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Molecular Identification and Biological Resistance to Local Isolate of the *Tomato Mosaic Virus (ToMV)* on the Sweet Pepper Plants.

Basma Dhabab Ayed* **and Maadh Abd Al- Wahab Al Fahad**
Plant Protection Dep. / College of Agriculture/ Tikrit Uni.-Iraq

Key word:
Spirulina platensis, *Ganoderma lucidum*, peroxidase enzyme, *ToMV*, Molecular identification.

Corresponding author:
Basma D. Ayed
E-mail:
dadiaplek@gmail.com

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ABSTRACT

The study aimed to identification the *ToMV* virus in some Salah aldin governorate fields from locally infected plants using RT-PCR technique, which gave clear bande at 318 bp and 512bp. It also the study including the use of *Spirulina platensis* and *Ganoderma lucidium*, and their interaction (Ga. +S.P.). Their effect on stimulating the resistance against *ToMV* virus and some characteristics of growth also studied. The treatment showed that *S.platensis* and *G.lucidum* with highest rate in stimulating the production of peroxidase enzyme at 4.8 and 4.6 unit/ ml respectively, while for the treatment without infection it was 2.2 unit / ml for both treatments. The intraction treatment (Ga +S.P.) increased percentage of the fruiting and dry weight was (85.4%, and 80.97 g respectively, compared to control treatment (62% and 39.35 g respectively), while the treatment with *S.platensis* gave an increase in the height of pepper plants reached to 22.2 cm compared to control treatment 16.8 cm.

التشخيص الجزيئي والمقاومة الحيوية للعزلة المحلية من فايروس فسيفساء الطماطة *ToMV* على نباتات الفلفل البارد

بسمة ضباب عايد ومعاذ عبد الوهاب الفهد
قسم وقاية النبات/ كلية الزراعة/ جامعة تكريت

الخلاصــة

هدفت الدراسة الى تشخيص فايروس *ToMV* من النباتات المصابة به من بعض حقول محافظة صلاح الدين بالاعتماد على تقنية RT-PCR حيث أعطت حزمتين واضحة بحجم 318bp و512bp. كما تضمنت الدراسة تقييم كفاءة عوامل احيائية مختلفة تضمنت استخدام طحلُب *Spirulina platensis* والفطر الريشي *Ganoderma lucidium* والتداخل بينهما (*Ga.+S.P.*) ودُرِست مؤشرات تأثيرها في تحفيز المقاومة ضد فايروس *ToMV* وبعض صفات النمو، أظهرت معاملة الطُحلب *S.platensis* والفطر الريشي *G.lucidum* في حالة الاصابة أعلى معدل في تحفيز انتاج أنزيم البيروكسيديز أذ بلغت 4.8 و4.6 وحدة/مل على التوالي أما بالنسبة للسليم فقد كانت2.2 وحدة/مل لكلا المعاملتين، كما أدت المعاملة التكاملية المشتركة (*Ga.+S.P.*) الى زيادة معنوية في صفة نسبة العقد والوزن الجاف 85.4% و 80.97 غم على التوالي مقارنة بمعاملة السيطرة 62% و 39.35غم على التوالي بينما أعطت المعاملة بطُحلب *S.platensis* زيادة في أطوال نباتات الفلفل وبلغت 22.2 سم مقارنة بمعاملة السيطرة 16.8 سم.

الكلمات المفتاحية:
التشخيص الجزيئي، المقاومة الحيوية، فايروس فسيفساء الطماطة، الفلفل البارد.

للمراسلة:
بسمة ضباب عايد
البريد الالكتروني:
dadiaplek@gmail.com

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Introduction:

Sweet pepper (*Capsicum annuml* L.) is considered a crop of high economic and nutritional value (Hassan, 2001). Its importance for its contribution to supplying the body with important energy compounds for building (proteins, carbohydrates, fats), also a good source of vitamin B in addition to its high content of vitamin C and vitamins E, A (Baloch *et al*., 2008). A study indicated that fruits contain highly effective compounds against microbes (Wahba *et al*., 2010). Its production has spread in the central and southern regions from Iraq with in protected environmental green houses and tunnels (Al-Mohammadi, 1990).

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The *ToMV* virus spreads epidemiologically during some seasons with an infection rate of 50% (Duarte, 2001). The infection also threatens the work of farmers and researchers in the production of healthy seeds (Madhusudhan *et al*., 2008). Since the plants possess different defensive means against pathogens and are biologically stimulating (Murphy, 2003), diseases resistance in general, as well as viral diseases in particular, with the difficulty of controlling diseases caused by viruses and due to the spread of mosaic virus lack of previous studies in this field, The aim of this study:

Molecular identification of *ToMV* isolation and induction of resistance against the virus infection using various biological agents, including *Spirulina platensis*, *Ganoderma lucidium* and the intraction complementary treatment (Ga+ S.p.)

Materials and methods:

Collect samples of infected plants and obtain pure isolation of ToMV virus

Sample from infected tomato plants were collected from different fields of Salahalddin province showing clear mosaic symptoms. Then diagnostic study's were conducted using RT-PCR reverse transcription polymerase chain reaction.

Molecular diagnosis of *ToMV* virus

1-Isolation of Ribosomal nucleic acid RNA

RNA was isolated according to the working method prepared by (Bio-Nier Company).

2. Measuring concentration and purity of RNA extract

The concentration and the purity of the extracted RNA was measured using a DNA concentration and purity device (NANO DROP 2000).

3. Step 1 / Convert Total RNA to cDNA

The following materials were added to the RT-Premix type supplied by BIO Neer Company and according to the recommended concentrations by the manufacturer as followed:

Material	Size required
Sample RNA	1 µl
Poligo-dte	1 µl
DEPC- D.W	16 µl
Total volume	20 µl

Put the interaction sample in the PCR (Veriti type) for the incubation procedure with the primers used in the diagnosis of the virus and according to the following table(1)

Table (1): The primers that were used to diagnose the virus

Source	The Sequence	Concentration	package size (bp)
Kumar *et.al* 2011	**5 CGAGAGGGGCAACAAACAT -3** **5 ACCTGTCTCCATCTTTGG -3**	100Pmole/ µl	508 bp
Silva *et.al* 2008	**5 CTCCATCGTTCACACTCGTTACT-3** **5 GATCTGAAGTCTGAGAAACTT -3**	100Pmole/ µl	318 bp

The following program was used:

The Steps	temperature	Time
Primer Anneling	37	10 min.
cDNA synthesis	42	30 min
Heat inactivation	95	5 min.

Total RNA was detected by conducting an electrode transfer on the agarose gel with a 1% concentration (Thalig, 2013).

4. Step 2 / multiplying DNA fragments using PCR technique:

The processed sample was added to the type of PCR-Premix supplied by BIO Neer Company, according to the recommended concentrations by the manufacturer in the work method, as follows:

Material	Size required
1-RT-PCR sample	5 µl
2- Primer(R+F)	2 µl
3- D.W	11 µl
Total volume	20 µl

PCR-Premix PCR-Premix sample was inserted into the PCR (Veriti type) for the double-reaction process and according to the program mentioned by Kumar et al, (2011).

Steps	temperature	Time
Denaturation	94	30 s.
Annealing	62	45 s.
Elongeation	72	10 min.
Cycle	35	-

DNA was detected by electrolyte transfer at 2% concentration of alkaloose gel according to the method described in 2-3-3-C, adding 5 µl of Marker to the first hole on one side.

Treatment used in resistance *Tomato Mosaic Virus (ToMV)*

Brought experimental Biomaterials from Parmaceutical Sdn Bhd - DXN Malaysia is (is specialized in the production of organic supplements), which included the following materials**:**

1- Treatment of the fungus *G. Lusidum* (Ga.) + *ToMV* inoculation.
2 - Treatment of *Spirolina platensis* mulch (S.p.) + *ToMV* inoculation.
3 - Treatment of fungus G.L. and algae S.p (Ga + S) + *ToMV* inoculation.
4 - Treatment of control infected with *ToMV* virus and has not been added to any treatment.
5 - Treatment of control control non-infected with the virus *ToMV* and did not add any treatment.
6 - Treatment of the fungus only *G. Lusidum* (Ga.) without infection with the virus.
7-Treatment S.*platensis* (S.p.) without virus infection.

Treatment of substances used in the experiment

1 - Treatment of the fungus *G. lusidum*

Add mushroom powder 1 g solved in 10ml sterile distilled water and seeds soaked for 24 hours. The second treatment was at the third leaf stage. The third treatment was at flowering stage**.** The treatment was applied on three varieties of pepper plant (Ca., P. and M.f1).

2- Treatment with *Spirulina platensis* algae

Pepper 30 seeds were soaked for 24 hours into 10ml sterile distilled water content 1 g of algae powder and then cultured in the plates. The second treatment was during the vegetative growth

stage at the age of the third real paper by adding 10 g / liter. The third treatment was during the flowering stage as in the second treatment method. The treatment was applied on three varieties of pepper plant (Ca., P. and M.f1).

Measure some effect indicators in *ToMV* virus and stimulate pepper plant resistance:

1-Determination of Peroxidase enzyme in different treatment:

Adopted Hammer Schmidt *et al*. (1982). The enzyme readings were taken after zero time and then 1 to 3 minutes. The enzymatic activity was calculated by subtracting the readings in the third minute of the readings at zero time and then divided by 0.01 units / ml.

2 - Calculation of the percentage of fruiting:

The percentage of fruiting was calculated by the following equation:

$$\text{Percentage of fruiting} = \frac{\text{number of flowers set fruiting}}{\text{total flowers number}} \times 100$$

Measuring some indicators of growth yield

1-Plant height measurement (cm)

The height of the plant was measured from soil contact to the highest growing peak of the plants obtained from each experimental unit from all treatments during stage was the readings after one week of adding the treated substances to the plants at the age of the Twelfth real leaf.

2- The dry weight determination

The dry weight of the shoot system (leaves and stems) was measured at the end of the season by taking three plants randomly from each experimental unit, and then weighing the dried plants by blance (samples were dried in the oven at 70 ±5 °C) for two days and until the weight was stable.

Results and discussion

Diagnosis of *ToMV* on sweet pepper using PCR technique

1- Results of RNA isolation

The process of isolating the RNA from the leaf leaves of the *ToMV* infected pepper leaves was succesiful and obtaining an appropriate amount of RNA ranging from 949.3 and 864.8 ng / µl. These results were obtained at high purity of the RNA extract, which ranged between 1.80 and 1.94. Due to the RNA's ability to break down with ribonucleic enzymes, RNA isolation was more difficult than DNA isolation (Hassan, 2004). The RNA isolation process required careful attention to minimize the activity of these enzymes using chemicals that inhibit RNase acts as a diethyl pyrocarbonate (DEPC) previously prepared by the materials used in the extraction process.

2- Results of PCR poly merase chain reaction

This required the use of DNA extracted from infected the plant tissue with the *ToMV* virus to make multiple attempts to reach the optimal conditions needed because high of sensitivity to the reaction conditions and that the best results were obtained through the optimal level of factors which were:

Concentration of the RNA used:

The concentration showed 949.3ng / µl clear band and was the best.

Primer concentration:

use the pmole 25 concentration, which showed packages and gave good measurable results

Electrical relay results:

After adjusting all conditions and conducting diagnostic experiments on the RNA, the used primers showed different results and as shown in Figure (1). The primer referred to as the symbol (A) in the form of (1) a bande size 318 bp and this corresponds to what Kumer (2011) found, A 512 bp bande was obtained from the use of the second primer referred to as (B) in figure (3) and this corresponds to what was reached (Silva *et al*., 2008). The reliance on several diagnostic primers is one of the important steps that enhance the accuracy of virus diagnosis. This study confirmed that the

diagnosis of the virus was accurate and this was also reinforced by the use of the specialized primer on the virus specifically *ToMV* and the general *TMV* virus

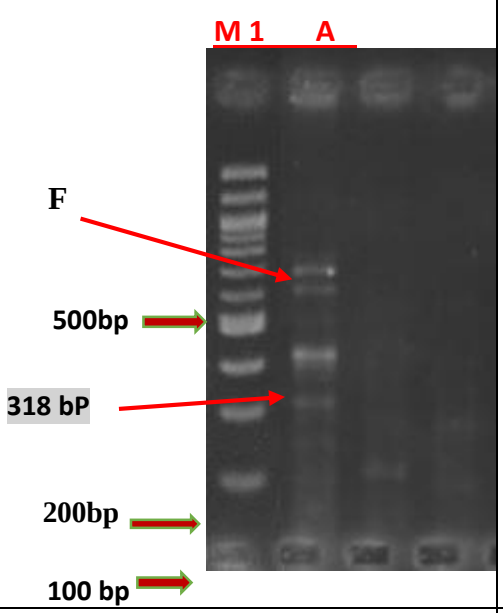

Figure (1) Detection *of Tomato mosaic virus (ToMV)* in sweet pepper by RT-PCR with size primer 318 bP

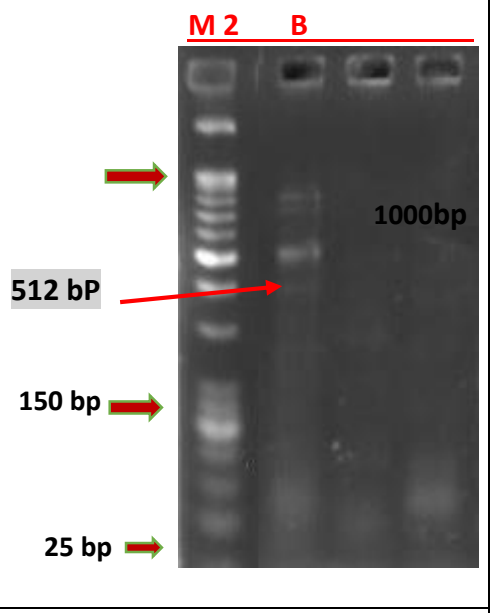

Figure (2) Detection of *Tomato mosaic virus (ToMV)* in sweet pepper by RT-PCR with size primer 512 bP

M1, M2 represents marker 1 and 2
(A) - represents a primer of 318 bp
(B) - represents a primer of 512 bp
(F) – Non-specific bands

The effect of the *ToMV* virus on some resistance and growth indicators

1. Effect of different treatments on the specific efficacy of Peroxidase (unit/ml) on healthy infected pepper plants and *ToMV*

Figure (3) shows the effect of treatments on healthy and infected plants. The treatment of *S.platensis* and *G.lucidum* was the highest in the case of infection with 4.8 and 4.6 unit/ ml respectively. While for the healthy plant it was 2.2 unit / ml for both treatments and the least was the treatment of control for healthy and infected treatment, Most of the treatments stimulated the production of the enzyme in pepper plants and thus increased the resistance of the pathogen, which is one of the enzymes related to the disease, as it increases its concentration after infection with pathogens (Strobel *et.al*, 1999) and participates in the construction of the cell wall and works to strengthen it such as oxidation of phenols and addition of Lignin reaction to cellular wall proteins ransfer of the virus to form compounds increase the hardness of the cell wall against invanding of biot (Hibar et al., 2007; Chittoor et al., 1999) suggested that the increased enzymatic activity in the presence of disease is significant resistance indication to having an antidote to pathogens. This is in greement with (Madhusudhan et al. 2009) and (Azzawi, 2014).

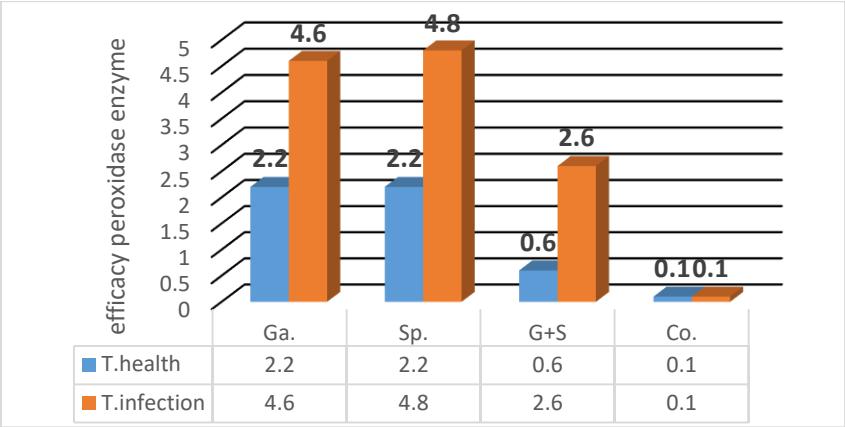

Figure (3) Effect of different treatments on specific efficacy of peroxidase enzyme.

2. Effect of different treatments in percentage of fruiting / plant

The results of Table (2) indicate that there were significant differences between the treatments. The highest percentage was achieved with the treatment of algae with the fungus (Ga +Sp), which reached 85.4%, which was not significantly different from the *spirulina platensis* and *G.lucidium*, which reached 84.4%. Treatment that gave the lowest percentage of fruiting control treatment at the rate of effect of transactions which reached 62.0% g. The results of the interaction between the treatments and the percentage of infection (healthy and infected) for the infected treatments exceeded the treatment of the algae with the fungus (Ga. + S.p.) giving the highest infection percentage rate of 77.1%, while the control treatment was lower which reached 44.3%. In both of which gave the control transaction the lowest percentage of fruiting in the transaction rate of influence it can be seen in the chemical composition of the algae *S.platensis* and *G.lucidium* that there are vitamins macro and micronutrients auxins and cytokines that are important for activation of flowering and stability of fruiting .This factor enhance the equal increase of vegetative growth (due to increase of absorption of water and nutrients rate alonge with sun energy to form necessary compounds for flowering and fruiting , and this is agread with (Serror, 2012. Ibrahim, 2013), as they stated the anions are important growth regulator to improve flowering and fruiting rates.

Table (2) Effect of the studied transactions in the percentage of the fruiting

treatment / Infected+ varieties		CO.	G.+S.	S.p.	Ga.	Infected + varieties
Health Treatment	Ca.	76.16 F-K	87.86 B-D	93.0 AB	92.36 A-C	84.40 A
	M. f1	76.53 E-K	94.53 AB	93.1 AB	93.33 AB	84.32 A
	P.	78.46 E-J	99.0 A	92.53 A-C	93.67 AB	86.72 A
Infected Treatment	Ca	46.20 M	78.0 D-J	74.0 F-K	75.70 F-I	69.12 BC
	M. f1	45.0 M	74.40 F-K	71.23 H-L	74.70 K	66. 84 C
	P.	40.40 M	78.98 D-I	82.67 D-F	74.63 F-K	71.51 B
treatment / infected		CO.	G.+S.	S.p.	Ga.	Effect infected
Health treatment		77.74 B-D	93.80 A	92.97 A	93.12 A	85.15 A
Infected treatment		44.30 G	77.1 CD	75.96 D	75.01 DE	69.12 B
Treatment effect		62.06 D	85.45 A	84.47 A	84.06 A	

3. Effect of treatments used in plants height / cm

The results of Table (3) indicate that the treatment of *Spirulina platensis* was the highest in the effect rate of the treatments at 22.2 cm while the least was the control treatment which gave 16.8 cm.

The combination of infection effect with the treatments resulted in significant differences between them. The height of the plant in the treatment of *S.platensis* algae was 19 cm and the the heightest compared to the control treatment, which gave to 14.9 cm. The treatment with algae resulted in an increase of plant *S.platensis* algae increased the height of the plant to providing the essemtial materials needed for plant growth. Studies have indicated the possession of more than a group of growth-promoting substances such as dioxins and cytokines and amino acids and vitamins, which have the role in the process of growth and stimulate cell division and activation of enzymes that stimulate plant growth and this is consistent with what he said (Kazem and Hadi, 2015, Abdel Hafez, 2011).

Table (3) Effect of treatments used in plants height / cm

treatment / Infected+ varieties		CO.	G.+S.	S.p.	Ga.	Infected+ varieties
Health treatment	Ca.	19.66 G-J	21.33 D-G	25.83 A	22.5 CD	21.15 A
	M. f1	19.16 I-L	21.83 DE	25.33 A	25.66 A	21.52 A
	P.	17.66 K-O	22.66 CD	24.83 A	23.66 BC	21.04 A
Infected treatment	Ca	15.16 R-T	16.5 O-S	19 I-M	17 N-R	16.38 B
	M. f1	14.5 T	17 N-R	18.16 J-O	17.33 M-P	16.15 B
	P.	15 ST	17.16 N-Q	19.83F-J	17.5 L-O	16.54 B
treatment / infected		CO.	G.+S.	S.p.	Ga.	Effect infected
Health treatment		18.83 D	21.94 C	25.3 A	23.94 B	21.2 A
Infected treatment		14.89 H	16.88 EF	19.0 D	17.28 E	16.4 B
Treatment effect		36 EF	19.42 C	22.17 A	20.6 B	

4. Effect of treatments used in dry weight of shoot system /g

The results of Table (4) show that there were significant differences between the rate of treatments. The treatment of the fungus with the algae (G. + S.) gave end creation of resistance, which reduces the infection symptoms and increases the fruiting and plant productivity. These results in agreement to our findings. The highest percentage of dry weight was 80.97 g, while the control treatment gave the lowest percentage 39.35 g. As for the interaction of the infection with the treatments, the fungus treatment with algae (G. + S.) gave the highest percentage of dry weight 65.1 g followed by the treatment of algae and fungus which did not differ significantly between the two treatments, 27.0 g. The effect of the interaction between the fungus and the algae (G. + S.) has a positive effect that may be due to the same effect mentioned in the study of the individual. Algae increase growth vegetative due to the presence of growth regulators such as cytokines that encourage plant growth. The activation of element absorption, which is reflected in the weight of the plant and this is agreed with the findings of (Crouch 1990) and what (Sheweili ,2013), reported as well as for the important fungus mushrooms, which directly affect the growth and overcome the effects of pathophysiology.

Table (4) Effect of the treatments used in the dry weight (gm) of shoot system

treatment / Infected+ varieties		CO.	G.+S.	S.p.	Ga.	Infected + varieties
Health Treatment	Ca.	49.83 L-O	97.67 A	92.67 AB	91.67 AB	78.89 A
	M. f1	51.67 LM	97.0 A	94.67 AB	90.0 B	78.50 A
	P.	49.50 L-P	95.67 AB	91.33 AB	92.33 AB	77.04 A
Infected Treatment	Ca.	23.33 V	66.67 G-I	62.33 IJ	55.33 KL	47.52 C
	M. f1	30.67 TU	70.67 FG	63.33 H-J	63.33 H-J	50.9 B
	P.	27.0 UV	58.16 JK	59.33 JK	60.0 JK	48.65 C
treatment / infected		CO.	G.+S.	S.p.	Ga.	Effect infected
Health treatment		50.33 H	96.77 A	92.88 B	91.33 B	78.14 A
Infected treatment.		27.0 L	65.16 F	61.67 G	59.55 G	49.04 B
Treatment effect		39.35 F	80.97 A	77.27 B	75.44 B	

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