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Broad bean stain virus: identification, detectability with ELISA in faba bean leaves and seeds, occurrence in West Asia and North Africa, and possible wild hosts*

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Abstract

During a survey of faba bean viruses in West Asia and North Africa a virus was identified as broad bean stain virus (BBSV) based on host reactions, electron microscopy, physical properties and serology. An antiserum to a Syrian isolate was prepared. With this antiserum the direct double antibody sandwich ELISA (DAS-ELISA) and dot-ELISA were very sensitive in detecting BBSV in leaf extracts, ground whole seeds and germinated embryos. Sensitivity was not reduced when the two-day procedure was replaced by a one-day procedure. Using ELISA the virus was detected in 73 out of 589 faba bean samples with virus-like symptoms collected from Egypt (4 out of 70 samples tested), Lebanon (6/44), Morocco (0/7), Sudan (19/254), Syria (36/145) and Tunisia (8/69). This is the first report of BBSV infection of faba bean in Lebanon, Sudan, Syria and Tunisia. Fourteen wild legume species indigenous to Syria were susceptible to BBSV infection, with only two producing obvious symptoms. The virus was found to be seed transmitted in *Vicia palaestina*.

Introduction

Worldwide, faba bean (*Vicia faba* L.) is known to be naturally infected by some 44 viruses (Schmidt et al., 1980; Bos, 1982; Cockbain, 1983; Bos et al., 1987). In West Asia and North Africa the crop is infected by a number of these viruses (Kaiser et al., 1968; Fischer and Lockhart, 1976; Allam et al., 1979; Makkouk et al., 1982). Broad bean stain virus (BBSV), a beetle- and seed-transmissible virus, has been reported to infect faba bean plants and cause seed staining in Europe (Lloyd et al., 1965; Tapio, 1970; Schmidt et al., 1977; Rohloff, 1980; Musil, 1981), Australia (Moghal and Francki, 1974), Asia (Xu Zhiang et al., 1985) and North Africa (Fisher and Lockhart, 1976; Allam et al., 1979; Tolba, 1980).

During a survey of faba bean viruses in the region served by ICARDA (West Asia and North Africa) we found isolates resembling BBSV. After their indentification as

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BBSV an antiserum was produced and was tested for virus detectability in seeds and leaves by ELISA. It was thereafter used to study the occurrence of the virus in several countries of the ICARDA region. Information on the occurrence of BBSV on faba bean in the region is scarce but faba been seeds often show staining reminiscent of infection by the virus. Some wild plants were tested for their susceptibility to the virus to identify possible natural sources of infection other than faba bean.

Materials and methods

Field collections and virus isolates. Samples of faba beans showing virus-like symptoms were collected from farmers' fields and experimental plots of agricultural research stations in Egypt, Lebanon, Morocco, Sudan, Syria and Tunisia during 1985 and 1986. Samples were brought to the laboratory in Tel Hadya, Aleppo, Syria, and each sample was split into two portions. One was desiccated over calcium chloride and the other was extracted in 0.2 M phosphate buffer, pH 6.0, using a motorized tissue extractor. Leaf extracts and desiccated tissue were stored at −20 °C until further testing in Aleppo, Beirut or Wageningen. A faba bean seed lot (cv. Syrian Local) which contained seeds with characteristic seed-coat staining was used for seed testing. Two representative virus isolates, i.e. one from Syria (SV173-85) and one from Tunisia (TV73-85), were identified, and the Syrian isolate was purified for antiserum production.

Host range. The Syrian and/or Tunisian virus isolates were inoculated to *Chenopodium quinoa, Cicer arietinum* (cvs ILC 263, ILC 482, ILC 3279), *Gomphrena globosa, Lens culinaris* (cvs Syrian Local, ILL 4400, ILL 4401, ILL 4605, ILL 5588), *Pisum sativum* (cvs Koroza, Rondo, and/or Syrian Local), and *Phaseolus vulgaris* (cvs Bataaf, Black Turtle, Monroe and Sutter Pink), *Trifolium subterraneum, Vicia sativa, V. ervilia, Vigna unguiculata* (cv. California Blackeye No. 5), and *Vicia faba* (cvs Kompakta and Syrian Local). Forty-four wild legume species were collected from Syria, mechanically inoculated with the Syrian isolate and maintained in the glasshouse where temperature ranged from 25 to 35 °C. Symptomless infection in non-inoculated leaves was detected by ELISA.

Virus purification. The Syrian isolate was propagated in faba bean cv. Syrian Local. Leaves from infected plants showing mild mottle were harvested 5 weeks after inoculation. The purification procedure followed was similar to the one used to purify a potyvirus from *P. vulgaris* (Azzam and Makkouk, 1986), but Triton X-100 was not added for tissue homogenization and 6% polyethylene glycol plus 0.3 M NaCl were used instead of high-speed centrifugation to concentrate the virus.

Serology and antiserum production. A BBSV antiserum prepared to a local isolate was provided by Dr A.A. Brunt of the Glasshouse Crops Research Institute, Littlehampton, England. The antiserum against the purified isolate from Syria (SV173-85) was prepared by giving a rabbit four weekly intramuscular injections containing 1-2 mg virus. The virus preparation for the first injection was emulsified with an equal volume of Freund's complete adjuvant, and for subsequent injections Freund's incomplete adjuvant was used. A booster injection was given eight weeks after the fourth injection.

The rabbit was bled 12 times, at weekly intervals, starting one week after the fourth injection.

Electron microscopy. Small leaf samples, either fresh or desiccated over calcium chloride, were chopped in sodium phosphotungstate (PTA, pH 6.5) for negative staining and viewing in the electron microscope.

Virus detection. The ELISA procedure was used to detect the virus in faba bean leaves and seeds. Gammaglobulins were fractionated from the antiserum prepared against the Syrian isolate by the caprylic acid method (Steinbuch and Audran, 1969) which was compared with the DE22 cellulose method (Clark and Adams, 1977). The direct double antibody sandwich ELISA (DAS-ELISA) was compared with the dot-ELISA described by Banttari and Goodwin (1985), where samples are placed on a nitrocellulose membrane. The plastic templates used for dot-ELISA were 10 mm thick and the sample holes were 5 mm in diameter. The NC membranes used were from BIO-RAD Laboratories, Richmond, California (No. 152-0116). For both methods samples were extracted in 0.2 M phosphate buffer, pH 6.0.

Samples from seed lots containing stained seeds were used for virus detection in seeds. Stained and normal-looking (unstained) seeds were selected from such lots for further testing. The seeds were either ground to a fine powder or germinated in moistened sterile sand and placed in a seed germinator where the temperature was maintained around 24 °C. Seven days after germination, seeds were dissected into coat, cotyledons and developing axis, and each was tested separately for the presence of the virus by both DAS- and dot-ELISA. Mixtures of infected and healthy seeds in different ratios were also tested for BBSV by DAS-ELISA.

Results

Host range. In inoculation tests, the isolates SV173-85 from Syria and TV73-85 from Tunisia infected systemically all the cultivars tested of chickpea, faba bean, lentil, pea, vetches (*V. sativa* and *V. ervilia*) and subterranean clover. Except for faba bean (mild mottle) and pea (diffuse mottle), all other infections were symptomless. Latent infections in non-inoculated leaves were detected by ELISA. In *P. vulgaris* cv. Bataaf SV173-85 produced small necrotic local lesions, usually only visible after the inoculated leaves turned yellow. Sometimes the isolate was recovered from non-inoculated leaves. In cv. Monroe TV73-85 caused chlorotic local lesions which developed into necrotic rings when the leaves turned yellow; the virus could not be recovered from non-inoculated leaves of such plants. Among the 44 wild leguminous species tested, the reactions obtained could be grouped into three categories:
(i) 30 species were not susceptible to BBSV infection:
Astragalus hamosus L., *Biserrula pelecinus* L., *Coronilla scorpioides* (L.) Koch., *Hymenocarpus circinnatus* (L.) Savi, *Medicago blancheana* var. *blancheana* Boiss., *M. disciformis* D.C., *M. minima*, (L.) Bart., *M. polymorpha* var. *polymorpha* L., *M. rigidula* vars. *agrestis* Burmat., *cinerascens* (Jord.) Rouy and *submitis* (Boiss.) Heyn, *M. rotata* Boiss., *M. scutellata* (L.) Miller, *M. turbinata* var. *spinulosa* (L.) All., *Onobrychis crista-galli* (L.) Lam., *O. aequidentata* (Sibth & Sm.) d'Urv., *Ornithopus compressus* L., *Scorpiurus muricatus* L., *Securigera securidaca* (L.) Degen & Dorf.,

Trifolium arvense L., *T. batmanicum* Katzn., *T. cherleri* L., *T. glanduliferum* Boiss., *T. hirtum* All., *T. pauciflorum* d'Urv., *T. pilulare* Boiss., *T. scutatum* Boiss., *T. stellatum* L.
(ii) 12 species were infected without producing obvious visible symptoms: *Medicago constricta* Dur., *M. polymorpha* var. *brevispina* L., *Trifolium aintabense* Boiss. & Hausskn., *T. bullatum* Boiss. & Hausskn., *T. lappaceum* L., *T. nigrescens* Viv., *T. resupinatum* L., *T. subterraneum* L., *T. tomentosum* L., *Trigonella arabica* Del., *Vicia palaestina* Boiss., *V. dasycarpa* Ten. In tentative tests on seed transmission in these species the virus was detected with ELISA in seedlings of *V. palaestina*.
(iii) Two species were found infected and had obvious (mosaic) symptons: *Medicago arabica* (L.) Huds. and *Trifolium spumosum*. L.

Electron microscopy. Isometric virus particles, c. 25 nm in diameter and occurring either singly or in aggregates (Fig. 1), were easily detected in field samples desiccated over calcium chloride and in plants inoculated and maintained in the glasshouse.

Virus purification and antiserum production. After sucrose density-gradient centrifugation, one very faint upper and two distinct lower bands were obtained. The two lower bands were collected, subjected to a second gradient centrifugation (Fig. 2), and the virus was then recovered by high-speed centrifugation. Virus pellets were resuspended in 0.05 M citrate buffer, pH 7.5. The UV absorbance ratio A_{260}:A_{280} of

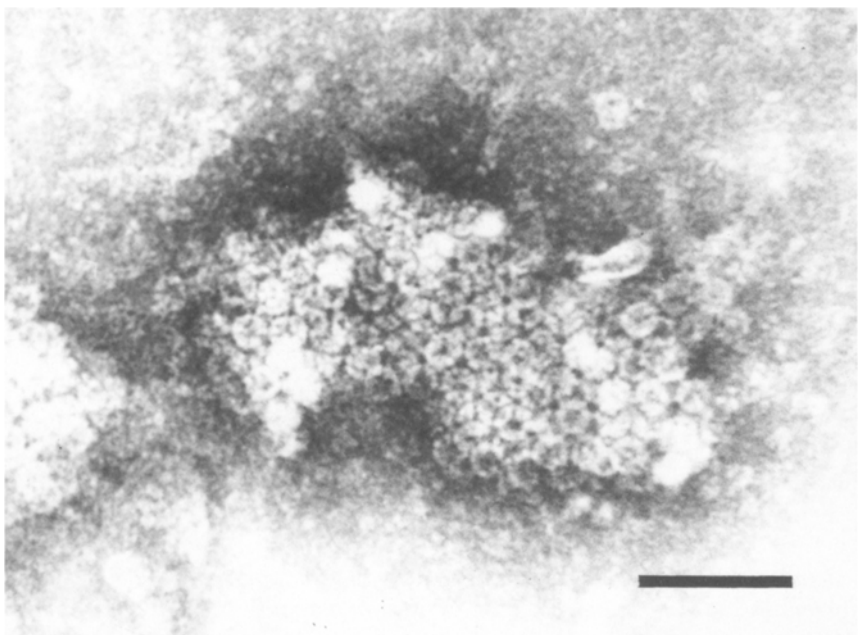

Fig. 1. Electron micrograph of an aggregate of broad bean stain virus particles in crude sap preparation of faba bean inoculated with the Tunisian isolate TV73-85 after staining with PTA. Bar represents 100 nm.

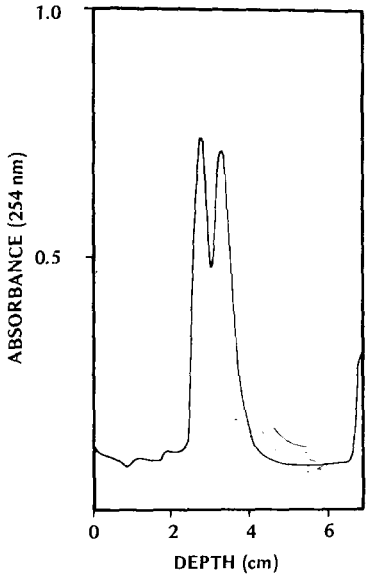

Fig. 2. Rate-zonal sucrose density gradient centrifugation analysis of purified broad bean stain virus. Virus previously fractionated on sucrose gradient was centrifuged for 2 h at 23 000 rpm in a 10-40% sucrose gradient in a Sorvall AH 627 rotor.

the purified preperation was 1.64 (average of three preparations). The yield of purified virus obtained was 1-2 mg kg^{-1} of faba bean leaves. When gammaglobulins (1 μg ml^{-1}), fractionated from antisera of different bleedings, were used for coating the ELISA plates, high A_{405} values were obtained for all bleedings (range 1.0-2.0) using a 1:10 dilution of BBSV-infected tissue. Low values (< 0.1) were obtained for healthy tissue. The gammaglobulins isolated by the caprylic acid method were used for preparing the conjugate. These gammaglobulins were of the same quality as those fractioned by the DE22 cellulose method, although the amount recovered was less. However, since the caprylic acid procedure is simpler and less time consuming, it was adopted for the isolation of BBSV gammaglobulins.

Virus detection in leaves. The sensitivity of ELISA in detecting BBSV in faba bean leaf extracts was high and the virus was detected in infected extracts at dilutions up to 1:16 000. Detectability was the same using either DAS-ELISA or dot-ELISA, but visual assessment in dot-ELISA was easier than in DAS-ELISA. Dots for sap dilutions of 1:16 000 were visible on the nitrocellulose as compared to those of healthy extract, whereas the corresponding wells in DAS-ELISA were not clearly visible (A_{405} = 0.05-0.07). When reaction times in both ELISA systems were reduced from two days to one day, the sensitivity of the test was not reduced (Table 1).

Virus detection in seeds. BBSV was reliably detected in infected whole ground faba bean seeds using ELISA. When mixtures of infected and healthy seeds in different ratios were tested, BBSV was detected in mixtures of up to 1:400 (Table 2).

In one seed sample (Table 3) BBSV was detected only in the cotyledons but not in the developing axis of the embryo. This was also the case when single seeds were tested. However, the percentage of seeds which contained detectable virus in the cotyledons and not in the axis of the embryo was variable.

Table 1. Detection of broad bean stain virus (BBSV) by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) on polystyrene plates and on nitrocellulose membranes (dot-ELISA) using one-day and two-day procedures.

Sap dilution[a]	DAS-ELISA[b] (A_{405} values)		dot-ELISA[b] (visual)	
	two-day procedure	one-day[c] procedure	two-day procedure	one-day[c] procedure
1:20	1.22	0.56	+	+
1:100	nt[d]	0.31	+	+
1:1000	0.67	0.26	+	+
1:4000	0.23	0.15	+	+
1:8000	0.10	0.10	+	+
1:16000	0.07	0.05	+	+
Healthy	0.02	0.01	−	−

[a] Dilutions were volumes of sap from infected leaves over volumes of sap from healthy leaves. Extraction buffer was 0.2 M phosphate, pH 6.0, and 1 g of tissue was extracted in 10 ml of buffer.
[b] Polystyrene plates and nitrocellulose membranes were coated with 1 μg ml^{-1} gammaglobulins and the enzyme-conjugate dilution used was 1:1000.
[c] Gammaglobulin coating was for 1 h, sample addition for 3 h at room temperature and enzyme conjugate for 1 h. Substrate incubation time was 30 min for the one-day procedure and 1 h for the two-day procedure.
[d] Not tested.

Table 2. Detectability of broad bean stain virus (BBSV) in mixtures of infected and healthy faba bean seeds using the enzyme-linked immunosorbent assay[a,b].

Ratio of infected to healthy seeds	A_{405}
1:10	1.55
1:50	1.10
1:100	0.68
1:200	0.42
1:400	0.27
1:800	0.17
1:1600	0.11
Healthy seed	0.05

[a] Extraction buffer used was 0.2 M potassium phosphate, pH 6.0.
[b] Plates were coated with 1 μg ml^{-1} gammaglobulins; enzyme-conjugate dilution used was 1:1000 and substrate incubation was for 1 h.

Table 3. Detection of broad bean stain virus (BBSV) in seed coats, cotyledons and embryo axes of stained and unstained (normal) seed samples (25 seeds each) when tested by DAS- and dot-ELISA.

Seed sample	Number of seed samples tested	Number of seed groups in which BBSV was detected					
		embryo axes		cotyledons		seed coat	
		DAS-ELISA	dot-ELISA	DAS-ELISA	dot-ELISA	DAS-ELISA	dot-ELISA
Stained	9	5	5	6	6	0	0
Unstained	9	1	1	1	1	0	0

When seed components were extracted by grinding in a Waring blender with extraction buffer for 30 sec, no virus was detected in seed coats of either stained or unstained seeds (Table 3). This was true even when trituration was prolonged to 1 min.

Virus survey. When 589 faba bean samples with virus-like symptons, collected in Egypt, Lebanon, Morocco, Sudan, Syria and Tunisia, were tested for the presence of BBSV by ELISA, 73 samples were found to be infected. BBSV was detected in 5.7, 13.6, 0.0, 8.6, 24.8 and 11.5% of the samples tested from each country, respectively. The figures were 4/70 (4 out of 70 samples tested) for Egypt, 6/44 for Lebanon, 0/7 for Morocco, 19/254 for Sudan, 36/145 for Syria and 8/69 for Tunisia. The number of samples tested from Lebanon and Morocco was small and further testing is therefore required for more reliable information on the relative occurrence of BBSV in these countries. Results reported here show that BBSV naturally infects faba bean in Egypt, Lebanon, Sudan, Syria and Tunisia.

Discussion

The rather narrow host range and the symptoms of the virus isolated in this study, its particle size and morphology and the physical properties revealed by ultracentrifugation readily suggested a close resemblance to the BBSV described by Lloyd et al. (1965) and Gibbs et al. (1968). Its identity was proved by using BBSV antiserum provided by A.A. Brunt.

The purification procedure yielded a fairly pure virus preparation as indicated by well-separated virus bands in sucrose gradients. The A_{260}:A_{280} ratio of the combined lower bands was 1.64 which is very close to the value reported for other comoviruses (Semancik, 1972; Van Kammen and De Jager, 1978). In addition, when the antiserum produced against the purified virus was used in ELISA, high A_{405} values (1.0-2.0) were obtained after 0.5-1 h of substrate incubation when testing BBSV-infected faba bean tissue and in low readings (< 0.10) when testing healthy tissue.

ELISA proved to be a very sensitive method for detecting BBSV in leaves, ground seeds and germinating embryos. Although dot-ELISA was as sensitive as DAS-ELISA, the visual assessment of the reactions in dot-ELISA was, in general, much

easier than in DAS-ELISA when samples contained low virus concentrations. This agrees with previous findings on TMV and PVX in tomato tissues (Parent et al., 1985). Thus dot-ELISA seems to be very useful for BBSV detection in laboratories where ELISA readers are not available.

Even though BBSV was detected in mixtures of infected and healthy seeds with a ratio of 1:400, but to avoid missing low virus concentrations in seed indexing, groups of 50 or 100 seeds could be tested as one sample by ELISA. The fact that in some faba bean seeds BBSV was detected in the cotyledons but not in the developing axis of the embryo indicates that testing germinated embryos could give a more precise value of the actual seed transmission rate of BBSV. Further testing is required to determine whether or not the virus in the cotyledons can eventually reach the growing seedling.

When using the prepared antiserum in ELISA, the virus was detected in 73 of 589 samples from 5 of 6 countries where samples were taken. This is the first report of BBSV infection of faba bean in Lebanon, Sudan, Syria and Tunisia, but it has been previously reported in Egypt and Morocco (Allam et al., 1979; Fischer and Lockhart, 1976).

Fourteen wild leguminous species naturally present in Syria were susceptible to BBSV infection, although the majority did not show obvious symptoms. Such wild species may therefore be important BBSV reservoirs especially if the virus is seed-borne in some of them, as already found for *Vicia palaestina*.

Samenvatting

Tuinbonezaadvlekkenvirus: identificatie, aantoonbaarheid met ELISA in bladeren en zaden van veldboon, voorkomen in het Midden-Oosten en Noord-Afrika en mogelijke wilde waardplanten

Tijdens een inventarisatie van virussen in veld- of tuinbonen (*Vicia faba*) in het Midden-Oosten en Noord-Afrika werden virusisolaten verkregen, die op grond van toetsplantreacties en fysische eigenschappen en van elektronenmicroscopische en serologische waarnemingen werden herkend als het met zaad overgaande en door snuitkevers verspreide tuinbonezaadvlekkenvirus (broad bean stain virus).

Met een nieuw antiserum, gemaakt tegen een Syrisch isolaat, bleek de aantoonbaarheid van het virus in bladextracten, vermalen zaden en gekiemde embryo's erg hoog te zijn, zowel bij toepassing van DAS-ELISA als dot-ELISA. De gevoeligheid van de methode werd niet geringer wanneer de procedure werd bekort van twee dagen tot één dag.

Met ELISA werd het virus aangetoond in 73 van de 589 bladmonsters van veldboon, met symptomen die deden denken aan virusinfectie, verzameld in Egypte, Libanon, Marokko, Soedan, Syrië en Tunesië. Het virus werd aangetroffen in monsters uit alle genoemde landen behalve Marokko, maar het aantal uit dat land afkomstige monsters was slechts gering. Dit is de eerste maal dat het virus wordt gerapporteerd in Libanon, Soedan, Syrië en Tunesië.

Veertien wilde soorten vlinderbloemigen uit Syrië bleken bij toetsing door inoculatie vatbaar voor infectie. Slechts twee ervan vertoonden symptomen. Het virus kan dus onzichtbaar voorkomen in de wilde vegetatie. In *Vicia palaestina* ging het zelfs over met zaad.

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