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IDENTIFICATION AND PURIFICATION OF *Cowpea mosaic virus* Comovirus ISOLATED FROM INFECTED COWPEA (*Vigna unguiculata* L.) IN NORTHERN EGYPT

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Abstract

Cowpea mosaic virus (CPMV) naturally infected Cowpea plants showing different mosaic, mottle, dwarfing, and vain clearing symptoms were separately collected from naturally infected cowpea plants grown at certain locations of Alexandria governorate during the growing seasons from 2011 to 2012. CPMV was parcialy purified using PEG 6000 and differential centrifugarion. The ratio of A 260/280 was 1.716 and A 280/260 was 0.583. Whereas, the ratio of A max/min was 1.445. Concentration of virus in the preparation was estimated using an extinction coefficient of $E_{260}^{0.1\%}$ *= 8.1. Yield of purified virus was about 73.1 mg/100 g fresh weight of leaves of cowpea. Antiserum titer was determined by indirect ELISA. Positive ELISA values were obtained up to dilutions of 1:204800, the virus was detected in infected sap at 8, 16 and 24 days after inoculation by indirect ELISA and 8, 16 and 24 days after inoculation by TBIA on nitrocellulose membrane. The incidence of CPMV was determined in 21 day old cowpea seedlings grown from infected seeds for comparing with TBIA and indirect ELISA, Data concerning detection of virus antigen in 21 day old seedlings Infection percentage was 62% as determined by TBIA, while ELISA was reached 75% percentage.*

Key Words: *Cowpea mosaic virus*, *Vigna unguculata*, Antiserum titer, indirect ELISA and TBIA

INTRODUCTION

Cowpea (*Vigna unguiculata* Walp-Holl) has an important role as a feeding crop. It can be used as green or dried seeds as a good diet for its high protein content. In Egypt (Abd El Aziz, 2015), Under field conditions, this crop is subjected to infection with *cowpea mosaic virus* (CPMV) (Thottappilly and Rossel, 1992). CPMV is the type member of the Genus *Comovirus* in the family *Comoviridae*. (Bruening and Agrawal, 1967). It is one of the most commonly reported virus diseases of cowpea, in which it causes mosaic and decreases leaf area and flower production (Chant, 1960). Symptoms induced by CPMV vary from light green mottle to distinct yellow mosaic, distortion of leaf, and premature death of the plant (Bliss and Robertson 1971).The objective of this work was aimed to detect and isolate CPMV infecting cowpea plants. As well as biological and serological characterization of CPMV isolate. Antiserum production, purification and Detection of CPMV isolate in seedlings stage and registration the isolate in gene bank.

MATERIALS AND METHODS

Leaf samples of Cowpea (*Vigna unguiculata*) showing different mosaic, mottle, dwarfing, and vain clearing symptoms were separately collected in plastic bags from naturally infected cowpea plants grown at certain locations of Alexandria and El-Beheira governorates during the growing seasons from 2011 to 2012. virus inoculum was prepared by grinding infected leaf tissues 1:10 (W/V) with a mortar and pestle in 0.1M phosphate buffer, pH 7.0, containing 0.5% 2-mercaptoethanol. Leaves of healthy plants in seedling stage were first dusted with carborundum (600 mesh) and then inoculated with a freshly prepared inoculum using forefinger method and kept in insect proof greenhouse under observation.

Diagnostic hosts and symptomology: The diagnostic hosts as *Vigna unguiculata*, *Chenopodium amaranticolor*; *Glycine max* , *Nicotiana glutinosa* L. and *N. rustica* were used to give characteristic symptoms for were used for tentative identification of the isolated virus. Five seedlings of each tested plant species or cultivar were mechanically inoculated with CPMV isolate and kept under greenhouse conditions. Plants were examined daily, for four weeks for symptoms expression. Inoculated plants that did not show any symptoms were cheeked for latent infection by back-inoculation to the indicator host *Chenopodium*

amaranticolor. (Chant, 1959; Van Kammen, 1971; Agrawal, 1964 and Hampton *et al*., 1978).

Serological Diagnosis: Serological diagnosis was carried out using indirect ELISA.

Source of antisera: Antisera of *cowpea mosaic virus* (CPMV), *cucumber mosaic virus* (CMV), *alfalfa mosaic virus* (AMV), *cowpea aphid borne mosaic virus* (CABMC) and *tobacco ring spot virus* (TRSpV) used in this study were kindly supplied by Antiserum – Bank, Institute of seed pathology for Developing Countries, Denmark

.**Indirect ELISA:**The indirect ELISA was carried out as described by Younes, (1995) and Fegla *et al.* (1997). Disposable polystyrene flat bottom micro-ELISA plats were used. The plant samples were ground in coating buffer (0.05 M carbonate, pH 9.6) 1:10 (W/V) using a mortar and pestle, then strained through double layered cheesecloth. Wells were coated with antigens by adding 100 µl of each sample to the bottom of the well and incubated for 3 hours at 37˚C or over night at 4˚C. The plates were rinsed three times by flooding wells with phosphate buffer saline and tween 20 (PBST), 5 minutes each. To reduce non specific reactions, antisera requiring cross adsorption were diluted 1:500 with filtered extract from healthy tissue 1:20 (W/V) in serum buffer [PBS-tween 20 (PBST) containing 2% soluble polyvinyl pyrolidone (PVP) and 2% bovine serum albumin (BSA)], and incubated for 45 minutes at 37˚C. The precipitate which had formed was removed by centrifugation for 10 minutes at 5000 rpm., 100 µl aliquots from the diluted antisera were added to each well, after which the plates were incubated at 37^0C for 2 hours or at 4^0C overnight, then washed as before.Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase (whole molecule, enzymatic activity 475 units/ml) obtained from Sigma chem. Co. St Louis, Mo (Production # A8025) was diluted 1:1000 in serum buffer and 100 µl were added to each well, followed by one hour incubation at 37^0C then washed as before.One hundred µl of the enzyme substrate, 1 mg/ml para-nitrophenyl phosphate (Sigma # 104) in 10% diethanolamine buffer, pH 9.8, were added to each well and incubated at room temperature (25^0C) for about 30 minutes. The enzyme activity was stopped by adding 50 µl of 3 M NaOH. The ELISA values, measured by Sunrise ELISA reader, were expressed as absorbance at 405 nm. Absorbance valued of at least double that of healthy control, were considered positive. In each set of test, wells lacking antigen (coating buffer only) were included as blanks.

Purification of CPMV isolate: CPMV isolate was purified according to a method of Van Kammen, (1967). One hundred grams of frozen systemically infected cowpea leaves were collected 16–20 days after inoculation and homogenized with a double amount of 0.1 M phosphate buffer pH 7.0 in a blender. The homogenate was pressed through cheesecloth and the extract was stirred for 15 min with equal volume of 1:1 cold mixture of n-butanol and chloroform. The emulsion was broken by low speed centrifugation at 10000 rpm for 15 minutes using Beckman rotor 50.2 Ti. Then PEG 6000 was added to final concentration of 4% (W/V) and NaCl was added to give concentration of 0.2 M. The mixture was stirred at room temperature to dissolve the PEG and NaCl after 1 hour the precipitate was collected by centrifugation at 10000 rpm for 15 minutes and the pellets were resuspended in 0.01 M phosphate buffer pH 7.0, this suspension was clarified by centrifugation at 10000 rpm for 15 minutes and the virus suspension was further purified by two cycles of differential centrifugarion at 33000 rpm for 3 hours to sediment the virus, and at 10000 rpm for 10 minutes in 0.01 M phosphate buffer pH 7.0 to clarify the virus suspension. Virus presence was checked biologically by inoculating of *Chenopodium amaranticolor*. U.V. absorption spectrum of the purified virus at a range of wave length 230 - 320 nm with 10 nm interval was recorded spectrophotometrically using ultra violet spectrophotometer Jon Way 6405 UV/VIS spectrophotometer A 260 / 280, A 280 / 260 and A max / A min as well as virus concentration were estimated. Virus concentration was calculated by assuming an extinction coefficient $E_{260}^{0.1\%}$ of 8.1 at 260 nm (Van Kammen, 1967 and Mc Laughlin *et al*. 1977).

Ultra violet absorption spectrum of the purified CPMV isolate: The absorption spectra of CPMV isolate purified suspension at the range of wavelength 230 – 320 nm with 10 nm interval were recorded spectrophotometrically using Ultra Violet Jon Way 6405 UV/VIS spectrophotometer. The ratios of A260/280, A280/260 and A max/min as well as virus concentration were estimated. Virus concentration was calculated using the absorbance value at 260 nm for CPMV virus and the extinction coefficient of 8.1 for CPMV (Van Kammen, 1967 and Mc Laughlin *et al*. 1977).

Preparation of antiserum to purified CPMV: An antiserum against CPMV was prepared according to the schedule given by Mc Laughlin *et al*. (1977). Two white New Zealand rabbits were immunized with purified virus preparation, first intravenously with the purified virus diluted with saline solution (0.85% NaCl) to give the dose of 2 mg of purified virus followed by four intramuscular injections of 1 mg virus emulsified with an equal volume of Freund's adjuvant (Difco Lab, U. S. A.) administered weekly for 4 wks. Complete adjuvant was used in the first injection and incomplete adjuvant thereafter. Bleedings were made from marginal ear veins 2 wks after the final injection for immune serum and the blood was left for 2 hr at room temperature to clot, and then stored in refrigerator overnight. The separated antiserum was clarified by centrifugation at 5000 g/35 min, divided in aliquots and kept frozen until needed for different serological tests. Antiserum titer was determined using indirect ELISA as described by Fegla *et al*., (1997). Extracts from infected and healthy cowpea plants were diluted with coating buffer to 1: 10. Serial dilutions of

double fold up to 1:256000 of antiserum from cross–adsorption with filtered extracts from healthy tissues diluted 1: 20 in serum buffer were used according to Younes, (2003).

Serological detections

Tissue blot immunoassay (TBIA): TBIA as described by Lin *et al*. (1990), Makkouk and Kumari, (1996) and Fegla *et al.* (2003) were used. Stem or leaves of healthy and virus infected plants were tested. Rolled leaves and stem pieces of cowpea plants, the positive reaction of TBIA was indicated by the development of purple color on the blots and the negative reaction development no more or green color. Infected and healthy samples of cowpea plants (*Vegna unguculata*) C.V. Kareem7 with CPMV isolate were checked serologically for virus presence by tissue blot immunoassay (TBIA).

Detection of CPMV in seedlings stage: Collected seeds from infected cowpea plants (Kareem7) with CPMV and sawed and cared till seedlings stage, Samples of apparently infected and healthy seedlings raised from seeds of CPMV isolate infected plants *Vegna unguculata* C.V. Kareem7 were checked serologically for virus presence detected by TBIA and indirect ELISA as previously described.

RESULTS

Serological detection revealed the involvement of virus namely *cowpea mosaic virus* (CPMV) with symptoms of collected samples (table 1). CPMV was isolated from cowpea plants (from Alexandria Governorate) showing mosaic, severe mosaic, mottling and leaf deformation with vein clearing (Fig. 1: a and b). CPMV was isolated and was used in the study, caused mosaic, blisters and severe distortion, vein clearing and leaf deformation on *Vegna unguculata* (Fig. 1: a and b), Chlorotic local lesions was exhibited on inoculated leaves of *C. amaranticolor* (Fig. 1: c) and necrotic local lesions on primary leaves of *Glycine max* (Fig. 1: d) and milled mosaic appearance on leaves of *Glycine max*.

Ultra Violet absorption spectra of purified CPMV: CPMV was purified using Van Kammen's method (1967). The absorption spectrum of the purified virus as determined with Jon Way 6405 UV/VIS spectrophotometer was typical for nucleoprotein (Fig. 21). The ratio of A 260/280 was 1.716 and A 280/260 was 0.583. Whereas, the ratio of A max/min was 1.445. Concentration of virus in the preparation was estimated according to Van Kammen, (1967), using an extinction coefficient of $E_{260}^{0.1\%} = 8.1$. Yield of purified virus was about 73.1 mg/100 g fresh weight of leaves of *Vigna unguculata*. The ultra violet absorption spectrum of the collected virus has A minimum at 240 nm and A maximum at 260 nm. When the purified virus was tested biologically on *Chenopodium amaranticolor* leaves, numerous local lesions were observed.

Production of purified CPMV antiserum: An antiserum against CPMV was produced according to MC Laughlin et al (1977). Antiserum titer was determined by indirect ELISA. Positive ELISA values were obtained up to dilutions of 1:204800 and not with 1:409600 (Table 2).

Detection in infected plants after different periods of inoculation: Indirect ELISA and TBIA were used to detect the isolated virus CPMV in infected *Vigna unguculata* plants at different periods (1, 2, 4, 8, 16 and 24 days) after mechanical inoculation. Dilutions of 1:10 in carbonate buffer pH 9.6 from CPMV infected *Vigna unguculata* plants at different periods (1, 2, 4, 8, 16 and 24 days) after inoculation were tested. Obtained results showed that CPMV antiserum 1:500 could detect the virus in infected sap at 8, 16 and 24 days after inoculation by indirect ELISA (Table 3) and 8, 16 and 24 days after inoculation by TBIA on nitrocellulose membrane (Fig. 3: a).

Detection of CPMV in a seedling stage: The incidence of CPMV was determined in 21 day old cowpea seedlings grown from seeds collected from infected cowpea plants for comparing with TBIA and indirect ELISA, Results are presented in table (9) and Fig (20, A and B). Data concerning detection of virus antigen in 21 day old seedlings were presented in Table (4) and illustrated in Fig. (3: b and c), Infection percentage was 62% as determined by TBIA, while ELISA was reached 75% percentage.

Table 1: Detection of some viruses infecting cowpea in leaf samples by Indirect ELISA determined as absorbance values at 405 nm.

No. of represented tested samples	Indirect ELISA absorbance values (E 405 nm)				
	CPMV	CMV	AMV	CABMV	TRSpV
I	0.614	0.319	0.254	0.193	0.167
H	0.232	0.280	0.196	0.145	0.153

The experiment was repeated twice and ELISA absorbance values at 405 nm are average of two replicates each. Absorbance values of at least double that of the healthy control were considered positive. H = Healthy Bold = Positive reaction

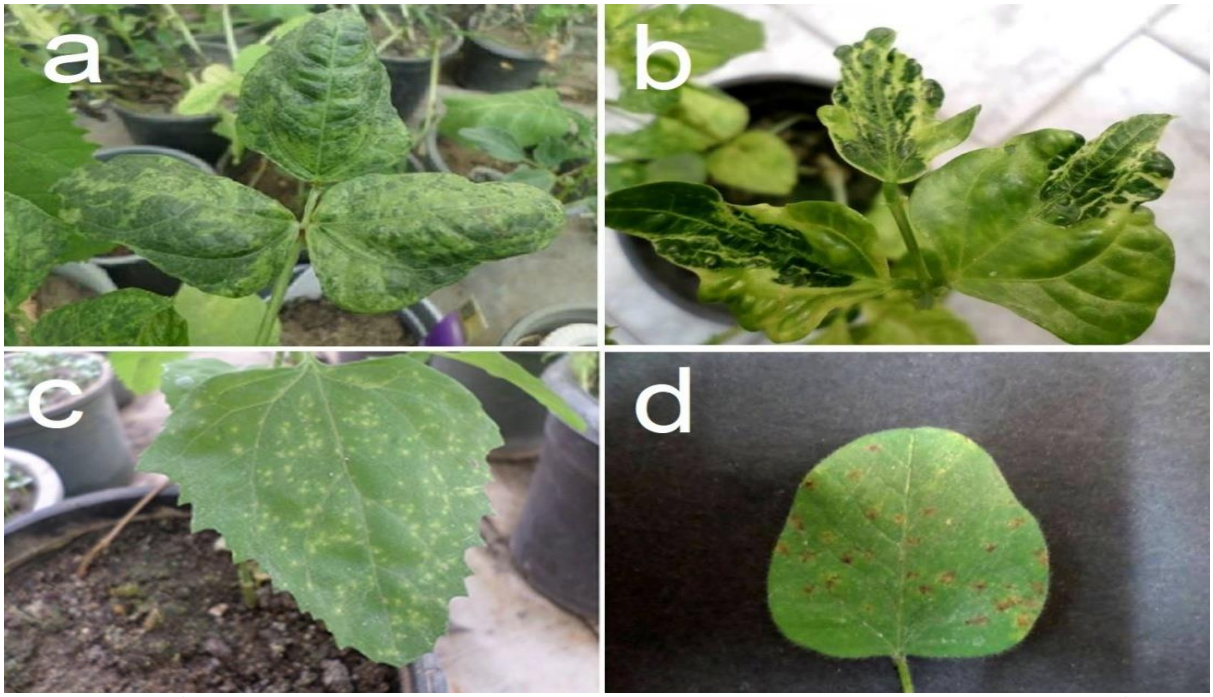


Fig. 1: a) Natural infection caused by CPMV on cowpea Vigna unguculata cv. Red cowpea leaves showing mosaic, crinkle and malformation, b) Symptoms caused by CPMV on cowpea (Vigna unguculata) cv. Kareem7 leaves showing mosaic, vein clearing, blisters and deformation, c) Chlorotic local lesions caused by CPMV on Chenopodiuam amaranticolor, inoculated leaf, d) Necrotic spots caused by CPMV on inoculated primary leaf of Glycine max.

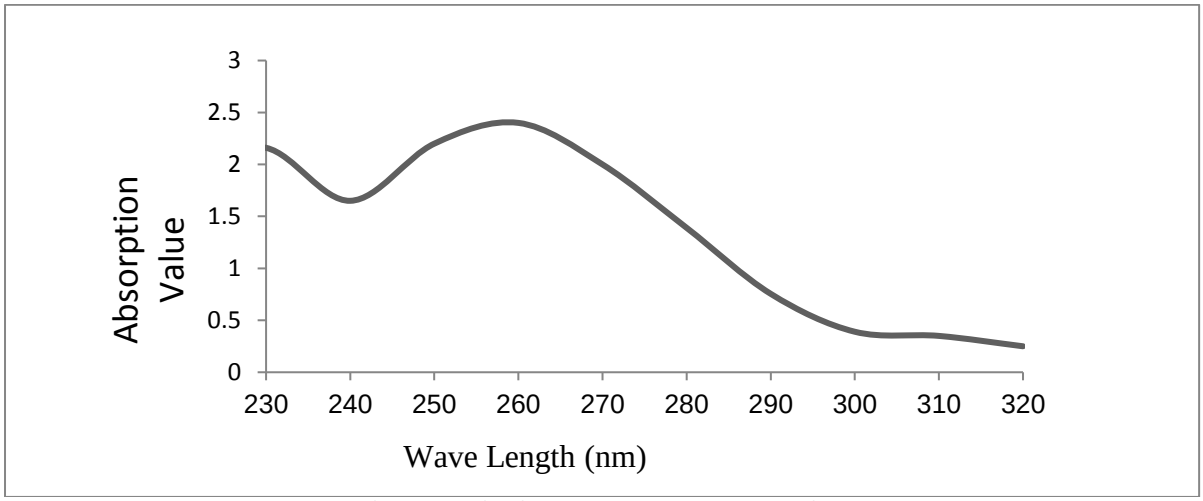
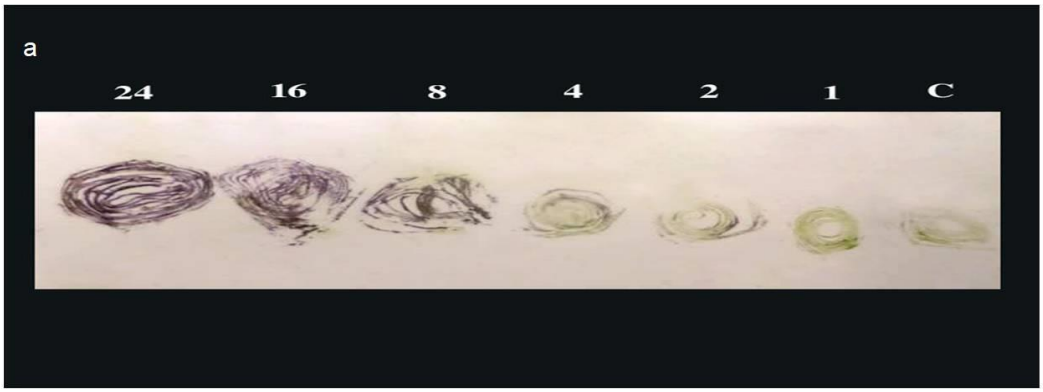


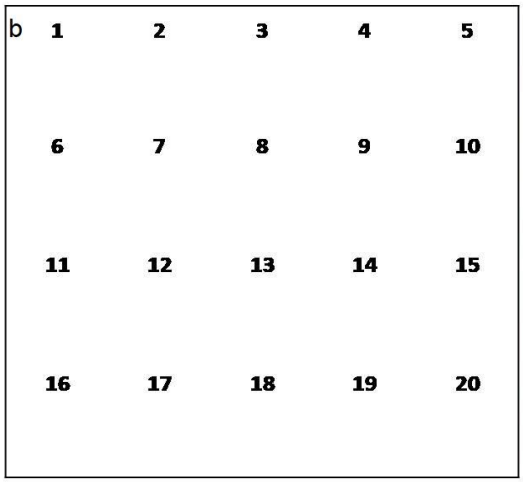
Fig. 2: Ultra Violet absorption spectrum of CPMV purified from cowpea Vigna unguculata infected leaves.

Table 2: Indirect ELISA absorbance values (E 405 nm) of extract of CPMV infected cowpea plants in various dilutions of CPMV antiserum.*

Antiserum dilution	Infected	Healthy
$1:4x10^2$	1.614	0.718
$1:8x10^2$	1.531	0.612
$1:1.6x10^3$	1.28	0.547
$1:3.2x10^3$	0.972	0.459
$1:6.4x10^3$	0.935	0.431
$1:1.28x10^4$	0.862	0.398
$1:2.56x10^4$	0.822	0.377
$1:5.12x10^4$	0.811	0.331
$1:1.024x10^5$	0.803	0.311
$1:2.048x10^5$	0.78	0.307
$1:4.096x10^5$	0.489	0.306

*The experiment was repeated twice and indirect absorbance values at 405 nm are average of two replicates each.
*Absorbance value of at least double that of the healthy control were considered positive.





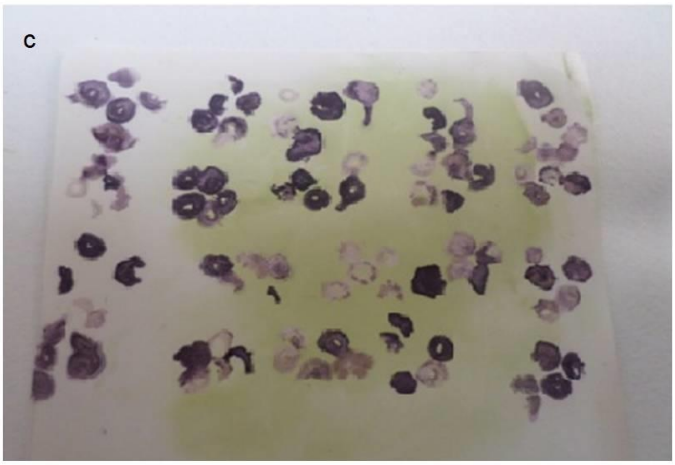


Fig: 3: a) Sensitivity of TBIA for detection of CPMV on NCM in infected cowpea plants after different periods of mechanical inoculation, b) Drawing showing blot distribution of 21 day old seedling groups of cowpea cv. Kareem7 on nitrocellulose membrane tested for CPMV infection by TBIA, c) Detection of CPMV in 21 day old seedling groups by TBIA. A positive reaction (infected seedlings) was indicated by the development of purple color on the blots, while the negative reaction (uninfected seedlings) was colorless.

Table (3) Indirect ELISA absorbance values (405 nm) for CPMV in 1:10 dilution of sap extracted from infected plants at different periods after inoculation.

Days	Indirect ELISA absorbance values (E 405 nm)	Days	Indirect ELISA absorbance values (E 405 nm)
24	0.454	2	0.214
16	0.394	1	0.203
8	0.357	H	0.126
4	0.291		

Table: 4: Incidence of CPMV in 21 day old seedlings of cowpea cv. Kareem 7 detected by TBIA and ELISA.

Group NO.	Indexing values of infected seedlings	
	*TBIA	**ELISA
C	--	0.350
1	5	0.799
2	5	0.949
3	3	0.851
4	3	0.748
5	3	0.755
6	2	0.689
7	5	0.805
8	4	0.831

Continued table 4

9	3	0.899
10	3	0.645
11	3	0.572
12	2	0.441
13	0	0.417
14	3	0.709
15	2	0.746
16	5	1.215
17	3	0.605
18	1	0.484
19	4	0.786
20	3	0.803
Total %	62%	65%

**: No. of infected seedlings out of 5 tested.*
***: Extracts of groups each of 5 were used for ELISA.*

DISCUSSION

Cowpea (*Vigna unguiculata* Walp-Holl) is considered as one of the most economically important Fabaceae crops cultivated in different regions in Egypt. Under field conditions, cowpea plants are subjected to attack by CPMV. It can be transmitted by mechanical inoculation and sometimes by seeds (Fan, 2008). Data concerning serological diagnosis revealed the presence of CPMV with different frequencies during the growing seasons 2011 and 2012 in cowpea leaf samples collected from naturally infected cowpea plants from different regions in northern Egypt, by using indirect ELISA with specific antiserum. This virus was reported in other countries (Agrawal, 1964; Chant, 1959; Hampton *et al*., 1978; and Thottappilly and Rossel, 1992). It is very important to recognize a sensitive, simple, reliable, inexpensive method for detection of the virus in the different parts of the infected plant (Thompson *et al*., 1995). Data concerning host range revealed that CPMV (it was easily inoculate on cowpea c.v. Kareem 7) induced chlorotic local lesions on *Chenopodium amaranticolor*; mosaic, blisters and severe distortion on *Vegna unguculata*; necrotic local lesions on primary leaves of *Glycin max* and milled mosaic appearance on leaves of *Glycin max* agreed with (Chant, 1959; Van Kammen, 1971 and Hampton *et al*., 1978). Evaluation was carried out after different periods of virus inoculation. Results revealed that, TBIA was more sensitive than indirect ELISA since they detected CPMV after 2 and 4 days, respectively of inoculation. Such results generally are not agreed with those reported by Abd El-Aziz, (2000) and Fegla *et al.*, (2001), who found that indirect ELISA was more sensitive than DBIA with CMV and PVY. TBIA has been used by many investigators for surveys, diagnosis and detection of viruses in different parts of the plants, since it was found to be cheaper, it could be completed in less than four hours without sacrificing sensitivity, not require sophisticated facilities and it was sensitive enough to detect the virus in all parts of infected plants (Lin *et al*., 1990; Hsu and Lawson, 1991; Makkouk and Kumari, 1996; Fegla *et al.*, 2000 and Fegla *et al*., 2003).The purification of the isolated CPMV was carried out to determine the photometrical characters of the purified virus and to prepare specific antiserum. The yield of *cowpea mosaic virus* as well as its specific photometrical data such as A260/280, A280/260 and A mix/min fall in the range that reported for CPMV (Van Kammen, 1967). The titre of antiserum obtained for CPMV was 1: $2.096x10^5$; similar results were obtained by Mc Laughlin *et al*. (1977).

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