Aust. J. Biol. Sci., 1974, **27**, 341–8

Occurrence and Properties of Broad Bean Stain Virus in South Australia

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Abstract

A polyhedral virus was isolated from commercial broad bean seedlings in South Australia and identified as broad bean stain virus on the basis of host range, symptom expression, morphology and serology. The virus is shown to have a divided genome and the data available confirm that it should be included in the comovirus group.

Introduction

In an electron microscopic examination, preparations of pea mosaic virus purified from leaves of broad beans were found to contain small polyhedral particles in addition to the typical rod-shaped particles of pea mosaic virus. Investigations reported in this paper demonstrate that the polyhedral particles are those of broad bean stain (BBS) virus originating from infected broad bean seed. BBS virus appears to be present in commercially available broad bean seed in South Australia.

Materials and Methods

Virus Culture and Assays

BBS virus was separated from pea mosaic virus by passage through single lesions on *Phaseolus vulgaris* L. cv. Brown Beauty and was maintained by mechanical inoculation to broad bean seedlings (*Vicia faba* L. cv. Leviathan Long Pod) that had been tested serologically for freedom from BBS virus. Infectivity was assayed on the local lesion host, *P. vulgaris*.

Host Range Studies

Leaf sap from infected broad bean plants was inoculated mechanically to a range of plant species grown in an insect-proof glasshouse. The plants were observed for symptoms and after 3–4 weeks were indexed by mechanical sap inoculation to *P. vulgaris*.

Virus Purification

Systematically infected broad bean leaves from plants inoculated 3–4 weeks previously were harvested, cooled to 4°C and homogenized in a Waring blender with chloroform and 0·2M phosphate buffer, pH 7, containing 0·15% thioglycollic acid (1 g : 1 ml : 2 ml). The emulsion was broken by centrifugation at 10000 *g* for 15 min and the buffer phase was passed through a Whatman No. 4 filter paper. Virus was precipitated from the filtrate by addition of polyethylene glycol 6000 to a concentration of 6% (w/v) and NaCl to 0·3M. After 1 h at 4°C the precipitate was collected by centrifugation at 10000 *g* for 10 min and the virus was suspended in 0·2M phosphate buffer, pH 7, for 2 h. After clarification at 10000 *g* for 10 min, virus was sedimented by centrifugation at 78000 *g* for 90 min and the pellet was resuspended in 0·02M phosphate buffer, pH 7, containing 0·005M EDTA.

On the following day the differential centrifugation was repeated and the final virus pellet was suspended in 0·02M phosphate buffer, pH 7.

Sucrose Density-gradient Centrifugation

Virus preparations were examined in linear 5–25% sucrose gradients prepared in 0·02M phosphate buffer, pH 7, and centrifuged for 3·5 h at 24000 rev/min in an SW25.1 Spinco rotor. The gradients were fractionated with an ISCO model D density-gradient fractionator and recording flow densitometer assembly.

Estimation of Sedimentation Coefficient

The relative sedimentation rates of BBS virus components were determined by centrifugation in sucrose gradients. Suspensions of BBS virus and tobacco ringspot virus, both separately and mixed together, were centrifuged in an SW50 rotor at 50000 rev/min for 30 min. The sedimentation coefficients of the marker tobacco ringspot virus components, top (57 S), middle (99 S) and bottom (136 S), as reported by Randles and Francki (1965), were used to estimate the sedimentation coefficients of the BBS virus components.

Spectrophotometry

Ultraviolet absorption spectra of virus preparations were determined over the range 230–300 nm in a Shimadzu QR50 spectrophotometer, using quartz cuvettes of 1 cm path length. An extinction coefficient ($E^{0{\cdot}1\%}_{260\,nm}$) of 8·1, as reported by van Kammen (1967) for cowpea mosaic virus, was used to calculate virus yield.

Electron Microscopy

Virus preparations were stained by mixing equal volumes of virus suspension with 2% phosphotungstic acid (adjusted to pH 6·8 with KOH). The mixture was applied to carbon-coated copper grids and excess liquid was removed with filter paper. The suspensions were examined in a Siemens Elmiskop 1A electron microscope.

Serology

The bottom component of BBS virus was isolated by sucrose density-gradient centrifugation and emulsified with an equal volume of Freund's complete adjuvant. A rabbit was injected subcutaneously with approximately 2 mg of antigen twice at 10-day intervals and a third injection was administered 1 month later. The rabbit was bled 10 days after the final injection. All serological tests were carried out by double immunodiffusion in 0·75% agar containing 0·005M phosphate buffer (pH 7), 0·14M NaCl and 0·2% sodium azide.

Results

Host Range and Symptoms

Plant species found to be susceptible to BBS virus are listed in Table 1. Several hosts additional to those listed by Gibbs and Smith (1970) were infected.

As already reported by Lloyd *et al.* (1965), symptoms of BBS virus disease in broad beans include mild to severe mosaic, leaf puckering, stunting, apical dieback, and poor pod and seed setting. Symptoms were very severe when young seedlings were mechanically inoculated with the virus (Fig. 1*a*).

The virus failed to infect the following plant species: some cultivars of *P. vulgaris* (Tweed Wonder, Pinto, Tender Crop and Blue Lake); *P. aureus* Roxb.; *Glycine max* (L.) Merr.; *Cyamopsis tetragonoloba* (L.) Taub.; *Trifolium pratense* L.; *T. repens* L.; *Medicago sativa* L.; *M. lupulina* L.; *Lotus corniculatus* L.; *Cassia occidentalis* L.; *Datura stramonium* L.; *Nicotiana tabacum* L.; *N. glutinosa* L.; *N. glauca* L.; *N. clevelandii* Gray.; *Lycopersicon esculentum* Mill.; *Chenopodium amaranticolor* Coste

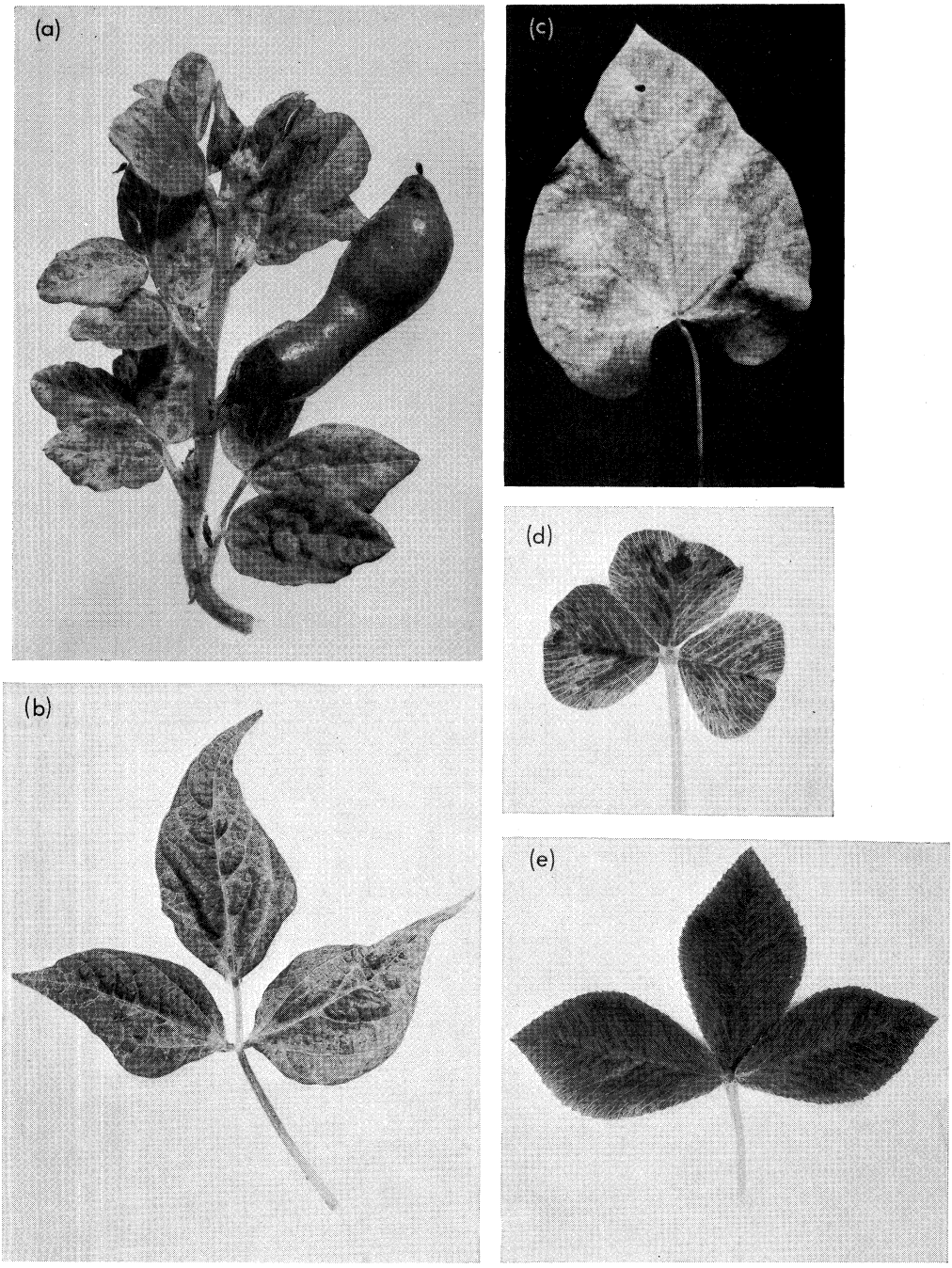
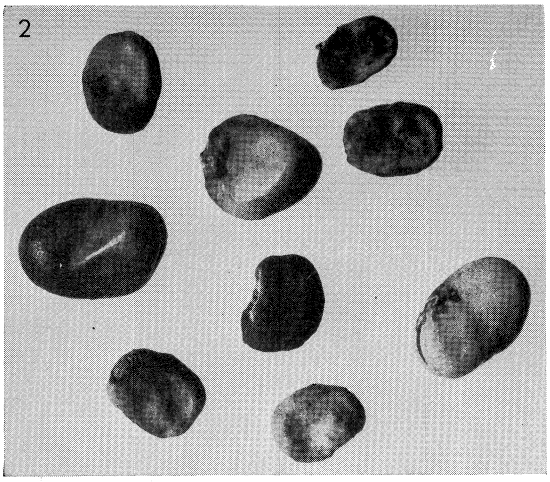


Fig. 1. Disease symptoms in plants infected with broad bean stain virus. (*a*) Mosaic in *Vicia faba* (cv. Leviathan Long Pod) with a stunted pod. (*b*) Systemic mosaic in *Phaseolus vulgaris* (cv. Tender Green). (*c*) Local infection in *P. vulgaris* (cv. Brown Beauty). (*d*) Mottle with vein clearing in young leaves of *Trifolium subterraneum*. (*e*) Vein clearing and mild mosaic in leaves of *T. resupinatum*.

& Reyn.; *C. album* L.; *C. quinoa* Willd.; *C. murale* L.; *Brassica pekinensis* Rupr.; *Cucumis sativus* L.; *Gossypium hirsutum* L.; *Helianthus annuus* L.; *Lactuca sativa* L.; *Spinacia oleracea* L.; *Vigna sinensis* (L.) Endl.; *Sonchus oleraceus* L.; *Solanum melongena* L.; *Capsicum frutescens* L.; *Gomphrena globosa* L.



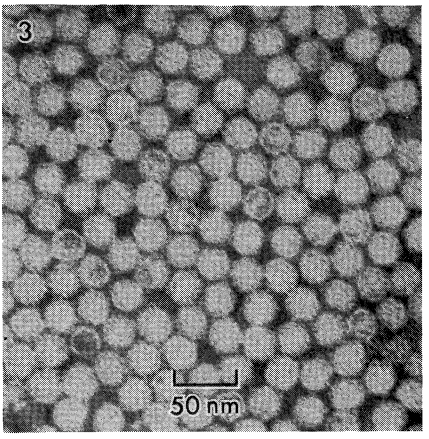


Fig. 2. Seeds harvested from infected broad bean plants.
Fig. 3. Electron micrograph of virus particles from bottom component from sucrose density gradients, mounted in 2% phosphotungstic acid.

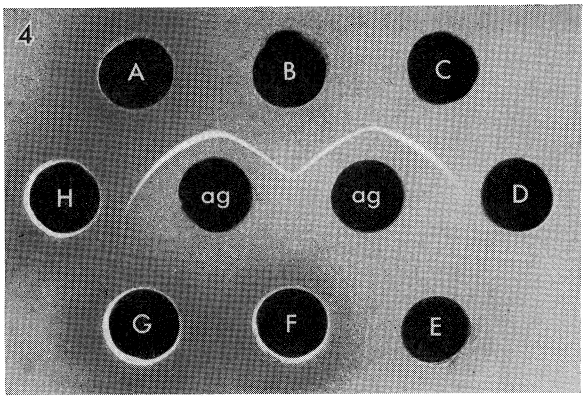


Fig. 4. Serological reactions of BBS virus in agar-gel diffusion tests. Central wells charged with purified BBS virus preparation (*ag*), outer wells charged with antisera to homologous BBS virus (*A*), BBS virus from U.K. (Hollings) (*B*), BBS virus from U.K. (Govier) (*C*), broad bean true mosaic virus (*D*), cowpea mosaic virus (*E*), broad bean mottle virus (*F*), tobacco ringspot virus (*G*) and normal serum (*H*).

Seed Transmission

About 500 plants raised from commercially available broad bean seed were examined for BBS virus infection by serological testing. The results presented in Table 2 indicate that imported, as well as locally produced, seed was carrying the virus to an extent of about 5%. When seeds were harvested from the inoculated broad bean plants, 3 out of 17 seeds, i.e. 11·8%, produced infected seedlings with mild or unnoticeable symptoms. The seeds from infected plants were small and had a characteristic necrosis of the testa (Fig. 2). Virus was also detected in pollen from the infected plants by mechanical inoculation to *P. vulgaris*.

Properties of Purified Virus Preparations

Purified virus preparations were clear and opalescent. When examined in an electron microscope, polyhedral particles were observed with a mean diameter of $28 \pm 1 \cdot 3$ nm based on measuring 300 particles (Fig. 3).

Table 1. Reactions of susceptible plant species to inoculation with broad bean stain virus

Symptoms are expressed as follows: CM, chlorotic mottle; La, latent; LC, leaf curl; LL, local lesion; LN, leaf necrosis; Ma, malformation; Mo, mosaic or mottling; 0, virus not recovered; St, stunting; TN, top necrosis; VC, vein clearing

Plant species	Symptoms: Local infection	Systemic infection
Vicia faba L. cv. Leviathan Long Pod	La	Mo, LC, TN
Pisum sativum L. cv. Green Feast	La	CM, St, LN
P. arvense L.	La	Mo, St
Lathyrus odoratus L.	La	Mo, St,
Trifolium incarnatum L.	La	VC, Mo
T. resupinatum L.	La	VC, Mo
T. subterraneum L.	La	Mo
Melilotus alba L.	La	Mo
Lupinus hirsutus L.	La	Mo
Cicer arietinum L.	La	CM
Lens esculenta Moench.	La	La
Phaseolus vulgaris L.		
cv. Tender Green	La	Mo
cv. Canadian Wonder	LL	Ma
cv. Brown Beauty	LL	0
cv. Bountiful	LL	Mo, Ma
cv. Redlands Pioneer	La	Ma

Table 2. Incidence of broad bean stain virus in commercial broad bean seed (cv. Leviathan Long Pod)

Source		Number of plants: Tested	Infected	Percentage of seed infected
South Australian grown	1970	132	6	4·5
South Australian grown	1971	116	7	6·0
Imported seed	1971	204	9	4·5

The u.v. absorption spectra of virus preparations were characteristic of nucleoprotein with a minimum at 240 nm and a maximum at 260 nm. The average ratios of the absorbances at 260 : 240 and 260 : 280 nm of eight preparations were $1 \cdot 46 \pm 0 \cdot 01$ and $1 \cdot 75 \pm 0 \cdot 03$ respectively. A yield of 150–200 mg of virus per kilogram of leaf tissue was consistently obtained.

In sucrose density gradients virus preparations separated into three components, top (T), middle (M) and bottom (B), with sedimentation rates of about 60, 100 and 127 S respectively. In some preparations the T component could not be detected.

It appears that T particles are devoid of RNA, as the absorbance ratio at 260 : 280 nm was 0·8. The 260 : 240 and 260 : 280 nm ratios of M component were 1·36 and 1·66, and those of B component were 1·57 and 1·81 respectively.

Infectivity

Virus preparations were submitted to sucrose density-gradient centrifugation and the distribution of infectivity in the fractionated gradients was determined. Results of a typical experiment (Fig. 5*a*) show that the bulk of infectivity did not coincide with the distribution of u.v. absorption but occurred in a zone where M and B components overlap. In subsequent experiments M and B components were collected from the gradients and after concentration by ultracentrifugation were subjected to a second cycle of density-gradient centrifugation. Again, the infectivity was associated with the fractions between M and B components (Figs 5*b*,*c*), indicating that both particles are required for infectivity. Results summarized in Table 3 confirm this conclusion.

Table 3. Infectivity of broad bean stain virus components compared with the infectivity of mixtures of components and unfractionated virus

Middle (M) and bottom (B) components were purified by two cycles of sucrose density-gradient centrifugation. T = top component. Numbers of lesions are the average number of local lesions in six half-leaves of *P. vulgaris*

Inoculum	Concentration ($E_{260\,nm}$)	Number of lesions: Observed	Expected[A]	Obs : Exp
T	0	0	—	—
M	0·2	4	—	—
M	0·4	6	—	—
B	0·2	5	—	—
B	0·4	8	—	—
T+M+B	0+0·2+0·2	48	9	5·3
M+B	0·2+0·2	45	9	5·0
M+B	0·4+0·2	58	11	5·2
M+B	0·2+0·4	64	12	5·3
Unfractionated virus	0·35	73	—	—

[A] Sum of lesions produced by inocula containing the individual components.

Serology

The prepared antiserum had a homologous titre of 2048 in gel-diffusion tests. It gave a single band of precipitation when tested against purified virus or sap from infected plants. M and B components were found to be serologically indistinguishable. The virus reacted strongly with undiluted or diluted BBS virus antiserum from the United Kingdom (Fig. 4). However, no reaction was observed when BBS virus was tested against antisera to broad bean true mosaic, cowpea mosaic, broad bean mottle or tobacco ringspot viruses.

Discussion

The host range studies of the virus isolated from broad beans in South Australia indicate that it is BBS virus (Table 1 and Figs 1*a–c*). Gibbs and Smith (1970) reported that BBS virus can be confused with broad bean true mosaic (Echtes Ackerbohne-

mosaik) virus. However, the virus from South Australia failed to react with antiserum to broad bean true mosaic virus (Fig. 4) but produced positive reactions with antisera to BBS virus from the United Kingdom (Fig. 4), providing conclusive evidence that it is BBS virus.

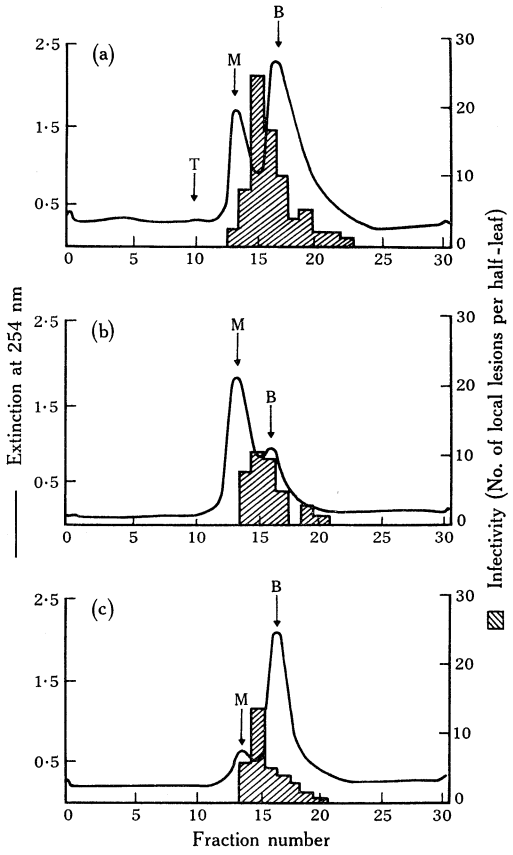


Fig. 5. Ultraviolet absorption spectra and infectivity distribution profiles of BBS virus following sucrose density-gradient centrifugation. (*a*) Overall infectivity of virus after one cycle of centrifugation at 24 000 rev/min for 3·5 h; (*b*) middle and (*c*) bottom component fractions after a second cycle of centrifugation.

The Australian BBS virus has biological properties (Tables 1 and 2 and Figs 1 and 2), particle morphology (Fig. 3) and sedimentation coefficients of the component particles (Fig. 5) very similar to BBS virus from the United Kingdom (Gibbs *et al.* 1968). However, the Australian virus appears to produce very much fewer T component particles than the British isolate (Gibbs *et al.* 1968). It seems possible that production of T component may be influenced by the purification procedure or by environmental conditions, as Clark (1972) in New Zealand also observed very small amounts of T component in his preparations of the BBS virus isolate obtained from Gibbs *et al.* (1968).

Data on the infectivity of BBS virus (Fig. 5 and Table 3) indicate that the viral genome is divided between the M and B nucleoprotein particles. These data, as well as those already discussed by Gibbs *et al.* (1968), indicate that BBS virus is very similar to cowpea mosaic virus (van Kammen 1967), although they appear to be serologically unrelated (Fig. 4). There seems little doubt that BBS virus should be included in the comovirus group as suggested by Harrison *et al.* (1971).

Judging by the apparent infection of broad bean seed with BBS virus (Table 2) it would seem that the virus is being established in Australia and that it may have been introduced into the country through commercial seed importation. In addition to its potential economic importance in broad bean crops, BBS virus is a potential danger to Australian pastures by virtue of its pathogenicity to clovers (Table 1 and Figs 1*d*,*e*). As yet no vectors of the virus have been identified in Australia, but weevils have been reported to be natural vectors of BBS virus in the United Kingdom (Cockbain *et al.* 1971).

Acknowledgments

We are indebted to Drs M. Hollings, D. A. Govier and R. M. Lister for their generous gifts of antisera. We also wish to thank Mrs L. Wichman for preparing the figure, Mr C. Grivell for technical assistance and Mr K. Jones for maintaining plants in the glasshouse. One of us (S.M.M.) is from Pakistan under the Commonwealth Scholarship and Fellowship Plan.

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Manuscript received 26 October 1973