

Production of Polyclonal Antibodies to Bean Yellow Mosaic Virus Isolates Affecting Legumes and Ornamental Plants In Taif Province

Eman A. H. Khattab¹ , Hayam S. Abdelkader*²

1,2. Biology department, Faculty of Science, Taif University, KSA.

1,2. Plant Pathology Research Institute, Virology Department, ARC., Giza, Egypt.

2. Microbiology and Immunology Department, Faculty of Pharmacy, Modern University for Technology & Information (MTI), Egypt.

*Correspondence author email: hayamabdelkader68@yahoo.com

ABSTRACT: Bean yellow mosaic virus (BYMV) was detected in *Phaseolus vulgaris* and *Vigna unguiculata* L. plants in different local farms at Al-Haweia, AL-Hada and Al-Shefa, during 2014, throughout Taif Province, Saudi Arabia. The virus was also detected for the first time in up to 10% of commercially grown ornamental plants (Taif Roses) *Rosa damascena*, in which it caused mosaic symptoms on leaf and moderate flower breaking. Large quantities of highly specific polyclonal antibodies was produced against BYMV and other serological related isolates for both capture and detection. The yield of purified virus was 2.0 mg/100 gm of infected *Phaseolus vulgaris* L. plants. Production of specific antiserum was obtained by immunization of New Zealand white rabbits with a total of 10 mg purified virus preparations using three different methods of injections. The titer of the produced antiserum as determined by an indirect ELISA was 1/2048. Results of enzyme-linked-immunosorbent serologic assay disclosed that the prepared antiserum had cross reactivity with different BYMV isolates like clover yellow vein virus (CIYVV) and Pea mosaic virus (PMV). Purified IgG and IgG conjugate polyclonal antibodies made against BYMV proved that it's as extremely economical as compared with the commercially accessible antibodies. Polyclonal antibodies derived from animals immunized with purified viral proteins are particularly valuable for use in the laboratory.

Keywords: BYMV; CIYVV; PMV; Polyclonal antibodies; IgG; *Rosa damascena* ; *Vigna unguiculata*; *Phaseolus vulgaris*; *Vicia faba*; Taif Province.

INTRODUCTION

Since few years ago, the need for commercial production of antibodies to a wide variety of antigens become of a substantial importance. One of the important ways of reducing the virus associated risks is to develop high quality diagnostics for the viruses. High quality diagnostics will provide effective management strategy, biosecurity for imported plant materials and credible phytosanitary certification for exported agricultural produces and planting materials. Bean yellow mosaic virus (BYMV) belongs to genus Potyvirus and family Potyviridae was recently observed for the first time on some leguminous and ornamental plants cultivated in Taif region, Saudi Arabia during 2014. It was thought that BYMV was restricted to leguminous plants but now known to infect several non-legumes (Bos, 1970). Like other members of the Potyvirus genus, BYMV is a monopartite strand of positive-sense, single-stranded RNA surrounded by a capsid made for a single viral encoded protein. The virus is a filamentous particle that measures about 750 nm in length (Brandes and Wetter 1959). This virus is transmitted by species of aphids and by mechanical inoculation (Anderson, 1955).

Considering the importance of the current virus diagnosis, the present research proposal aimed to produce diagnostic polyclonal antibodies against BYMV isolates and its use in screening the bean crops and ornamental plants cultivated in Taif province using ELISA techniques. We believe that the evidence that higher numbers of the insect vectors were present in the area cultivated with beans in Taif governorate may cause an outbreak of bean yellow mosaic virus (BYMV) which severely affected beans and ornamental plants. Both *Aphis fabae* (black bean aphid) and *Myzus persicae* (green peach aphid) were shown on these plants in some local farms in Al-Hada and Al-Shefaa. These vectors are common on sugar beets, adjacent to some of the bean fields and *Rosa damascena* which mostly affected by BYMV.

The virus, now known to form a distinct subgroup (BYMV-subgroup) from other potyviruses, also includes clover yellow vein virus (CIYVV). A close serological relationship between the virus isolates has been demonstrated by Goodchild (1956) and Bercks (1960). The identification of BYMV has been confusing because the BYMV-subgroup viruses are more similar to each other than to other potyviruses. Although BYMV has been an important pathogen of leguminous and ornamental plants in Taif province, KSA., for many years, no studies have assessed variation among BYMV isolates.

Immunological protocols are not standard but it differ from laboratory to another according the animal immunosystem and the type of antigen used for injection. Polyclonal antisera raised in rabbits offer advantage of the ready availability and general high quality of a wide variety of anti-rabbit secondary detection reagents especially, if the antibodies are to be used for western blotting or ELISA (Hancock and O'Reilly, 2005).

Serologically related isolates of BYMV infected tissues (PMV & CIYVV) were tested by in direct ELISA against produced antibodies. The antibodies produced were evaluated for sensitivity and specificity compared to commercial BYMV antibodies.

MATERIAL AND METHODS

Physical Properties

Virus isolates

Naturally infected plants with typical symptoms of BYMV; *Phaseolus vulgaris*, *Rosa damascena*, and *Vigna unguiculata*, were collected from different local farms at Al-Haweia, AL-Hada and Al-Shefa, during 2014, Taif Province, KSA. For host range studies and for producing virus for purification, plants were inoculated with the forefinger wetted with inoculum obtained by grinding infected tissue in phosphate buffer (0.05M, pH 7-6) in the approximate proportions 1: 10 (w/v). The plants were dusted with carborundum before inoculation and the inoculums rinsed off with tap water immediately after. All plants were grown in an insect-free glasshouse at appropriate temperatures.

Host Range and Symptomatology

The virus isolate was biologically purified from single lesions developed on *Chenopodium amaranticolor* leaves and propagated in *Vicia faba* plants for the subsequent experiments. The virus was identified as BYMV on the basis of host range, electron microscopy, transmission experiments, serological tests and some biological studies.

Inspection of infected plants using Tissue Print Immunoassay (TBIA)

TBIA was applied as described by Lin et al. (1990). Healthy and infected leaf samples were ground in phosphate buffer pH 9.0 and pressed steadily on the membrane

Modes of Transmission

Mechanical Transmission

Leaves of infected *Phaseolus vulgaris* L. was ground in a mortar and pestle with 0.01 M Phosphate buffer, pH 7.6, mixed with carborandom. A total of 18 plant were inoculated mechanically, and kept in the glasshouse at 20-30 °C for three weeks. Symptomless plants were also tested by TBIA for the presence of BYMV using the specific antiserum.

Virus Purification

Two to three weeks after inoculation 500-1000 g of leaf and stem tissue were harvested and cooled to 4 °C, and maintained at this temperature throughout the purification process. BYMV isolates were purified from *Phaseolus vulgaris* L. according to the method given by Azzam and Makkouk (1986) with some modifications as following: Triton X-100 was not added during tissue homogenization and 8% PEG and 0.3 M NaCl were used to concentrate the virus. After high speed centrifugation, the virus pellet was suspended in 0.02 M borate buffer, pH 8.0 containing 0.1% 2-mercaptoethanol instead of 0.05 M sodium citrate buffer, pH 7.5 without additives. The pellets were resuspended and clarified as described by Taylor and Smith (1964), except that phosphate buffer was used when the pellet was to be emulsified in adjuvant, because preparations in borate buffer emulsified poorly in Freund's incomplete adjuvant. The U.V. absorption spectra of the supernatants containing virus was estimated spectrophotometrically using an extinction coefficient of 2.4 ($E^{0.1\%}_{260} = 2.4$) as described by Puricfull et al. (1975) with a spectrophotometer.

Electron Microscopy

Purified virus preparation was negatively stained with 2% uranyl acetate, pH 7.0 and examined by electron microscope, Faculty of Science, Taif University as described by Puricfull et al. (1975).

Serological Tests

Rabbit Immunization and antiserum production

Antisera to BYMV isolates were prepared by applying seven weekly consecutive injections of purified virus into four Newzealand rabbits as described by Khattab, (2006). A total yield of 10 mg purified virus were used for injection (three Intramuscular, two subcutaneous, and two intravenous). One ml (2mg/ml) of purified virus was emulsified with an equal volume of Freund's incomplete adjuvant for subcutaneous injections. One mg/ml of purified virus was injected intravenous in the left ear at the marginal vein using 1 ml insulin disposable syringes. Rabbits were bled 10 days after last injection from the right ear. The blood was left to coagulate for 2-3 hrs at 37°C then kept at 4°C overnight. Each antiserum was separated through centrifugation at 4000 rpm for 15 min and stored at -20 °C until used.

Determination of antiserum titer

Antiserum titer was measured with direct ELISA technique. employing antigen- coated plates as described by Clark and Adams (1977). Clarified sap of virus infected and un-infected (healthy) *Phaseolus vulgaris* leaves were diluted at 1/5, using phosphate buffer, pH 7.2, containing 0.85% NaCl and placed in 96 well microtitre plate. BYMV/antiserum preparation was 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. Also, the prepared antiserum was used to determine the antigen dilution end point of BYMV clarified sap using direct ELISA. The crude sap of *Phaseolus vulgaris* leaves infected with BYMV was diluted with phosphate buffer pH 7.2 to 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200 and 1/6400 and used in direct ELISA as described by Khattab, (2006).

IgG purification

The immunoglobulin G (IgG) fraction for the obtained antisera in this study was separated according to the technique described by (Mckinney and Parkinson, 1987; Temponi et al., 1989; Perosa et al., 1990). The IgG fraction was purified from rabbit sera with caprylic acid to precipitate albumin and other non-IgG proteins. Then, the IgG fraction is precipitated with ammonium sulfate. Equal volume of 120 mM sodium acetate buffer (pH 4.0) was added to the rabbit serum placed on magnetic stirrer. Caprylic acid was slowly added with continuous stirring for 30 min at room temperature. The tubes were centrifuged at 5000g for 10 min and the supernatant was saved. The supernatant was transferred to dialysis tubing and dialyzed versus PBS. The obtained IgG was further concentrated and purified by ammonium sulfate precipitation.

Sensitivity of IgG and IgG conjugate

Anti-Rabbit IgG was conjugated to alkaline phosphatase by protein cross-linking with 0.2% glutaraldehyde as described by Avrameas, 1969; Wisdom, 2005). The sensitivities of the IgG and IgG conjugate for BYMV were determined by DAS-ELISA using end-point dilution of a BYMV-IgG conjugate. BYMV- infected and healthy *Phaseolus vulgaris* tissue of 0.5 g, each, were ground in 2 ml of extraction buffer (0.01 M phosphate buffer, pH 7.0, with 0.05% Tween 20). The concentrations of IgG tested were 0.5, 1.0, 2.0 and 4.0 µg/ml and the IgG conjugate was diluted 1:250, 1:500, 1:1000 and 1:2000, and tested for the presence of BYMV as described by Banttarri and Godwin (1985).

Cross-Reactivity of BYMV/Ab with related isolates

The BYMV antibodies were tested against CIYVV and PMV infected clover and pea plants respectively by indirect ELISA. Sample leaves were macerated in phosphate buffered saline (PBS)-Tween 20 (0.01 M phosphate buffer, pH 7.0, with 0.05% Tween 20). The wells were coated with 2 µg/ml of BYMV IgG in 200 µl of 0.05 M sodium carbonate (pH 9.6) and incubated at 37°C for 2 h. After washing, 200 µl of sap was added and incubated at 4°C overnight. A second rabbit-derived antibody was added at a concentration of 1 µg/µl in 200 µl of PBS-Tween 20 and incubated at 37°C for 4 h. Alkaline phosphatase conjugated anti-BYMV prepared in this study was added at a dilution of 1:2000 in 200 µl of PBS-Tween 20 and incubated at 37°C for 4 h. After addition of 200 µl of substrate solution (pNpp), ELISA values were read after 15 to 30 min with optical density at 405 nm. The cross reactivity of the antibodies in an indirect ELISA and their ability to distinguish among virus isolates was evaluated by comparing its avidity with the commercially available BYMV from BIOREBA AG, Switzerland. The absorbance values were recorded using HumaReader HS, GmbH.

Statistical analysis

Analysis of obtained data was performed using the one way ANOVA and is presented as mean ± standard deviation. Efficiency and avidity of prepared polyclonal antibodies to BYMV isolates were compared with the commercial antibodies bought from BIOREBA AG, Switzerland and its O.D. values and ELISA tests were measured in triplicate for accuracy.

RESULTS

Isolation of the Studied Viruses

Source of the Virus Isolate and Observed Symptoms

Naturally infected plants with typical symptoms of BYMV; *Rosa damascena*, *Vigna unguiculata* L., and *Phaseolus vulgaris* were collected from different local farms at Al-Haweia, AL-Hada and Al-Shefa, during 2014, Taif governorate, KSA. The infected plants showed yellow mosaic on *Rosa damascena*, Blistering on *Vigna unguiculata* L. plants and severe systemic yellow mosaic & necrosis on *Phaseolus vulgaris* L (Fig.1).

Identification of the Studied Viruses

Host Range and Symptomatology

BYMV was purified from *Phaseolus vulgaris* as described under the Material and Methods. Ultra violet spectra of the purified virus particle were similar and characteristic of nucleoprotein with a minimum at 243 nm and a maximum at 260 nm. The $A_{260/280}$ and $A_{max/min}$ ratios were 1.4 and 1.2, respectively. Virus yield was 2 mg /100 g of infected tissue calculated on the basis of an extinction coefficient of 2.4. This high yield of the virus in infected *Phaseolus vulgaris* L tissues reflects the simplicity of mechanical transmission of the virus (Dawoud, 1999).

It was identified as BYMV on basis of host range, modes of transmission, electron microscopy and serological reaction studies. For host range studies and for producing virus for purification, BYMV isolate was mechanically inoculated on *Phaseolus vulgaris* L., *Pisum sativum* L. and *Vicia faba* L. The symptoms of BYMV on *Phaseolus vulgaris* L was strong systemic yellow mosaic while it was strong dark green to light green mosaic on *Pisum sativum* L. and *Vicia faba* L. The plants were inoculated with the forefinger wetted with inoculums obtained by grinding infected tissue in phosphate buffer (0.05M, pH 7.6) in the approximate proportions 1:10 (w/v). The plants were dusted with carborundum before inoculation and the inoculums rinsed off with tap water immediately after. All plants were grown in an insect-free glasshouse at temperatures fluctuating between 25 and 30 °C. Electron microscopic examination of purified virus preparation stained with 2% Uranyl-acetate revealed the presence of filamentous flexuous virus particles of about 750 nm long like all the member of the Potyviridae. These results were similar to those reported by Puricfull et al. (1975); Rodríguez-Alvarado et al. (2002), and Khattab (2006).

Tissue Print Immunoassay (TBIA)

Tissue blot immunoassay (TBIA) was used to detect BYMV in naturally infected tissues (Fig. 2). TBIA found to be sensitive enough for detection of BYMV in infected plants. A purplish blue colour was obtained from tissues in positive reactions, whereas extracts from healthy plants remained green representing negative reactions. The same results were obtained by Chaicharoen et al. (2003), El-Sharkawy (2005) and Khattab (2006).

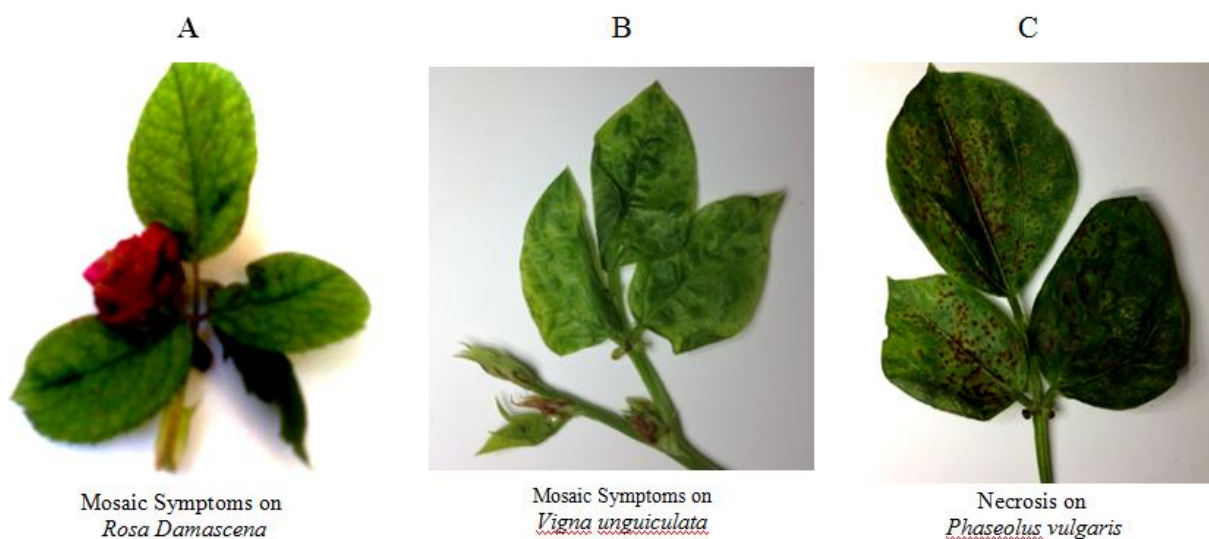


Figure 1. Symptoms of Bean Yellow Mosaic Virus (BYMV) on naturally infected plants: Mosaic on (A) *Rosa damascena*, Blistering on *Vigna unguiculata* L. plants (B) and Severe systemic yellow mosaic & necrosis on *Phaseolus vulgaris* L.

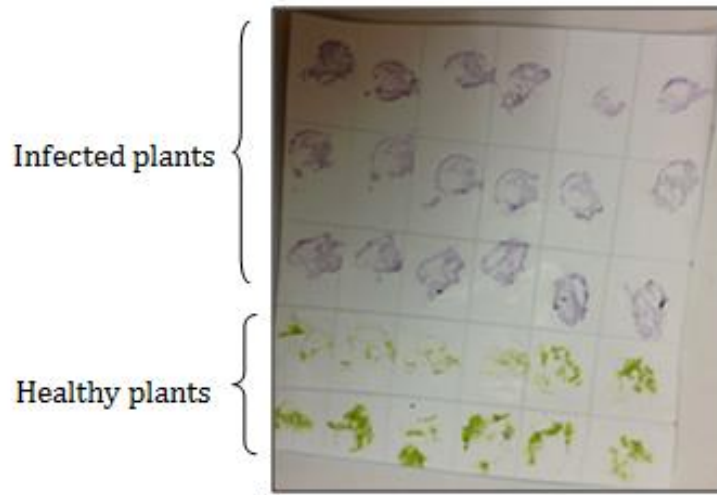


Figure2. Detection of BYMV by tissue blotting immunoassay.

Rabbit Immunization

The immunization of laboratory animals to induce a humoral and/or cellular immune response, is a routine procedure performed worldwide. Although several animal species are used for the production of antibodies, rabbits and mice are the species most frequently used for the production of polyclonal antibodies (pAbs) and monoclonal antibodies (mAbs), respectively.

The viral antigen was prepared as described by Zhuang et al., (2013). Complete Freund s Adjuvant is used in the first injection only. The FCA and ICFA were mixed in equal volume to the antigen, making a 1:1 mix. ICFA was used for booster immunizations. Four female rabbits were used for intravenous, subcutaneous and intramuscular injection as described in Fig. (3) because they are more docile and are reported to mount a more vigorous immune response than males.

Our immunization scheme showed that the intramuscular route of injection in combination with intravenous and subcutaneous rabbit immunization was successful and continued without any problems. Female rabbits with large muscles, large volumes of material can be accommodated (Hanly et al., 1995). Herbert, (1978) considered intravenous administration to be the route of choice for small particulate antigens such as viruses (where danger of anaphylaxis is low), because the antigen distribution is broad and capture by lymphoid tissues is high.

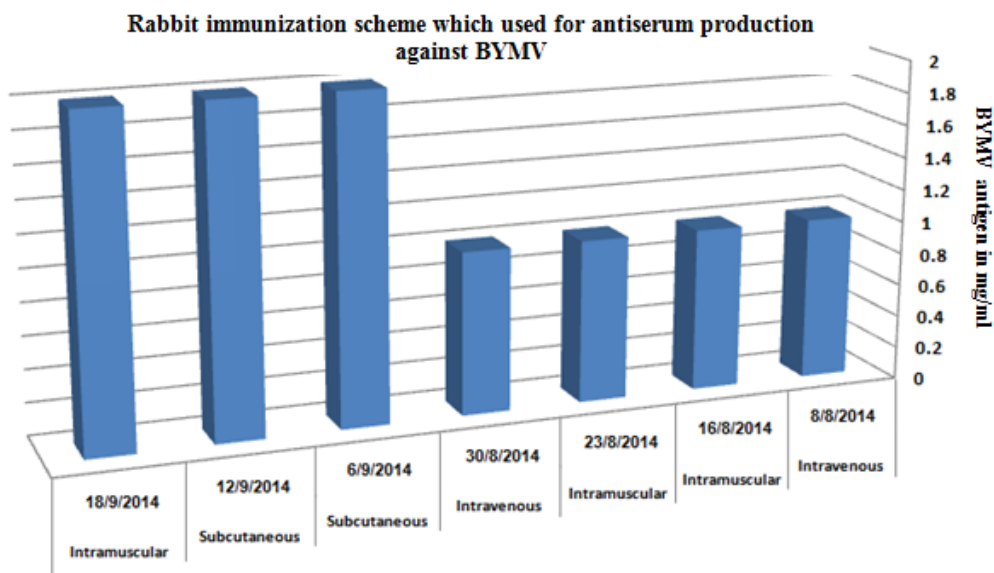


Figure 3. Rabbit immunization scheme used for BYMV antiserum production. Y axis show the injected concentration of BYMV (mg/ml). X axis show the date of injection and the route of immunization.

Serological tests

Antiserum titration

In an indirect ELISA results, the antiserum produced diluted 1:1 was the most reactive while antiserum diluted 1:2048 showed the least activity (Table 2 & Fig. 4). However, all the antisera were effective in distinguishing healthy and infected plant materials using indirect ELISA.

Antigen end point dilution

Antigen end point dilution was determined by direct ELISA as shown in (Table 3). The diluted antigen coated directly on the plate (by using coating buffer pH 9.6) on incubation with BYMV antiserum in sample buffer, showed higher absorbance values with infected samples than with healthy controls (Fig. 5). The antiserum allowed detection of BYMV-infected samples in dilutions ranging from 1:50 to 1:6400. Initially the mean absorbance at 405 nm of the infected sap decreased slowly from 0.499 (1:50) to about 0.015 (1:1600) but dropped more rapidly to 0.028 at 1:3200 and 0.015 at 1:6400. The readings of healthy controls were lower at all the dilutions (0.220 at 1:50 and 0.015 at 1:6400) (Fig. 5). However, the absorbance value of infected sample was more than twice that of healthy sample at all the dilutions tested.

IgG purification

Greater than 80% of the IgG in rabbit serum was isolated by caprylic acid and ammonium sulphate procedure, with a purity equal to rabbit IgG purified by anion-exchange chromatography described by Grodzki and Berenstein (2010). In addition to its simplicity and low cost, the procedure described offers several advantages over other methods to purify IgG.

Table 2. Determination of BYMV antiserum titer

Antiserum dilution	Indirect ELISA absorbance values (405 nm)	
	Infected	Healthy
1/1	1.892	0.701
1/2	1.789	0.622
1/4	1.591	0.501
1/8	1.344	0.422
1/16	1.022	0.401
1/32	0.997	0.382
1/64	0.898	0.280
1/128	0.785	0.255
1/256	0.699	0.221
1/512	0.584	0.201
1/1024	0.492	0.182
1/2048	0.284	0.154

ELISA readings were recorded after 30 min incubation with the pNpp substrate at 405 nm wave length. ELISA readings greater than twice absorbance value of healthy controls was considered positive. O.D. data recorded are mean of optical absorbance.

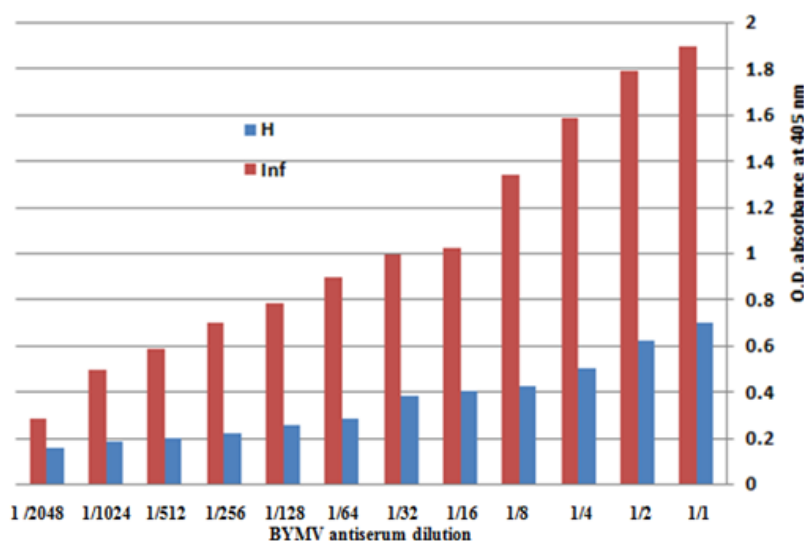


Figure 4. Direct ELISA using different dilutions of crude BYMV rabbit antisera. Healthy and BYMV infected Phaseolus vulgaris (1:5, w/v) were tested. Goat-anti rabbit AP-conjugate was used as secondary antibody. O.D. data recorded are mean of optical absorbance.

Table 3. Determination of antigen dilution end point using prepared BYMV-antiserum

Dilution of tissue extract	Direct ELISA absorbance values (405nm)	
	Infected	Healthy
1/50	0.499	0.22
1/100	0.389	0.178
1/200	0.259	0.109
1/400	0.196	0.054
1/800	0.105	0.048
1/1600	0.044	0.02
1/3200	0.028	0.019
1/6400	0.015	0.015

ELISA readings were recorded after 30 min incubation with the pNpp substrate at 405 nm wave length. ELISA readings greater than twice absorbance value of healthy controls was considered positive. O.D. data recorded are mean of optical absorbance.

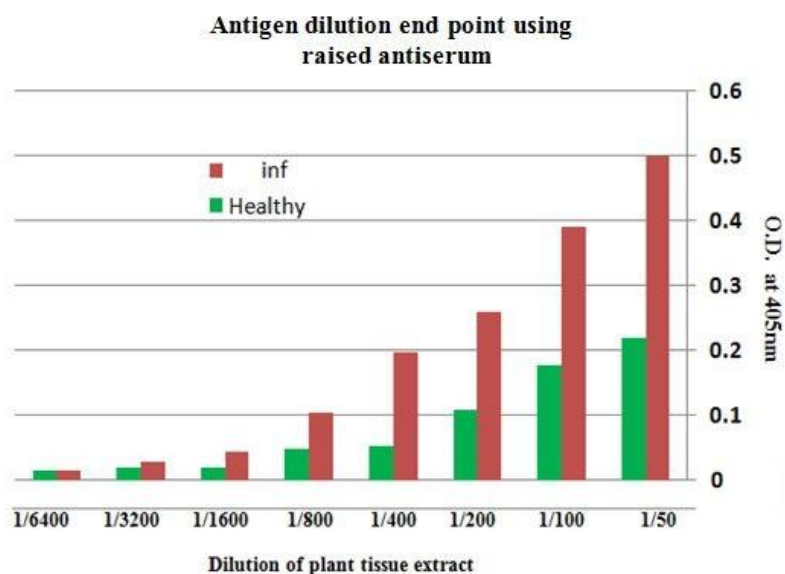


Figure 5. Direct ELISA using a crude sap of infected and Healthy Phaseolus vulgaris diluted (1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, and 1/6400). BYMV- antiserum was used at dilution 1:2000 as first antibody and the Goat anti-rabbit IgG/AP conjugate (1 µg/ml) (BIOREBA AG, Switzerland) was used as secondary antibody. O.D. data recorded are mean of optical absorbance.

Purification of immunoglobulin is a standard approach to reduce non-specific background, but the purification method itself can alter the quality and yield of recovered Ag-specific antibodies (Bergmann-Leitner et al., 2008).

Enrichment of total immunoglobulin by a two step sequential caprylic acid depletion of serum proteins followed by ammonium sulfate precipitation of immunoglobulins was performed as essentially described earlier (Temponi et al., 1989; Perosa et al., 1990).

Antibody specificity & sensitivity

The purified rabbit IgG were tested for their binding strength to their specific Ag (BYMV) and related virus isolates using an indirect ELISA. The quality of the recovered IgG, i.e., whether the IgG can still bind to their specific antigen and whether the secondary detecting Ab in the ELISA still recognizes the IgG was measured (Table 4 & Fig 6). Samples from IgG purification experiments were tested and no changes in the binding avidity. Comparison of BYMV/ IgG titers with commercial BYMV IgG did not distinguish significant differences when using an indirect ELISA method. The purified BYMV-IgG titer which exhibit a higher absorbance value in an indirect ELISA was 1: 2000 while the IgG conjugate titer was 1:1000 (Fig. 9).

Antibody evaluation & cross-reactivity

The prepared polyclonal antibodies were tested with crude saps of different plant species infected with BYMV, PMV and CIYVV using an indirect ELISA. These also displayed a higher absorbance value compared to their respective healthy controls (Table 5 & Fig. 7). The S/H ratios were 2.4 for Phaseolus vulgaris infected with

BYMV, 2.661 for *Rosa damascena* infected with BYMV, 2.373 for *Pisum sativum* infected with PMV and 2.3 for *Trifolium repense* infected with CIYVV, respectively. We present evidence that the obtained antibodies have cross reactivity against CIYVV and PMV isolates. The polyclonal antisera developed against BYMV were evaluated for their specific reaction with purified BYMV as well as with PMV and CIYVV infected plant tissues by indirect ELISA.

Table 4. Schematic diagram of checker board for determination of approximate working dilution of IgG and IgG conjugate against BYMV by DAS-ELISA.

Conc. of IgG (µg/ml)	Dilution of IgG conjugate									
	1:250		1:500		1:1000		1:2000			
	In.	H.	In.	H.	In.	H.	In.	H.		
0.5	0.986	0.422	0.875	0.382	0.713	0.324	0.459	0.259		
1.0	0.807	0.334	0.721	0.298	0.624	0.285	0.382	0.242		
2.0	0.642	0.301	0.632	0.232	0.521	0.242	0.246	0.189		
4.0	0.502	0.290	0.404	0.220	0.362	0.195	0.202	0.169		

H: Healthy plants In. : Infected plants O.D. data recorded are mean of optical absorbance.

Determination of approximate working dilution of IgG and IgG conjugate against BYMV by DAS-ELISA

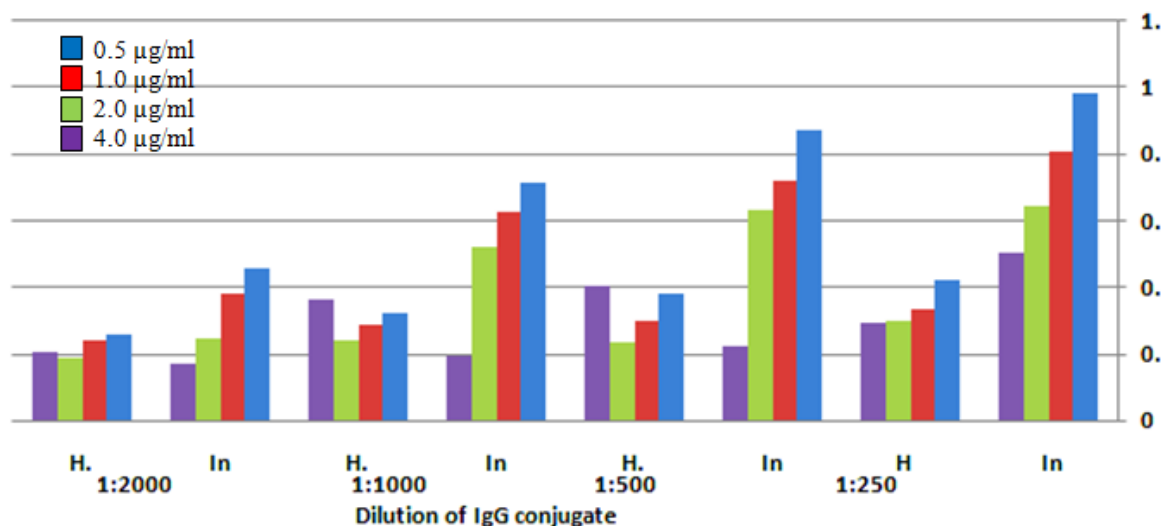


Figure 6. DAS- ELISA using different concentrations of BYMV IgG antibody (0.5, 1.0, 2.0, and 4.0 µg/ml). Healthy and BYMV infected *Ph. vulgaris* (1:4, w/v) were tested. IgG/AP conjugate diluted 1: 250, 1:500, 1:1000 and 1:2000 was used as secondary antibody. O.D. data recorded are mean of optical absorbance.

Table 5. Cross reactivity of obtained IgG with their specific Ag (BYMV) and related virus isolates (CIYVV and PMV) by an indirect ELISA.

	Trifolium repens infected with CIYVV			Pisum sativum infected with PMV			Rosa damascena infected with BYMV			Phaseolus vulgaris infected with BYMV		
	S/H	H.	S	S/H	H.	S	S/H	H.	S	S/H	H	S
	2.54	0.259	0.659	2.5	0.324	0.813	2.55	0.382	0.975	2.573	0.422	1.086
	2.4	0.242	0.582	2.19	0.285	0.625	2.52	0.298	0.751	2.71	0.334	0.907
	2.35	0.189	0.446	2.4	0.242	0.581	2.853	0.232	0.662	2.46	0.301	0.742
	1.82	0.202	0.369	2.36	0.21	0.497	1.04	0.104	0.421	1.73	0.29	0.502
Mean Value	2.3	0.223	0.514	2.373	0.265	0.629	2.661	0.263	0.7	2.4	0.336	0.809

S: Infected plant H: Healthy plant S/H: Infected value/Healthy value
 S/H ratios of greater than 0.5 are considered positive
 O.D. data recorded are mean of optical absorbance.

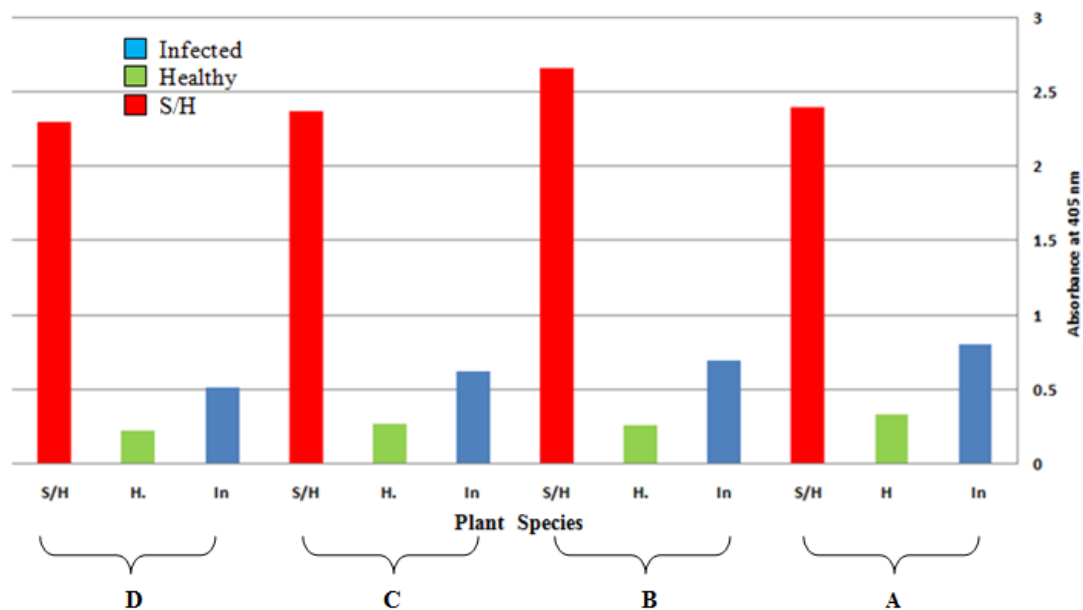


Figure 7. Detection of different BYMV-infected plant species with an indirect-ELISA using Polyclonal antibody prepared against BYMV (1:1000) and (4 µg/ml) of alkaline phosphatase-conjugated IgG. A: Phaseolus vulgaris infected with BYMV; B: Rosa damascena infected with BYMV; C: Pisum sativum infected with PMV; D: Trifolium repens infected with CIYVV.

DISCUSSION

Legume viral diseases have to be controlled in order to improve yields and increase food and especially protein supply to the ever-increasing population all over the world.

Due to a lack of an effective direct control method involving the use of viricides, control options are aimed at reducing infection sources from within or outside the crop. This is often achieved by developing high quality diagnostics for the viruses to provide effective management strategy, biosecurity for imported plant materials and credible phytosanitary certification for exported agricultural produces and planting materials. The use of virus-free seeds or breeding for resistance to seed transmission (Bowers and Goodman 1982) may also be used for effective control. Also, vector control especially through the use of systemic pyrethroides which have been shown to reduce the transmission of non-persistently transmitted viruses (Atiri et al., 1987; Roberts et al., 1993) or breeding for resistance to vector transmission may be used. Limited control has been achieved by the use of cultural practices including crop rotation, modification of planting dates, roguing, and removal of alternative hosts.

This paper briefly describes one of the important viruses and viral diseases affecting legumes and ornamental plants in Taif Province and the preparation of polyclonal antibodies for early detection.

We prepared rabbit polyclonal antibodies against BYMV field isolates from Saudi Arabia, Taif Province for the first record.

Rabbit immunization with pure BYMV, followed by purification of polyclonal antisera have been reported for a number of plant viruses.

The prepared antisera during this study have been used successfully for BYMV detection by different format of ELISA.

There are few recent reports, however, in which antibodies produced against recombinant viral proteins of Tomato spotted wilt virus (TSWV) (Vaira et al., 1996), Grapevine Leafroll associated closterovirus-3 (GLRaV-3) (Ling et al., 2000) and Citrus tristeza virus (CTV) (Abdelkader and Rifaat, 2007), were found to be effective in detecting the viruses using DAS-ELISA. Eni et al., (2010) produced rabbit polyclonal antibodies against purified preparations of a yam isolate of CMV from Nigeria. CMV antibody titre was determined by Protein-A sandwich (PAS) enzyme linked immunosorbent assay (ELISA) and antigen-coated plate (ACP) ELISA. Abdel-Salam et al., (2008) have also demonstrated the successful production of polyclonal antiserum against Prunus necrotic ringspot virus (PNRSV) infecting Rosa hybrida L. in Egypt for the detection of the virus by DAS-ELISA.

Our results confirmed that the antisera produced against BYMV had high titer and high affinity and were able to detect BYMV viral proteins and antigens of serologically related virus isolates (PMV and CIYVV) in native forms by indirect ELISA.

The use of recombinant proteins is an attractive strategy for the production of antibodies against viruses, which are present in low concentrations in infected plants, or are difficult to purify. However, the use of antibodies against recombinant structural proteins in diagnostic tests seems to be impeded by their inefficiency in recognizing native epitopes.

The conventional purification procedure method of BYMV in this study, was easy, fast, with very high yields and can recognize the native epitopes of BYMV, CIYVV, and PMV in infected tissues efficiently. One thousand gram of infected *Ph. vulgaris* yielded about twenty mg of BYMV, an amount sufficient for a repeated immunization of laboratory animals for antisera preparation.

We think that the conventional way for virus purification and rabbit immunization still have great potential as a source of antigens for raising specific antibodies to plant viruses. These polyclonal antibodies can be used for research purposes in many areas of biology, such as immunoprecipitation, histochemistry, enzyme linked immunosorbent assays (ELISA), diagnosis of disease, immunoturbidimetric methods, western blots and Biochip technology. Polyclonal antibodies are ideally suited for use in sandwich assays as second stage antigen detectors. Our polyclonal antibodies prepared against BYMV during this study were tagged with alkaline phosphatase (AP) so that the virus can be detected by yellow colour when positive infected tissues be used in serological assay.

The prepared BYMV polyclonal antibodies during this study were inexpensive and in large quantities. They were capable of recognizing multiple epitopes on the virus and its related isolates. This capability increased the signal produced by the viral proteins as the antibody bound to more than one epitope, sensitive to related antigens, and can recognize both native and denatured proteins.

In conclusion, the induction of pAbs usually takes 4 to 8 weeks. The serum is suitable for many applications, for example, the immunostaining of western blots, ELISA and immunoprecipitation complement fixation. In most cases, polyclonal sera are of high titre, and permit substantial dilution; however, there may be batch-to-batch variability. The fact that a polyclonal antiserum can be obtained within a short time with little financial investment favours its use. In research, many questions can be answered with the assistance of a polyclonal antiserum.

ACKNOWLEDGEMENTS

This research was supported by the grant no. 1-435-3203 of the Grant Agency at Taif University. The authors wish to thank the Dean of Scientific Research at Taif University for funding this research project. All the authors take responsibility for the manuscript's final content.

REFERENCES

- Abdelkader HS, Rifaat MM. 2007. Molecular Cloning, Expression, and Characterization of Mouse Single-Chain Fragment Variable Antibody against Citrus Tristeza Virus. *Arab J. Biotech.* 10 (2):355-368.
- Abdel-Salam AM, Ibrahim AM, Abdelkader HS, amah Mokbel SA, El-Shazly MA. 2008. Biological, serological and molecular studies on Prunus necrotic ring spot virus infecting Rosa hybrid L. in Egypt. *Arab J. Biotech.* 11(1): 125-138.
- Anderson CW. 1955. Vigna and Crotalaria viruses in Florida II. Notations concerning cowpea mosaic virus (Marmor vignae). *Pl. Dis. Repr.* 39: 349-353. 8.
- Atiri GI, Thottappilly G, Ligon D. 1987. Effects of cypermethrin and deltamethrin on the feeding behavior of *Aphis craccivora* and transmission of cowpea aphid-borne mosaic virus. *Annals of Applied Biology.* 110(3): 455-461.
- Avrameas S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochemistry.* 6: 43-52
- Azzam OI, Makkouk KM. 1986. Purification Of The Potyvirus Isolates Infecting *Vulgaris* In Lebanon. *Phytopathol. Medit.* 25: 125-130.
- Banttarri EE, Godwin PH. 1985. Detection Of Potato Viruses S, X And Y By Enzyme-Linked Immunosorbent Assay On Nitrocellulose Membrane (Dot Elisa). *Plant Dis.* 69:202-205.
- Bercks R. 1960. Serologische Untersuchungen Zur Differenzierung Von Isolaten Des Phaseolus-Virus 2 Und Ihrer Verwandtschaft Mit Phaseolus-Virus 1. *Phytopathol. Z.* 39: 120-128.
- Bergmann-Leitner ES, Duncan EH, Burge JR, Spring M, Angov E. 2008. Short Report: Miniaturization Of A High-Throughput Pldh Based Plasmodium Falciparum Growth Inhibition Assay For Small Volume Samples From Preclinical And Clinical Vaccine Trials. *Am J Trop Med Hyg.* 78(3):468-71.
- Bos L, Lindsten K, Maat DZ. 1977. Similarity Of Clover Yellow Vein Virus And Pea Necrosis Virus. *Neth. J. Pl. Pathol.* 83: 97-108.
- Bowers GRJ, Goodman RM. 1982. New sources of resistance to seed transmission of soybean mosaic virus in soybeans. *Crop Science.* 22(1): 155-156.
- Brandes J, Wetter C. 1959. Classification Of Elongated Plant Viruses On The Basis Of Particle Morphology, *Virology* 8: 99-115 .
- Clark MF, Adams AN. 1977. Characteristics Of The Micro Plate Method Of Enzyme-Linked Immunosorbent Assay For The Detection of Plant Viruses. *J. Gen. Virol.* 34: 475-483.
- Dawoud Rehab M. 1999. Preparation of antisera for some plant viruses. M.Sc. Thesis, Fac of Agric., Ain Shams Univ., 117pp.
- Eni AO, Hughes JA, Rey MEC. 2010. Production Of Polyclonal Antibodies Against a Yam Isolate of Cucumber Mosaic Virus (CMV). *Research Journal of Agriculture and Biological Sciences*, 6(5): 607-612.
- Goodchild DJ. 1956. Relationships Of Legume Viruses In Australia. *Aust. J. Biol. Sci.* 9:213 -230.
- Grodzki AC, Berenstein E. 2010. Antibody Purification: Ion-Exchange Chromatography. *Methods Mol Biol.* 588:27-32.
- Hancock DC, O'reilly NJ. 2005. Production Of Polyclonal Antibodies In Rabbits. *Methods In Mol. Biol.* 295: 27-39.
- Hanly WC, Artwohl JE, Bennett BT. 1995. Review Of Polyclonal Antibody Production Procedures In Mammals And Poultry. *Ilar Journal* 37(93):118.

- Herbert WJ. 1978. Mineral-Oil Adjuvants And The Immunization Of Laboratory Animals. In Handbook Of Experimental Immunology, 3rd Edn (Ed. D.M. Weir), Pp. A3.1.3.15. Oxford, UK:Blackwell.
- Khattab EAH.2006. Biological, Serological And Molecular Detection Of Pepper Mottle Virus Infecting Pepper Plants In Egypt. Egypt. J. Phytopathol., 34(2): 121-138.
- Ling KS, Zhu HY, Jiang ZY, Gonsalves D. 2000. Effective Application Of DAS-ELISA For Detection Of Grapevine Leafroll Associated Closterovirus-3 Using A Polyclonal Antiserum Developed From Recombinant Coat Protein. Eur J Plant Pathol. 106, 301–309
- Mckinney MM, Parkinson A.1987. A Simple, Non-Chromatographic Procedure To Purify Immunoglobulins From Serum And Ascites Fluid. J. Immunol. Methods. 96: 271-278
- Perosa F, Carbone R, Ferrone S, Dammacco F.1990. Purification Of Human Immunoglobulins By Sequential Precipitation With Caprylic Acid And Ammonium Sulphate. J Immunol Methods. 128: 9-16.
- Petrzik K, Mraz I, Kubelkova D. 2001. Preparation Of Recombinant Coat Protein Of Prunus Necrotic Ringspot Virus. Acta Virol. 45: 61-63.
- Purcifull DE, Zitter TA, Hiebert E. 1975. Morphology, Host Range, And Serological Relationships Of Pepper Mottle Virus. Phytopathol. 65: 559-562.
- Roberts JMF, Hodgson CJ, Jackai LEN, Thottappilly G, Singh SR.1993. Interaction between two synthetic pyrethroids and the spread of two nonpersistent viruses in cowpea. Annals of Applied Biology. 122: 57–67.
- Smith H, Taylor J, Eds. 1964. Microbial Behaviour, "In Vivo" And "In Vitro" 296 Pp, Cambridge Univ. Press, Lond.
- Temponi M, Kageshita T, Perosa F, Ono R, Okada H, Ferrone S.1989. Purification Of Murine Igg Monoclonal Antibodies by Precipitation With Caprylic Acid: Comparison With Other Methods of Purification. Hybridoma. 8:85-95.
- Vaira AM, Vecchiati M, Masenka V, Accotto GP.1996. A Polyclonal Antiserum Against Recombinant Viral Protein Combines Specificity And Versatility. J Virol Methods 56: 209-219.
- Wisdom GB.2005. Conjugation Of Antibodies To Alkaline Phosphatase. Methods Mol Biol. 295:123-126.