Biological and Serological Studies on *Pea Mosaic Virus* (PMV) on Cowpea Forage Crop (*Vignaunguiculata L. Walp*)

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ABSTRACT

In the present work, *Pea mosaic* (PMV) was first reported on cowpea (V*ignaunguiculata* (L.) Walp).PMV was isolated from naturally infected cowpea plants, showing mosaic and choloratic ring spot symptoms, which had been grown in the experimental fields of Giza Agricultural Research Experimental Station (A.R.E.S) in 2008. Identification studies based on host range, symptomatology and seed transmission through cowpea and different hosts belong to Fabaceae.The results indicated that the host range of the virus was expanded to seven different plant families. The virus was mechanically transmitted, and the percentage of seed transmission was 6%. PMV was partially purified for its antiserum production. The absorption spectrum of the purified virus had a min at 245 nm and a max at 260 nm. The ratios of A260/280, A280/260 and Amax/min were 1.16, 0.85, and 1.04, respectively. Yield of PMV was 1.7 mg/100g of infected leaves. Electron micrographs of the partially purified virus preparation revealed the presence of filamentous flexuous virus particles about 700- 750nm long. Titers of the antisera after first, second and third bleeding were 1/800, 1/1600 and 1/3200 respectively.The optimum concentrations of IgG and IgG conjugate were 1.0 µg/ml and 1/1000, respectively. The antigen dilution end point was 1:500.The produced antiserum was evaluated by ELISA and DBIA. Electron microscopy f ultrathin-section of PMV- infected leaf tissue revaled several morphological changes and aggregated virus particles associated with pinwheel inclusion bodies. Salicylic acid and Parahydroxy benzoic acid were used for induction of systemic acquired resistance, whereas camphor oil was used as an antiviral agent to reduce PMV infection. Parahydroxy benzoic acid was effective at 0.001% concentration, exhibited 88.7% reduction when had applied 2h before inoculation. Also, salicylic acid was effective as it reduced infection to 77.4% at 0.01% concentration when applied 3h before inoculation. Whereas camphor oil exhibited lesser reduction being (74.6) when used at 0.001% concentration before 2 h from inoculation.

Key words: *Pea mosaic virus*, Host range, Seed transmission, Purification, Electron microscopy, IgG, ELISA, DIBA, Salicylic acid, Parahydroxy benzoic acid, Camphor oil.

INTRODUCTION

Viral diseases in field-grown cowpea plants are the major concern for the forage crops, feed leguminous growers and the rate of production (Cuttings yield). Cowpea growing areas have been affected by several viruses (Gillaspie *et al*., 1995 and Taiwo *et al.*, 2007).

Pea mosaic virus (PMV), an economically significant seed – transmitted virus of pea has commonly found in pea germplasm collection of many countries. The virus is suspected to have spread world- wide due to the exchange of infected germplasm material (Khetarpal and Maury, 1987).

Various percentage of seed transmission of PMV was recorded by several authors depending on different factors like growth conditions, cultivar genotype, seed size and the severity of PMV strain .(Hmpton and Mink, 1975 and Makkouk *et al.*1988)

Different serological methods were reported to detect *Pea mosaic virus* including enzyme linked immune sorbent assay (ELISA), Dot-blot immunoassay (DBIA) and Tissue blot immunoassay (TBIA) on nitrocellulose membrane.

Aiming to minimize the negative effect of pesticides are been development the alternative control of plant disease, which includes the induction of resistance and the use of natural products with induction of resistance and /or with direct antimicrobial activities. In the latter include the use of extracts and essential oils from medicinal plants (Stangarlin*etal.*,1991)

Several chemical and natural compounds including salicylic acid, benzoic acid and natural oils were reported to use for plant virus control purposes and effective results was obtained (Kobeasy & Zein 2005).

The aim of the current study was: Firstly, isolation and identification of the causal agent found in collected cowpea samples using the traditional(biological)methods. Secondly, purification and production of ELISA reagents which can be used as a rapid serological diagnosis for PMV. Thirdly cytological studies using electron microscopy. Finally, the control measures using different applications such as antiviral agent and induced systemic resistance against the virus isolate.

MATERIALS AND METHODS

Part I: Isolation and identification:

1. **Isolation:**

Samples of naturally infected cowpea (*Vignaunguiculata* L.) plants showed mosaic and cholorotic ring spot symptoms were collected from Giza Agricultural Research experimental Station (A.R.E.S) in 2008. The collected samples were screened for viruses by ELISA test using antisera of Cucumber *mosaic virus* (CMV), *Broad bean true mosaic virus* (BBTMV), *Bean yellow mosaic virus* (BYMV), *Pea mosaic virus* (PMV), *Pepper mild mottle virus* (PMMoV) and *Bean common mosaic virus* (BCMV). Polyclonal antibodies obtained from Serology Lab., Virus Research Dept., Plant Pathology Research Institute were used in indirect ELISA as described by Clark and Adams (1977). The virus isolate was biologically purified by single local lesion technique *(*Kuhn, 1964). The virus isolate was propagated in pea plants as a main host while Ch.*amaranticolor* was used as local lesion host. Cowpea plants reacted positively only against PMV antiserum were served as source of virus inoculum.

2- Identification of the virus isolate:

2.1. Host rangeand diagnostic host reactions:

The isolated virus was mechanically inoculated in 39 selected hosts including certain diagnostic hosts belonging to *Alliaceae*, *Cucurbitaceae*, *Chenopodiceae*, *Fabaceae*, *Graminaceae, Brassica ceae*and *Solanaceae*. Seedlings of each host plant were maintained four weeks in the greenhouse for symptoms development at temperature average 25-30^{o} C. An equal number (10 seedlings) of healthy seedlings of the same age and species were left as a control. Symptomless plants were assessed biologically and serologically by indirect ELISA method.

2.2. Transmission through cowpea (V.*unguiculata* (L.) Walp) seeds:

Healthy cowpea plants (Buffalo.cv) were mechanically inoculated with PMV. The inoculated plants were kept inside an insect-proof greenhouse. Two weeks after inoculation, indirect ELISA was used to check the virus presence in cowpea plants. Plants, which gave positive reaction, were labelled and kept till seed formation. Two hundred seeds were collected from infected plants and tested for seed transmission as they were sown in pots (25 cm diameter) containing sterilized clay soil. Seedlings were kept in the greenhouse. Percentage of seed transmission was calculated as number of seedling showing symptoms and gave positive reaction with Indirect ELISA

divided by No. of immerged seedlings X 100.

3- Serologigal detection of the virus isolate:

Indirect ELISA technique was performed to confirm the identity of the virus isolate

Part II-Virus purification and ELISA reagents production:

1. Purification of the virus isolate:

The inoculatedpea plants by the virus isolate were used as a source for virus purification using a modified method described by Gamal El-Din *et al.*(1997).

1.1. U.V. absorptionspectrum of the purified virus:

U.V. absorption spectrum of the purified virus preparation was estimated spectrophotometrically with Spectronic 2000 at range from 220 to 320 nm. Virus concentration was calculated using the extinction coefficient of 2.4 (purcifull,1990).

1.2. Electron microscopy:

Partially purified virus preparations were negatively stained with 2% urinal acetate, mounted on, formvar-coated grids, examined with a JeolTEM-1400 in the Electron Misrcope Unit, Faculty of Agriculture, Cairo University and virus particles were photographed (Noordam,1973)

2. Antiserum production

2.1. Rabbit immunization and bleeding

Polyclonal antibodies were prepared by injecting New Zealand white rabbit intravenously eight times with 11mg.of purified PMV preparations. Serum was collected 7 days after the last injection along 3 weeks. Antiserum was separated by centrifugation at 5,000 rpm for 3 min. (Hampton *et al.,* 1990). The antisera was collected and stored at 4 C^{o} until used.

2.2. Determination of antiserum titre:

Antiserum was cross-absorbed with healthy plant proteins using a method by Hampton *et al*. (1990). Antiserum titer was determined using indirect ELISA technique. Clarified sap of virus infected (PMV) and healthy tobacco (*N. tabacum*, White Burley) plants, was diluted at 1/3. PMV antiserum was diluted to 1/100, 1/200, 1/400, 1/800, 1/1600 and 1/6400 (Lommel*et al*., 1982).

2.3. Purification of the immunogamaglobulin G (IgG).

IgG was purified from PMV antiserum using the caprylic acid method described by Steinbuch and Audran (1969).

2.4. Conjugation of IgG with alkaline phosphatase:

IgG was conjugated with alkaline phosphatase (AP) according to protocol given by **Clark and Adams (1977).**The concentrations of IgG and IgG conjugate were determined by a checkerboard test (Converse and Martin,1990) evaluating a series of conjugate dilutions against a series of trapping globulin dilutions using infected and healthy plant preparations of the same species. IgG conjugate was diluted to 1/250, 1/500, 1/1000 and 1/2000 with the conjugate buffer, while IgG was diluted with the coated buffer to concentration of 0.5, 1.0, 2.0 and 4.0 µg/ml, respectively. Controls of healthy and infected cowpea sap were used. The reaction was done between IgG and IgG conjugate using DAS ELISA.

2.5. Determination of antigen dilution end point:

ELISA reagents specific to PMV were assembled from IgG and IgG conjugated with alkaline phosphatase, positive and negative controls of the virus were diluted with the coated buffer to concentrations 1/500, 1/1000, 1/1500 and 1/2000.

2-6- Serological detection of PMV by DAS-ELISA and DBIA using the ELISA reagent produced:

2-6-1- Enzyme linked immunosorbent assay (ELISA):

The produced ELISA reagents were usd in detection of PMV in seed lots of different plant species as described by Convers Martin (1990).

This experiment was carried out at Agricultural Research Experimental Station. Mature seeds were collected from lots to estimatepercentage of natural infection with PMV through seeds of Chickpea (*Cicerarietinum),* Lentil (*Lens culinaris*), Pea (*Pisumsativum***),** Faba bean (*Viciafaba***)** and Peanut (*Arachishypogaea*)**.** ELISA reagents produced were used to detect the presence of PMV in the seeds after they were washed in running tap water and kept for 48 hr. in Petri dishes with wet cotton before homogenized and assayed by DAS- ELISA. Percentages of seed infection were calculated.

2-6-2- Dot- Blotting Immunobinding Assay on nitrocellulase membrane (DBIA):

The DBIA on nitrocellulose membranes was essentially similar to those described by Hsu and Lawson(1991)and Azzam *et al*. (2007)for the serological detection of antigen using authentic antiserum for PMV.

Part III- cytological studies using electron microscopy (EM).

Ultra-thin sections taken from mechanically inoculated tobacco leaves as well from cowpea seedlings immerged from infected seeds. The used technique was the same as describedby Bozzala and Russell (1999).This work was done in EM-Lab in Faculty of Agriculture- Cairo University Research Park (FARP).

Part IV- Virus control:

Salicylic acid, parahydroxy benzoic acid and camphor oil were used to test their inhibitory effect of PMV infection. Ten seedlings at 2 weeks stage for each treatment were sprayed with the compounds under investigation. The salicylic acid solution was prepared as described by Yalpani, *et al*. (1991) and Kobeasy & salwa, Zein (2005), benzoic acid (White & Antoniw *et al*., 1986 and Smith-Backer *et al*., 1998) Camphor oil (Ahmed, Amal *et al*. , 2010) were used as foliar sprays Each of the inhibitor were applied by spraying one 1h, 2h, 3h, and 4 h before mechanical inoculation using different concentrations i.e., 0.0001, 0.001, 0.01, respectively.

Fifteen leaves of the local lesion host plant dusted with carborundum (600 mesh) for each treatment were inoculated with the virus isolate. Total number of local lesions for each treatment was counted two weeks post virus inoculation. Equal number of the plants were inoculated with the virus isolate and kept without spraying in the greenhouse as control. Efficiency of the inhibition was determined as described by **Devi *et al.* (2004)** using the following equation:

Inhibition % = A-B/A × 100

Where: A= Control, B= treatment

RESULTS

Part I: Isolation and identification:

1- Isolation:

The virus isolated from infected (*V. unguiculata*) plants was biologically purified through single local lesion transfers on *C. amaranticolor*. The resulting virus was propagated on *pea plants*. It was identified by Indirect ELISA test using authentic polyclonal antisera obtained from the serological lab, Virus and Phytoplasma Research Department.

2- Identification of the virus isolate:

2-1- Host rangeand diagnostic host reactions:

Result in Table (1) and Fig (1) show the reaction of forty plant species and cultivar belong to seven different families to virus infection. Inoculated plants

showed a great variability in symptoms expression, such as mosaic, mottling, deformed leaves, chlorotic and necrotic local lesions.

It was also indicated that the tested hosts belonging to family Solanaceae and Fabaceae susceptible to infection with PMV. Some hosts showed no obvious symptoms and when checked by ELISA gave positive reaction (symptomless).

It is obvious from Table (1) that species and caltivars tested can be classified into four general groups:

(1) Plants reacted only with local symptoms: *Chenopodium quinoa* , *C. amaranti color* Coste & reyne *Datur ainnoxia L.* and *Nicotiana glutinosa L*

(2) Plants reacted only with systemic symptoms: *Vignaunguiculata* Cv. beef, *Pisumsativum*cv. Litel Marvel, *Viciafaba*. cv. Giza 3, *Cicerarietinum* .cv. Giza 531, *Lens culinaris.* cv. Kordy1, *and N. tabacum*cultivars *White, Samsun and Xanthi.*

(3) Plants didn't show symptoms , but from which PMV could be recovered either by back inoculation onto the indicator host plants or by ELISA test include the following: *Glycine max* cv. Crowford**,** *Phaseolus vulgaris*L. cv. Bronc, *Viciafaba*L. cv. Giza 3, *Cicerarietinum* .cv. Giza 531, *Lens culinaris.*cv.Kordy1**,** *Hordeumvulgare*L.cv. G123**,** *Tritcumaestivum*cv.sods1**,**

(4) Plants didn't show any symptoms and PMV couldn't be recoverd by nither by back inoculation nor by ELISA, these are**:**

*Allium cepa*cv.Giza 6, *Allium sativum*cv.Baladi**,** *Beta vulgaris*cv. Pleno**,** *Glycine max*cultivars Giza 35and Clark**,** *Triflium alexandrium* cv. Giza 6**,** *Zea mays*cv. T. W.C-360**,** *N. rustica,* *Lycopersico nesculentum* cv. *Casle Rock***,** *Solanum melongena*cv. *Balady***,** *Capsicum annuum*cv. California Wonder, *Solanum tuberosum*cv. sponta**,** *Brassica oleraceavar* . Capitatacv. Baladi**,** *Brassica oleracea. L.* var. *botrytis*cv. soltany**,** *Brassica olercea.* var. italic, *Brassica Rapa*cv. Baladi and *Eruca Sativa*cv. Baladi**.**

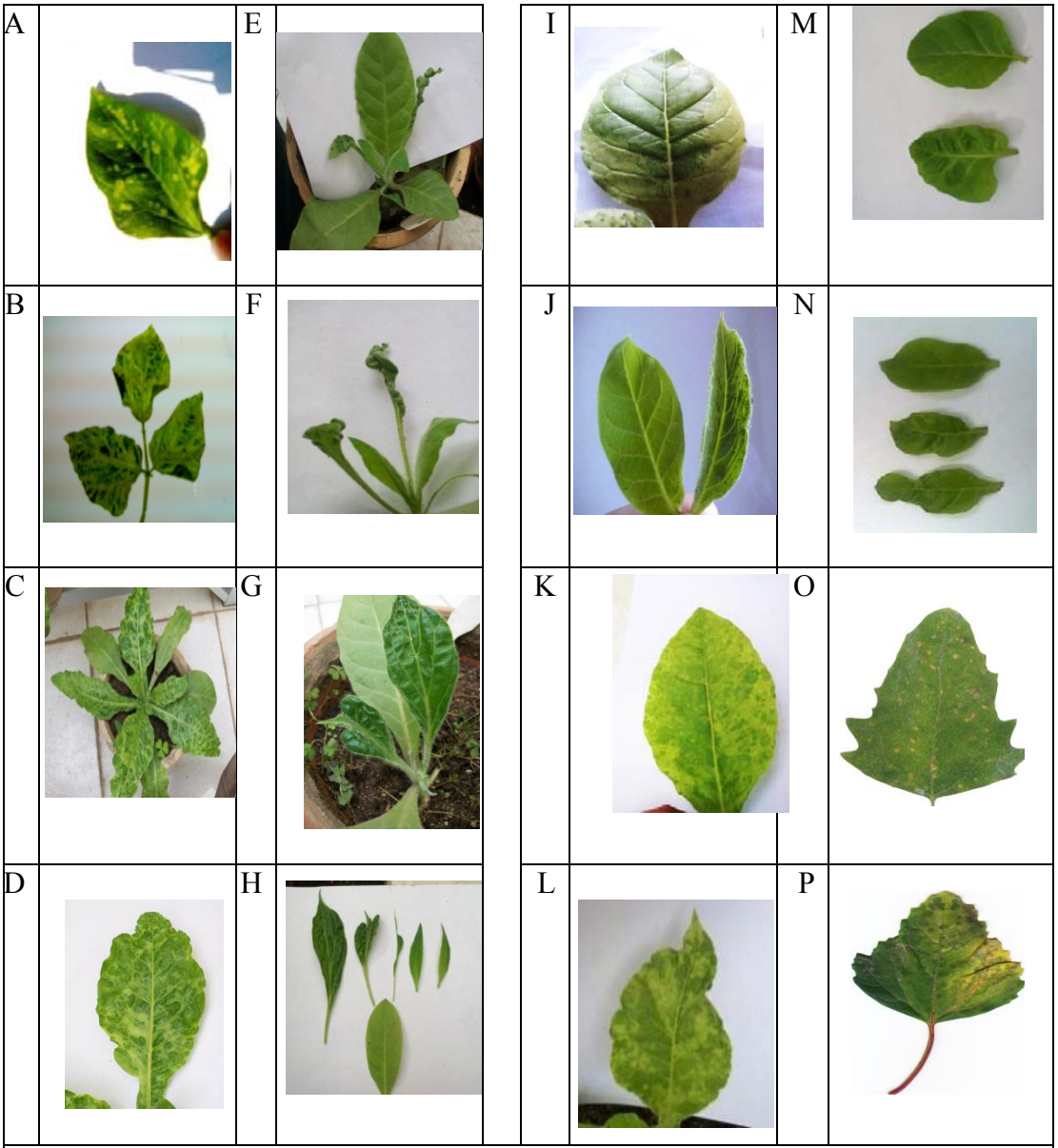

Fig (1): Symptoms of PMVas a result of artificial inoculation on some hosts (A,B) ***V. unguiculata***systemic mosaic ; (C and D) ***N. tabacum*****cv Xanthi** systemic mosaic; (E ,F,G and H) ***N. tabacum***deformed leaves, (I,J, K and L) systemic mosaic ; (M and N) ***N. tabacum*****cv. Samsun**deformedleaves ; (O) ***C. quinoa L*** local lesion *;* (P)***C. amaranticolor*****Coste&Reyn**local lesion.

Table (1) Symptoms of *Pea mosaic virus* in different host as a result of artificial inoculation

Scientific name of Tested plant	English name	Variety	Observed symptoms	ELISA
Alliaceae				
*Allium cepa*L.cv.Giza 6	Onion	Giza 6	NS	-
*Allium sativum*L.cv.Baladi	Garlic	Baladi	NS	-
Chenopodiaceae				
Beta vulgaris L cv.Pleno.	beet	Pleno	NS	-
Chenopodiumquinoa Wild	quinoa		LL	+
*C. amaranticolor*Coste&reyne	large lambsquarters	Coste&reyne	LL	+
Cucurbitaceae				
Cucumissativus L.cv. Beta Alfa	Cucumber	Beta Alfa	NS	-
*Arumpalaestinum*L.cv. Baladi	Arum	Baladi	NS	-
*Cucurbitamoschata.*cv.winterlecsery	Pumpkin	winter lecsery	NS	-
Citrullsvulgaris L. cv.Giza1	Watermelon	Giza1	NS	-
Cucumismelo cv. Reteculatus	Melon	Reteculatus	NS	-
Cucurbita pepo L.cv. Escandrani	Squash	Escandrani	NS	-
Fabaceae				
*Vignaunguiculata*L. Cv. beef	Cowpea	beef	SM	+
*Glycine max*L.cv Giza 35	Soybean	Giza 35	NS	-
Glycine max L. **cv.** Crowford	Soybean	Crowford	NS	+
Glycine max L.**cv. clark**	Soybean	**clark**	NS	-
*Phaseolus vulgaris*L.cv.Bronc	Bean	Bronco	NS	+
*Pisumsativum*L.cv.Litel Marvel	Pea	LitelMarvel	SM	+
Vicia faba L.cv.Giza 3	Broad bean	Giza 3	SM	+
Cicerarietinum .cv.Giza 531	chickpea	Giza 531	SM	+
*Lens culinaris.*cv.Kordy1.	lentil	Kordy1	SM	+
*Trifliumalexandrium*L.cv.Giza 6	Berseem clover	Giza 6	NS	-
*Arachis hypogea*L.cv.Giza 5	Peanut	Giza 5	NS	+
Graminaceae				
*Hordeumvulgare*L.cv. G123	Barley	G123	NS	+
*Tritcumaestivum*cv.sods1	Wheat	sods1	NS	+
Zea mays cv.T.W.C-360	Maize	T.W.C-360	NS	-
Solanaceae				
Daturainnoxia L.	Sacred datura		NLL	+
Nicotianaglutinosa L.	wild tobacco		NLL	+
N. rustica L.	wild		NS	-
N. tabacum L. White Burley	tobacco	*White Burley*	SMD	+
N. tobacum L. Samsun	tobacco	*Samsun*	SMo	+
***N**. tabacum cv Xanthi*	tobaco	*Xanthi*	SM	-
Lycopersiconesculentum L. cv.*Casle Rock*	Tomato	*Casle Rock*	NS	-
Solanummelongena L. cv.*Balady*	eggplant	*Balady*	NS	-
Capsicum annuum L. cv. *California Wonder*	pepper	California Wonder	NS	-
Solanumtuberosum L. cv.sponta	potato	sponta	NS	-
Brassicaceae				
Brassicaoleraceavar .Capitatacv.Baladi	cabbage	Baladi	NS	-
Brassica oleracea.L. var. *botrytis*cv.soltany	Cauliflower	soltany	NS	-
*Brassica olercea.*var . italica	Broccoli	italica	NS	-
*Brassica Rapa*cv.Baladi.	Turnip	Baladi	NS	-
ErucaSativa. cv.Baladi	Arugula	Baladi	NS	-

SM: Systemic mosaic,**SMo**: Systemic mottling., **SMD**: systemic mosaic and deformed leaves.,**NLL:** Necrotic local lesions, **LL:** local lesions, **+ : Positive, - : Negative NS :** NoSymptoms

2.2. Seed transmission through cowpea (V.*unguiculata* (L.) Walp):

Results showed that, PMV could be transmitted through cowpea *(V. unguiculata* (L.) Walp) seeds of local cultivar (buffalo). It was noticed that the percentage of transmission of PMV was reached 6%.

3- Serological detection:

The virus isolate was detected by indirect ELISA. Positive reaction was obtained only between the virus and its corresponding antiserum but not reacted with the other antisera reported earlier.

II- Virus purification and ELISA reagent production:

1- Purification of the virus isolate:

In the present work PMV was partially purified for antiserum production following the method mentioned before.

1.1.UV-absorption spectrum of the purified virus:

The absorption spectrum of the purified isolate was typical for nucleoprotein, with a maximum at 260 nm and a manimum at 245 nm. The ratios of A260/280, A280/260 and Amax/min ratios were 1.16, 0.85, 1.04, respectively. Yield of the partially purified virus was 1.7 mg/100g of infected leaves.

1-2-Electron microscopy:

Electron micrographs of the partially purified virus preparation obtained from infected plants revealed the presence of numerous unaggregated filamentousflexuous virus particles**(Fig. 2)** of about700-750nm long.

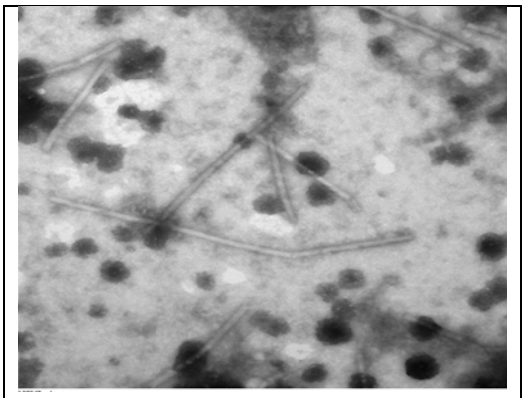

Fig (2): EM micrograph of partially purified virus particles of BSbMV, negatively stained with 2% uranyl acetate, pH 7.0 .(60.000 x)

2. Production of polyclonal antibodies produced against to BSbMV:

2.1.Determination of antiserum titer:

Antiserum developed against BSbMV after rabbit immunization from bleeding taken 3 times at weekly intervals after the last injection had antibody dilution titer of 1/800 in the first, rose to 1/1600 in the second bleeding (Table 3), and then to 1/3200 in the third bleeding, respectively, when infected sap was diluted to 1/10 was used in indirect

ELSA. Antiserum obtained after the 3rd bleeding (1\3200) was used in the subsequent experments.

2.2. Purification of IgG:

Purification of IgG was done using caprylic acid method as described before. Its concentration was adjusted to 1mg/ml (A280= 1.4).

2.3. Conjugation of IgG with alkaline phosphatase:

Positive reactions obtained when purified IgG and IgG conjugated with alkaline phosphatase were tested for PMV- infected plants using DAS-ELISA. The optimum concentrations of IgG and IgG conjugate were 1.0 µg/ml and 1/1000, respectively according to the schematic diagram of checkerboard arrangement test (Table 3).

2.4. Determination of antigen dilution end point:

It was found that, IgG and IgG conjugated with alkaline phosphatase can be readily applied for virus detection in tobacco plants extracts at dilutions up to 1:500 (Table 4).Reading after 30 min incubation with the substrate.

2.5. Serological detection of PMV by DAC ELISA and DBIA using the ELISA reagents produced:

2.5.1. DAS –ELISA:

Results in Table (5) showed that,PMV could be transmitted through peaseeds cv. master B, lentil cv. Kordy 1 and peanut cv. Giza 5 with percentage of transmission 7%, 6% and4% respectively. It was noticed that PMV was not transmitted through seeds of chickpea cv. Giza 1, and faba bean cv. sakha 1.

2.5.2. Dot- Blotting Immunobinding Assay on nitrocellulasemembrane (DBIA).

PMV was readily detected immunologically using DBIA (Fig.3). Positive reaction was indicated by development of purplish- blue colour, whereas in negative reaction, tissues from healthy plants remain green.

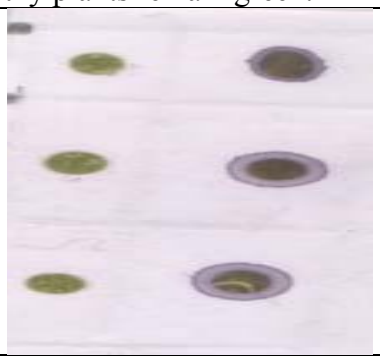

Fig (3): Dot blot immunoassay (DBIA) using poly polyclonal antibodies. (1) Healthy and infected cowpea cv. Buffalo plant samples of PMVand (2)*N. tabacum*, cv White Burley (3) *N. tobacum* L *Samsun* L. Purplish blue color indicates positive reaction

Table (2): Determination of PMV antiserum titer in relation to time of blood collection

Antiserum dilution	ELISA reading of PMV Antisera collected at weekly intervals (A405nm)					
	1st week		2nd week		3rd week	
	Inf.	H.	Inf.	H.	Inf.	H.
1/100	1.123	0.555	0.873	0.365	0.892	0.350
1/200	0.892	0.517	0.726	0.225	0.728	0.337
1/400	0.756	0. 371	0.603	0.262	0.730	0.261
1/800	0.674	0.362	0.580	0.256	0.669	0.230
1/1600	0.418	0.284	0.488	0.221	0.573	0.205
1/3200	0.390	0.216	0.297	0.232	0.424	0.212
1/6400	0.311	0.181	0.202	0.194	0.286	0.208

Reading after 30 min incubation with the substrate.
Inf. = Infected plants H. = Healthy plants

Table (3): Schematic diagram of checkerboard arrangement determination of approximate working dilutions of IgG and IgG conjugate to BSbMV for ELISA test.

Dilution of IgG conjugate	Concentration of IgG (µg /ml)							
	4.0		2.0		1.0		0.5	
	I	H	I	H	I	H	I	H
1/250	0.892	0.350	0.731	0.321	0.634	0.290	0.554	0.280
1/500	0.851	0.341	0.672	0.290	0.623	0.272	0.509	0.256
1/1000	0.697	0.292	0.604	0.259	0.587	0.232	0.461	0.236
1/2000	0.442	0.242	0.401	0.221	0.399	0.211	0.300	0.193

I = Infected plants ; H= Healthy plants

Table (4): Determination of antigen end point:

Antigen dilution	Absorbance at (405 nm) ELISA reading	
	I	H
1/250	1.099	0.266
1/500	0.640	0.169
1/1000	0.408	0.255
1/2000	0.260	0.151

Table (5): The use of the produced ELISA kit in detection of PMV In seed lots of different hosts .

Seedlings of	scientific name	No.infected seedlings/No.tested seedlings	% seed transmission
Chickpea	*Cicerarietinum*	0/100	0%
Lentil	Lens culinaris	6/100	6 %
Pea	*Pisumsativum*	7/100	7 %
Faba bean	*Viciafaba*	0/100	0%
Peanut	*Arachis hypogea*	4/100	4%

Part III- Cytological studies using electron microscopy:

Electron microscopy of ultra-thin sections prepared from infected tobacco leaf tissues revealed that almost all the inspected tissues were drastically affected with infection. The nucleus was misshapen, the nuclear membrane is irregular, and the plasma membrane is characterized with multiple invaginations(Fig.5). On the other hand, the aggregates of virus particles (VP) are found in the cytoplasm next to the chloroplast (Fig.6). The starch granules assumed to be large in the affected plasteds .the thylakoids as will as intergrana lamella appered as if not well fixed and showed different pathological anatomical symptoms (Fig.6). The phloem of the infected plants was also affected, Theparenchymatous of the vessel cylinder showed unusual vesicle like structure. The membrane of the affected cells showed different degrees of degeneration.

The content of the companin cell assumed as if not fixed well(Fig4). Concerning leaf cell of cowpea seedlings, the changes were dis organized chloroplast and its content, the envelop of plastid is not observed and the thylakoids exhibited signs of disintegration **(Fig.11).**

The mitochondria (M) wereaggregated and degenerated, also abnormal structures in the cells were observed **(Fig.9, 10)**These cytological changes as described above were not observed in the cells of healthy control plants (Figs8 and 12).

Part IV- Virus control:

Two different compounds were tested for their ability to make induce resistance against PMV in local lesion formed on *C. amanticolar*. Whereas camphor oil was used as antiviral agent Results demonstrated in Table (6) reveal that all these compounds induced resistance to virus

Infection, when applied to the plant as spray before virus inoculation

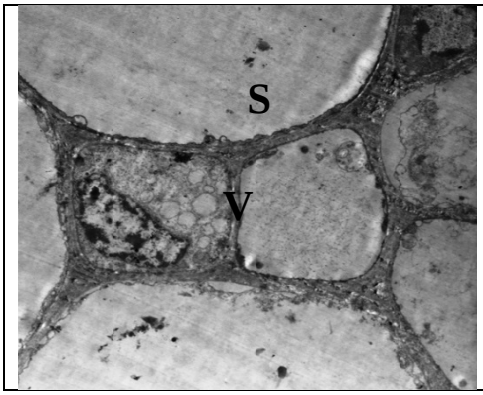

Fig (4): EM micrograph of vascular cylinder of tobacco leaf artificially infected with PMV. The parenchymatous of the vessel cylinder showed unusual vesicle like structure. The membrane of the affected cells showed different degrees of degeneration (5000X). **S**sieve tube, **V**vessel.

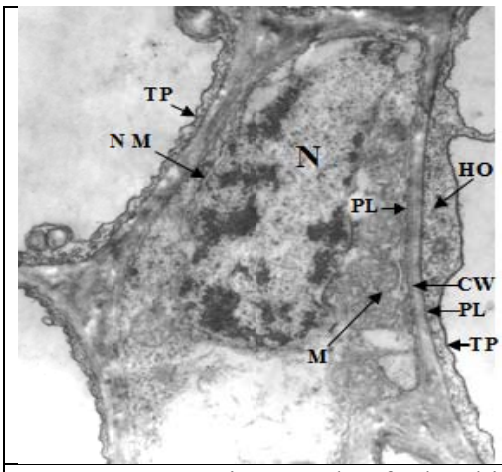

Fig (5): EM micrograph of ultrathin section of tobacco leafartificiallyinfected with **PMV**show misshaped uncles(N) , the nuclear membrane (NM) is irregular and the plasma membrane also affected in the central cell compared with the right cell in the current figure (15000X). **TP:**tonplast , **N:**nuclear, **NM:**nuclear membrane, , **CW:** cell wall, **PL:**plasma membrane, **M:** mitochondria

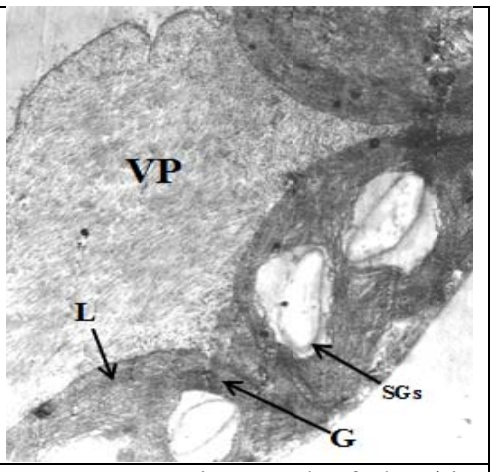

Fig (6): EM micrograph of ultrathin section of tobacco leaf artificially infected with **PMV.**The aggregates of virus particles **(VP)** are found in the cytoplasm next to the chloroplast . The starch granules **(SGs)** assumed to be large in the affected plasteds.the thylakoids as will as intergrana lamella **(L)** appered as if not well fixed and showed different pathological anatomical symptoms . (12000X).

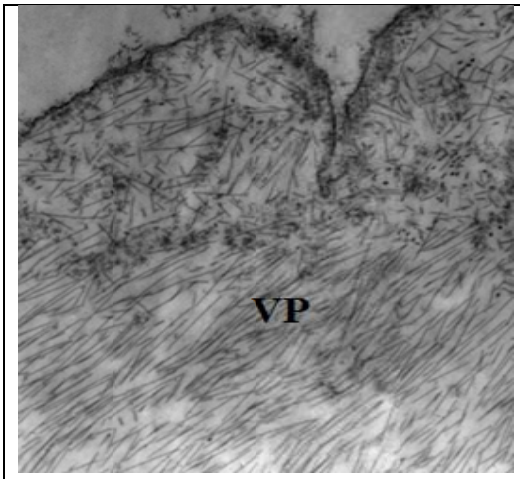

Fig (7): Magnified part of Fig .(6)showing several particles of rod shape of PMV(50000X)

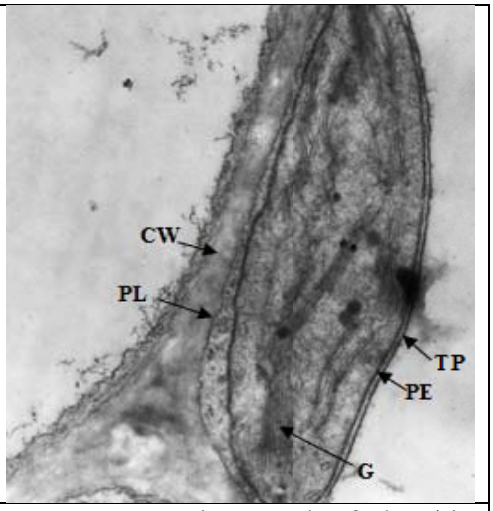

Fig (8): EM micrograph of ultrathin section in healthy cell of tobacco leaf (30000X).**TP**: tonplast,**CW**: cell wall, **PL:**plasma membrane,**G:g**rana

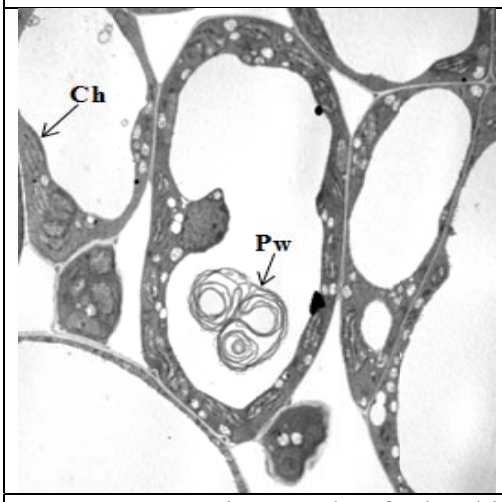

Fig.(9): EM micrograph of ultrathin section on seedling of cowpea plant(7days after germination), growing from seeds which harvested from artificially inoculated plants with PMV. Note Pinwheel inclusions **(Pw)** in the cells .**(2500X)**

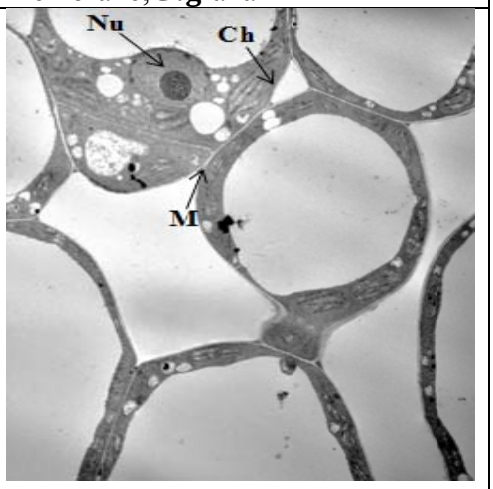

Fig:(10): EM micrograph of ultrathin section on seedling of cowpea plant 7days after germination showing natural structure of mesophyll cells in healthy cowpea leaf **(2500X)**.**Ch:**chloroplast , **Nu:** nucleus , **M:** mitochodria

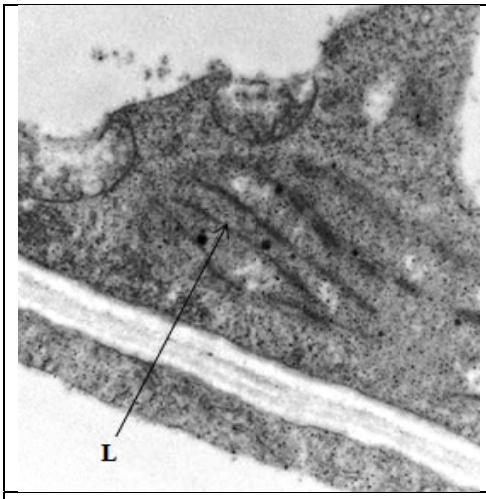

Fig (11): EM micrograph of ultrathin section in leaf cells of cowpea plant (7days after germination), growing from seed which harvested from artificially inoculated plants with PMV. Note the disorganized plastid and its content, also the envelop of plastid is not observed and the thylakoids expiated signs of disintegration. **(25000X).**

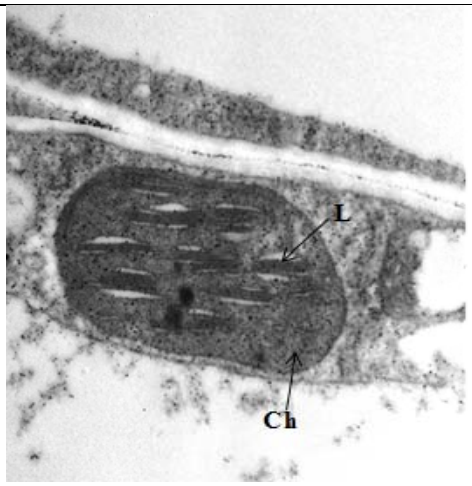

Fig (12): EM micrograph of ultrathin section on healthy seedling of cowpea plant (7days after germination) showing natural structure of plastid in cowpea leaf **(30000X).Ch:**chloroplast, L: intergrana lamella.

Table (6): Effect of salicylic acid (SA) and parahydroxy benzoic acid (PHBA) and camphor oil (CO) on PMV infection expressed as average number of local lesion.

Chemical Concentration %	Percentage of inhibition after treatment with								
	Salicylic acid (SA) after			Parahydroxy benzoic (PHBA) acid after			Camphor oil (CO) after		
	1h	2h	3h	1h	2h	3h	1h	2h	3h
0.1	46.4	49.2	50.7	63.3	74.6	63.3	47.8	40.8	63.3
0.01	16.9	46.4	77.4	42.2	74.6	52.1	57.7	66.1	71.8
0.001	60.5	35.2	50.1	74.6	88.7	69.0	29.5	74.6	73.2

Average number of local lesions induced in 15 leaves with SA, PHBA and CO

Control = unsprayed Plant

88.7% reductions before 2h of inoculation than 1 h (74.6%). Also, salicylic acid was effective as it reduced infection by 77.4% when used 0.01% concentrationbefore 3hours of infection . Moreover camphor oil gave a little like reduction as salicylic acid (74.6) when used with 0.001% concentration before 2 h.

Fortunately, all compounds were effective to inhibit PMV.

DISCUSSION

In this work *Pea mosaic Potyvirus* was isolated from naturally infected cowpea plants cultivated in A.R.E.S. It was identified on the bases of symptomology, hostrange, and serology and particles morphology. Symptoms vary depending on virus or strain, host plant, time of year and environmental conditions (Matthews,1991). The virus was found to infect several hosts resamble those produced by some other PMV isolates especially *Chenopodiaceae* and *Fabaceae* . These agree with the results obtained by several workers (Hmpton and Mink,1975,Alconero *et al*., 1986 and Brunt *et al*., 1996).It was noticed that some hosts did not produced any symptoms but when checked by ELISA gave positive reactions. Apola *et al*. (1974)mentioned that non-leguminous host are infected without producing symptoms . This might be attributed to the low concentration of the virus in these plant species . Some differences in host reaction were also observed ,so these hosts can be used to differentiate between our PSbM isolate(cowpea isolate) and other PMV isolates.

Knowledge of the ways in which viruses are transmitted from plant to plant is important for recognized particulat viral disease and development of satisfactory control measurres (Matthews,1991). The virus uneder stady was transmitted through the seeds of cowpea ,lentil, pea and peanut at 6,6,7and 4% ,respectively , but not through chickpea and faba bean. Khatarpal and Maury(1987) found that PMV was transmeitted in the lentils at frequencies of 32-44% and through a low percentage of seeds of faba bean . Wherea Shukla *et al*. (1994) reported seed transmission of the virus in lintel at range of 0.2 -44% and in pea at range of 0.3- 80%.

The use of ELISA technique gratly facilitated the identification of the virus and provide more accurate and consistent results than did symptomatology and host range (Matthew,1992). Positive reaction obtained with the virus and its corresponding antiserum using indirect ELISA provided further evidence that the virus under instudy is indeed PMV.

In th present investigation PMV was partially purified for antiserum production following the method described earliar. The estimated yield of the purified virus was 1.7 mg\ 100g of infected leaves. The yield was lower than that of the other Potyvirus (Awad,Maisa *et al* .,2005, Farag,,Azza *et al*.2005 and El-Kady *et al*.,2010). It wasshould be pointed out that a technique which succeeds with one virus isolate may fail with other one because of such factors as host species ,growing conditions and

isolation procedure (Matsubara, 1985).

Electron micrograph of the partially purified virus preparation should numerous unaggregated filamentus flexuous virus particles of about 700-750 nm long. Such length is within the range reported for PMV by other invistegtors (Alconero *et al* .1986,Franki *et al.* ,1991 and El-banna *et al.*,2008). This type of virus particles is chractaristic for Potyvirus (Bos,1970)

In the present study polyclonal antybody raised against PMV was prepared aftr rabbit immunization from bleding taken1to 3 weeks after the last injection using indirect ELISA. The titer of the antisera was 1/800 for the 1st , 1/1600 for the 2nd and 1/3200 for the 3rd bleeding . The latter is higher than that reported by (Brunt et al. 1996) for PMV. The concentration of Ig G and IgG conjugate were 1.0 mg /ml and 1/1000, respectively. These results are agree with the results obtained by Salama (1998) and EL-kady *et al.*(2010).

Our study showed that the procedure of DAS-ELISA and DBIA could be readily applied in detection of PMV in the either infected plants or infected seeds for routine indexing of large number of samples .

Several invistegators used both technique for detection of their isolated viruses (Salama,1998, Awad,Maisa *et al*. ,2005 and El-kady *et al.*, 2010)

External symptoms are reflection of disturbed cell metabolism leading to modifications in tissues, cell and cell organelles (Franck, *et al*., 1985).Ultrathin sections of infected cells showed several cytological changes ,these include chloroplast which were disorganized and fragmented , grana and inergrana were not fixed will and degenerated. The mitochondria were aggregated and degenerated. Intracellular structures sush as inclusion bodies or pinwheels were observed. Beside these cytological abnormalities , aggregates of virus particles are also found in the cytoplasm next to the chloroplast . PMV has been reported by different works to induce cylindrical cytoplasmic inclusion (CCI). CCI seen in transverse section are described as pinwheels and their presence in disease plant is of diagnostic value as they are characteristic of Potyvirus group (Edwrdson *et al*. 1984, and Shukla *et al*.1994).

Important advances in virus chemotherapy have been made during the last few years. A varity of compounds with potent and selective antiviral activity had been found. These antiviral agents affected viral synthesis. Other compound also resuls in increase risistance of the trated areas to some viruses (Gupta *et al*. , 1980, and Sherwood, 1985). In this work salsilc acid (SA) ,parahydroxy

benzoic acid (BHBA) and camphor oil (CO) were used for reduce PMVinfecition . The three cpmpounds were found effective in this respect .Nevertheless, applicatoion gave supersession results (88.7 %) followed by CO (74.6%) when applied 2h before inoculation , whereas ,SA had the lower effect (60.5%) at concentration ,of 0.001 before 1h of inoculation .Several invistgators used SA and PHBA for thir ability to stimulate immune system and offer some indirect protection agents viral infection through these effects (Hooir *et al.*1986, Kobeasy and salwa, Zein,2005 and El-Kady*et al.*2010)

On the other hand essemtial oils such as camphor oil were mainly used to control plant pathogens viz , fungal , bacteria and viral pathogens and act as antiviral agents (Verma *et al.*, 1998, Romeilah *et al*., 2010 and Modhusudhan *et al.*, 2011).

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