

## Induction of Systemic Resistance to *Bean yellow mosaic virus* (BYMV) Infecting Bean Plants Using Plant Extracts and Salicylic Acid

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### ABSTRACT

*Bean yellow mosaic virus* (BYMV) was appeared to be in the last years the most frequently destructive virus of bean crop causing mosaic with bright yellowing symptoms. The virus was isolated in different locations in Egypt, and detected by using DAS-ELISA and Dot-blot immunobinding assay (DBIA). Initiative or introductory screening of inducers against BYMV infection on bean plants directed by using the local lesion assay (LLA) *Chenopodium amaranticolor* L. All used inducers were effective in decreasing the local lesion formation by the virus inoculation. Biochemical and ultrastructural modifications of bean plants (*Phaseolus vulgaris*, L. cv. karnk) leaves in response to Systemic Acquired Resistance (SAR) to BYMV infection. Treating of all inducers on infected bean leaves were effective in banning or obstruction of destroying and smashing effects that resulted after virus infection. ELISA was used as a diagnostic tool at the end to SAR. Extract of *Dianthus caryophyllus* L. was the most effective against BYMV as a factor of resistance induction.

**Keywords:** Bean, BYMV, ELISA, DBIA, induced systemic resistance, plant extracts, Salicylic acid.

### INTRODUCTION

Bean (*Phaseolus vulgaris*, L) is a vegetable crop widely grown in Egypt and many countries of Africa. It considered an important food legume crop for human and animal nutrition in developing countries (Sameh, 2005). Many viral diseases could be attack bean plants causing serious problems worldwide. Virus infection on bean plants causes yield reduction as well as economic losses (Beute, 1970). Zaumeyer and Meiners (1975) mentioned the most important viruses that attacks bean plants during growth stages and cause a great reduction in yield quantity and quality. *Bean yellow mosaic virus* is the type member of the genus *Potyvirus* in the *Potyviridae* family of plant viruses, which have a broad distribution worldwide causing greater overall yield reduction, despite it induced mild symptoms (Pierce, 1934). It naturally infects a wide range of legume species as well as some non-legumes. These viruses transmitted in non-persistent manner by numerous aphid species (Bos, 1970). Tolba (1980) reported that BYMV infects a wide members of legume species, moreover some nonlegumes. BYMV consider the most widespread of all the viruses affecting broad bean crops in Egypt. As a result of infection, many changes has happened in the ultrastructure contents and size as well as the number of organs, i.e. chloroplast, in infected leaves by BYMV and other *Potyviruses*. Changes in chloroplast number of spongy parenchyma and palisad cell layers as a result of infected leaves of *Styrain pumpkin* by ZYMV. Changes in chloroplast, inner structure like decrease in area of thylakoid systems were noticed. Also, enhanced stromal area, starch accumulation and plastoglobuli induction were noticed by (Zechmann *et al.*, 2003). Infection of *Potato virus Y* (PVY) resulted changes in the number of mitochondria and microbodies (Hinrichs-Berger *et al.*, 1999). The same changes in chloroplast ultrastructure, increasing plastoglobuli and dilation of thylakoid members were noticed in peach plants infected by *Plum pox virus* (PPV) (Hernandez *et al.*, 2004). Mahgoub *et al.*, 1997 and Pompe *et al.*, (2001) indicated that they found after viral infection many ultrastructural changes; i.e. reduced grana stack height, occurrence of small vesicles, loosened thylakoid structure, deformation of membranes and many other virus infection reactions. Ultrastructural and biochemical changes in relation to viral infection are still not clear. Management of viral disease could be achieved through induction the natural

defense of plants, e.g., systemic acquired resistance (SAR). SAR is a form of induced resistance that activated by the viral pathogen that reacted as localized necrotic disease lesions or a hypersensitive response. These necrotic lesions revealed the presence of a zone around each local lesion on tobacco which was more resistance to a second infection by the same virus, this reaction called local acquired resistance (LAR) Ross (1961a, b). The leaves of the same plant, showed resistance to the second infection; but called systemic acquired resistance (SAR). The phenomenon of SAR against disease in plants following infection has been recorded, but often not been documented as early as the 19<sup>th</sup> century, the natural phenomenon of resistance in response to pathogen infection or plant immunity was first recognized in 1901 by Ray, when they worked on *Botrytis cinerea*. Acquired resistance, first described by Gilpatrick and Weintraub (1952) on *Dianthus barbatus* when the lower leaves were inoculated with *Carnation mosaic virus* (CarMV), the upper leaves were appeared resistant to the infection. Abo El-Nasr *et al.*, (2004), Galal (2006) and Elbadry *et al.*, (2006) reported that (SAR) against virus infection has been sureness using biological and chemical inducing agent, i.e. botanical, plant extracts that had been found effective against many plant pathogen. Botanicals; over a wide range have been used in the management of pathogenic plant viruses. Plant extracts also, have been found effective against a lot of pathogens (Chakraborty and Chakraborty, 2010). Dugger and Armstrong (1925) used the Juice or crude extract of Pokeweed (*Phytolacca decandra* L.) against *Tobacco mosaic virus*; they found that all treatments markedly inhibited the infectivity of this virus. Also, Arslan and Erkan (2012) studied the inhibitory effect of 14 plant extracts on the infection of *Tobacco mosaic virus* (TMV). According to their results of experiments on tested plants, the highest inhibition leaves were obtained with three plant extracts (*Dianthus caryophyllus*, *Capsicum annum* and *Yucca elephantipes*). The systemic inhibitory effect of some plant extracts on *Bean yellow mosaic virus* in faba bean plants under greenhouse conditions was tested by Mahdy *et al.*, (2007), the fresh leaf extracts of *Dianthus caryophyllus* showed antiviral and induced systemic resistance in faba bean plants reacting hypersensitivity to BYMV. Many chemical compounds have been reported as resistance inducers in plants, between these chemicals Salicylic acid (SA). (SA) well regarded one of the key components of

defense signal transduction. It known that SA is a natural product of numerous plants and induce resistance to viral infection. SAR is dependent on SA signaling and associated with systemic expression of pathogenesis- related Protein genes and other putative defenses. Once induction happened, SAR-expressing plants are primed to respond to subsequent pathogen infection by induction of defenses that are localized at the site of attempted pathogen ingress. SA induces a full set systemic acquired resistance genes application influence physiological processes such as transpiration rate, stomata closure, membrane permeability, growth, antioxidant capacity and photosynthesis (Deya Eldeen *et al.*, 2006 and 2008). In plant resistance, SA play an important role belong to varius biotic like virus infection (Borsani *et al.* 2001; Radwan *et al.* 2006, 2007 and 2008). SAR establishment in tobacco and Arabidopsis get together with SAR genes (Ward *et al.*, 1991) that correlate with some of these encoding pathogenesis- related (PR) proteins (Van Loon and Van Strien, 1999). These (PR) proteins revealed as acidic  $\beta$ -1,3-glucanases and chitinases, moreover, these PR proteins accumulate as the molecular basis for SAR (Van Loon and Van Strien, 1999). Therefore, the cumulation of PR proteins has often been suggested as the molecular basis for SAR. However, the past few years it became widely appreciated that the accumulation of PR proteins does not per se explain the SAR phenomenon (Van Loon, 2000). Cloning of PR genes and plant transformation have not provided an inducible acidic glucanase or chitinase alone or in combination, enhance resistance to fungal pathogens. Because of these reasons, the contribution of PR portions to SAR appears to be minor (Van Loon, 2000).

The present study aims to isolate and identify *Bean yellow mosaic virus Potyvirus*; control and reduce legumes virus-infections including natural and chemical alternatives as systemic resistance inducers for induction of systemic acquired resistance (SAR) and enhancing protection in greenhouse.

## MATERIALS AND METHODS

### 1. Isolation of *Bean yellow mosaic virus* (BYMV):

*Bean yellow mosaic virus* (BYMV) was isolated from common bean (*Phaseolus vulgaris*, L. cv. Karnk) plants, which collected from different localities in Menoufia governorate, Egypt, during Nile and winter seasons of 2013. Leaf samples showing yellow mosaic, blisters, leaf curling, malformation and often stunting symptoms, doubted to be due to virus infection were suspected in the subsequent experiments.

### 2. Biological identification

The samples (doubted to be due to virus infection) were homogenized in a sterilized mortar, after adding phosphate buffer (1:5 w/v, 0.1 M, 0.1 ML, pH 7.2, (Mahdy *et al.*, 2007) then the extracted sap was passed through a double layer of cheesecloth. The saps used for mechanical inoculation into healthy differential hosts as: *Chenopodium quinoa*, *Chenopodium album*, *Chenopodium amaranticolor*, *Nicotiana tabacum* and *Nicotiana glutinosa*, they were inoculated into host range as: *Vicia fabae* L.cv Balady , *Pisum sativum* L.cv Mister B, *Vigna unguiculata* L.cv Black eye, *Phaseolus vulgaris* L. cv. Karnk, *Glycine max* L.cv. Giza 21 and *Trifolium alexadrium* L. Inoculated plantes were maintained in

greenhouse and examined daily for symptoms expression. At least, ten seedling of each species and variety were inoculated with the virus isolate. Four weeks later, symptoms on infected plants were checked for virus infection by back inoculation onto healthy bean plants. All the following experiments were carried in Faculty of Agriculture, Monofiya University and Virus and Phytoplasma Research Section, Phytopathology Research Institute, Agriculture Research Center (ARC), Giza.

### 3. Serological detection of *Bean yellow mosaic virus* (BYMV):

#### DAS-ELISA:

Naturally viral infected bean samples directly detected with the double antibody sandwich-enzyme linked immuno-sorbent assay (DAS-ELISA) using antisera specific to BYMV as described by Clark and Adams (1977).

#### Dot-blot immunobinding assay (DBIA):

Dot blot immunobinding assay was used for identification of BYMV isolate as described by Lin *et al.*, (1990).

### 4. Preparation of standard virus inoculum:

The leaves showing mosaic yellowing symptoms and examined by serological testes were homogenized in phosphate buffer (pH 7.2, 100 mM) as described by Madhusudhan *et al.*, (2011) in a sterilized mortar. Sap extract was squeezed through two layers of muslin cloth and the solution was centrifuged at 5000 rpm for 10 min. The clear supernatant was used as the source of inoculum.

### 5. Preparation of Salicylic acid (SA) as chemical inducer:

The SA solutions (25 mM, 50 mM and 100 mM) were prepared by dissolving (0.25 & 0.50 and 1.0 g) in 100mL of chilled distilled water, respectively as mentioned by Madhusudhan, *et al.*, (2011).

### 6. Preparation of aqueous plant extracts:

Dried leaves, flowers, fruits, buds and rhizomes of different plants belonging to 6 families were collected from the local market and the botanical garden of Faculty of Agriculture, Menoufia University. The aqueous extraction of water-soluble compounds was done using the method which is described by Mbata *et al.*, (2006). Each of the ground ingredients (15 g) were extracted by soaking for two days using 150 ml of distilled water in a glass flask (250 ml) at room temperature on a rotary shaker. The water extracts were filtered using filter paper (Whatman No.1). The extracts were concentrated using vacuum freezer-dryer. The obtained concentrates were stored in test tubes (50 ml) and kept at 4°C in refrigerated prior to use. From each stock aqueous crude extract, three concentrations were made *i.e.*, 5, 10, 15 % using distilled water.

### 7. Induced systemic resistance against BYMV:

#### Effect of tested inducers on infected indicator plant with BYMV:

In this trail, six botanical extracts of *Dianthus caryophyllus*, *Glycyrrhiza glabra*, *Psidium guajava*, *Solanum nigrum*, *Spinacea oleracea* and *Thymus vulgaris* were used at 5, 10 and 15 % concentrations in addition to Salicylic acid at 25 mM, 50 mM and 100 mM as inducers against BYMV. The tested inducers were sprayed using a hand automizer sprayer (1L) on ten *Chenopodium amaranticolor* leaves for each treatment 24 hrs pre-inoculation with BYMV sap where each plant received about 20 ml solution. Leaves of *Ch. amaranticolor* were

dusted with fine carborandum (600-mesh), then they mechanically inoculated with virus inoculum. The control plants were sprayed with distilled water. Total number of local lesions on the treated tenth leaves of each treatment was counted at 5-10 days post virus inoculation (Mahdy *et al.*, 2007). The percentage of inhibition of local lesions formation by each treatment over the control was calculated based on the number of local lesions produced using the following formula described by Devi *et al.*, (2004).

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

Where:

A= Control B= Treatment

**Effect of tested inducers on infected bean plants with BYMV:**

After 21 days of growth, bean plants with similar size were selected and divided into different groups. Each group consists of three replicates (replication is one pot containing three healthy plants). Eight groups were treated as follows; (G1) healthy plants (control) without any treatments, (G2) Inoculated plants with BYMV and sprayed with distilled water at the same time as other tested groups. The other groups from 3 to 8 pre-treated with 15% of each extract as: (G3) *Dianthus caryophyllus*, (G4) *Glycyrrhiza glabra*, (G5) *Psidium guajava*, (G6) *Solanum nigrum*, (G7) *Spinacea oleracea* and (G8) *Thymus vulgaris*. As for the effect of SA on BYMV, the second experiment was carried out using the different concentrations (25, 50 and 100 mM) of Salicylic acid (SA) with the same treatments in first experiment as mentioned above by spraying the leaves until run off. All leaves were mechanically inoculated with BYMV before treatment. The control treatment was inoculated with BYMV and sprayed with distilled water only. Inoculation of virus was performed a day before spraying. Spraying was performed on the entire plant shoots until the leaves looked fully saturated the inducers. To improve spread of various reducers, two drops of Tween 80 were added (Deya Eldeen *et al.*, 2008). Treated and untreated plants were kept under greenhouse and observed for the appearance of systemic symptoms formation.

**8. Protein Electrophoresis:**

This trail was done to exhibit the reacted antiviral proteins against BYMV infection which initiated because of treating bean leaves with different plant extracts comparing with those initiated in bean leaves of healthy and infected only with BYMV. In this respect, electrophoretic analysis using SDS-PAGE technique as described by Laemmli, (1970) was used for this purpose. Screening for induced systemic resistance against BYMV infection by making rapid isolation of the initiated antiviral proteins and others according to the described protocol by Bollag *et al.*, (1996) who used SDS-PAGE analysis.

**9. Histopathological changes in treated bean tissues with tested inducers using Transmission electron microscopy (TEM) technique:**

Small segments were taken from treated and untreated leaves and were cut into small pieces about 1-2 mm, then fixed in 2% Glutaraldehyde in phosphate buffer, pH 7.2 and subjected to a vacuum for 1-4 minutes every 15 minutes for 2 hours on ice. Prior to a vacuum treatment, floating samples were poked under the buffer surface with pointed metal pokers. Rinsing took place in 0.1 M Na-Cacodylate buffer, pH 7.2 for 45 min, with buffer changes

at 15 and 39 min. Further fixation in 1% Osmium Tetraoxide in Na-Cacodylate buffer, under intermittent vacuum and poking, took place for 1.5 hours. Samples were then rinsed again in the Na-Cacodylate buffer (Williams and Carter, 1996).

Samples were dehydrated through an Ethanol series in buffer: 35% - 50%- 70%- 80%- 95%- 100% for 60 minutes each. Then infiltrate with Resin. Semi thin sections were prepared on glass slides through cutting at 1um using the ultramicrotome. Sections were stained with Toluidine blue for 5 min. Examined by light microscope model M-200M (Hanschke and Schauer, 1996).

Ultra-thin sections were cut using ultramicrotome Leica model EM- UC6 at thickness 90nm, mounted on copper grids (400 mesh). Sections were stained with double stain (Uranyl acetate 2% for 10 min followed by Lead citrate for 5 min and examined by transmission electron microscope JEOL (JEM-1400) at the candidate magnification. Images were captured by CCD camera model AMT, optronics camera with 1632 x 1632 pixel format as side mount configuration. This camera uses a 1394 fire wire boared for acquisition (Jakstys, 1999). The work was done in TEM lab FARP. Faculty of Agriculture Research Park-Cairo University.

**RESULTS**

**1. Isolation of Bean yellow mosaic virus (BYMV):**

*Bean yellow mosaic virus* was isolated from naturally infected common beans (*Phaseolus vulgaris*, L. cv. karnk) plants growing under field conditions in Menoufia governorate, Egypt which showing yellow mosaic, blisters, leaf curling, malformation and often stunting symptoms as clear in Fig. (1A).

**2. Serological detection of Bean yellow mosaic virus (BYMV):**

**DAS-ELISA**

This test using DAS-ELISA was done as a rapid diagnostic tool to confirm the presence of BYMV either as pure form or in mixed form with BCMV and BLRV in some infected bean leaves samples. Three leaves samples were used from three different districts (Menouf, El Bagour and Ashmon) represent Menoufia governorate. Table (1) and Fig (1) showed that Ashmon sample was the only positive reaction according to DAS-ELISA test. The other samples showed negative reaction

**Table 1. DAS-ELISA test on bean leaves from three different locations represent Menoufia governorate.**

Location	Symptoms	ELISA
Menouf	+	-
El Bagour	+	-
Ashmon	++	+
ELISA	+ Positive reaction	- Negative reaction
Symptoms	+ weak symptoms	++ moderate symptoms

**Dot-blot immunobinding assay (DBIA):**

As clear in Fig. 1(B,C), the technique dot-blot immunobinding assay (DBIA) was successful to make readily detection of BYMV on nitrocellulose membrane which probed 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 reaction was indicated by a purplish-blue color whereas negative reactions remained green.

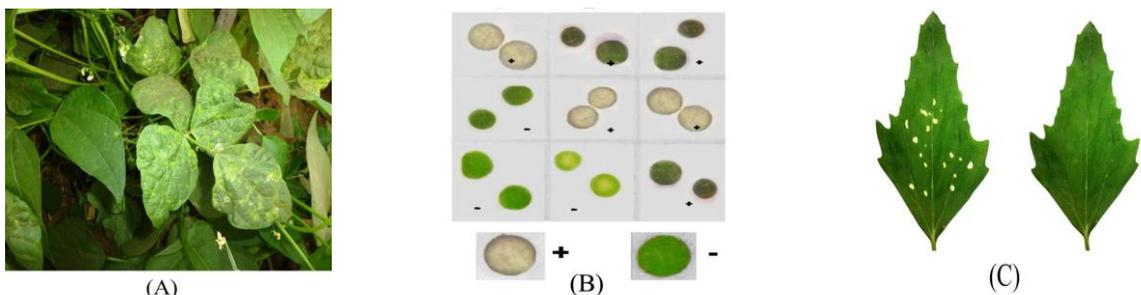


Fig 1. (A) Symptoms of BYMV on naturally infected common bean plants cv. Karnk, showing yellow bright, mosaic, vein banding, blisters and malformation of leaves, (B) Dot Blot Immunoassay for BYMV precipitation against specific IgG- BYMV polyclonal. + = Positive samples - = Negative samples and (C) Chlorotic local lesions on *Ch. amaranticolor* L.

**3. Induced systemic resistance against BYMV: Effect of tested inducers on infected indicator plant with BYMV:**

Data in Table (2) indicate that spraying leaves of the indicator *Chenopodium* plant with the different six tested plant extracts in addition to SA as resistance inducers, 24-hrs pre-inoculation with BYMV sap inhibited effectively the number of local lesions on treated leaves of *Chenopodium* comparing with control treatment (inoculated only with BYMV sap). In this respect, the least recorded number of local lesions on treated leaves of *Chenopodium* was scored on treated plants with *Dianthus caryophyllus* extract with inhibition% reached 95.8% at 15% concentration followed by those sprayed with *Solanum nigrum* extract and Salicylic acid (SA) as chemical inducer. Also, increasing the concentration of six tested plant extracts from 5-15% and SA from 25-100 mM increased gradually the inhibition% and minimize the appeared local lesions on inoculated

*Chenopodium* leaves with BYMV sap. On the other hand, the least effective extract among the six tested ones was of *Psidium guajava* where the recorded inhibition% at the three tested concentrations were the least.

**Efficacy of inducers on detection in systemically infected plant (*Phaseolus vulgaris* L. cv. karnk) against BYMV.**

Infected bean plants (*Phaseolus vulgaris* L. cv. karnk) by BYMV revealed severe symptoms in comparison to healthy one. Young leaves showed clearer symptoms, which more newly developed than fully expanded older ones. In addition to mosaic, another viral symptoms appears on infected bean leaves, i.e. severe mottling, crinkling, blisters and deformation. Reduction of symptoms appearance by plant extract application was noticed especially; harmful symptoms caused by virus developed; the most effective plant extract was *Dianthus caryophyllus* that affecting BYMV infection. Fig. 2.

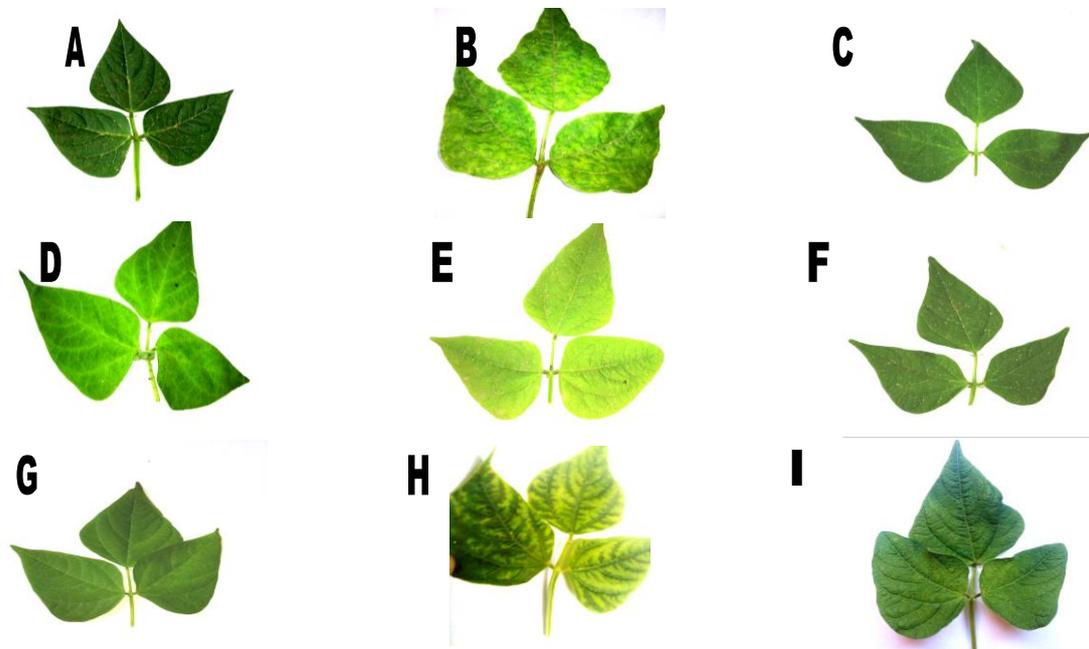


Fig. 2. Effect of BYMV infection and treatments on leaf morphology of *Phaseolus vulgaris* L. All plant extracts decreasing disease severity, disappearing totally symptoms in (*Dianthus caryophyllus* treat) and showing normal morphological appearance.

- A. Healthy *Phaseolus vulgaris* L plants, without any treatments.
- B. Infected plants with BYMV, show malformation, blisters and yellow mosaic
- C. Sprayed plants with *Dianthus caryophyllus* extract.
- D. Sprayed plants with *Glycyrrhiza glabra* extract.
- E. Sprayed plants with *Psidium guajava* extract.
- F. Sprayed plants with *Solanium nigrum* extract.
- G. Sprayed plants with *Spinacea oleracea* extract.
- H. Sprayed plants with *Thymus vulgaris* extract.
- I. Sprayed with salicylic acid

**Table 2. The inhibitory effect of different plant extracts on BYMV infection using the indicator plant *Ch. amaranticolor*.**

Tested natural and chemical inducers	Used part	Conc. (% & mM)	No. of local lesions on treated leaves of <i>Chenopodium</i> *	Inhibition (%)
<i>Dianthus caryophyllus</i>	Flowers and buds	5	25	73.7
		10	15	84.2
		15	4	95.8
<i>Glycyrrhiza glabra</i>	Rhizomes	5	73	23.2
		10	60	36.8
		15	41	56.8
<i>Psidium guajava</i>	Leaves	5	79	16.6
		10	70	26.3
		15	50	42.1
<i>Solanum nigrum</i>	Leaves and Fruits	5	23	75.8
		10	18	81.05
		15	9	90.5
<i>Spinacea oleracea</i>	Leaves	5	43	54.7
		10	35	63.3
		15	18	81.0
<i>Thymus vulgaris</i>	Leaves	5	60	37.8
		10	51	46.3
		15	30	68.4
Salicylic acid (SA)		25 mM	33	65.3
		50 mM	20	78.9
		100 mM	8	91.5
Control (un-treated)			95	

\*Total number of local lesions on ten leaves of *Chenopodium* plant used in each trial

**Antiviral protein (SDS-PAGE)**

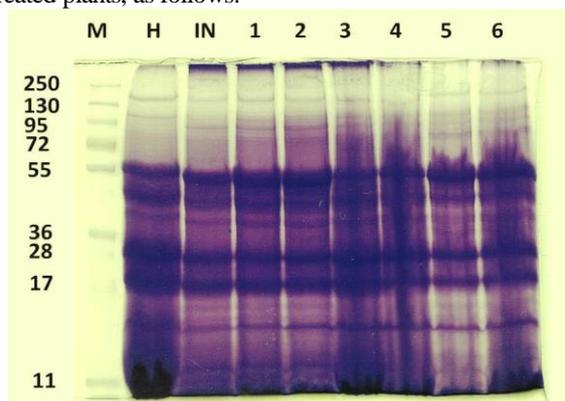
Using bean leaves (healthy, infected and treated with inducers) in Fig (2), SDS-PAGE analysis is used to analysis the protein profiles of healthy, infected and treated with biotic inducers pre- BYMV inoculation plants in.

**Table 3. Zero, one of healthy plants, infected plants and extract treatments (*Dianthus caryophyllus*, *Glycyrrhiza glabra*, *Psidium guajava*, *Solanum nigrum*, *Spinacea oleracea* and *Thymus vulgaris*).**

Total MW	healthy plants	Infected plants	<i>Dianthus caryophyllus</i>	<i>Glycyrrhiza glabra</i>	<i>Psidium guajava</i>	<i>Solanum nigrum</i>	<i>Spinacea oleracea</i>	<i>Thymus vulgaris</i>
111	+	+	+	+	+	+	+	+
97	+	+	+	+	+	+	+	+
92	-	+	+	+	+	-	+	+
84	+	+	+	+	+	+	+	+
69	-	-	+	-	-	-	-	-
67	+	+	+	+	+	+	+	+
64	+	+	+	+	+	+	+	+
56	+	+	+	+	+	+	+	+
55	+	+	+	+	+	+	+	+
48	+	+	+	+	+	+	+	+
42	-	-	-	+	-	-	+	+
37	+	+	+	+	+	+	+	+
36	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+
26	-	+	+	+	-	-	+	+
23	+	+	+	+	-	-	+	-
22	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+

- The infected plants showed different bands than healthy plants, at molecular weight (92 and 26 kDa).
- The pre- treated with *Dianthus caryophyllus* extract showed only band, it did not appear in healthy, infected and other treatments at molecular weight (69kDa).

[Fig (3) shows a considerable difference in protein profiles of all treatments with molecular mass from 11 kDa to 250 kDa. All treatments contain the main protein band between (15 to 111 kDa).The SDS-PAGE protein gel is analyzed using Clustering analysis using Jaccard's average (UPGAMA) clustering method, version (3.1): The zero-one (Table 3) was done for the gel according to the molecular mass of marker and existence of each band in the same molecular mass in all lanes of the gel. The zero one results showed that there were differences in molecular weights (23, 42, 26, 42, 69, and 92 kDa) between healthy, infected and treated plants, as follows:



**Fig. 3. SDS-PAGE of total plant protein of all treatments: M: Protein marker (kDa). H: Healthy plants. In: Infected plants. 1-6: Lanes of common bean sprayed with plant extract of *Dianthus caryophyllus*, *Glycyrrhiza glabra*, *Psidium guajava* extract, *Solanum nigrum*, *Spinacea oleracea* and *Thymus vulgaris*; repectively.**

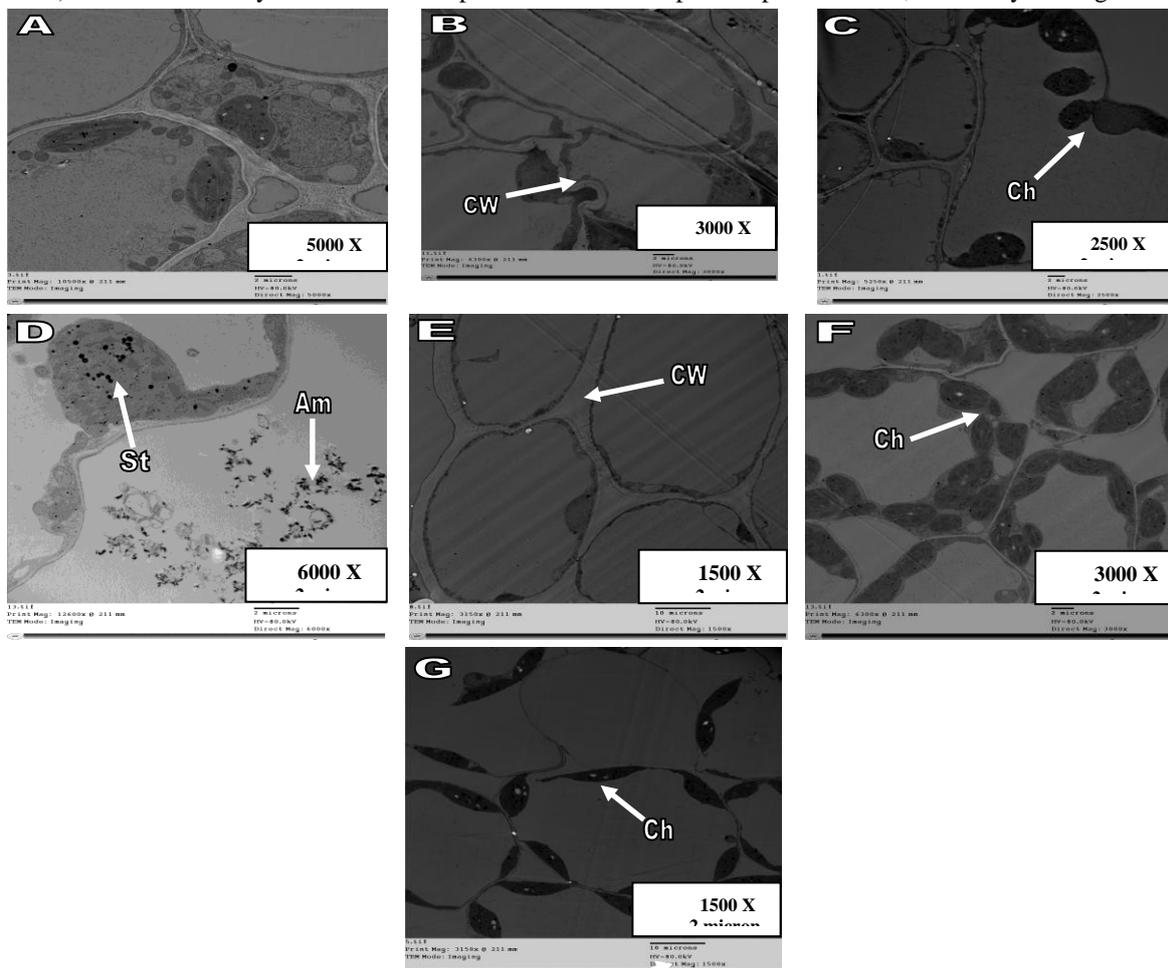
- The pre-treated with *Glycyrrhiza glabra* extract, showed a band at molecular weight (42 kDa), it is not present in infected and healthy plants.

- The pre-treated with *Psidium guajava* extract showed absence a band when protein molecular weight of 23 and 42 kDa, was present in healthy and infected plants.
- The pre-treated with *Solanum nigrum* extract, showed absence a band, that was present in infected and other treatments, similar to healthy plants at molecular weight (26 kDa). Also, disappeared a band at molecular weight (23 kDa), was present in healthy and infected plants.
- The pre-treated with *Spinacea oleracea* extract, showed a new band at molecular weight (42 kDa), appeared in some of the treatments but not present in the healthy and the infected plant.
- The pre-treated with *Thymus vulgaris* extract, showed absence a band when molecular weight (23 kDa), that was present in infected, healthy and other treatments. Also a new band at molecular weight (42 kDa), appeared in some of the treatments but not present in the healthy and the infected plant.

**Histological changes (Transmission electron microscopy)**

Control, infected, healthy plants treated with *Dianthus caryophyllus* extract (the highest treatment) and plants treated with *Psidium guajava* extract (the lowest treatment) were examined by electron microscope. Control

samples (healthy plants without any treatments) revealed as normal tissues without any changes in cell organelles or chloroplast shape or structure (Fig 4A). In the infected leaves by BYMV; ultrastructural investigation, showed deformations and wrinkles in cell wall (Fig 4B). Chloroplasts in infected cells lost their normal arrangement and appeared spherical or longish and curved in shape as well as accumulation of many starch grains and amorphous inclusions. Some chloroplast had partially or totally damaged envelopes, resulting in stromal matrix exclusion in the cytoplasm (Fig4C, D). Ultrastructural investigations of *Dianthus caryophyllus* extract -treated samples showed that the extract can slightly regain the harmful effects of BYMV infection. *Dianthus caryophyllus* extract application caused the irregularity in cell wall (Fig.4E). And caused significantly increased the chloroplast number per cell. Chloroplast structure and organization appeared normal and well developed (Fig 4F). Generally, cells of *Dianthus caryophyllus* extract – treated samples, like controls, showed well- developed chloroplasts with well-organized cell organelles . On other hand, *Psidium guajava* extract-treated samples, like infected cells showed some modifications in chloroplast shape or structure, with many starch grain.



**Fig 4. (A) Ultrastructural modifications of healthy (*Phaseolus vulgaris* L.cv. karnk) leaves performing no cytological changes in cell organelles or chloroplast shape or structure. (B) In BYMV-infected leaves, deformation and wrinkles in cell wall (CW). (C, D) Chloroplasts (Ch) lost their normal arrangement, some chloroplasts had partially or fully damaged, resulting in stroma matrix exclusion in the cytoplasm and appeared spherical or longish, curved in shape, many starch (St) grains and amorphous inclusions (AM) were observed. (E, F, G) Increasing in the chloroplast number per cell and irregularity in cell wall with *Dianthus caryophyllus* treatments (E), some modifications in chloroplast shape or structure (F), with many starch grains with *Psidium guajava* treatments (G).**

## DISCUSSION

BYMV severely affects many crops including bean (*Phaseolus vulgaris*), the infected bean plants with BYMV great affected by virus infection; destroyed plants and resulting great loss in bean yield. In our study, many visually symptoms were detected and noted during BYMV infection; these symptoms including sever mosaic, yellowing, deformation, size reduction and blisters, as well as these symptoms were more marked in young leaves than in older one. The same symptoms had been recorded by the infection of BYMV in faba bean (*Vicia faba*), soybeans (*Glycine max*) and through infection with other *Potyvirus*es (Deya El-deen *et al.*, 2008).

In the present study, BYMV isolate was detected using serological tests (DAS-ELISA and dot blot technique), which were successfully used. Positive reaction was obtained when specific IgG of BYMV was used. Many investigators have used ELISA test for serological detection of BYMV from different hosts (Campos *et al.*, 2014, Kumar *et al.*, (2015) and Pradeep *et al.*, 2015).

In the dot blotting technique, the specific antigen was immunologically localized with enzyme labeled antibody on nitrocellulose membranes. This technique has been found to have much higher sensitivity for the detection of BYMV. These result in agreement with those recorded by Muthana *et al.*, (2001).

In this work, the inhibitory effects of some plant extracts and chemical inducers were determined in greenhouse for virus elimination. Investigation was carried out using materials obtained from six plants belonging to six families, i.e. *Caryophyllaceae*, *Chenopodiaceae*, *Lamiaceae* (*Labiatae*), *Myrtaceae*, *Papilionaceae* and *Solanaceae* family and Salicylic acid (SA) inducer to induce acquired resistance to BYMV. Different plant organs and their extract represented the materials tested and SA. All inducers tested in this study inhibited the infection with BYMV with different degrees.

The first criterion to judge the occurrence of SAR in *Ch. amaranticolor* plant treated with plant extracts and SA inducers, the reduction of percentage of infection. All inducers were able to reduce the local lesions count on *Ch. amaranticolor* leaves, that indicated the plant resistance to the virus infection. The obtained results in our experiments showed that all inducers reduce BYMV infection in the range between (42.1 - 95.8 %) at 15% concentration. *Dianthus caryophyllus* extract was the best inducer by (95.8 %) followed by *Solanum nigrum* extract (90.5%), *Spinacea oleracea* extract (81%), *Thymus vulgaris* extract (68.4 %), *Glycyrrhiza glabra* extract (56.8%) and *Psidium guajava* extract (42.1%).

On the other hand, pre BYMV inoculation, the spraying of SA can delay or prevent the disease symptoms. Interestingly, 100mM of SA- treated plants showed highest increase in reducing BYMV infection (91.5%). This result came in harmony with those recorded by Tolba (1980) and Mahdy *et al.*, (2007).

Our results were consistent with Madhusudhan *et al.*, (2011), they were tested the effect of Salicylic acid against *Tobamovirus*es infection in Bell pepper and Tomato plants. The results presented that the inducer used for testing was efficient in decreasing the number of local lesions formed by the challenge inoculation of *Tobacco mosaic viruses* (TMV). Also Carl *et al.*, (2005) reported that (SA) revealed as resistance inducer to *Cucumber mosaic virus* (CMV) in Tobacco (*Nicotiana tabacum*) prevent the systemic virus action and stimulate a signal transduction pathway and can be triggered by antimycin A, an inducer of the mitochondrial enzyme alternative oxidase (AOX).

Quantitative proteins of induced bean plants were determined using SDS-PAGE, the results indicated that, a new pattern of proteins were produced, as well as, different increasing in the density of bands among biotic inducers treatments. It has been proposed that, the induced proteins may help to stop virus diffusion or multiplications (Mahmoud, 2000 and Chen *et al.*, 2006). The localization of viral infection may be helped by the continuous of newly-induced proteins; the contrary is not correct, since the existence of insignificant amount of induced proteins is a needful condition to the observed systemic infection.

Plant extracts may induce resistance or act role in suppression of viral replication. In this respect; ribosome inactivating proteins (RIPs) and glycoproteins may prevent the sites of replication. After the botanical treatment, the signal inducer may be produced in treated leaves with the host plant surface. This signal creates an inhibiting factor for the virus in the entire plant system. Specific low molecular weight pathogenesis related proteins might also play a role in the induction of (SAR). In order to these reasons, biologically active compounds present in plant products act as elicitors and induce resistance in plant hosts in reduction of disease development (Verma *et al.*, 1998). Stripe *et al.*, 1981 reported that ribosome inactivating protein (RIP) called Dianthin antiviral protein (DAP) was found in *Dianthus caryophyllus* (*Caryophyllaceae*) and play role against infection by *Tobacco mosaic virus*. Spraying of (DAP) on tested plants 24h pre virus inoculation inhibited infection by about 100%. The tow active proteins named dianthin (30) and (32) depended on the molecular weight were noticed in *Dianthus caryophyllus* leaves purified extract, these proteins inhibit protein synthesis in a cell-free system by damaging ribosomes, but have little effect on whole cells, meanwhile it's have strong inhibition activity on the replication of *Tobacco mosaic virus*. These two proteins similar to another two antiviral proteins purified from carnation leaves by Ragetli and Weintraub (1962a, b).

Mahdy *et al.*, (2007) show that the flower buds of *Dianthus caryophyllus* show high inhibitory activity against plant viruses, most of these substance have been found to be proteinaceous nature and are known as antiviral proteins (AVPs). They can manifest their effects either by inactivating the viral pathogen or acting indirectly by inducing host resistance. The presence of such AVPs in extracts of several higher plants like *Dianthus caryophyllus*, *Mirabilis jalapa*, *Clerodendrum* spp. and *Bougainvillea* spp., have been reported.

Histopathological changes (as indicator to SAR) in bean leaf tissues sprayed with inducers and pre-virus inoculation were examined. The inoculation of bean leaves by BYMV changed leaf morphology, yellowing, mosaic and photosynthesis properties, reduction of pigments concentrations; the ultrastructure researches were concentrated firstly on chloroplasts. After BYMV inoculation by 25 days, most of chloroplast turned spherical in shape, others noticed without envelopes and internal structure e.g. grana and thylakoids were dilated (Shalitin and Wolf, 2000). Many starch grains were observed in BYMV-infected cells, virus infection inhibit decomposition of starch into dissolved sugars and thus prevent transmission to the outside of the leaf in the form of decomposed products accumulate inside the leaf, a result of its impact on enzymes analyzing starch (Fekry, 2006). Chloroplast modification plus endoplasmic reticulum were noticed ruptured in infected leaves with BYMV as well as wrinkles and deformations in cell wall were noticed (Deya Eldeen *et al.*, 2008).

No obvious modifications were revealed in leaves that treated pre-virus infection with the extract of *Dianthus caryophyllus* incomparing to control. Photosynthesis processes were improved and the destroy effects were recovered in addition disappear of cytological alteration that believing general evidence for *Dianthus caryophyllus* extract's role in resistance induction towards virus infection (Arfan *et al.*, 2007 and Deya Eldeen *et al.*, 2008).

Virus infection and *Dianthus caryophyllus* extract treatments were greatly affected chloroplast structure and numbers. The number of chloroplast was reduced significantly in BYMV- infected leaves and vice versa *Dianthus caryophyllus* treatments caused induction of chloroplast number. This number of chloroplast closely reverse related with mosaic and yellowing symptoms. The decreasing in chloroplast number and thylakoid system reduction may be due to virus- induced changes in metabolism (Goodman *et al.*, 1986). Furthermore, the lower number of chloroplasts is thought to be immediately responsible for the reduction of chlorophyll content revealed in BYMV-infected leaves. Improving of the photosynthesis with *Dianthus caryophyllus* extract treatment through increasing chloroplast number, photosynthesis rate as well as chlorophyll contents (Arfan *et al.*, 2007; Deya Eldeen *et al.*, 2008 and Radwan *et al.*, 2010).

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**إستحثات المقاومة الجهازية لفيروس الموزيك الأصفر في الفاصوليا والذي يصيب نباتات الفاصوليا باستخدام المستخلصات النباتية وحمض السالسليك**  
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فيروس الموزيك الأصفر في الفاصوليا BYMV ظهر في السنوات الأخيرة كأكثر الفيروسات المدمرة لمحصول الفاصوليا محدثاً أعراض الموزايك المصحوبة بالإصفرار. تم عزل الفيروس من عدة مناطق لزراعات الفاصوليا داخل محافظة المنوفية وتم إثبات تواجده باستخدام الإليزا المباشرة DAS- ELISA وكذلك تقنية Dot- blot immunobinding assay (DBIA). بالفحص المبدئى عن مستحثات المقاومة ضد هذا الفيروس باستخدام تحليل النقاط الموضوعية على نبات كشاف *Chenopodium amaranticolor* دلت النتائج أن كل المستحثات للمقاومة كانت فعالة في تقليل عدد النقاط الموضوعية والتي تكونت كرد فعل للحقن بالفيروس. التحورات البيوكيميائية والتشريحية التركيبية في أوراق نباتات الفاصوليا صنف كرنك كإستجابة للمناعة الجهازية المكتسبة SAR للإصابة بفيروس BYMV. كما إتضح أن رش كل المستحثات على أوراق الفاصوليا ساعدت في تقليل أو منع التأثير الضار الذى يظهر على النباتات عقب العدوى بالفيروس. وقد تم إستخدام تقنية إليزا كوسيلة تشخيصية لعملية المناعة الجهازية المكتسبة SAR. وكان مستخلص نبات *Dianthus caryophyllus* أكثر المستخلصات المستخدمة في عملية الدفع لتكوين المناعة الجهازية المكتسبة SAR في نباتات الفاصوليا المصابة بفيروس BYMV.