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Identification and detectability of broad bean stain virus in broad bean seeds and effects on nodulation

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During a survey for broad bean viruses in Sohag Governorate, a virus was identified as Broad bean stain virus (BBSV), a member of the genus Comovirus, based on host reactions, symptomatology, electron microscopy and serologically by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) technique. The virus was mechanically transmitted to some members of Leguminosae and also through seeds. The ultraviolet absorption spectrum of the purified virus showed a typical curve of nucleoprotein with an $A_{260/280}$ ratio of about 1.25–1.34. The yield of purified virus was 0.61–0.63 mg/kg infected tissue. Electron microscopy of purified preparations revealed the presence of isometric virus particles, 27 nm in diameter. The polyclonal antibodies against BBSV were produced and the antiserum titre of three bleedings was determined by indirect DAS-ELISA technique. Gross reduction of nodulation was achieved by virus inoculation on broad bean plants cv. Giza 402. It produced smaller, fewer nodules and reduced its leghaemoglobin content. As well as seed yield quality and quantity was strongly affected due to infection. When cells of root nodules in BBSV-infected broad bean plants were investigated by transmission electron microscopy, a decrease of number, volume of bacteroids in nodule cells and the space between the bacteroid and its membrane envelope (ME) were observed when compared with healthy cells. This difference was accompanied with the presence of BBSV particles in the root nodule cells. Seeds taken from these plants were tested for the presence of the virus by DAS-ELISA and symptoms development on the seedlings produced. There was a good correlation between ELISA detection of BBSV in tissue taken from single broad bean seeds and subsequent development of infected plants grown from the same seeds. The ELISA detected BBSV in the cotyledons and developing axis of the embryo, but not in seed coat tissues. When mixtures of infected and healthy seeds in different ratios were tested, BBSV was detected in mixtures up to 1:100 (infected: healthy). The ELISA technique is reliable for selecting BBSV-free stocks of broad bean seeds.

Keywords: BBSV; identification; electron microscopy; broad bean; nodulation

Introduction

Broad bean (*Vicia faba* L.) is an important food crop in many countries. It is considered as the main protein source for a large part of the population. Productivity

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of the crop is affected by a number of factors including viruses. The crop is subjected to infection with different viruses all over the world. In Egypt, many viruses have been identified, i.e. Pea mosaic; Bean yellow mosaic; Alfalfa mosaic; Broad bean stain; Broad bean leaf roll; Broad bean mottle; Broad bean wilt; Pea enation mosaic and Pea seed-borne mosaic viruses (Allam et al. 1979, 1985, 1986a, 1986b; Makkouk et al. 1988; Omar et al. 1990). Broad bean stain virus (BBSV) is an economically important virus infecting broad bean and other legume plants over the world, such as Europe (Musil 1981; Haaek 1990; Mali et al. 2003); North Africa (Tolba 1980; Makkouk et al. 1987; Salama 1998); Africa (Abraham and Makkouk 2002) and Asia (Makkouk et al. 1987; Al-Mabrouk and Mansour 1998a, 1998b). The virus is transmissible by seed (El-Hammady et al. 2004), easily transmitted by sap (Gibbs et al. 1968) and with a shape of isometric particles of 25–30 nm in diameter according to Makkouk et al. (1987) and Omar et al. (1990). The embryo of seeds has protection against invasion by viruses but nevertheless, a number of viruses are seed borne. It usually carried in or on the seed testa and cotyledons (El-Dougdoug et al. 1999). The earlier works on virus infection of plants in relation to root nodulation have shown that the number of nodules was significantly reduced in broad bean (Hammouda et al. 1990; Sidaros et al. 1991), but few studies have been undertaken to find out the effects of viral infection on the fine structure of the nodule cells and change in their activity particularly N_2-fixation (Tu 1973, 1977; Gomaa et al. 2006). Therefore, the objective of this study was to isolate and identify BBSV, as well as, the detectability of the virus in seeds by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and study the differences between bacteroidal cells in root nodules in healthy and BBSV-infected broad bean plants.

Materials and methods

Virus isolation and identification

Isolation and mechanical inoculation

Samples of broad bean plants showing virus-like symptoms were collected from the open fields in Sohag Governorate, Egypt. The presence of BBSV was preliminary detected by DAS-ELISA as described by Clark and Adams (1977), using the BBSV-ELISA kit obtained from Agricultural Genetic Engineering Institute (AGERI), Agricultural Research Centre (ARC), Giza, Egypt. The samples which gave positive reaction were used as virus source for mechanical inoculation of virus-free broad bean seedlings (cvs. Giza 402 and Giza planka) kept in insect-proof greenhouse (25–28°C). For further isolation, the inoculated plants showing symptoms were used as a virus source to inoculate *Phaseolus vulgaris* cv. Lima plants. After the appearance of local lesions, the virus isolate was biologically purified by three cycles of single local lesion isolation, and then mechanically transmitted to virus-free broad bean seedlings cv. Giza 402 which used to inoculate broad bean seedlings once more and host range determination.

Virus purification

BBSV was propagated in broad bean cv. Giza 402. Leaves from the infected plants showing mild mottle were harvested 3 weeks post-inoculation. The purification procedure was similar to that used by Damsteegt et al. (1999), and the virus purification protocol involved the use of 100 mM sodium citrate extraction buffer

with 10 mm ethylenediaminetetra-acetic acid (EDTA), pH 8; precipitation of chloroform/butanol-clarified supernatant with 8% polyethylene glycol (PEG) 6000 with 1% NaCl and centrifugation of the resuspended high-speed pellets on 30% sucrose pad. The resultant pellets were resuspended overnight at 4°C in 1.0 ml of 0.1 mM sodium phosphate pH 7.6. Virus preparations were layered onto 10–40% sucrose gradients and centrifuged in Beckman 28.1 rotor at 26,000 rpm for 2 h. After sucrose density-gradient centrifugation, fractions were separated manually and subjected to spectrophotometry at 254 nm. Fraction which showed virus-like peak recovered by high-speed centrifugation at 60,000 rpm for 90 min. Absorbance peak fraction was collected, concentrated by centrifugation at 50,000 rpm for 2 h, then resuspended in 250 μl of resuspension buffer for 12 h and stored frozen at −20°C. The virus concentration was estimated spectrophotometrically using an extinction coefficient of 3.2 for cowpea mosaic virus according to Van Kammen and De Jager (1978).

To distinguish shape and size of BBSV particles, the purified virus preparations were negatively stained by using 2% aqueous uranyl acetate, and then examined in a JEOL-1010 transmission electron microscopy at EM-unit, Sohag University, Sohag, Egypt.

Antiserum production

Antiserum raised against BBSV was prepared according to the method described by Abdel-Ghaffar et al. (1998). Two adult New Zealand white rabbits were injected with purified viral preparation. Before starting the injection schedule, the rabbits were bled to obtain the normal serum. Initially, 200 μg/ml of purified virus was emulsified with an equal volume of Freund's complete adjuvant and then injected subcutaneously, followed after 4 weeks by five intramuscular booster injections consisting of 200 μg/ml of the purified virus for each emulsified in an equal volume of incomplete adjuvant at weekly intervals. The rabbits were bled three times, starting 1, 2 and 3 weeks after the last injection. The antiserum was separated by incubating the blood at 37°C overnight. After low-speed centrifugation, the supernatant containing the clarified antisera was collected and the antisera titre of the three bleedings was determined by indirect DAS-ELISA.

Effect of virus infection on nodulation and broad bean yields

This experiment was carried out at the farm of Faculty of Agriculture, Sohag University, Sohag. The experiment design was split plot design, with two rhizobial inoculation treatments as main plot, two viral infection treatments as sub plots. Twenty seeds of broad bean cultivar (Giza 402) were sown in each plot. The seeds of the inoculation treatment were soaked in a 3-week-old yeast–mannitol broth culture of *Rhizobium leguminosarum* biovar *viceae* (1 ml/10 seeds). The 0.5% (w/v) of powdered acacia gum was added to the *Rhizobium* broth to increase adherence to the seed. Seeds inoculated by this manner acquired about 10^3 rhizobial cells per seed, according to Orellana and Fan (1978). BBSV inoculum was mechanically inoculated 15 days after sowing, and then covered with Agril net. For sampling, five replicates of plants in each treatment were uprooted at the age of 8 weeks, and the number and fresh weight of nodules were determined. Nodule function from the standpoint of leghaemoglobin (LH) content was also studied on nodule samples collected from the same plants. Broad bean LH was determined, according to Orellana et al. (1978), as

total cyanmethemoglobin (CMH) in milligrams per gram-nodule (fresh weight). This method is based on the oxidation of LH to CMH with potassium cyanide (KCN). Actual broad bean CMH concentration was determined by measuring the optical density at 540 nm and comparing it with that of a standard CMH curve. On the other hand, seed yields were estimated at the end of experiment. Tukey test for multiple comparisons among means was utilised according to Neler et al. (1985).

Detection of virus in nodules and its effect on bacteroidal cells

Random nodule samples were collected from BBSV-infected and healthy broad bean plants from the previous experiment. The presence of virus in these nodules was assessed by DAS-ELISA, also, the effects of BBSV infection on ultrastructure of nodule cells in broad bean root nodules were studied according to Tu (1977) and Gomaa (2006).

Detection of BBSV in broad bean seeds

All seeds tested in this study were harvested from field-infected and non-infected plants from the previous experiment.

Relationship between detection of BBSV in seed and production of diseased plantlets

Forty-five seeds (showed stained coats) from infected plants and the same number from healthy plants (showed unstained coats) were collected for tested in this experiment. Seeds were soaked in sterile distilled water for 1 h. Fragment (approximately 100 mg) was cut from the basal end of the cotyledon of each seed. The fragment was ground separately in 0.2 M potassium phosphate buffer saline containing 0.05% Tween-20, pH 6.0 (PBS-T), (1/10, w/v) and the homogenate was decanted, then tested by DAS-ELISA. The remains of each labelled seed were germinated in moistened sterile sand and the appearance of symptoms on seedlings was monitored daily for 4 weeks. The extract from a single leaf at constant position of each plant was tested by DAS-ELISA for the presence of BBSV. Another 90 seeds from infected and healthy plants were used as intact seeds for combination.

Virus location in broad bean seed

Stained and normal-looking (unstained) seeds were selected from infected and healthy plants, respectively. The seeds were germinated in moistened sterile sand at 25°C. Five days after germination, the seeds were dissected into coats, cotyledons and developing embryo axis. The embryo axis (about 10 mg), cotyledon (300 mg) and coat (10 mg) of each seed were ground in PBS-T (w/v of 1/30, 1/10 and 1/30, respectively). After low speed centrifugation (3.000 rpm), the supernatant of each part from each seed was tested separately for the presence of the BBSV by DAS-ELISA. Another set of infected and healthy seeds were used as intact seeds for combination.

Detection of BBSV in pooled seed samples

Ten, 50, 100, 200, 400 and 800 seeds from known virus-free plants and one seed from infected plant were blended, resulting in six seed groups. Five replicates of each

group were ground in PBS-T (phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20) (w/v of 1/10), using commercial food blender. The supernatant of each seed group was tested by DAS-ELISA.

Results

Virus isolation and identification

Host range

The data in Table 1 show that 14 out of 27 plants belonging to five families were varied in their response to infect with virus isolate. The data of infection by mechanical inoculation indicated that the *Vicia faba* cultivars, *Pisum sativum, Trifolium hybridum, Lupinus termis, Vigna unguiculata* cv. Blackeye and *Nicotiana debneyi* give systemic infection, i.e. mottle, diffuse mottle, vein banding and stunting (Figure 1A, B, G, and H). On the other hand, *Lens culinaris, Cicer arietinum* and *Glycine max* plants were infected without producing visible symptoms (symptomless infection), while *P. vulgaris* cv. Lima, *Chenopodium quinoa* and *C. murale* show necrotic local lesions (Figure 1C, D, and F), but *Chenopodium album* shows chlorotic local lesions (Figure 1E). *Medicago sativa, P. vulgaris* cv. Pinto, *Nicotiana glutinosa, N. tabacum* var showed no symptoms. White burley, *N. clevelandii, Datura stramonium, D. metel, Petunia hybrida, Lycopersicon esculentum* cv. Pito 86, *Beta vulgaris* cv. Ras poly, *Chenopodium amaranticolor, Cucurbita pepo* cv. Eskandarane and *Zea mayz* cv. Giza 160 after 30 days from virus mechanical inoculation. The infection was checked by DAS-ELISA.

Virus purification

Virus pellets were resuspended in 0.05 M citrate buffer, pH 6.5. The UV absorbance ratio $A_{260/280}$ of the purified preparation ranged from 1.25 to 1.34 (Figure 2). The yield of purified virus obtained was 0.61–0.63 mg/kg infected broad bean leaves.

Negative staining

The electron micrograph of BBSV-purified preparation showed isometric particles, 27 nm in diameter and occurring either singly or in aggregates (Figure 3).

Production of BBSV-antiserum

The polyclonal antiserum against BBSV isolate was produced and its titre was determined by indirect DAS-ELISA. The data presented in Table 2 show that when antisera of three bleedings were used for coating the ELISA plates, positive and high A_{405} values were obtained for all bleedings (range 0.27–1.35) up to dilutions of 1/100, 1/200 and 1/400 for the first, second and third bleedings, respectively. It was observed that third bleeding gave the highest ELISA values followed by the second and first bleedings. On the other hand, low values (<0.15) were obtained for healthy tissues.

Effect of virus infection on nodulation and broad bean yields

The data revealed that either rhizobial inoculation or viral infection significantly affected nodulation (Table 3). The number and fresh weight of nodules on

Table 1. Reaction of the differential hosts to mechanical inoculation with BBSV-isolate.

Test plant	Symptom	DAS-ELISA reaction
Family: Leguminaceae		
Vicia faba		
cv. Giza 402	Mo	+
cv. Giza planka	mMo	+
Pisum sativum	dMo	+
Lens culinaris	–	+
Cicer arietinum	–	+
Glycine max	–	+
Medicago sativa	–	–
Trifolium hybridum	Mo, Vb	+
Phaseolus vulgaris		
cv. Lima	NLL	+
cv. Pinto	–	–
Lupinus termis	Mo, Vb, Y, St	+
Vigna unguiculata cv. Blackeye	mMo	+
Family: Solanaceae		
Nicotiana glutinosa	–	–
N. tabacum var. White burley	–	–
N. clevelandii	–	–
Nicotiana debneyi	mMo, Y	+
Datura stramonium	–	–
D. metel	–	–
Petunia hybrida	–	–
Lycopersicon esculentm cv. Peto 86	–	–
Family: Chenopodiaceae		
Beta vulgaris cv. Ras poly	–	–
Chenopodium amaranticolor	–	–
C. murale	NLL	+
C. album	CLL	+
C. quinoa	NLL	+
Family: Cucurbitaceae		
Cucurbita pepo cv. Eskandarane	–	–
Family: Graminaceae		
Zea mayz cv. Giza 160	–	–

Mo, mottle; mMo, mild mottle; dMo, diffuse mottle; Vb, vein banding; Y, yellows; St, stunting; NLL, necrotic local lesions; CLL, chlorotic local lesions.

BBSV-infected plants were less than the healthy ones. Regarding viral infection, the data showed that even without rhizobial inoculation, the BBSV-uninfected plants outstripped the infected plants for the number of nodules, fresh weight of nodules and seed yields. This difference was not significant except for fresh weight. Similarly, inoculation with rhizobia without viral infection increased the nodulation and plant yield. On the other hand, DAS-ELISA results indicated the presence of BBSV in nodules obtained from BBSV-infected plants. Mean LH content determined as CMH in milligrams per gram-nodule fresh weight is shown in Figure 4. LH content was reduced from 11.65 in virus-free nodules to 7.41 in BBSV-infected ones taken from rhizobial inoculated plants and from 11.46 to 9.35 taken from non-rhizobial inoculated plants.

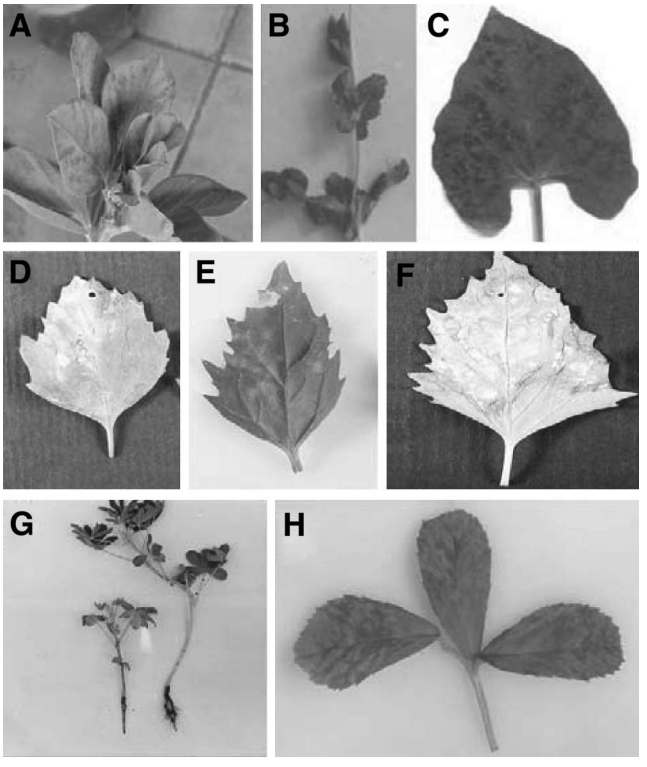

Figure 1. Symptoms induced in test plants by BBSV inoculation. (A, B, G and H), systemic infection on *Vicia faba*, *Pisum sativum*, *Lupinus termis* and *Trifolium hybridum* leaves, respectively. (C, D, E and F), local infection on *Phaseolus vulgaris*, *Chenopodium quinoa*, *C. album* and *C. murale* leaves, respectively.

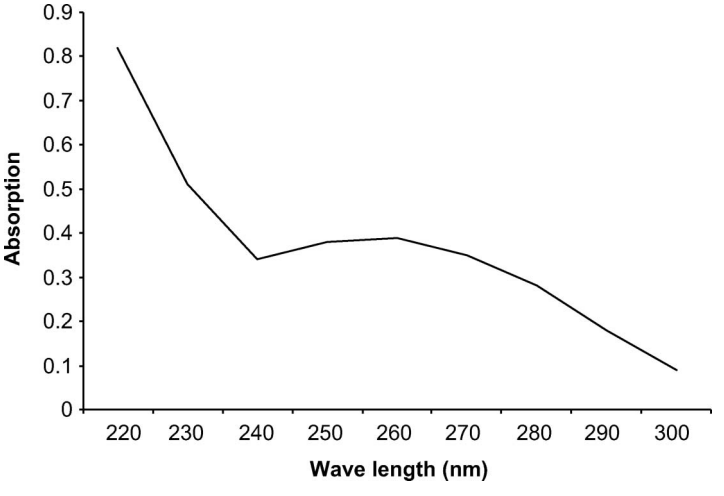

Figure 2. Ultraviolet-absorption spectrum of BBSV purified preparation.

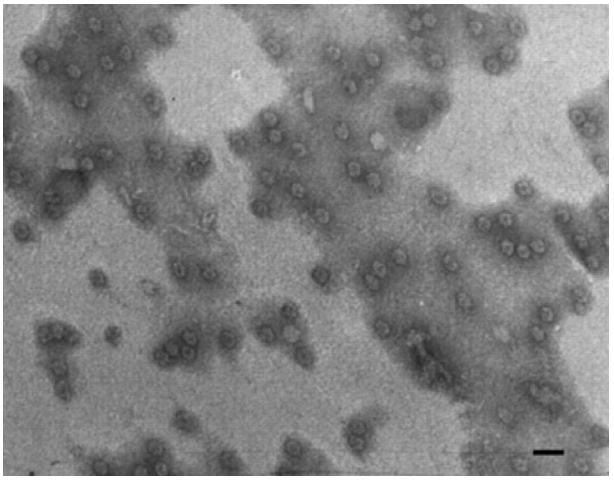

Figure 3. Electron micrograph of purified-BBSV stained with 2% aqueous uranyl acetate. (Bar = 60 nm).

Table 2. Determination of BBSV antiserum titre by indirect DAS-ELISA.

Antiserum dilutions	Normal serum		Indirect DAS-ELISA detection in three bleedings					
			First		Second		Third	
	EV	R	EV	R	EV	R	EV	R
1 10	0.065±0.02	−	0.759±0.01	+	0.947±0.04	+	1.358±0.02	+
1:20	0.071±0.01	−	0.528±0.02	+	0.715±0.01	+	0.984±0.02	+
1:50	0.068±0.03	−	0.441±0.01	+	0.584±0.03	+	0.856±0.01	+
1:100	0.062±0.03	−	0.318±0.01	+	0.334±0.02	+	0.643±0.01	+
1:200	0.073±0.01	−	0.202±0.02	−	0.275±0.021	+	0.550±0.01	+
1:300	0.075±0.03	−	0.154±0.02	−	0.182±0.02	−	0.325±0.01	+
1:400	0.066±0.01	−	0.073±0.02	−	0.084±0.15	−	0.301±0.01	+
1:500	0.068±0.01	−	0.077±0.02	−	0.072±0.01	−	0.182±0.02	−
[a]Negative control 10^{-1}	0.115±0.08	−	0.142±0.01	−	0.138±0.02	−	0.146±0.02	−
[b]Positive control 10^{-1}	0.128±0.03	−	0.649±0.02	+	0.958±0.02	+	1.367±0.03	+

EV, ELISA value at 405 nm (average of three replicates) after incubation for 45 min at 30°C; R, result.
[a]Healthy broad bean tissues.
[b]BBSV-infected broad bean tissues.
The ELISA values that equal two-folds of healthy tissues were considered as a positive (+) result.

Detection of BBSV in nodules and its effect on the ultrastructure of nodule cells

BBSV was successfully detected within broad bean nodules by the means of DAS-ELISA. Sections were made in the early nodulation stage. In this stage, each bacterium in the host cytoplasm is enclosed in a membrane envelope (ME). At early stage, the differences between BBSV-infected and healthy bacteroidal cells are most noticeable. Figure 5 revealed greater numbers of bacteroids in healthy nodule cells than in BBSV-infected cells. The next important difference is the greater space

Table 3. Effect of BBSV-infection on nodulation and seed yield of rhizobial-inoculated or uninoculated broad bean plants.

Treatments		Nodulation			Broad bean yield	
Rhizobium inoculation	Viral infection	No/ plant	Fresh weight (g)/plant	Virus infection	Seed yield (g)/plant	100 seed wt (g)
Inoculated	Infected	18.4a*	1.01b	+	71.6a	58.6a
	Uninfected	23.7b	3.27c	−	103.4b	75.4a
Uninoculated	Infected	15.6a	0.75a	+	59.9a	43.7a
	Uninfected	19.1a	1.44c	−	76.1a	58.4a

*Values with same letter(s) in the same column are not significantly different at 5% level using Duncan's multiple range test.
+ virus detected; − virus not detected.

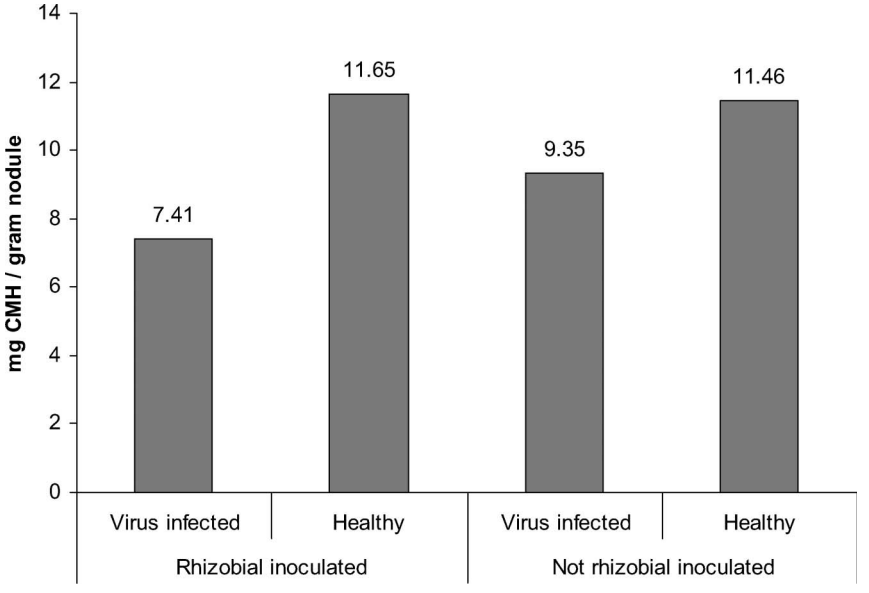

Figure 4. Leghemoglobin (LH) concentration in nodules taken from BBSV-infected and virus-free broad bean plants measured as CMH.

between the bacteroidal cells and ME in healthy cells than in BBSV-infected cells (compare Figure 5C with 5D). Also, ME is deteriorated in virus-infected cells (Figure 5D). The presence of BBSV particles in the BBSV-infected bacteroidal cell is readily discernible.

Detection of BBSV in broad bean seeds

Detection of BBSV in whole seed

The comparison between the percentages of seed transmission estimated by DAS-ELISA on whole seed (Table 4) and by symptoms on the produced plantlets, gave a

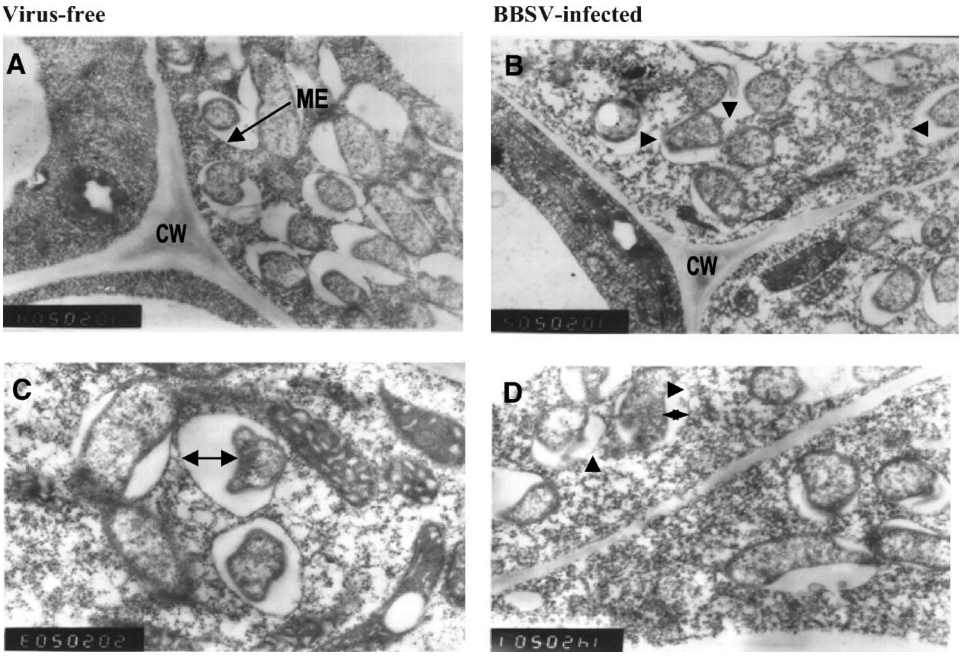

Figure 5. A portion of nodule cells observed in virus-free root nodules (A and C) compared with BBSV-infected ones (B and D). (A) A portion of nodule cell having high density of bacteroids with normal ME, each bacteroid is embedded in ME (arrow) and tends to aggregate beside cell wall (CW). (B) Bacteroids are decreased in number and ME is deteriorated (arrowheads). (C) A close-up view of a portion of a nodule cell showing bacteroids in a ME. It shows a clear-cut ME containing bacteroidal cell with wide space between the bacteroid and ME (double arrows). (D) The ME of bacteroids are lacking (arrowhead) and the space between the bacteria and ME is very narrow, while most of the remaining space is occupied by cellular organelles (arrowhead).

Table 4. Testing BBSV-seed transmission by symptom development and ELISA.

Seed sample	No. of tested seeds	BBSV-detection by			
		Symptoms[a] on plants (%)	ELISA[a] on plants (%)	ELISA[b] on cotyledons (%)	ELISA[c] on intact seeds (%)
Stained	45	12.5	17.5	15.6	15.5
Unstained	45	0.0	0.0	0.0	0.0

[a]Plants originated from seeds where a fragments of a cotyledon have been removed.
[b]DAS-ELISA test was performed on extracts of cotyledon fragments.
[c]DAS-ELISA test was performed on extracts of intact seeds (other seeds).

very close correlation. When the same comparison was made using the same individual seeds for both estimates (a fragment of cotyledons were tested by DAS-ELISA and a plantlet derived by germinating the rest of the seed), it was similar (Table 4). Approximately each seed which tested positive in DAS-ELISA developed a diseased plant (Figure 6B).

Virus location in broad bean seed

BBSV was reliably detected in infected whole ground broad bean seeds using DAS-ELISA (Table 5). In one seed sample, BBSV was detected in the cotyledons and developing axis of the embryo. BBSV could not be detected in seed coat tissue of either stained or unstained seeds. The number of cotyledons which tested positive was approximately equal to the number of whole seeds in separate experiment which tested positive (Table 5).

Detection of BBSV in pooled seed samples

BBSV was reliably detected in infected whole ground broad bean seeds using DAS-ELISA (Table 6). When mixtures of infected and healthy seed in different ratios were tested, BBSV was detected in mixtures up to 1:100 (infected:healthy) (Table 6).

Discussion

An attempt to identify the BBSV isolate from Sohag Governorate, Egypt was carried out by simple and rapid methods. These methods are based on host range, serological reactions, mechanical and seed transmission, particle size and

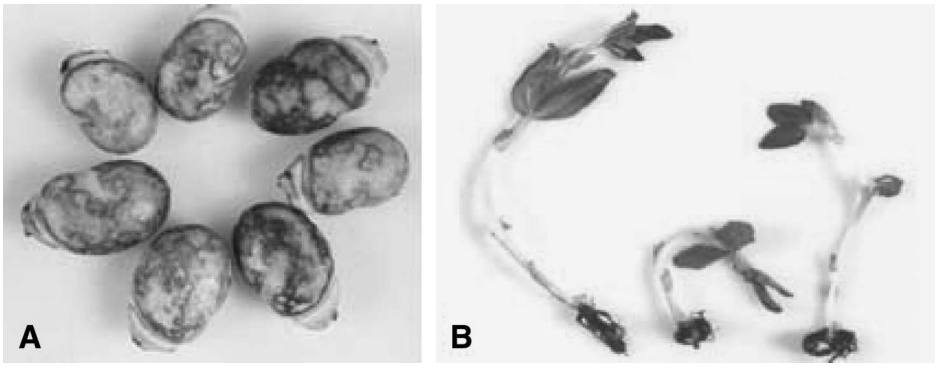

Figure 6. Symptoms induced by BBSV. (A) Stained broad bean seeds harvested from mechanically inoculated plants 'BBSV detected by ELISA in cotyledon parts' (B) Size reduction and mosaic of seedlings resulted from the same BBSV-infected seeds compared with control one on the left.

Table 5. Detection of BBSV in seed coats, cotyledons and embryo axis of broad bean seed samples.

Seed sample	No. of tested seeds	No. of seeds giving positive results			Intact[a] seed
		Seed coat	Cotyledon	Embryo axis	
Stained	15	0	9	4	11
Unstained[b]	15	0	1	1	2

[a]No. of seeds that give positive results in another 15 seeds.
[b]Normal seeds taken from uninoculated plants.

Table 6. Detectability of BBSV in pooled broad bean seeds.

Ratio[a] of infected to healthy seeds	1:0	1:10	1:50	1:100	1:200	1:400	1:800
Seed group size	1	11	51	101	201	401	801
No. of groups giving positive ELISA	5	5	5	5	0	0	0

[a]No. of infected to healthy seeds, five groups per seed group size were tested.

morphology. The present data indicated that the viral isolate has a narrow host range. Also, its particle size and morphology suggested a close resemblance to the BBSV described by Gibbs et al. (1968), Omar et al. (1990), Fiedorow et al. (2002) and Salama et al. (2003). Serologically, the virus was proved to be BBSV by using a BBSV ELISA kit. ELISA proved to be a very sensitive method for detecting BBSV in ground seeds and germinating embryos. Similar results were indicated by El-Dougdoug et al. (1999). Although no significant interaction effects were detected between rhizobial inoculation and viral infection on nodulation, the results indicated that rhizobial inoculation would enhance nodulation. These findings are confirmed by those reported by Hammouda et al. (1990). Rhizobial root nodules of broad bean appear to be affected by BBSV. It produces smaller, fewer and less effective root nodules than healthy plants. It also showed that the virus is usually present in root nodule cells. This result is in agreement with Tu (1973, 1977) and Gomaa et al. (2006). The present data indicated the presence of less number of bacteroid in nodule cells or they were deteriorated in some cases. On the other hand, the micrographs indicated the presence of very narrow space between the bacteroid and the ME in the BBSV-infected nodules compared with healthy nodules. This may be significant in light of the results of Tu (1977), which indicate that LH is located in the space between the symbiotic bacteria and the ME in the bacteroidal cells. The data indicated the reduction in LH content in BBSV-infected nodules. LH, the haeme protein complex, is involved to function in the symbiotic fixation process by regulation of O_2 for the respiration of the nitrogenase-containing *Rhizobium* bacteroids in the nodule (Burns and Hardy 1975). Since the ME is known to be involved in N_2-fixation and BBSV affects N_2-fixation, it is conceivable that the structure of the ME is altered by BBSV. The present data indicated that every infected seed (when embryo and cotyledon tissues were tested) capable of producing a diseased plant gave a positive ELISA value. Similar correlations were reported for lettuce and soybean seeds infected with lettuce mosaic virus and soybean mosaic virus according to Falk and Purcifull (1983) and Maury et al. (1985), respectively. Hence, the present study indicated that in the absence of ELISA facilities, grow out test is sufficient to determine the extent of infection in seed lot. The fact that in some broad bean seeds BBSV was detected in the cotyledons but not in the developing axis of the embryo indicated that testing germinated embryos could give more precise value of the actual seed transmission rate of BBSV. Other seed-transmissible viruses behave differently, for example, Sweet and Barbara (1979) reported that the high concentration of apple mosaic virus (ApMV) in embryos of chestnut seeds was not correlated with the development of diseased plantlets. In the case of BBSV, seed transmission is very efficient which may help to explain why it plays a major part in the epidemiology of the virus, since no reservoir–host of the virus has been identified among wild plant species as recorded by Makkouk et al. (1987). Transmission of BBSV through broad bean seeds is important in the introduction and epidemiology

of the virus; hence there is a need for its detection in seed lots which are meant for sowing. The percentage of cv. Giza 402 seeds which ELISA tested positive using extracts from 1, 11, 51 and 101 seeds was similar but not for seed lots larger than 100 seeds.

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