Production of specific antisera against *Beet mosaic virus* and *Beet necrotic yellow vein virus*

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ABSTRACT

The ultraviolet spectrum of purified *Beet mosaic virus* **(**BtMV) particles obtained by density gradient centrifugation revealed that, the concentration of virus preparation estimated using spectrophotometric measurements at 260 nm was 3.004 mg/ml. The min, max nm, $A_{max.}/A_{min.}$, A_{260}/A_{280} and A_{280}/A_{260} ratios were 244, 260, 1.276, 1.512 and 0.662, respectively for BtMV. The corresponding figures of the purified virus preparation of *Beet necrotic yellow vein virus* (BNYVV) were 2.196 mg/ml, and the min, max nm, $A_{max.}/A_{min.}$, A_{260}/A_{280} and A_{280}/A_{260} were 248, 264, 1.606, 1.237, and 0.785, respectively. The polyclonal antibodies raised against BtMV and BNYVV by 5 injection doses each, using different injection types obtained from rabbit bleedings 10 days after the last injection had the titer of 1:2048 and 1:1024, respectively by indirect-enzyem linked immunosorbent assay (I-ELISA) technique. The produced antisera were evaluated and compared with foreign antisera using tissue printing immunoassay (TPIA) and dot blot immunoassay (DBIA). The produced antisera were found more efficiency in development the purplish blue color than the positive reaction when the foreign antisera applied, therefore it should be use produced antisera from Egyptian isolates specific for detection of sugar beet viral infections as a good replacement of foreign ones.

Key words: BtMV, BNYVV, Polyclonal antibodies, DBIA, TPIA

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is considered as the second sugar crop for sugar production in Egypt after sugar cane. Sugar beet crop has been an important position in Egyptian crop rotation as a winter crop in fertile, poor, saline, alkaline and calcareous soils. It could be economically grown in newly reclaimed soils (Gobarah and Mekki, 2005). It is known to be infected with a number of viruses and virus-like diseases. BtMV is a member of family *Potyviridae*, genus *Potyvirus* (Mayo *et al.*, 2005). It has filamentous particles, monopartite, and positive sense single stranded RNA gnome (Brunt *et al*., 1996). It is transmitted by mechanical inoculation (Mali, 2000) and in non-persistent manner by aphid (Omar *et al.,* 2006). BNYVV is the etiological agent of rhizomania disease (Tamada *et al.,* 1989). It was first reported in Italy and has since been reported in more than 25 countries. It is the type member of the genus *Benyvirus*, rod shape particles, transmitted by widely spread soilborne protocystist *Polymyxa betae* Keskin (family *Plasmodiophoraceae*) (Smith *et al.,* 2013) and has multipartite genome process a fifth RNA species (Pavli *et al.,* 2011). Till recently, there were no exclusive or reliable methods for detection and identification of viral pathogens except only the traditional methods to detect viral diseases are many and takes several months. These methods are biological detections such as external symptoms, host rang, virus stability, virus transmission, inclusion bodies and morphology of virus particles (Noordam, 1973). Nowadays both serological and nucleic acids techniques are increasingly developing accuracy, rapid detection and identification methods in plant viruses.

The present study aims to produce of specific polyclonal antibodies which can be used as a rapid serological method for the most serious sugar beet viruses such as BtMV and BNYVV as serologicaly for agricultural quarantine in Egypt.

MATERIALS AND METHODS

Source of virus isolates:

The BtMV and BNYVV isolates were obtained from the Virology Laboratory, Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt (Megahed, 2013) and maintained in *B. vulgaris* cv. Gazella plants as propagative host a propagative host.

BtMV and BNYVV purification:

The purification of BtMV and BNYVV was carried out in Virus and Phytoplasma Research Dept., Plant Pathology Research Institute, Agricultural Research Center. The BtMV isolate was purified according to Glasa *et al.* (2000) and modified by Sayed *et al.* (2008); while BNYVV was purified by the method described by Žižytė *et al.* (2009)**.**

UV. spectrum of purified BtMV and BNYVV particles:

The purified BtMV and BNYVV preparations were diluted individually in 0.01 M sodium borate buffer, pH 7.2 and 0.01 M borate buffer, pH 8.0, respectively at 10^{-1}. The diluted purified viruses were measured at serial wave lengths ranged from 220-300 nm with 2 nm intervals in Shimadzu UV-2401 PC UV-Vis recording spectrophotometer (Molecular Biology Lab. NRC). The same buffers were used as a blank, and the UV spectrum properties and the $A_{max.}$ $A_{min.}$, A_{260}/A_{280} and A_{280}/A_{260} as well as the purity of the virus were determined. The virus yield was estimated using extinction coefficient of BtMV

2.4 (Stace-Smith and Tremaine, 1970) and 3.2 for BNYVV (Bouzoubaa, 1998) using the following equation

$$\text{Virus yield (mg/ml)} = \frac{A_{260}\text{ nm} \times \text{Dilution factor}}{\text{Extinction coefficient}}$$

Production of antisera:

Rabbit immunization:

Four adult New Zealand white rabbits, each one about 3 kg weight (2 rabbits for each virus isolate) were used for antiserum raised against BtMV and BNYVV. A total of 7 mg purified BtMV and 2.4 mg purified BNYVV were used after dialysis in 0.01 M sodium borate buffer, pH 7.2 and 0.01 M borate buffer, pH 8.0, respectively for injection as described in Tables (1 and 2). For intramuscular and subcutaneous injections of purified virus was emulsified with an equal volume of Freund's incomplete adjuvant. Intravenous injection without adjuvant was made in the left ear at the marginal vein using 1 ml insulin disposable syringes. Intramuscular injection was performed in the right and the left leg thighs, respectively using a 5 ml disposable syringe and the virus was diluted with phosphate buffer, pH 7.2 (v/v) as illustrared in Tables (1 and 2).

Blood collection and separation of antisera:

Rabbits were bled 10 days after the last injection, rabbit right ear was shaved and wiped with 70 % ethanol; small longitudinal cut was made by a new blade along the external marginal vein. Blood was collected in sterilized glass tube. The blood was left to coagulate for 2-3 hr at 37 ^{0}C then kept at 4 ^{0}C overnight.

The antiserum of each virus (immunogen) was separated through centrifugation at 4000 rpm/20 min, and then antiserum was collected, divided and stored in glass vials at -20 ^{0}C until using for titer determination and other serological studies (Sayed *et al.,* 2008).

Titration of antisera:

The antiserum titer specific to each virus was measured with I-ELISA according to Koening (1981). BtMV and BNYVV-antiserum preparations were diluted individually with coating buffer to two-fold dilutions, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048, 1/4096 and 1/8192.

Evaluation of the produced antisera using DBIA and TPIA on nitrocellulose membranes:

Dot blot and tissue printing immunoassays (DBIA and TPIA) were performed as described by Lin *et al.* (1990). BtMV and BNYVV isolates were used to evaluate the locally produced antisera against BtMV and BNYVV, and compared with foreign ones (BtMV, 1:200; and BNYVV, 1:500) in 12 sugar beet cv. Gazella plants mechanically inoculated individual with both viruses.

Table (1): Scheme for raising BtMV antiserum in rabbit immunization

Injection No.	Date of injection	Route of injection	Injection dosage (mg/ml) purified virus
1	18-3-2012	Subcutaneous	1.0
2	25-3-2012	Intravenous	1.0
3	7-4-2012	Intramuscular	1.0
4	15-4-2012	Intramuscular	2.0
5	22-4-2012	Intramuscular	2.0

Table (2): Scheme for raising BNYVV antiserum in rabbit immunization

Injection No.	Date of injection	Route of injection	Injection dosage (mg/ml) purified virus
1	27-8-2012	Subcutaneous	0.2
2	3-9-2012	Intramuscular	0.2
3	17-9-2012	Intramuscular	0.5
4	24-9-2012	Intramuscular	0.5
5	1-10-2012	Intramuscular	1.0

RESULTS

UV. spectrum of purified viruses:

The yield of highly purified BtMV was 3.004 mg/ml based on the purification method of BtMV isolate and the UV. spectrum was determined by measuring the absorption of UV using spectrophotometer at wave length from 220 to 300 nm. The obtained results in Table (3) and illustrated by Fig. (1-A) showed that, A_{Min} at 244 nm, A_{Max} at 260 nm and, $A_{max.}/A_{min.}$, A_{260}/A_{280} and A_{280}/A_{260} ratios were 1.276, 1.512 and 0.662, respectively.

The highly purified BNYVV yield was (2.196 mg/ml) as judged by the UV. Absorption spectrum by spectrophotometer (Fig. 1-B). The results in Table (3) illustrated the UV spectrum of BNYVV nucleoprotein with a minimum absorption at 248 nm and a maximum at 264 nm. The values referred to the absorption ratios of $A_{max.}/A_{min.}$, A_{260}/A_{280} and A_{280}/A_{260} were 1.606, 1.237 and 0.785, respectively.

Production of antisera:

Titration of antisera:

The polyclonal antibodies raised against BtMV and BNYVV were obtained from rabbit bleedings 10 days post the last injection. Data presented in Table (4) showed that, the titer of antiserum against BtMV was 1:2048 as determined by I-ELISA technique. On the other hand, BNYVV had a virus-specific titer of at least 1:1024 as positive reaction by I-ELISA technique.

Table (3): UV. spectrum of BtMV and BNYVV purified particles.

Purified virus	A_{260}	(A) A_{280}	$A_{260/280}$	$A_{280/260}$	Max (nm)	Min (nm) (B)	$A_{Max/Min}$	Yield mg/ml
BtMV	0.721	0.477	1.512	0.662	260	244	1.276	3.004
BNYVV	0.703	0.552	1.237	0.785	264	248	1.606	2.196

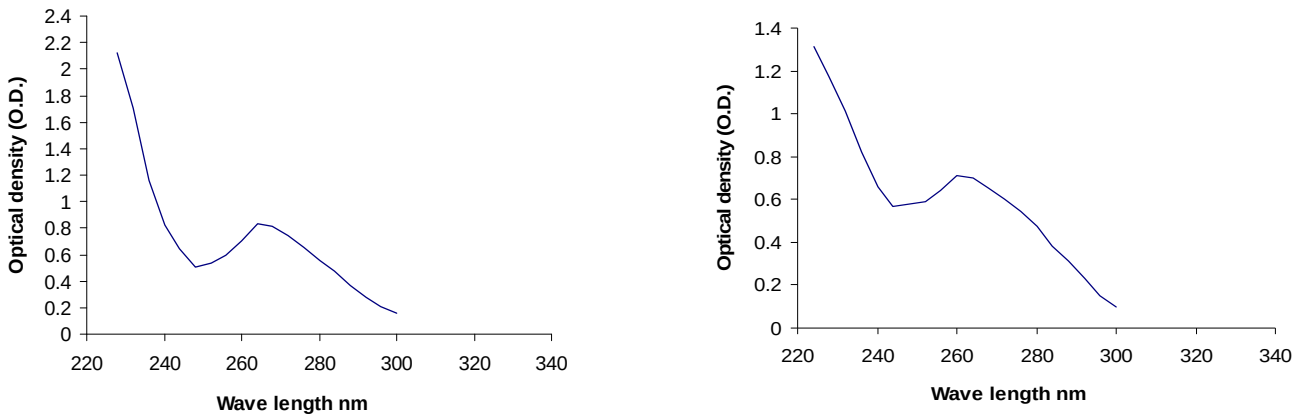

Fig. (1): UV. spectrum of purified BtMV (A) and BNYVV (B) preparations

Table (4): Titration of antisera specific to BtMV and BNYVV using I-ELISA technique.

Antiserum dilution	A_{405} values specific to BtMV antiserum		A_{405} values specific to BNYVV antiserum	
	Infected samples	Healthy samples	Infected samples	Healthy samples
1/1	0.986	0.450	0.855	0.400
1/2	0.815	0.335	0.785	0.365
1/4	0.740	0.290	0.619	0.310
1/8	0.665	0.230	0.588	0.266
1/16	0.555	0.200	0.520	0.220
1/32	0.495	0.140	0.489	0.198
1/64	0.450	0.113	0.441	0.188
1/128	0.410	0.100	0.400	0.164
1/256	0.377	0.086	0.237	0.089
1/512	0.300	0.066	0.184	0.064
1/1024	0.284	0.052	0.150	0.049
1/2048	0.225	0.045	0.083	0.036
1/4096	0.070	0.038	0.050	0.020
1/8192	0.036	0.025	0.013	0.009

Reading greater than twice the A_{405} values of healthy controls were considered positive.

Evaluation of locally produced antisera:

A. Dot blot immunoassay (DBIA):

Dot blot immunoassay was performed on infected sugar beet sap using specific locally produced

antisera to BtMV and BNYVV. The infected plants gave positive results in the form of purple color dots with different degree (Fig.2, A and B). The negative result was obtained as colorless with healthy sugar beet sap, (Fig. 2, C and D).

B. Tissue printing immunoassay (TPIA):

TPIA test was used to evaluate the locally produced BtMV and BNYVV antisera via directly printed leaves petioles on a nitrocellulose membrane. The specific antibodies precipitate the virus particles in parenchyma phloem in the form of purple color (Fig 3, A and B), while no color was detected in healthy ones, Fig. (3 C and D). The infected sugar beet plants exhibited vein necrosis gave strong positive reaction than sugar beet plants exhibited mottling. The technique was more specific with vascular viruses than parenchyma viruses.

Comparison between BtMV and BNYVV locally produced and foreign antisera:

DBIA and TPIA tests were used to compare between the efficiency of the specific locally produced polyclonal antibodies and foreign antisera against BtMV and BNYVV. The two tests were found to be sensitive serological tests to detect BtMV and BNYVV in all 12 infected sugar beet plants cv. Gazella and evaluated the efficiency of specific locally produced and foreign polyclonal antibodies against BtMV and BNYVV, Fig. (2), Fig. (3) and Table (5), respectively.

A purplish blue color was developed with all infected plants in two immunobloting tests when applied both locally produced and foreign antisera specific to BtMV and BNYVV. It was noticed the dark purplish blue with local produced antisera due to the strong serological precipitation reaction between viral protein and locally produced antisera as 75 and 83 % for BtMV and 83 % for BNYVV in both DBIA and TPIA membranes compared with foreign antisra which were reacted with the same samples in the percentages 66 and 58 % on DBIA and TPIA for BtMV, and 58 and 66 % on DBIA and TPIA for BNYVV, respectively (Table 5).

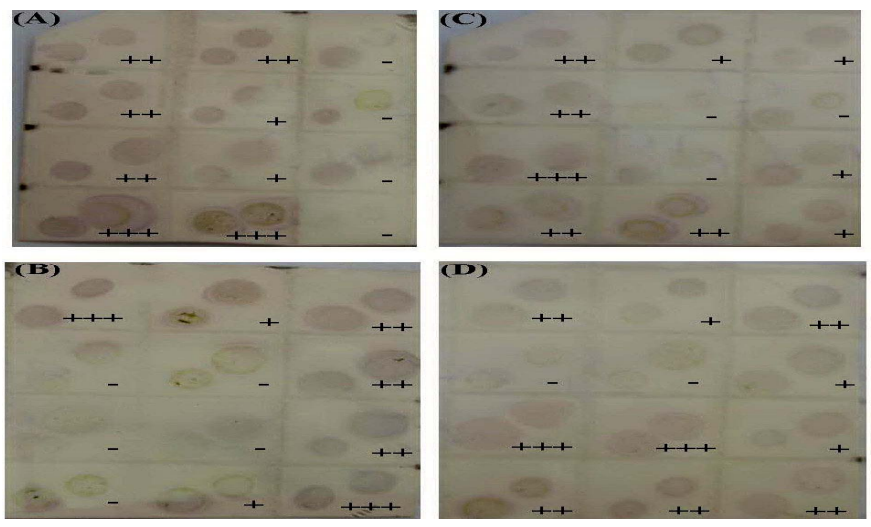

Fig. (2): Dot blot immunoassay illustrates the comparison of foreign and locally produced antisera specific to BtMV and BNYVV.
(A): Foreign BtMV antiserum (C): Locally produced BtMV antiserum
(B): Foreign BNYVV antiserum (D): Locally produced BNYVV antiserum

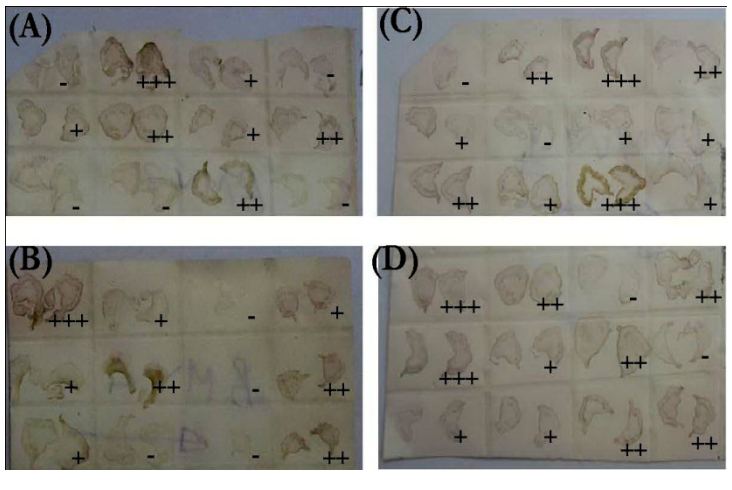

Fig. (3): Tissue printing immunoassay illustrates the comparison of foreign and locally produced antisera specific to BtMV and BNYVV.
(A): foreign BtMV antiserum (C): locally produced BtMV antiserum
(B): foreign BNYVV antiserum (D): locally produced BNYVV antiserum.

Table (5): Evaluation percentage of locally produced and foreign antisera specific to BtMV and BNYVV.

Viruses	Produced antiserum		Foreign antiserum	
	DBIA (%)	TPIA (%)	DBIA (%)	TPIA (%)
BtMV	75	83	66	58
BNYVV	83	83	58	66

The percentage calculated to positive samples for 12 tested samples in all membranes.

DISCUSSION

In the present work, two methods were attempted to purify *Beet mosaic virus* (BtMV) and *Beet necrotic yellow vein virus* (BNYVV) in sufficient quantities for antiserum production. The first two methods i.e. Glasa *et al*.(2000), and modified by Abdel-Ghaffer *et al*.(2003), and Sayed *et al*.(2008) was used to purify BtMV. After sucrose density gradient centrifugation one zone was detected in the gradient column. This zone gave a typical UV-absorption spectrum of other *Potyvirus* nucleoproteins with a min. at 244 nm and a max. at 260 nm. The Amax/min, $A_{280/260}$ ratios were 1.276,1.509 and 0.662, respectively. The yield of virus zone as shown from UV-absorbance data was 3.004mg/ml. These results almost agree with the results reported by other investigators (Abdel-Ghaffar *et al*., 2003; Awad *et al*., 2005 and El-Kady *et al*., 2012). Abdel Gaffar *et al*. (2003) found that UV. Absorption spectrum of BtMV revealed that A min. ranged from 244 to 246 nm and A max. from 258 to 260 nm and its yield was 25-30 mg/kg of infected tissues based on the extinction coefficient of 2.4. Whereas, Awad *et al*. (2005) worked with *Watermelon mosaic virus* found that, the max and min absorptions of the purified virus were 260 and 245 nm, respectively. The Amax/min and A 260/280 ratios were 1.11 and 1,01 respectively. The estimated yield of the virus was 3.88 mg/100g of squash tissue. whereas El-Kady *et al*.(2012) mentioned that, the absorption spectrum of the purified *Pepper mottle virus* had a min. at 245 and a max. at 260 nm. The ratios of $A_{260/280}$, $A_{280/260}$ and Amax/min were 1.30, 0.67 and 1.29, respectively. The estimated virus yield was 3.35mg/100 g of leaf tissues.

The second method of purification described by Žižytė *et al*. (2009) was used for purification of BNYVV. Maximum and minimum absorbances and Amax/min, $A_{260/280}$ and $A_{280/260}$ ratio were 264,248 nm and 1.606, 1.273 and 0.785, respectively. The yield of the purified BNYVV preparation was 2.196 mg/ml. These results were almost in agreement of Koenig *et al*. (1984), Tamada *et al*. (1989) and Žižytė *et al*. (2009). The intensity of light scattering depends on the degree of aggregation of the virus particles (Noordam, 1973).

In the current investigation, we focused on the potential efficiency of purified preparations of BtMV and BNYVV for local production of specific polyclonal antibodies which can be used for detection and identification of the most serious sugar beet viruses as a serological techniques in agriculture quarantine.

The produced antisera against BtMV and BNYVV had titers of 1:2048 and 1:1024, respectively using I-ELISA technique . Awad *et al*. (2005) produced antiserum against *Watermelon mosaic virus* from bleeding taken 3 weeks after the last injection had a titer of 1,2048 using I-ELISA. While it was disagree with Sayed *et al*.(2008) whose found a titer of 1,4096 BtMV antiserum obtained from the 2nd bleeding using also I-ELISA. On the other hand Sukhacherva *et al*.(1996) and Iihan and Ertunc (1999)reported that BNYVV – antiserum had a titer of 1/1024 in ring precipitin test. However, Žižytė *et al*. (2009) reported a titer of 1: 600 using I-ELISA for antiserum of the same virus.

The evaluation of specific locally produced polyclonal antibodies against BtMV and BNYVV and their foreign antisera were compared using DBIA and TPIA. It was found to be sensitive serological tests to detect BtMV and BNYVV in all the same samples of infected sugar beet plants cv. Gazella, as well as for the efficiency of specific locally produced and foreign polyclonal antibodies against BtMV and BNYVV. These results indicated that the locally produced antibodies were related to stimulate Egyptian viral isolates as well as more specific for Egyptian viral isolates as agreed with those reported by Abdel-Salam and El-Shazly (2002), Omar *et al.,* (2006) and Sayed *et al.,* (2008).

Abdel-Salam and El-Shazly (2002) mentioned that, BNYVV was readily detected for the first time in Egypt using DBIA and TPIA. Several investigators were used DBIA and TPIA techniques for detection of their potyviruses (Awad *et al.*, 2005; Khatab *et al*., 2006 and El-Kady *et al*., 2012).

Conclusion

In present work we can be concluded that, the necessity for using of locally produced antisera from Egyptian isolates specific for detection sugar beet viral infections as a good replacement of foreign ones. In this way, application of locally produced antisera in agricultural quarantine in Egypt during cultivated seasons, on the same time to evaluate and certify the imported sugar beet seeds for production of free virus plants.

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