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PRODUCTION OF SPECIFIC ANTISERUM FOR BEAN YELLOW MOSAIC VIRUS AND BROAD BEAN STAIN VIRUS

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ABSTRACTS: Bean yellow mosaic Potyvirus (BYMV) and Broad bean stain Comovirus (BBSV) were isolated from El-Sharkia and Bani-Seuf governorates and purified using differential centrifugation. Ulteraviolate absorbance of purified viruses were typical for nucleoprotein with minimum at 243, 244 nm and maximum at 260, 260 for BYMV and BBSV respectively. The ratio of A 260 /A 280 and A $_{max/min}$ were 1.33 and 1.11for BYMV respectively and A 260 /A 280 and A $_{max/min}$ ratio were 1.68 and 1.23 for BBSV respectively. The yield of purified virus was 5.55 mg/100 gm infected leaves and 4.78 mg/kg infected leaves for BYMV and BBSV respectively. Production of specific antisera were obtained by rabbit immunization with different amount of antigen (1,2 and 3 mg/ml) for BYMV and (1 and 2 mg/ml) for BBSV using three different methods of injection. The titer of the prepared antisera as determined by indirect ELISA at dilution of tissue 1/5 were 1/2048 and 1/1024 for BYMV and BBSV respectively. Purification of the immunogamaglobulins (IgG) and conjugate of IgG with alkaline phosphatase were carried out to be used for ELISA detection. The concentration of IgG and IgG conjugated with alkaline phosphatase were 0.5 mg/ml and 1:2000 for BYMV and 1 mg/ml and 1/1000 for BBSV. ELISA kits specific to BYMV and BBSV were used for virus detection by using different serological diagnostic methods such as DAS-ELISA, tissue blot immunoassay (TBIA) and dot-blot immunoassay (DBIA) on nitrocellose membrane.

Key wards: BYMV, BBSV, IgG, ELISA, TBIA, DBIA, DAS-ELISA

INTRODUCTION

Faba bean is widely cultivated in Egypt for its value as food, forage and green manure. Several viruses have been reported to infect faba bean under Egyptian conditions such as bean yellow mosaic virus (BYMV) and broad bean stain virus (BBSV) which considered the most economically important viruses infecting faba bean (Salama, 1998 and Sallam *et al.*, 1999). Plant virologists have found, serological techniques to be extremely useful for the routine diagnosis of virus diseases and serological test provides rapid and convenient results for virus assay, detection and diagnosis in addition the main advantage of serological methods is the specific of the reaction. Since the work of Clark and Adams 1977, ELISA technique has been developed with many variants to increase the sensitivity of virus diagnosis. Thus this investigation aimed to : (1) Preparation of purified BYMV and BBSV for production specific antisera, and purification of IgG and conjugated of IgG with alkaline phosphatase for application of serological diagnostic methods. (2) Production of ELISA kit specific for the first time in Egypt to BYMV and BBSV to use it in different serological diagnostic methods such as direct and indirect-ELISA, TBIA and DBIA.

MATERIALS AND METHODS

1.Source of virus isolates

The virus isolates used in this study were isolated from naurally infected faba bean plants collected from El-Sharkia and Bani-Seuf Governorates and identified as BYMV and BBSV using DAS-ELISA tests described by Clark and Adams (1977) and Bar-Josef *et al.* (1979).

2. Viruses purification

2.1. Purification of BYMV

BYMV was purified according to the method given by Alconero *et al.* (1986) with some modifications. One hundred grams of harvested faba bean infected leaves, collected two weeks post inoculation, were mechanically pulverized to powdered tissue and

homogenized in a Braun blender with 300 ml of 0.5 M cold potassium phosphate buffer, pH 8.2, containing 0.5% 2-mercaptoethanol, 0.05 M disodium EDTA and Triton X-100 for two minutes.

The homogenized extract was strained through two layers of cheesecloth and clarified by adding an equal volume of cold chloroform (v/v) followed by low speed centrifugation LSC at 5000 rpm for 10 minutes at 4°C in a Beckman J–21 C Centrifuge using JA–20 rotor.

Virus from the clarified sap was sedimented by high speed centrifugation (HSC) at 35000 rpm for 90 minutes in Beckman L8–80M Ultracentrifuge using rotor 80 Ti. Pellets were taken up in 0.01 M borate buffer, pH 7.5, containing 0.05% 2-mercaptoethanol, 0.002 M disodium EDTA and 1% Triton X-100 with stirring at 4°C overnight. The upper phase was collected after LSC for 10 minutes at 3000 rpm at 4°C, and centrifuged at 35000 rpm for 90 minutes through a 30% sucrose cushion prepared in the borate buffer. Pellets were drained and suspended in 0.01 M borate buffer, pH 7.5, stirring at 4°C overnight. After centrifugation for 10 min. at 3000 rpm, two ml of the resultant virus suspensions were layered onto 10 – 40 % sucrose gradients in 0.01 M borate buffer, pH 7.5 and centrifuged 2.5 hr at 28000 rpm at 4°C in a Beckman SW 60 rotor. Gradient columns were stored overnight at 8°C prior to use.

The virus zones were collected with a bent tip hypodermal needle and syringe, diluted 1 : 1 with 0.001 M borate buffer, pH 7.5, without additives, then concentrated by centrifugation for 90 min. at 35000 rpm. Final pellets were suspended in 2 ml of 0.001 M borate buffer and stirred overnight. Infectivity was tested on leaves of *C. amaranticolor*.

2.2. Purification of BBSV

BBSV was purified according to the method described by Azzam and Makkouk (1986) with some modifications. One kilogram of faba bean leaves showing distinct symptoms, 4 weeks after virus inoculation were harvested and pulverized in liquid nitrogen.

The pulverized tissues were homogenized with a blender in 300 ml of 0.5 M potassium phosphate buffer, containing 0.005 M ethylene diamine tetra acetic acid

(EDTA), sodium sulfite (Na_2SO_3) and 0.01 M diethyldithiocarbamate (DIECA), pH 8.5. The homogenate was filtered through two layers of cheesecloth and subjected to LSC at 8000 rpm for 10 min. The supernatant obtained was stirred with 25% chloroform plus 25% carbon tetrachloride for 30 min. and the aqueous phase was separated by LSC at 8000 rpm for 10 min at 4°C in a Beckman J–21C Centrifuge using JA–20 rotor. The virus was precipitated from the supernatant with 6% polyethylene glycol (PEG 6000) and 0.3 M sodium chloride (NaCl) by stirring overnight at 4°C. The pellets were collected by 20 min. centrifugation at 10.000 rpm at 4°C and suspended in 70 ml of 0.01 M borate buffer, pH 7.5, containing 0.1% 2–mercabtoethanol (2-ME) and left overnight at 4°C with slowly stirring, then centrifuged for 10 min. at 8000 rpm at 4°C to eliminate any non–soluble materials followed by centrifugation for 90 min. at 30.000 rpm at 4°C in a Beckman L8–80 M Ultracentrifuge using rotor 80 Ti. The pellets were resuspended in 0.01 M borate buffer, pH 7.5, and then layered on top of 10–40% sucrose gradient prepared in 0.01 M borate buffer, pH 7.5, and centrifuged for 2 hours at 28.000 rpm in a Beckman SW 60 rotor at 4°C. Gradient columns were stored overnight at 8°C prior to use. The virus zones were collected with a bent tip hypodermal needle and syringe, diluted 1 : 1 with 0.001 M borate buffer, pH 7.5, without additives, then concentrated by centrifugation for 90 min. at 36.000 rpm. Final pellets were suspended in 1 ml of 0.001 M borate buffer and clarified. Infectivity was tested on leaves of *C. quinoa*.

2.3. Absorption of purified viruses and determination of virus concentration

The absorpation spectra of BYMV and BBSV purified suspensions were determined using spectronic 200 spectrophotometer by measure absorbance at serial wave length 220 up to 320 nm. The concentration was calculated using the absorbance value at 260 nm and extension coefficient of 2.4 and 2.8 for BYMV and BBSV respectively (Noordam, 1973). The values of a minimum and maximum for the purified virus were determined as well as the ratio of A 260 /A 280 and $A_{max/min}$ were calculated.

3.Rabbit immunization

Four New Zealand white rabbits, each one weighted 4 kg (2 rabbits for BYMV or BBSV) were used for antiserum raised against either BYMV or BBSV. A total of 15 mg purified BYMV and 8 mg purified BBSV were used for injection.

One ml (1mg/ml) of purified virus (BYMV or BBSV) was emulsified with an equal volume of Freund's complete adjuvant for subcutaneous and intramuscular injections at weekly intervals. Each rabbit recieved 7 injections from each virus tested.. Intravenous injection without adjuvant were given. Intramuscular injection was performed in the right and the left hand thighs, respectively using a 5 ml disposable syringe. Virus was diluted with phosphate buffer, pH 7.2, (v/v). Intravenous injection was made in the left ear at the marginal vein using 1 ml insulin disposable syringes.

3.1. Rabbit bleeding

Rabbits were bleed 10 days after the last injection from the right ear. The blood was collected, left to clot at 37°C in an incubator for 1-2 hrs, and then kept at 4°C overnight.

3.2. Blood collection and separation of serum

Antiserum was separated through centrifugation at 4000 rpm for 20 min. The antiserum was collected and stored at 4°C until used for titer determination and other serological tests.

3.3.Determination of antiserum titer

Antiserum titer was measured with the indirect ELISA test. Clarified sap of virus infected and control broad bean leaves were diluted at 1/5, 1/10, using phosphate buffer, pH 7.2, containing 0.85% NaCL.

BYMV and BBSV antiserum preparations were diluted with the serum buffer, 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and 1/2048, respectively. The reaction was done between infected clarified extract and its induced antiserum by indirect ELISA test according to methods described by Lommel *et al.* (1982).

4. Purification of immunogamagluline (IgG)

Immunogamaglobulins were purified from the antisera using the method recorded by Steinbuch and Audran (1969). One

ml of virus antiserum was added to 2 ml of 0.06 M sodium acetate buffer, pH 4.8. Then dialyzed against this buffer (0.06 M sodium acetate) for about 24 hours (three times). While stirring vigorously add drop wise 0.082 ml actonic acid (caprylic acid) continuous stirring for 30 min. at room temperature, then centrifuged at 8000 rpm for 10 minutes. The supernatant was collected and dialyzed twice against 0.05 M phosphate buffer, pH 7.2, for 4 hours each.

The resulting IgG was diluted with distilled water to make 4 ml and an equal volume of saturated ammonium sulphate solution were added at room temperature while stirring and left at continuous stirring for 30 min. After centrifugation at 8000 rpm for 10 min., the pellet was collected and suspended in 1 ml distilled water followed by dialysis three times against 0.05 M phosphate buffer, pH 7.2, for 24-48 hrs (three times). If necessary the IgG were centrifuged 10 minutes at 8000 rpm. The IgG concentration was then adjusted to 1 mg/ml (A_{280} nm =1.4) and stored at-20°C until use.

4.1. Conjugation of alkaline phosphatase with IgG

IgG was conjugated with alkaline phosphatase according to protocol given by Clark and Adams (1977). Two mg alkaline phosphatase were dissolved in 1 ml of the above IgG preparation and dialyzed extensively against PBS (0.02 M phosphate buffer saline, pH 7.4).

Glutaraldehyde was added to 0.05% final concentration and the mixture was incubated at 22°C for 4 hours. Glutaraldehyde was then removed by dialysis five times against PBS buffer. Sodium azied 0.02% was added as preservative. The volume obtained was measured and bovine albumin (Sigma A-4503) at the rate of 5 mg bovine albumin to 1 ml solution was added and stored at 4°C.

The concentrations of IgG and IgG conjugate were determined using direct ELISA. IgG conjugate was diluted to 1/250, 1/500, 1/1000 and 1/2000 with the conjugate buffer, while the IgG was diluted with the coated buffer to concentrations of 0.5, 1.0, 2 and 4 µg/ml respectively. Controls of healthy and infected broad bean sap were used. The reaction was done between IgG and IgG conjugate, by DAS-ELISA as follows.

100µl of purified gamaglobulin diluted in coating buffer (0.5, 1.0, 2.0 and 4.00µg/ml) were added to each tested well of microtiter plate. The plate was incubated at 37°C for 4 hours or overnight at 4°C, washed 4 times with PBS-Tween using washing bottle and left at least 5 min.

Two hundred µl aliquots of the test samples (extracted in 0.2 M phosphate buffer, pH 6.0) were added to duplicate wells and incubated overnight at 4°C, then the plate was washed as above.

Two hundred µl IgG conjugate diluted (1/250, 1/500, 1/1000 and 1/2000) by adding conjugate buffer to each well.

The plate was incubated at 37°C for 4 hours or overnight at 4°C and washed 4 times. Two hundred µl aliquots of freshly prepared substrate (5.0 mg Pnitrophenyl phosphate [Sigma 104-105] dissolved in 10 ml substrate buffer) were added to each well, then incubated at room temperature for 30-60 minutes. The absorbance was measured at 405 nm by ELISA reader. Reading twice those of healthy plant control average were considered positive.

5. Determination of antigen dilution end point

Production of an ELISA kit was the end product of this study. ELISA kit specific to each virus under this study (BYMV and BBSV) was assembled from IgG, IgG labeled with alkaline phosphatase, positive and negative controls for the virus, which can be applied for virus detection. Antigen was diluted with phosphate buffer, pH 7.2, to concentrations of 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200 and 1/6400.

6.Immunological detection of BYMV and BBSV by tissue blot immunoassay (TBIA) and dot blot immunoassay (DBIA) on nitrocellulose membranes

6.1.Tissue blot immunoassay (TBIA)

The technique of TBIA described by Lin *et al.* (1990) with some modification on nitrocellulose membranes was used for BYMV and BBSV detection. Nitrocellulose membrane, 0.45 mM pore size were marked with a lead pencil into squares of 1 x 1 cm.Rolled up fresh leaf tissues and/or stems were cut with razor blads for each sample. Exposed cut edges of tested tissues were pressed

steadily, but not firmly into NCM.

The membranes were washed three times with PBST at 5 min interval, incubated in 1 % bovine serum albumin + 2% nonfat dried milk in PBST for one hour at room temperature and washed after blocking with PBS-BSA (bovine serum albumin). The NCM were then incubated with virus specific antibodies diluted in PBS, 1:2000 for BYMV and 1 : 1000 for BBSV, for two hours at room temperature.

Following three successive washing in PBST, 5 min interval the NCM were incubated with alkaline phosphatase labeled with goat antirabbit immunoglobulins (dilution 1:1000 and incubated for one hour at room temperature followed by three times washing in PBST. Two membranes for both BYMV and BBSV were prepared for detection.

The membranes were incubated in substrate solution, Nitro Blue Tetrazolium (NPT) and 5-bromo-4 chloro-3-indolyl phosphate (BCIP) for 5 minutes. After color has appeared, membranes were rinsed quickly with H_2O and then air-dried.

6.2. Dot blot immunoassay (DBIA)

The technique of DBIA described by Lin *et al.* (1990) with some modification on nitrocellulose membranes was used for BYMV and BBSV detection. Nitrocellulose membrane, 0.45 mM pore size were marked with a lead pencil into squares of 1 x 1 cm. Healthy and BYMV, BBSV infected faba bean plant samples were ground in phosphate buffer pH 9.5 (1:10, w/v). Five microliters from each healthy and infected samples were placed on the membrane. The membranes were washed three times with PBST at 5 min interval, placed in the PBS-BSA blocking solution for one hour at room temperature, washed three times with PBST at 5 min interval and incubated with specific BYMV and BBSV antibodies separately in dilutions of 1: 2000 and 1 : 1000, respectively in PBS for one hour at room temperature with gently shaking. The membranes were then washed three times in PBST and incubated with antirabbit conjugate (dilution 1:1000 in conjugate buffer) for one hour at room temperature. The membranes were washed three times in PBST and incubated 5

min in a substrate solution. After color has appeared, membranes were rinsed quickly with H_2O and then air-dried.

RESULTS AND DISCUSSION

When plant pathologists, become involved in immunology, the goal, generally, is to generate an antibody probe which will significantly identify a target antigen in the assay. One of the most important objectives of this work is to produce ELISA kits specific to either BYMV or BBSV. In the present study, two different procedures of purification were undertaken to purify BYMV and BBSV. Methods used here achieved the purification of several viruses in adequate yields, high degree of purity, and little aggregated virus particles. BYMV was purified according to the method described by Alconero *et al.* (1986). After sucrose density-gradient centrifugation, purified BYMV migrated as a single zone, 3 cm below the minsicus of the density gradient column.

This zone was found infectious when tested in the local lesion host plant and gave a typical ultraviolet absorption spectrum of nucleoprotein with a maximum at 260 nm and a minimum at 243 nm, the A260/280 and max/min ratios were 1.33 and 1.11, respectively. The yield of viral zone, using the extinction coefficient of 2.4 was 5.55 mg/100 gm of infected faba bean leaves (Table1 and Fig. 1). The results suggested that, this method of purification was quite successful in purifying BYMV. These results are in agreement with several investigators (El-Kady 1983 and Morales *et al.* 1990).

BYMV was verified by the presence of filamentous flexuous virus particles, with a modal length of 750 nm and 15 nm width (Fig. 2). This type of virus particles is characteristic for Potyvirus group (Alconero *et al.*, 1986 ; Hampton *et al.*, 1992; and Gamal-El-Din *et al.*, 1997).

Concerning purification of BBSV, one faint upper and two distinct lower zones were collected from the sucrose density-gradient columns. The top zone was found non-infective, while the middle and bottom zones found infective and contain virus particles. The two lower zones were collected, pelted, resuspended and tested for purity by UV-absorption spectra.

Gibbs *et al.* (1968) found that, the purified preparations of BBSV produced three boundaries with sedimentation coefficients of 127S, 100S and 60S. The slowest sedimenting particles (top component) consist of protein only, the fastest sedimenting ones (bottom) contain more nucleic acid (36%) than those sedimenting at intermediate speed 25%. The maximum and minimum absorbances were at 260 nm and 244 nm. respectively. The UV-absorbance ratios of a max/min and $A_{260/280}$ were 1.18 and 1.68, respectively. The yield of the purified BBSV preparation was 1.78 mg/kg infected faba bean leaves (Table 1 and Fig. 1). The predominant particle in samples from the virus zone was isometric and about 28 nm in diameter (Fig, 3). Similar results were obtained by several workers (Gibbs *et al.*, 1968; Makkouk *et al.*, 1987 and Sallam *et al.*, 1999).

Antisera have been produced against plant viruses in a variety of animals. Rabbits have been used most often since they respond well to plant virus antigens, are easy to handle, and produced useful volumes of serum. Most traditional methods for using antisera with plant viruses involved direct observation of specific precipitates of virus and antibody, either in liquid media or in agar gels. Over about the past ten years these methods have been progressively superseded by the use of ELISA.

In the present work, polyclonal antibodies raised against BYMV and BBSV were prepared. The antisera produced against BYMV and BBSV had titers of 2048 and 1024, respectively as determined by indirect ELISA test (Tables 2 and 3). However, since the caprylic acid method is simpler and less time consuming (Makkouk *et al.*, 1987), it was adapted for the isolation for BYMV and BBSV immunoglobulins. The concentrations of IgG and IgG conjugated with alkaline phosphatase were 0.5 mg/ml and 1:2000 for BYMV test (Table 4) 1 mg/ml and 1:1000, (Table 5) for BBSV test respectively using direct ELISA technique. Results from direct ELISA test clearly showed that, the IgG and IgG conjugate can be readily applied for virus detection in infected faba bean extracts at dilutions 1:1600 for BYMV and 1:800 for BBSV (Tables 6 and 7).

Since the production of ELISA kits specific to either BYMV or BBSV one of the main objectives of this work to cover the

continuous increasing needs for accurate and fast virus detection and for production of virus-free materials, they has been assembled and readily used for many aspects of plant virus research and particularly for detection and diagnosis of BYMV and BBSV. ELISA method is very economical in the use of reactants adapted to quantitative measurements. It can be applied to viruses of various morphological types in both purified praparations and crude extracts. It is very sensitive, detecting concentrations as low as 1-10 ng/ml (Van Regenmortel, 1982 ; Clark & Bar Joseph, 1984 and Matthews, 1991). Salama (1998) produced ELISA kits specific to BYMV and faba bean necrotic yellows virus (FBNYV).

Our studies showed that, the technique of tissue blotting and dot-blot on nitrocellulose membranes could be readily applied for detection of BYMV and BBSV (Figs 4 and 5). In the tissue blotting technique, the specific antigens are immunologically localized with enzyme labeled antibodies on nitrocellulose membranes.

Although similar in principles to dotblot immunoassays of antigens on various membrane supports, the tissue blotting technique does not require mechanical disruption of tissues for the extraction of antigen or require different buffers for each antigen. Both techniques have been found to have much higher sensitivity for the detection of BYMV and BBSV. Wang *et al.* (1985) reported that, TBIA and DBIA are more sensitive than conventional ELISA technique.

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Table (1): UV-absorption spectra of purified preparations of BYMV and BBSV.

Virus isolates	Max. Absorbance (nm)		Min. Absorbance (nm)				$A_{max/min}$ ratio	$A_{260/280}$ ratio	Yield
	Wave length	Absorbance	Wave length	Absorbance	Wave length	Absorbance			
BYMV	260	1.333	243	1.201	280	1	1.11	1.33	5.55 mg/100 g tissue
BBSV	260	0.982	244	0.828	280	0.582	1.23	1.68	4.78 mg/kg tissue

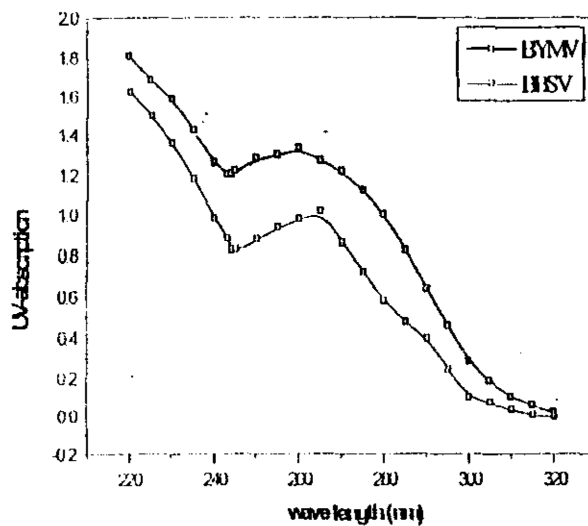

Fig. (1): Ultraviolet absorption spectra of purified BYMV and BBSV.

Fig. (2): Electron micrograph of purified BYMV negatively stained with 2 % uranyl acetate (X 80.000)

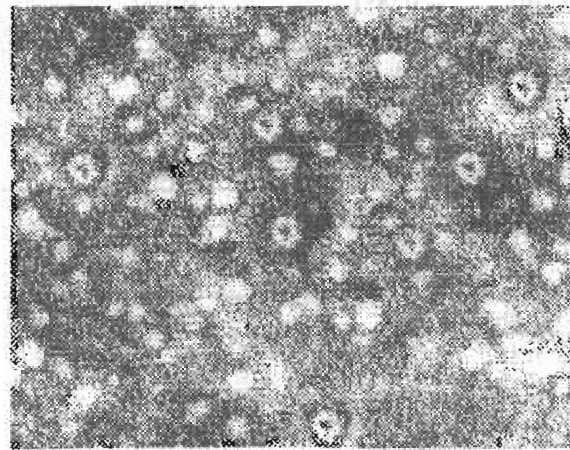

Fig. (3): Electron micrograph of purified BBSV negatively stained with 2 % uranyl acetate (X 67.000)

Table (2): Determination of BYMV antiserum titer.

Antiserum dilutions	ELISA reading at 405 nm			
	Dilution of tissue extract1/5		Dilution of tissue extrct 1/10	
	Infect	H	Infect	H
1 / 1	Over	0.830	1.333	0.622
1 / 2	Over	0.721	1.114	0.510
1 / 4	1.325	0.233	0.882	0.310
1 / 8	1.114	0.121	0.790	0.190
1 / 16	1.062	0.110	0.699	0.162
1 / 32	0.988	0.094	0.589	0.142
1 / 64	0.733	0.090	0.477	0.122
1 / 128	0.492	0.085	0.314	0.110
1 / 256	0.256	0.080	0.245	0.075
1 / 512	0.182	0.070	0.185	0.068
1 / 1024	0.140	0.060	0.130	0.060
1 / 2048	0.120	0.050	0.118	0.050

Reading after 15 min incubation with substrate.
Infect = Infected plant H = Healthy plant

Table (3): Determination of BBSV antiserum titer.

Antiserum dilutions	ELISA reading at 405 nm			
	Dilution of tissue extract 1/5		Dilution of tissue extract 1/10	
	Infect	H	Infect	H
1 / 1	1.922	0.720	1.333	0.610
1 / 2	1.322	0.611	0.839	0.444
1 / 4	0.982	0.520	0.689	0.343
1 / 8	0.831	0.411	0.512	0.220
1 / 16	0.790	0.301	0.442	0.119
1 / 32	0.688	0.209	0.390	0.100
1 / 64	0.599	0.190	0.250	0.089
1 / 128	0.472	0.099	0.190	0.066
1 / 256	0.390	0.080	0.098	0.044
1 / 512	0.279	0.070	0.059	0.022
1 / 1024	0.122	0.030	0.036	0.011
1 / 2048	0.025	0.020	0.010	0.010

Reading after 15 min incubation with substrate.
Infect = Infected plant H = Healthy plant

Table (4): Schematic diagram of checker for determination of approximate working dilution of IgG and IgG conjugate to BYMV for ELISA test.

Concentration of IgG mg/ml	Dilution of IgG conjugate							
	1 : 250		1 : 500		1 : 1000		1 : 2000	
	Infect	H	Infect	H	Infect	H	Infect	H
4.0	0.999	0.251	0.890	0.201	0.687	0.120	0.501	0.100
	0.989	0.250	0.882	0.211	0.683	0.125	0.499	0.099
2.0	0.870	0.203	0.785	0.199	0.590	0.099	0.401	0.095
	0.897	0.201	0.781	0.192	0.579	0.092	0.398	0.089
1.0	0.624	0.198	0.510	0.110	0.431	0.082	0.298	0.052
	0.618	0.192	0.520	0.103	0.391	0.080	0.289	0.059
0.5	0.404	0.101	0.301	0.092	0.109	0.052	0.096	0.021
	0.421	0.122	0.320	0.095	0.105	0.054	0.099	0.025

Infect = Infected plantH = Healthy plant.

Table (5): Schematic diagram of checker for determination of approximate working dilution of IgG and IgG conjugate to BBSV for ELISA test.

Concentration of IgG mg/ml	Dilution of IgG conjugate							
	1 : 250		1 : 500		1 : 1000		1 : 2000	
	Infect	H	Infect	H	Infect	H	Infect	H
4.0	0.820	0.341	0.739	0.210	0.541	0.190	0.0399	0.150
	0.810	0.242	0.729	0.222	0.532	0.181	0.381	0.142
2.0	0.612	0.244	0.501	0.199	0.401	0.120	0.291	0.098
	0.623	0.223	0.521	0.192	0.395	0.129	0.284	0.099
1.0	0.442	0.200	0.303	0.120	0.190	0.090	0.088	0.050
	0.420	0.201	0.309	0.123	0.187	0.089	0.085	0.045
0.5	0.198	0.109	0.088	0.025	0.045	0.022	0.012	0.010
	0.188	0.099	0.090	0.050	0.041	0.019	0.015	0.010

Infect = Infected plantH = Healthy plant

Table (6): Application of BYMV and BBSV Kits

Dilution of tissue extrat	ELISA reading at 405 nm			
	BYMV		BBSV	
	Infect.	Health	Infect.	Health
1/50	0,988	0,102	0,432	0,201
1/100	0,822	0,101	0,392	0,143
1/200	0,700	0,098	0,212	0,054
1/400	0,686	0,082	0,185	0,049
1/800	0,492	0,070	0,126	0,042
1/1600	0,200	0,050	0,030	0,026
1/3200	0,070	0,040	0,024	0,020
1/6400	0,040	0,025	0,009	0,009

Reading after one hour incubation with the substrate
Infect.= Infected plant Health= Healthy plant

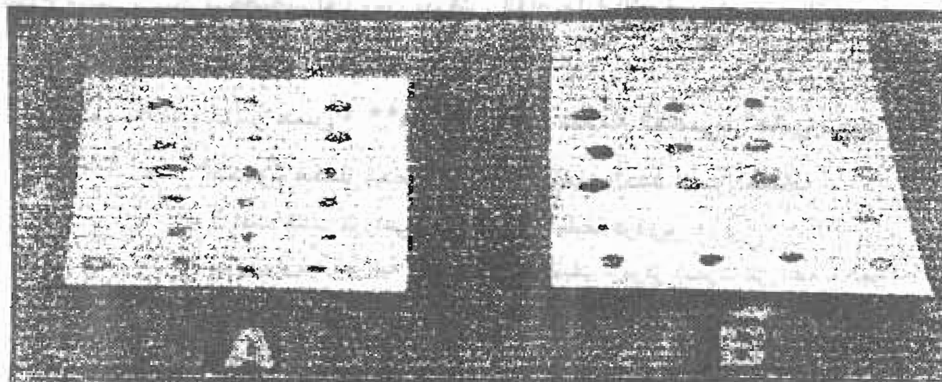

Fig. (4): Detection of BYMV (A) dot blot immunoassay (DBIA) and (B) tissue blot immunoassay (TBIA)

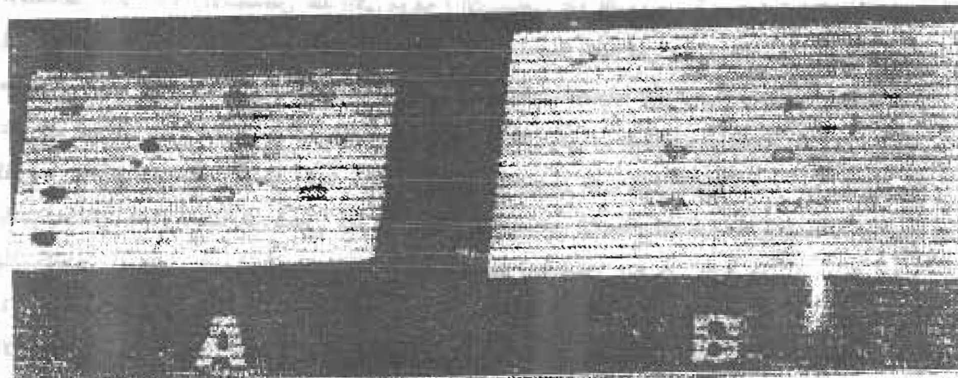

Fig. (5): Detection of BBSV (A) dot blot immunoassay (DBIA) and (B) tissue blot immunoassay (TBIA)

إنتاج مصل مضاد متخصص لفيروس تبرقش الفاصوليا الأصفر وفيروس تلون بذور الفول البلدي

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**وحدة الفيروس والميكروبيولوجيا الجزيئية – معهد أمراض النبات – مركز البحوث الزراعية – الجيزة

تم عزل فيروس تبرقش الفاصوليا الأصفر الذي يتبع مجموعة البوتي فيرس، وفيروس تلون بذور الفول الذي يتبع مجموعة الكوموفيرس من محافظتي الشرقية وبني سويف ثم أجريت تنقية للفيروسين باستخدام الطرد المركزي متفاوت السرعات . وكان طيف امتصاص الأشعة فوق البنفسجية للفيروسات المنقاة مطابقا للنيكلوبروتين مع أدنى امتصاص عند طول موجة ٢٤٣، ٢٤٤ نانوميتر وأعلى امتصاص عند طول الموجة ٢٦٠، ٢٦٠ نانوميتر لفيروس تبرقش الفاصوليا الأصفر وفيروس تلون بذور الفول على التوالي وكان الامتصاص عند ٢٦٠/ الامتصاص عند ٢٨٠ والامتصاص عند أعلى موجة/ الامتصاص عند أقل موجة = ١,٣٣، ١,١١ علي التوالي لفيروس تبرقش الفاصوليا الأصفر بينما كان الامتصاص عند ٢٦٠ / الامتصاص عند ٢٨٠ والامتصاص عند أعلى موجه / الامتصاص عند أقل موجة = ١,٦٨ ، ١,٢٣ على التوالي لفيروس تلون بذور الفول . وكمية الفيروس النقي المتحصل عليه كان ٥,٥٥ ملجم /١٠٠جرام أوراق مصابة ، ٤,٧٨ ملجم / كيلوجرام أوراق مصابة لفيروس تبرقش الفاصوليا الأصفر وفيروس تلون بذور الفول على التوالي . تم إنتاج أمصال مضادة متخصصة عن طريق حقن الأرانب بكميات مختلفة من الأنتيجين لفيروس تبرقش الفاصوليا الأصفر (١,٢ ، ٣ملجم / مل)، ولفيروس تلون بذور الفول(١ ، ٢ ملجم / مل) باستخدام ثلاث طرق مختلفة من الحقن .وتم تحديد أعلى تخفيف من الأمصال المضادة تعطي تفاعلا موجبا باستخدام طريقة الأليزا غير المباشرة وتخفيف عصير النسيج المصاب ١ : ٥ حيث كان ١ : ١٢٠٤٨ و ١٠٢٤ : ١ لفيروس تبرقش الفاصوليا الأصفر ، وفيروس تلون بذور الفول على التوالي . تم تنقية الأمينوجاماجلوبيولين وتم ربطه بأنزيم الفوسفاتيز القاعدي وذلك لاستخدامه في اختبار الأليزا .وكان تركيز الأمينوجاماجلوبيولين ، والأمينوجاماجلوبيولين المرتبط بإنزيم الفوسفاتيز القاعدي ٠,٥ ملجم / مل ، ١ : ٢٠٠٠ على التوالي لفيروس تبرقش الفاصوليا الأصفر ، ١ ملجم / مل ، ١ : ١٠٠٠ على التوالي لفيروس تلون بذور الفول . تم استخدام ELISA-Kits المتخصصة لفيروس تبرقش الفاصوليا الأصفر وفيروس تلون بذور الفول للتعرف على الفيروس باستخدام طرق سيرولوجية تشخيصية مختلفة مثل الأليزا ، TBIA ، DBIA على غشاء النيتروسليولوز . وهذه أول مرة يتم إنتاج ELISA-Kits في مصر لهذين الفيروسين .