compared to un-treated control (92%) and reduced disease severity by (15 %) compared to un-treated control (89.34%).

Key words: Bell pepper, ToMV, Serological tests, Electron microscope, systemic induced resistance, Plant extracts.

INTRODUCTION

Bell pepper (*Capsicum annum* L.) plants belongs to family *Solanaceae* are very important vegetables crops worldwide. Peppers give the variety that many low-income families require to supplement their otherwise boring diets. In many underdeveloped nations, peppers are also a good source of revenue for small farmers (Ismaeil *et al*., 2012). The family Solanaceae includes bell peppers, which play an essential role in Egypt agriculture. According to the report of Food and Agriculture Organization (FAO, 2020) the total cultivated area of chilies and peppers in Egypt reached 5840 ha which yielded 1055605 tons, with average of 18074 Kg/ha.

During all stages of growth bell pepper are attacked by numerous diseases causing considerable losses in the yield. Plant viruses are the second most common cause of economic losses in horticultural, houseplant, vegetable, and agricultural crops around the world. At least 65 different viruses have been reported to infect pepper plants, resulting reduction in yield (Premezny *et al*., 2003).

The following viruses have been found in pepper and are known to cause major losses in pepper production: Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), Cucumber mosaic virus (CMV), Tomato spotted wilt virus (TSWV), Pepper vein mottle virus (PVMV), Potato Y virus (PVY), Alfalfa mosaic virus (AMV), and Pepper ring spot virus (PRSV) (Arogundade *et al*., 2012).

ToMV belongs to the Tobamo virus genus. Its particle structure is rod-shaped. The virus is roughly 300 nm in length and 18 nm in width. Virus particles are composed of 5% nucleic acid and 95% protein. The genome is single strand. This virus can be spread between plants by workers' hands, clothes and tools with usual

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doings as plant tying and harvest. Seed transmission might be infected with the virus and pass it on to the plant. Tomato mosaic virus can survive for up to two years in infected root remains in the soil (Silva *et al*., 2011). Yield losses in pepper due to ToMV infection has been stated to be 1 to 90%. (Chitra *et al*., 2002).

Virus infections, their effects and symptoms differ depending on the species of pepper plant and the environmental conditions. That is why, main the detection of the viruses in cultivated plants, should be completed to decrease the damages produced by virus infection and to improve control methods. It is not correct to realize diagnosis based only on symptomless for virus detection (Kilic, 2019).

Structure changes of number and size of organelles (particularly chloroplasts) in infected cells have been described for other *Potyviruses*. For instance, BYMV-infected leaves of *Vicia faba* revealed changes in chloroplast number of both palisade spongy and spongy parenchyma and cell layers. Additionally, decreased thylakoid system area, increased stromal area, starch accumulation, and plastoglobuli induction were detected. (Efaisha, 2005).

Recently, there has been object attention in biologically active compounds established in plant species for inhibiting several viruses on the plant as they are very safe for human and environment (Bezic *et al.,* 2011). Systemic induced resistance (SIR) can be utilized as an alternative to the old methods of plant protection. Also these compounds were non-phytotoxic simply biodegradable, environment friendly and safe (Ebadollahi, 2011).

Systemic acquired resistance activated by the viral pathogen that reacted as localized necrotic disease lesions or a hypersensitive response (Mahdy *et al*., 2007). On the other hand, Elbadry *et al*., (2006) stated that biological inducing agent, i.e. botanical, plant extracts that had been establish effective against bean yellow mosaic virus.

Plant extracts can promote resistance or function as viral replication inhibitors on their own. Ribosome inactivating proteins (RIPs) and glycoproteins can block replication sites. A mobile made signal might be produced in treated leaves after the botanical resistance inducers bind with the host plant surface. In the overall plant system, this signal-producing virus-inhibiting agent (Verma *et al*., 1998).

The aim of this study is to isolate and identify of and ToMV in some bell pepper production areas of Menoufia governorate, Egypt. Also, screen promising some plant extracts against ToMV virus.

MATERIAL AND METHODS

Plant material:

The plant material of this study randomly collected from three pepper production regions in Menoufia governorate, Egypt. Leaf samples showing mosaic, leaf deformation, leaf curling and plant stunting, were observed and suspected to be because of virus infection. During surveys, there is no difference between sampling sites. Samples were homogenized in a mortar and pestle with phosphate buffer (0.1 M, 1:5 w/v, pH 7.2) then the extracted liquid was clarified through a double layer cheesecloth. Finally, the sap was back mechanically inoculated onto pepper (*Capsicum annum* L cv. Balady) plants and *Nicotiana glutinosa* (as indicator plants). Inoculated plants were reserved in the greenhouse, then used as a supplier of infection in the following experiments.

Serological test methods:

The isolated virus was recognized by (DAS-ELISA) as designated by Clark and Adams (1977). ELISA kits (completely ready to use) were supplied by LOEWE® Biochema GmbH, Germany. Tissue blot immunoassay (TBIA) and Dot blot immunoassay (DBIA) were used to detect the isolated virus as defined by Lin *et al*. (1990).

Electron microscopy:

Ultra histopathological changes due to virus infection were studied using pepper leaves, the work was done in TEM lab, Faculty of Agriculture, Research Park, Cairo University.

Preparation of solvent extracts:

Solvent extracts were carried by grinding 10 g of the leaves of four botanicals *viz Psidium guajava* (Myrtaceae), *Thymus vulgaris* (Lamiaceae), *Solanum nigrum* (Solanaceae) and *Spinacea oleracea* (Chenopodiaceae) in 10 ml of 95.5 % Ethanol (1: w/v). The extract was filtered over double-layer cheese cloth. The solvent was evaporated and residue was dissolved in a known volume of ethanol (Deepthi *et al.,* 2007). This was used further for studies. From each stock ethanol of crude extract, three concentrations were made i.e., 2.5, 5, 10 % using distilled water.

Efficacy of plant extracts on local infected plant (*Nicotiana glutinosa.*) against ToMV:

The four ethanol extracts with different concentrations was sprayed on ten *N. glutinosa* leaves. After one hour, challenge inoculation with the viruses was done. Untreated leaves were inoculated with the virus and saved as positive control.

Challenge inoculation of virus:

The treated leaves of *N. glutinosa* with solvent extracts , were inoculated with the virus inoculum. The inoculum was then cleansed onto the leaves of the treated plants After 30 minutes of virus inoculation, the leaves were cleaned with water. The leaves were inoculated and symptoms were observed 3-7 days after the inoculation for the formation of local lesions.

The percentage of inhibition of local lesions formation by each treatment over the control was calculated based on the number of local lesions using the following formula defined by Devi *et al*., 2004:

$$\text{Inhibition \%} = \frac{\text{A-B}}{\text{A}} \times 100$$

Where:
A= Control B= Treatment

Efficacy of plant extracts on systemically infected plant (*Capsicum annum* L) against ToMV under greenhouse conditions.

Trail was carried under greenhouse at Faculty of Agriculture at Menoufia University. The sterilized bell pepper seedlings (cv. Balady), were seeded in sterilized plastic pots (40 cm in diameter) containing clay loam soil and sand (2:1 v:v). The soil was heat-treated at 121°C for 20 minutes on two consecutive days, and then cooled before the use. The plants divided into six groups. Each group involves of five replicates (replication is one pot containing five healthy plants) as follows: (G1) healthy plants (control) without any treatments, (G2) Inoculated plants with ToMV and sprayed with water. s. The other groups from 3 to 6 pre- treated with 10% of each extract as: (G3) *Psidium guajava*, (G4) *Thymus vulgaris*, (G5), *Solanum nigrum* and (G6) *Spinacea oleracea*. Treated and untreated plants were kept under greenhouse and observed for the appearance of systemic symptoms formation.

Percentage of ToMV disease incidences and Disease severity were recorded 21 to 30 days post inoculation. Percentage of infection was based on the number of plants showing systemic symptoms in relative to the total number of planted seedlings. While the disease severity (DS) method was calculated as described by Yang *et al*. (1996).

$$\text{DS(\%)} = \frac{\text{disease grade x number of plants in each grade}}{\text{total number of plants x highest disease grade}} \text{x } 100$$

Statical analysis

The data was analyzed using one-way analysis of variance (ANOVA) following by LSD test for mean separation. Statically significance was defined as P value <0.05 (CoStat-statistic software, CoHort software).

Results:

Isolation:

ToMV was isolated from naturally infected bell pepper plants (*Capsicum annum* L. cv. Balady) in the Egyptian Governorate of Menoufia. Mosaic, leaf curling, severe stunting, and leaf and fruit deformation were all visible on the infected plants. (Fig 1, B, C). These symptoms were similar to those previously noticed on naturally infected bell pepper plants.

Symptoms of a chlorotic local lesion were noted on *N. glutinosa* one week after mechanical inoculation (Fig.1,D).

Serological tests:

- **DAS-ELISA**

The virus isolate well-detected by DAS-ELISA, this test using DAS-ELISA was done as a rapid diagnostic tool to confirm the presence of ToMV in some infected bell pepper leaves samples. Three leaves samples were used from three different regions (El-Bagour, Shebin El-kom and Ashmoun) represent Menoufia governerate. Table (1) showed that Ashmon and Shebin samples were exhibited a reaction according to DAS-ELISA test. El -Bagour sample showed negative reaction.

Fig (1): Disease symptoms on bell pepper plants: A: Healthy plants, B: Mosaic, leaf curling, leaf deformation and severe stunting on plants, C: Deformation on bell pepper fruit. E: Chlorotic local lesion on *N. glutinosa.*

Table1. DAS-ELISA test on bell pepper leaves from three different regions in Menoufia Governorate.

Regions	Symptoms	ELISA
Ashmoum	+++	+
El-Bagour	++	-
Shebin El –kom	++	+

Symptoms: ++ moderate degree of symptoms +++ highest degree of symptoms
ELISA : + Positive reaction - Negative reaction

- **Tissue and Dot-blot immunobinding assay :**

As clear in Fig. 2 (A&B) the procedure tissue - blot immunobinding assay and dot-blot immunobinding assay (DBIA) were successful to make readily detection of ToMV on nitrocellulose membrane which investigated with polyclonal antisera diluted 1/1000 in TPS buffer and goat anti rabbit alkaline phosphatase conjugate diluted 1/8000, was used as secondary antibody. Positive samples reaction was indicated by a purplish-blue color whereas negative samples reactions was remained green color.

Electron microscopy:

The electron microscope was used to investigate ultrathin sections of healthy, infected Bell pepper leaves. There were no cytological changes in cell organelles or chloroplast shape or structure in control samples (healthy plants) (Fig. 2 C). While infected leaves were studied for changes in the shape and structure of chloroplasts. Infected cells' chloroplasts lost their normal shape and became spherical or longish and curved. Accumulation of many starch grains and amorphous is observed. Some chloroplast had incompletely or completely damaged envelopes, resulting in stromal matrix exclusion in the cytoplasm (Fig.2 D & E).

Efficacy of plant extracts on local infected plant (*N. glutinosa*) against ToMV:

The antiviral activity was evaluated based on the number of local lesions appeared on control and treated *N. glutinosa* leaves. Our results recorded in Fig. (2, F) and Table (2) indicated that all the three concentrations (*i.e*., 2.5 , 5, 10 %) of all tested plant extracts, inhibited ToMV infection with different degrees. The inhibition was increased with increasing the concentration of all extracts. The concentration (10 %) was the most effective one, where it induced the highest systemic resistance against ToMV (as inhibitory percentage of local virus infection). The extract of *Solanum nigrum* gave the highest inhibition percentage of all concentrations (74.48, 86.73 and 94.90 %). The least inhibitory rate was obtained using leaf extract of *Thymus vulgaris* (58.16, 64.28 and 72.44%) at concentration (2.5, 5 and 10%), respectively.

Efficacy of plant extracts on systemically infected plant (*Capsicum annum* L) against ToMV under greenhouse condition.

Data present in Table (3) indicate that all tested extracts reduced percentage of infection (PI %) and percentage of disease severity (DS %) on bell pepper plants compared to inoculated and untreated control. Our results show that *Solanum nigrum*, *Spinacea oleracea*, *Psidium guajava* and *Thymus vulgaris* reduced percentage of infection by 20, 28, 44 and 64 %, respectively compared to un-treated control (92%). Also these extracts reduced disease severity by 15.00, 24.37, 33.33 and 48.33% compared to un-treated control (89.34%).

Discussion:

Plant virus infections have resulted in a significant reduction in yield and quality around the world. It is needed to first detected the viruses recognized in the cultivated culture plant to limit the damages due to viruses and to improve control strategies. The symptomatologic studies that will be conducted based on reflection to define the viruses should be accompanied by serologic and molecular tests. It is possible to take approaches for the control of this virus disease only after carrying out the diagnosis of this pathogen. Biological, serological, and molecular approaches are commonly used to identify plant viruses (Almeida *et al*., 2018).

Symptoms such as mosaic, leaf curling, leaf deformation, stunting, and fruit deformation were detected during surveys conducted in bell pepper growing areas and were consistent with symptoms described in earlier studies (Kumar *et al.*, 2011, Svoboda and Svobodova, 2012 and Kilic, 2019).

Symptoms that appeared on *Capsicum annuum* L. and *N. glutinosa* L. as a result of mechanical inoculation make known the

existence of ToMV in collected leaf samples. The induced symptoms were in harmony with previous study results and supports the view that the virus can be ToMV (Deepthi *et al*., 2007a , Aghamohammadi *et al*., 2013 and Ullah *et al*., 2017).

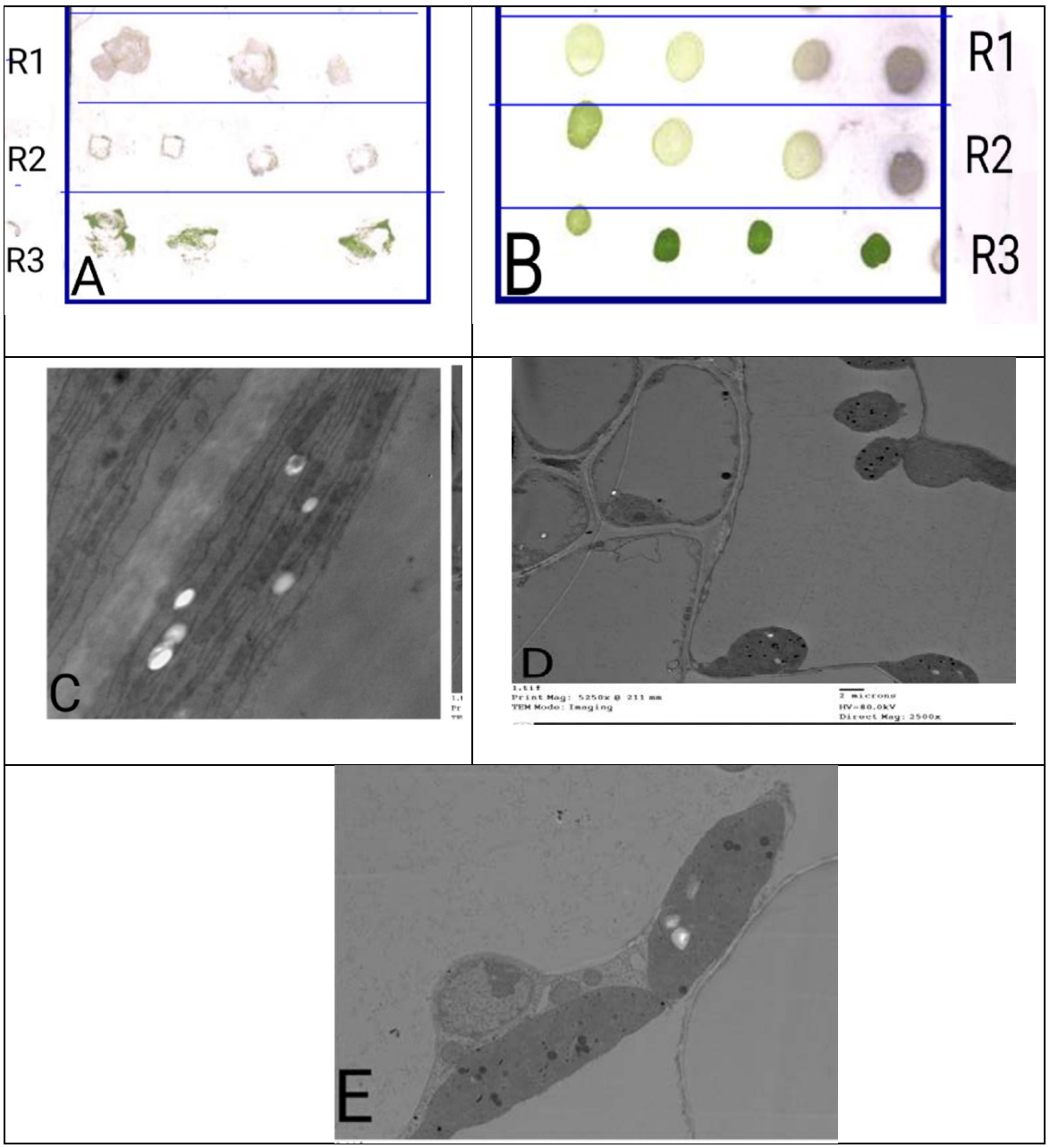

Fig (2): (A) Tissue blot Immunoassay for ToMV precipitation against specific IgG- ToMV polyclonal. R1, R2 = Positive samples R3 = Negative samples(B): Dot Blot Immunoassay for ToMV precipitation against specific IgG- ToMV polyclonal. R1, R2 = Positive samples R3: = Negative samples (C): Ultrastructural modifications of healthy bell pepper leaves, showing normal shape and structure of chloroplasts (D&E): Ultrastructural modifications of ToMV - infected bell leaves, showing ,chloroplasts (Ch) lost their normal arrangement and appeared spherical or longish and curved in shape and many starch grains (St) and amorphous inclusions (Am) were observed .

Table (2): The inhibitory effect * of different plant extracts on ToMV using local lesion leaves of *N. glutinosa*.

Solvent plant extract	Conc (%)	*No. of local lesions on treated leaves of *N. glutinosa*	Inhibition (%)
Psidium guajava	2.5	23	76.53^{f}
	5	18	81.63^{e}
	10	16	83.67d^{e}
Solanum nigrum	2.5	25	74.48^{fg}
	5	13	86.73^{c}
	10	5	94.90^{a}
Spinacea oleracea	2.5	28	71.42^{h}
	5	15	84.69^{cd}
	10	8	91.83^{b}
Thymus vulgaris	2.5	41	58.16^{j}
	5	35	64.28^{i}
	10	27	72.44^{gh}
Control (untreated)		98	00.00^{k}
L.S.D.			2.05

* Total number of local lesions on ten leaves used in each trial.

Table (3): Percentage of infection and disease severity noted on bell pepper cv. Balady plants inoculated by ToMV and treated by different plant extracts as compared to controls after 30 days of inoculation and treatment.

Solvent plant extract	S/T * Replicates	Percentage of Infection %	Percentage of Disease severity %
Psidium guajava	11/25	44^{c}	33.33^{c}
Solanum nigrum	5/25	20^{e}	15.00^{e}
Spinacea oleracea	7/25	28^{d}	24.37^{d}
Thymus vulgaris	16/25	64^{b}	48.33^{b}
Healthy control	0/25	00^{f}	00.00^{f}
Infected control(un-treated)	23/25	92^{a}	89.34^{a}
L.S.D.	-	5.18	4.66

* = Number of symptomatic plant (S) / Total number of tested plants (T).

The positive results obtained during the virus's diagnosis serologic test indicate that ToMV is present in the plant. The ELISA method, which is used to diagnose numerous plant viruses, has been widely employed by researchers (Geyik, 2017 and Kapoor *et al*., 2018). It has been established in this study as a result of carrying out DAS-ELISA tests on three isolates that 2 of the 3 isolates were infected with ToMV. ToMV infection has been identified in studies carried out at various parts of our country (Abdelmoamen *et al*., 2018 and Safy *et al.*, 2021).

In the tissue dot blotting methods, the specific antigen was immunologically localized with enzyme labeled antibody on nitrocellulose membranes. This method was discovered to have a substantially higher sensitivity for detecting ToMV. These result in agreement with those recorded by (Dombrovsky *et al*., 2010).

Cytological changes also revealed the formation of different abnormal in size and shape of chloroplasts. It became longish and spherical in shape, whereas others, such as grana and thylakoids, lost their envelopes and internal

structures. These findings are consistent with previous research. (Gümüş and Paylan, 2013).

The goal of this study was to see if particular plant extracts could be used to control ToMV. Cytological changes also revealed Plant extracts gave increased importance in the recent years due to their safety and target specificity (Deepthi *et al*., 2007b). Four different plant species *viz, Psidium guajava, Solanum nigrum, Spinacea oleracea* and *Thymus vulgaris* were evaluated for their antiviral activity against ToMV. In a similar way, many workers have reminded the use of some of these plants for the control of different viruses (Mandal and Singh, 2001, Deepthi *et al*.,2007b and Awad and El-Helaly, 2017).The plants have their own defense mechanisms against viruses. Antiviral products' mechanisms of action are difficult to describe (Elsharkawy and El-Sawy, 2015).The antiviral action of the natural products used in controlling viruses, is associated to their components that may act directly by interaction with the virus particles in the primary stage of the infection and block the freeing of its nucleic acid that eventually lead to preventing viral replication. The curative action of these products may support this purpose (Radwan *et al*., 2010).

In our experiments, the extracts of four plants have proved effective in reducing the tobamovirus concentration on *N. glutinosa* leaves by reducing the number of local lesions.When compared to control leaf extracts of *Solanum nigrum* and *Spinacea oleracea* demonstrated the greatest reduction in the production of local lesions in *N. glutinosa* challenge inoculation with TMV. These results were in a agreement with report of (Awad and El-Helaly, 2017) when used these extracts against bean yellow mosaic virus (BYMV). Deepthi *et al*., (2007b) found that crude extracts of Guava and ThuJa lowered the number of local lesions developed on *N. glutinosa* infected with TMV and ToMV. Thymus oil (5% concentrate) has been shown to have antiviral properties towards TMV and ToMV in infected leaves (Madhusudhan *et al*., 2005). Secondary metabolic products such as alkaloids, anthocyanins, caratins, flavonoids, phenolics, and some types of oils, according to (Wink and Schimmer, 2010), have antiviral properties against several viruses.

When compared to inoculated and untreated control plants, the results of this investigation clearly showed that all plant extracts reduced the percentage of infection and the percentage of disease severity on bell pepper plants. Verma and Kumar (1980) indicated that *D. Jalapa* leaf extract suppressed the incidence disease on a few systemic hosts when the extract was used as a foliar spray 24 h prior to virus inoculation.

As reported by (Verma *et al*., 1998) application of plant extracts exhorts the plant system to produce new proteins which act against virus diseases. Different plant extracts were applied to effectively prevent green gram leaf curl infection, which is produced by tomato spotted wilt virus (TSWV). When compared to control, *Solanum nigrum*, *Cocos nucifera*, and *Prosopis chilensis* extracts were found to be beneficial in suppressing Tomato spotted wilt virus severity (Manickam and Rajappan, 1999)

Conclusion:

In this study, the actuality of ToMV in pepper production areas at Menoufia Governorate, Egypt, have been found out using biological, serological methods and electron microscopy examination. Because plant extracts may act as viral replication inhibitors, the findings of this study suggest that using them to minimize the incidence of ToMV could be a promising method.

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الطرق المختلفة في فيروس موزايك الطماطم واستحثاث المقاومة على نبات الفلفل الحلو بواسطة بعض المستخلصات النباتية

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الملخص العربي:

تم عزل فيروس موزيك الطماطم من نبات الفلفل الحلومن ثلاث مناطق مختلفة فى محافظة المنوفية وتم تعريف هذه العزلات بواسطة رد فعلها داخل النباتات المشخصة مثل نبات الدخان وكذلك نبات يصاب جهازيا مثل الفلفل الحلو (صنف بلدي) وأيضا تم تعريفها بالطرق السيرولوجيه المختلفة ومنها (DAS- ELISA- TIBA and DIBA) كما تم فحص التغيرات التي تحدث في الخلية بواسطه الميكروسكوب الإلكتروني بالإضافة إلى تقييم فاعليه أربعة أنواع من المستخلصات النباتية علي تقليل الإصابه الفيروسة وهي (مستخلص الجوافه وعنب الديب والسبانخ والزعتر) بثلاث تركيزات مختلفة (٢,٥ – ٥ – ١٠ % من المستخلص الإيثانولي الخام) وكانت أهم النتائج أن جميع المستخلصات بالتركيزات المختلفة قد أدت الي تقليل عدد البقع الموضعية علي نبات الدخان وكان مستخلص عنب الديب أفضل هذه المستخلصات أيضا تم قياس نسبه الإصابه والشدة المرضية كمقياس للمقاومه المستحثة أدت المعاملة بكل المستخلصات الي تقليل نسبه الإصابه والشدة المرضية مقارنه بالكنترول المعدي والغير معامل وكانت أفضل المعاملات هي مستخلص عنب الديب حيث أدى إلى خفض نسبة الإصابة إلى (٢٠%) مقارنه بالكنترول المعدي والغير معامل (٩٢%) كذلك أدى الي تقليل الشدة المرضية إلى (١٥%) مقارنه بالكنترول المعدي والغير معامل (٨٩,٣٤%).