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Purification and Serological Studies on *Barley Stripe Mosaic Hordevirus*

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Three different procedures of purification were performed to purify a local isolate of *Barley stripe mosaic Hordeivirus* (BSMV). The 1st procedure was successful in producing relatively high yield of virus preparation 3.7 mg/100 of barley leaves with adequate purity and one light-scattering zone was found in sucrose density-gradient column. The 2 nd and 3rd procedures yielded aggregated virions (2.3 and 7.0 mg/100 g of fresh barley leaves, respectively). Electron microscopy of negatively stained purified virus preparation showed rod –shaped particles with dimension of 150×25 nm. The polyclonal antibodies raised against the local isolate of BSMV had a specific titer of 1:2000. Positive reactions were obtained when purified IgG and IgG conjugate with alkaline phosphatase was 1:1000. The prepared antiserum was used for detection of BSMV by serological method i.e. Enzyme-linked immunosorbent assay (ELISA), dot-blot and tissues-blot immunobinding assays (DBIA and TBIA). The presence of the virus was confirmed in mature seed parts and non seed parts of the different five barley cultivars tested.

INTRODUCTION

Barley Stripe Virus (BSMV) is one of the few seed borne viruses known to occur naturally in barley, wild oats and in wheat (Najar *et al.*, 2000) and Pecchioni *et al.*, 2000). Visual detection of infected seedlings is not very efficient because seedlings germinated from infected seeds usually have very mild symptoms. So, more refined serological assays as ELISA have been used for detection and identifying plant viruses (Converse and Martin, 1990). Tissue blot immunobinding assays (TBIA) and dot-blot immunobindings assays (DBIA) techniques are now widely used for detection and identification of plant viruses (Dijkstra and De-Jaqer, 1998, Soliman, 2000 and Ghanem *et al.*, 2002). All the serological tests depend on how to obtain highly purified virus (antigen) for immunization and obtaining specific antiserum (Shivas *et al.*, 1989). As the virus under study is seed transmitted (Lister *et al.*,1981; Nutter *et al.*, 1984; Brunt *et al.*; 1996 and Zein, Salwa, 2002) and because it is well known that one of the determining factors involved in seed transmission is ability of the virus to infect male and female gametophytes (Wang and Maule, 1992). Therefore seed parts and non-seed parts were checked using DAS-ELISA.

The objectives of the present study are purifying of barley stripe mosaic virus (BSMV) and produce of ELISA Kits to cover the need for virus detection in barley grains and plants

MATERIALS ANDMETHODS

Virus purification

Three different procedures were undertaken to purify BSMV. One hundred grams of systemically infected barley leaves collected 2-3 weeks after

inoculation were used for virus purification. The different procedures are summarized in Table (1).

The purified and partially purified virus preparations were estimated spectrophotomerically using the extinction coefficient of 2.6 (Atabekov and Novikov, 1989). Samples of purified virus preparation were stained with 2% uranyl acetate and examined with a Philips 301 EM.

Production of antiserum specific to BSMV

A total of 7 mg of purified virus preparation were used for antiserum production. Three routs of injection were used in immunizing the rabbits, intravenous, in the first injection followed by subcutaneous and intramuscular injections. In the two latter of injection rotes, the virus was emulsified with an equal volume of Freund's complete adjuvant according to (Hampton *et al.*, 1990). Ten days from the last immunization, the rabbits were bled. The antiserum titer was determined by indirect ELISA (Brattey and Burns, 1998). In this method, the antiserum was added at dilutions of 1/500, 1/1000, 1/1500, 1/2000, and 1/2500 with PBS buffer pH 7.4. Anti-BSMV immunoglobulin was purified from the antiserum to BSMV and it was conjugated with alkaline phosphatase, using the method described by Brattey and Burns (1998). Optimum concentration of IgG and IgG conjugate with alkaline phosphatase was determined using a check board test (Converse and Martin, 1990) to optimize concentration for DAS-ELISA test (Kirby and Appleyard, 1981).

Serological detection

1-Detection of BSMV using DBIA, and TBIA.

DBIA and TBIA techniques were applied for detection of BSMV in infected barley plants according to the methods described by Hsu and Lawson (1991) and Makkouk *et al.* (2001) respectively.

Table (1): Methods used to purify BSMV.

Methods applied, Reference	Extraction buffer (pH)	Stabilizing additives	Buffer /weigh t (v/w)	Clarifying agent (v/v)	Concentration	Resuspendi ng buffer
The 1st Atabekov& Novikov (1989)	0.1M Phosphate buffer pH 7.2	0.02M Na2EDTA	3:1	25% CcL3	Precipitation by 22% A.S. then two cycles of D.C. 20% sucrose cushion then SDGC	0.05 M Tris-Hcl 7.5
The 2 nd Carroll *et al.* (1979)	0.05M sodium borate (8.2)	0.1% 2-ME	1:1	1.5 (v) n-butanol 1.5(v) Chloroform	Precipitation by 8% PEG then two cycles of D.C	0.01 M Sodium borate (8.2)
The3rd Lawrence& Jackson (1998)	0.5 M Sodium borate (9.0)	2.0% Triton X-100	3:1	-	20% Sucrose cushion then S.D.G.C	0.01M P.B (6.8)

A.S.= Ammonium Sulfate **D.C** = Differential Centrifugation
P.B = Phosphate buffer **2-ME** = 2-mercaptoetanol
PEG = Polyethylene glycol **CcL3** = Chloroform
SDGC = Sucrose density-gradient centrifugation
Na2EDTA = Disodium ethylene diamine tetracetic Acid

2-Presence of the virus isolate in infected non-seed parts

Direct-ELISA technique was applied to assay whether the virus under study infected non-seed parts of barley cvs. G117, G119, G121, G124 and G125 or not. Anther, stigma and lodicules were removed separately from fifty florets of each infected and healthy barley cultivars tested. Non-seed parts were prepared for ELISA as described by Kibry and Appleyard (1981).

3- Presence of the virus in mature barley seeds.

In mature barley seeds, fifty mature seeds collected separately from infected and healthy tested barley cultivars were prepared for DAS-ELISA. Embryos, testae, and endosperms were manually separated from barley seeds previously soaked in water for two successive days. Pooled embryos, pooled testae and pooled endosperms were ground separately in phosphate buffer saline containing 0.5 ml of Tween 20 per liter (PBST) at 1:20 dilution (w/v) with a mortar and pestle. Each extract was centrifuged for 10 min at 5000 rpm and the supernatant was collected and used as antigen in ELISA test.

RESULTS AND DISCUSSION

Purification of the virus isolate

Three different procedures of purification were performed to purify the Egyptian isolate of BSMV. The results of all absorbance spectra and yield of virus/100g fresh barley tissues are illustrated in Table (2) The 1st procedure of purification (Atabekov and Novikov, 1971) gave a relative high yield of virion with aqequate purity (3.7 mg/100g of barley leaves) using the extinction coefficient of 2.6 (Atabekov and Novikov, 1971). One light –scattering zone was observed 13-15 mm below the meniscus of the density-gradient column. The UV-absorption spectrum of the purified isolate had a maximum absorption at 270 nm and a minimum at 255 nm. The ratios of max/min and A_{260}/A_{280} were 1.14 and1.20, respectively. These ratios were calculated from values uncorrected for light scattering.

The two other procedures (Carroll *et al.*, 1979 and Lawrence&Jackson, 1998) failed to yield un-aggregated virus particles. Only pellets were observed at the bottom of the density-gradient column. These pellets were infectious and contained aggregated virus particles when examined with electron microscope. Many investigators reported that BSMV had an UV-absorption spectrum with a max. between 260 and 255 nm, a min between235-245 nm, A_{260}-A_{280} and A max/min. Ratios of 1.78-1.25 and 1.42-1.48.

Table (2):UV-absorption spectra and yield of the purified isolate of BSMV Using three different procedures of purification

Procedure applied	A maximum at/nm	A minimum	A max/min ratio	A_{260}/A_{280} ratio	Yield mg/100g leaves
1st	270	255	1.14	1.20	3.7
2nd	260	235	1.42	1.78	7.0
3rd	255	245	1.48	1.25	2.3

, respectively (Jackson&Brakke, 1973; Lane, 1974 and McFarland *et al.*, 1983). Virus yields in the second and third methods were 7.0 and 2.3mg/100g of barley leaves, respectively. It is well known that aggregation is a serious problem encountered during the purification of viruses (Atabekov *et al.*, 1968). The use of PEG in the second procedure of purification seems to be harmful since virions were aggregated at the bottom of the sucrose density gradient columns. Darirdage and Shepherd (1970) reported that aggregation become apparent and increased after one PEG precipitation followed by 2 cycles of differential centrifugation in the buffer alone, whereas the virus was much less aggregated in the buffer containing 0.5M urea and 0.1% 2-ME.They warned against such difficulty of purification procedures and suggested that each worker should try and develop his own set of purification, as a method that work well with one researcher might prove a failure with another. Little is known concerning the actual process that results in aggregation, though various suggestions have been made on the basis of treatments which initiate the process. The superiority of the 1st technique to other two ones may result from using of chloroform as a clarifying agent, sodium EDTA in the resuspended buffer and ammonium sulfate as a precipitate to concentrate the virus particles from the clarified extract. Chloroform and additives are known to decrease aggregation of viruses (Abdel-Salam&El-Kady, 1991, and Dijkstra&De-jager, 1998). The second method, of purification gave high yield of virions but the virus was aggregated with plant components forming pellets, hence the purity of the virus particles was not satisfactory as shown from the UV-absorption spectrum. The yield of purified virus preparation was rather low comparing to that obtained by Brakke (1979).

Electron micrograph of purified BSMV stained with 2% uranyl acetate, showed rod-shaped particles (Fig 1). Harrison *et al.* (1965); Brunt *et al.* (1996), and Lawrence&Jackson (1998) reported that BSMV is a rigid rod approximately 30 nm wide and average length of 130 nm. Brunt *et al.* (1996) recorded that BSMV particles were rod-shaped not enveloped, usually straight, with a clear modal length of 112-150 nm and 18-25 nm wide. The same was reported by Lawrence and Jackson (1998).

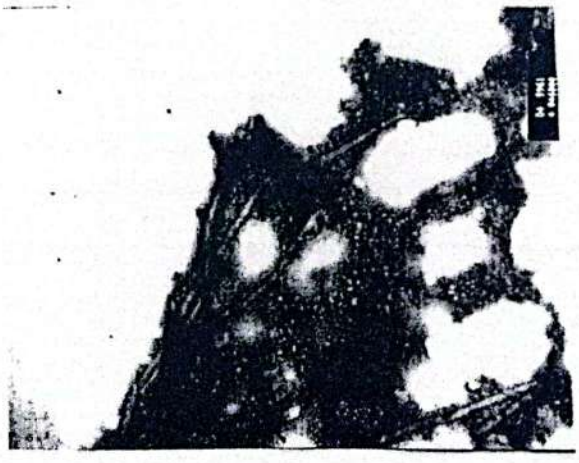

Fig (1):Electron micrograph of purified BSMV preparation stained with 2% uranyl acetate, PH 6.8. (Magnification 46,000X).

Production of antiserum specific to BSMV

The usefulness of antiserum produced was tested by using indirect ELISA technique. Data presented in Table (3) show that BSMV was detected at 1:2000 concentration of antiserum, when 1:10 dilution- infected barley plant extracts when used. Positive reactions were obtained when purified IgG and IgG conjugate with alkaline phosphatase was 1:1000.

One of the major goals in the present work is to produce ELISA Kit which can be used as a rapid method for BSMV detection in cereals cultivated areas, in seed production schemes and in post-entry quarantine (Shivas *et al.*, 1989).

Serological detection

1- Detection of BSMV using DBIA and TBIA techniques

TBIA and DBIA techniques were used to confirm the identification of the isolated virus (Fig 2). Positive reactions were obtained with BSMV – infected tissues as strong pink colour appeared, while negative reactions were observed with samples of healthy plants. The advantage of DBIA technique for detection of small amounts of antigen over strandard ELISA. DBIA is cheaper but TBIA combines sensitivity and red reliability of both ELISA and DBIA with simplification of the procedure, hence it is very suitable for routine-indexing of large number of samples (Dijkstra and De-Jager, 1998).

Table (3):ELISA values of BSMV antiserum related with infected barley plants

Antiserum dilution	A 405 nm	
	Infected sap	Healthy sap
1/500	0.683	0.130
1/1000	0.624	0.110
1/1500	0.451	0.092
1/2000	0.295	0.089
1/2500	0.031	0.021

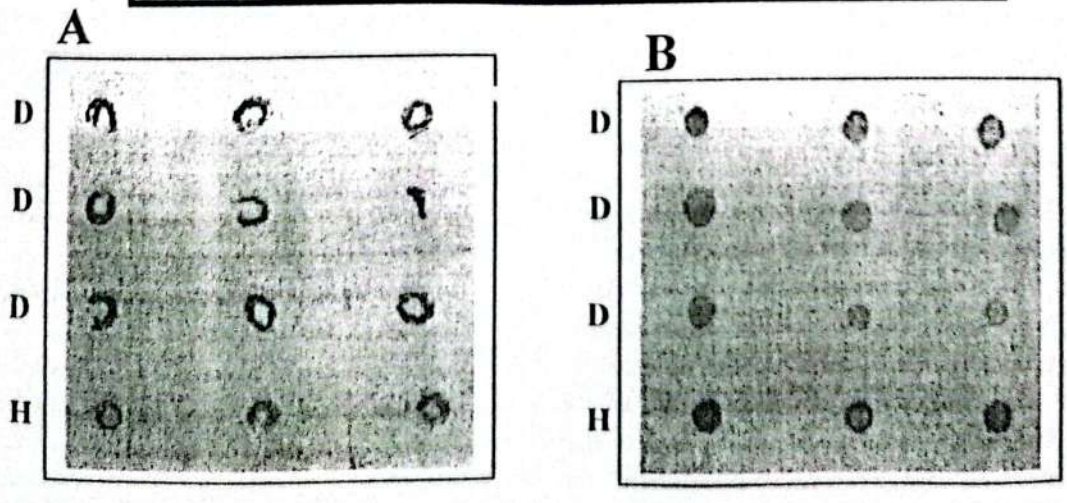

Fig (2): Detection of BSMV in both diseased (D) and healthy (H) Giza 119 barley plants by DBIA (A) and TBIA (B) pink colour indicates positive reaction.

Table (4): Serological assays of non-seed parts and mature seed parts of infected barley plants tested by DAS-ELISA.

Barley cultivar	Non-seed parts			Mature seed parts		
	Anther	Stigma	Lodicules	Endosperum	Testa	Embryos
G117	-	-	+	-	-	+
G119	-	-	+	+	-	+
G121	+	-	+	+	-	+
G124	-	-	+	+	+	+
G125	-	-	+	-	-	+

+ = positive - = negative

2- Presence of BSMV in infected non-seed parts

Using DAS-ELISA, BSMV was detected from lodicules of all the tested barley cvs (Table 4). On the other hand BSMV was not detected from stigmae of all the tested cvs. Only anther of G121 was found infected.

3- Presence of BSMV in mature seed parts

Data tabulated in Table (4) indicate the presence of the virus (antigen) in all embryos of tested barley cultivars, while differences were found in endosperm and testa among the tested barley cultivars. Endosperm of cvs. G119, G121 and G124 were found infected, while endosperms of cvs. G117 and G125 were not. The virus was detected only from testa of G124.

The differences observed in distribution of the virus in the different tissues tested might be attributed to the elimination or exclusion of the virus from tissues during seed ripening (Makkouk *et al.*, 1994). Anther reason is that the virus occurred at a concentration level too low to be detected by ELISA. (Sukhacheva *et al.*, 2000).

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