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Evaluation of Broad Bean Cultivars for Resistance to *Broad Bean Mottle Virus* using a Simplified Enzyme-linked Immunosorbent Assay

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ABSTRACT

Fifty vegetable-type broad bean cultivars were biologically indexed for resistance to *Broad bean mosaic virus* (BBMV) using pathogenicity tests and a simplified enzyme-linked immunosorbent assay (ELISA) procedure. While all the broad bean cultivars tested, except 'Assiut 12 , were vulnerable (i.e., susceptible and/or sensitive) to BBMV, there were variations in symptom severity among the cultivars and vulnerability varied from high to moderate. Insensitivity was detected only in 'Assiut 12 . BBMV concentrations in infected broad bean leaves suggest differences in vulnerability among the cultivars due to differences in sensitivity rather than susceptibility. When combined with BBMV symptom expression, the ELISA procedure may provide a useful biological index for rapid screening of broad bean cultivars for resistance to BBMV, and thus reduce time for simple field diagnosis of the virus.

RESUMEN

Se evaluó la resistencia biológica de 50 cultivares de haba al virus del mosaico del haba (BBMV) usando pruebas de patogenicidad y la prueba de ELISA. Todos los cultivares de haba estudiados fueron vulnerables al BBMV, excepto el cultivar 'Assiut 12'; sin embargo, se observaron variaciones en la severidad de los síntomas entre los cultivares y esta vulnerabilidad varió de moderada a alta. Se detectó insensibilidad sólo con el cultivar 'Assiut 12'. Las concentraciones del BBMV observadas en las hojas de haba infectadas sugieren diferencias en sensibilidad mas que en susceptibilidad. La técnica de ELISA, en combinación con la expresión de síntomas, puede brindar una evidencia biológica útil para la selección de cultivares de haba con resistencia a BBMV, y de esta manera simplificar y reducir el tiempo de detección del virus en campo.

Additional Index Words: Faba bean, Bromoviridae, virus disease, ELISA procedure

Broad bean mottle virus (BBMV) belongs to the family *Bromovirus*: *Bromoviridae* group and is serologically related to *Brome mosaic virus* and *Cowpea mottle virus*. BBMV is an important viral pathogen that reaches high infection levels and causes considerable reduction in yield of broad bean (*Vicia faba* L) (Makkouk et al., 1988). The virion contains 21-23% nucleic acid with three segments of linear stranded RNA and total genome size of 8.25 kilo-base (Pogany et al., 1997; Romero et al., 1993).

Typical symptoms of BBMV in broad bean are vein clearing of the youngest leaves, which appear 8-10 days after infection. Subsequent symptoms varied from vein-clearing fading to a bright yellow inter-venial mottle, to chiorosis, and extensive necrosis (Bawden et al., 1951). Symptom expression depends on broad bean cultivar resistance, strain of BBMV and the season of cropping (Bos et al.. 1992). Enzyme-linked immunosorbent assay (ELISA) is sometimes more reliable than symptoms for detecting viral infection (Clark, 1981).

Broad bean is an important legume crop used as human food in the Middle East, Mediterranean region, China, Egypt, Ethiopia, Sudan, Australia, and Canada (Bond et al. 1985). It can be used as a vegetable, green or dried, fresh or canned and cultivation worldwide occupies more than 150,000 ha (Duc, 1997). During growing season a mild to severe outbreak of a virus disease complex including BBMV may occur leading to an epidemic (Makkouk et al., 1994). Recent surveys have shown an early appearance of BBMV on broad bean, which could be attributed to seed transmission nature of the virus when it occurs in a complex infection with other viruses (Gibbs, 1972). Attempts to develop broad bean resistant to the viral disease complex including BBMY are underway. The present investigation was to evaluate the use of ELISA in combination with symptom expression for rapid screening of 50 broad bean genotypes infected with *Broad bean mottle virus*.

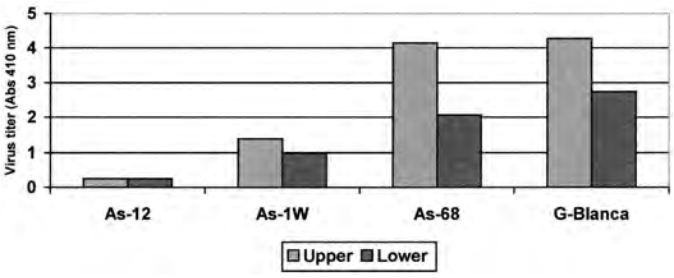

Fig. 1. Virus titers in upper and lower leaves of four broad bean cultivars. As-12 = 'Assiut-12 is apparently insensitive and cultivars As-1W = 'Assiut-1W , As-68 = 'Assiut-68 , and G-Blanca = 'Giza-Blanca are relatively sensitive to *Broad bean mottle virus* (BBMV).

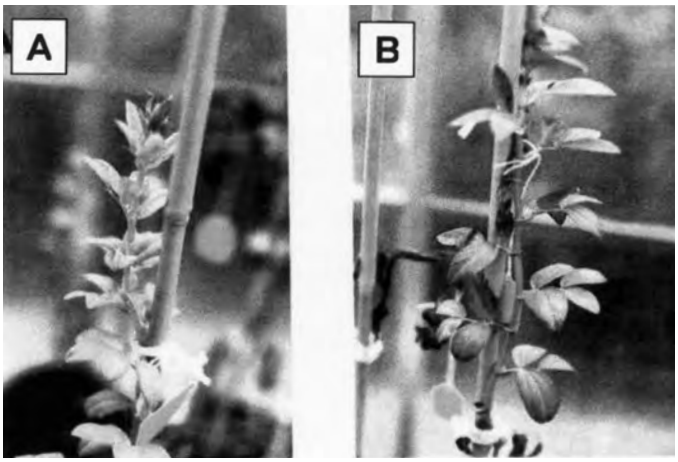

Fig. 2. *Broad bean mottle virus* symptoms on susceptible broad bean leaves 6 to 8 weeks after inoculation with the virus particles. Note the upper and lower leaf mottling **(A)** and chlorosis **(B)** after inoculation with virulent broad bean mottle virus.

MATERIALS AND METHODS

Virus and antiserum. Broad bean mottle virus (BBMV) was obtained from American Type Culture Collection (ATCC), Rockville, Maryland. The virus was maintained on tobacco (*Nicotiana tabacum*). The virus was purified by a chloroform-pH procedure (Dias and Allen, 1980) followed by rate-zonal density gradient centrifugation in 10, 15, 25, and 30% sucrose solutions buffered in 0.01 M phosphate buffer, pH 7.0. The fractions were pooled, pelleted with high-speed centrifutigation. and re-suspended in the phosphate buffer. Antiserum against BBMV was prepared by injecting New Zealand white rabbits intramuscularly with virus preparations emulsified with an equal volume of Freund's complete adjuvant. The 2-mg intra-muscular injections were given at 10-day intervals, followed by one 2-mg intravenous injection. The rabbits were bled 2 wk after the last injection. The titer of the antiserum as determined by double diffusion in Ouchterlonv agar gel (Ouchterlony, 1962) was 1/800.

Plant Inoculation. Fifty broad bean cultivars, obtained from Egypt, Australia, U.S.A. and Canada, were tested for reaction to BBMV. The inoculum was prepared by grinding lyophilized virus-infected leaf tissue in buffers using a mortar and pestle. The buffers used consisted of 0.05 M sodium carbonate (pH 9.6) and 10% diethanolamine adjusted to pH 9.8 with HCl; phosphate buffer saline (PBS), 0.02 M phosphate plus 0.15 M NaCl at pH 7.4; PBS with 0.05% Tween 20 (PBS-Tween), and 2% polyvinyl pyrrolidone (PVP. MW 40,000; Sigma Chemicals). All buffers contained 0.02% sodium azide as a preservative. The inoculum was squeezed through a double layer of cheesecloth plus one layer of Miracloth. A small amount of 22 µm Carborundum (600 mesh) was added and inoculum was applied to the primary leaves of 4-week-old faba bean seedlings using cotton tipped applicators that had been dipped into the inoculum. Plants were kept in the greenhouse for 8 wks, checked daily for symptoms development and compared with uninoculated healthy control plants.

The virus-infected and healthy plants were collected every 2 wks and prepared by grinding 1 g of foliar tissue in 100 ml of ELISA extraction buffer. Six twofold dilutions of virus-infected leaf macerates in extraction buffer were used in subsequent tests while healthy plant macerates were assayed without further dilution. The concentration of BBMV in leaves of *Nicotiana tabacum*, the propagative host, was estimated at 2.5 mg per 100 g of infected material.

ELISA procedure. The double antibody sandwich ELISA, as described by Clark and Adams (1977) was used. The excised leaf samples were extracted with 1:5 w/v ratio of PBS-Tween containing 2% PVP clarified by centrifugation at 1000 g for 5 min. The extracts were diluted 1:100; 1:200; 1:300; 1:400; 1:800 respectively using PBS-Tween as diluent. Virus detection was accomplished using polystyrene MicroELISA plates (Dynatech Laboratories Inc.. Alexandria. VA) with a minimum of three replicate wells for each broad bean genotype screened. Wells were coated with 200 1 of BBMV specific antibody (~ 3 µg/ml γ-globulins) suspended in a buffer and mixture incubated at 37º C for 2 to 6 hr. Approximately 200 µL leaf extract mixed in PBS-Tween was added to each well and incubated at 6º C. About 200 µL of enzyme-labeled-globulins (alkaline phosphatase) in PBS-Tween was added to plates and incubated for 3 to 4 h at 37ºC before adding 300 µL p-nitrophenyl phosphate substrate in diethanolamine buffer. Plates were incubated for ½ to 2 h before the reaction was stopped with 50 µL of 3.0 M NaOH. Absorbance was measured at 410 nm using an ELISA microplate reader (MRX, Microplate Reader, Dynex Technologies Inc., Chantily, VA). Positive or negative ELISA reactions were indicated by presence and absence of BBMV particles. Control wells consisted of extraction buffer and wells containing sap of healthy uninoculated plants. The experiment was repeated at least three times, and means for virus titer were then calculated.

RESULTS AND DISCUSSION

Reactions of the broad bean cultivars to BBMV based on ELISA and relative leaf symptom intensities on inoculated plants are presented in Table 1. Of the 50 cultivars screened, nine cultivars ('Assiut-1W, 'Assiut-8A, 'Assiut-13-9 , 'Assiut-48', 'Assiut-67, 'Assiut-69, 'Assiut-88, 'Assiut-106 and

Table 1. Reactions of broad bean cuitivars to Broad bean mottle virus (BBMV) based on enzyme-iinked immunosorbent assay (ELISA) at 1/200 dilution and leaf symptoms on inoculated plants.

	Absorbance at 410 nm (X±S.D.)[z]		Symbols for symptoms[y]			
Cultivar	Healthy control	Virus infected	Top leaf	Middle leaf	Bottom leaf	Symptom[x] intensity
Assiut-1W	0.25±0.01	1.47±0.01	C	—	C	++
Assiut-3	0.25±0.01	1.97±0.03	C, VC	C	C	+++
Assiut-8A	0.25±0.01	1.45±0.04	C	—	C	++
Assiut-12	0.25±0.01	0.25±0.u3	—	—	—	-
Assiut-13-9	0.25±0.01	1.39±0.03	C	—	C	++
Assiutt-34	0.25±0.01	2.38±0.05	C, VC, M	C, VC	VC, N	+++
Assiut-39	0.25±0.01	3.05±0.09	C, VC,	C, VC, M	VC, N, M	+++
Assiut-48	0.25±0.01	1.55±0.04	C	—	C	++
Assiut-54	0.25±0.01	2.73±0.03	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-65	0.25=0.01	3.05±0.09	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-67	0.25±0.01	1.55±0.02	C	C	VC	++
Assiut-68	0.25±0.01	4.15±0.08	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-69	0.25±0.01	1.34±0.04	C	—	VC	++
Assiut-84	0.25±0.01	4.05±0.10	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-85	0.25±0.01	3.30±0.04	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-88	0.25±0.01	1.65±0.02	C	C	C	++
Assiut-89	0.25±0.01	3.15±0.06	C, VC, M	C, VC, M	VC, N, M	+++
Assiut.94	0.25±0.01	2.75±0.10	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-95	0.25±0.01	4.00±0.07	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-99	0.25±0.01	3.46±0.17	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-99B	0.25±0.01	3.35±0.05	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-101	0.25±0.01	3.05±0.11	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-102	0.25±0.01	3.85±0.07	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-103	0.25±0.01	3.92±0.04	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-106	0.25±0.01	1.43±0.02	C	C	C	++
Assiut-110	0.25±0.01	3.05±0.09	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-112	0.25±0.01	3.93±0.54	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-114	0.25±0.01	3.07±0.07	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-119	0.25±0.01	2.64±0.08	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-123	0.25±0.01	3.73±0.09	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-131	0.25±0.01	3.87±0.07	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-147W	0.25±0.01	2.60±0.04	C, M	C, VC	VC, M	+++
Assiut-149	0.25±0.01	1.50±0.10	VC	—	C	+++
Asslut-159	0.25±0.01	4.04±0.13	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-195	0.25±0.01	3.16±0.07	C, VC, M	C, VC, M	VC, N, M	+++
Assjut-215	0.25±0.01	2.83±0.06	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-226	0.25±0.01	3.06±0.01	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-502	0.25±0.01	3.93±0.12	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-902	0.25±0.01	2.95±0.03	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-1104	0.25±0.01	4.10±0.11	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-3101	0.25±0.01	3.17±0.07	C, VC, M	C, VC, M	VC, N, M	+++
Assiut-Gold	0.25±0.01	2.87±0.05	C, VC, M	C, VC, M	VC, N, M	+++
Giza-2	0.25±0.01	3.16±0.09	C, VC, M	C, VC, M	VC, N, M	+++
Giza-123A	0.25±0.01	3.02±0.04	C, VC, M	C, VC, M	VC, N, M	+++
Giza-402	0.25±0.01	3.69±0.03	C, VC, M	C, VC, M	VC, N, M	+++
Giza-429	0.25±0.01	3.05±0.15	C, VC, M	C, VC, M	VC, N, M	+++
Giza-461	0.25±0.01	2.94±0.03	C, VC, M	C, VC, M	VC, N, M	+++
Giza-674	0.25±0.01	2.94±0.08	C, VC, M	C, VC, M	VC, N, M	+++
Giza-716	0.25±0.01	4.23±0.13	C, VC, M	C, VC, M	VC, N, M	+++
Giza-Blanca	0.25±0.01	4.27±0.07	C, VC, M	C, VC, M	VC, N, M	+++
Aguadulce	0.25±0.01	2.83±0.02	C, VC, M	C, VC, M	VC, N, M	+++

[z]Twenty four wells were used in each treatment.

[y]Symbols for leaf s\mptoms: C = chlorosis: M = mottling; N = necrosis; VC = v\ein clearing.

[x]Symptom intensity (-, +, ++, +++) represents no to relative mild to severe symptoms.

'Assiut-149) were found to possess some degree of tolerance to BBMV as indicated by lower virus titer and reduced symptom intensity in leaves of inoculated plants. One broad bean cultivar, 'Assiut-12' showed no apparent BBMV symptoms and had OD values (0.25 ± 0.01 absorbance at 410 nm) indistinguishable from healthy controls throughout the 8 wk duration of the experiment. This reaction indicates a possible insensitivity or resistance to BBMV. However, the rest of the broad bean cultivars tested appeared to be relatively sensitive or susceptible to BBMV. No virus was detected from the leaves of control healthy plants, which were symptomless for the duration of the experiment. BBMV titers were higher in upper than lower leaves of inoculated broad bean plants except in 'Assiut-12 (Fig. 1). These observations indicate that BBMV movement within the broad bean plant may be mostly toward the apical meristem. When a virus infects a plant, it multiplies within the parenchyma cell and moves from one cell to another through plasmodesmata. The rate of movement between cells may be 8 to 10 cells per day (Hull, 1989). The first visible symptoms of BBMV on inoculated plants were observed approximately 14 d after inoculation in the upper leaves, indicating that the virus is probably moving via the phloem and rapidly translocating into the upper leaf tissues. Typically, mottling symptoms were observed on the upper leaves and chiorosis on the lower leaves (Fig. 2). The BBMV symptoms expressed by broad bean cultivars in this greenhouse study agree with symptoms observed in BBMV field outbreaks (Bawden et al., 1951; Fortass and Bos, 1992; Gibbs, 1972). BBMV symptoms of field-grown susceptible broad bean cultivars vary with season. However at all seasons, the vein clearing may fade and be replaced by a bright inter-veinal mottle and extensive necrosis (Gibbs, 1972). A range of symptoms and infection levels for BBMV was demonstrated among the broad bean cultivars examined in this study. BBMV needs to be studied further in order to determine which genotypes can be grown successfully where BBMV virus is endemic.

The present study confirms BBMV pathogenicity on susceptible broad bean cultivars as well as differential reaction to the virus among these cultivars. The study also confirms that when combined with symptom expression, enzyme-linked immunoassay (ELISA) procedure is useful for biological indexing of plant viruses and may be used for a rapid screening of plant genotypes for resistance to the virus, thus reducing time for simple field diagnosis of the virus (Hu et al.. 1995).

While some broad bean cultivars tested in this study show potential as breeding materials, a number of promising breeding lines and accessions of broad bean with some level of resistance to BBMV have been developed at various laboratories in the world. Other factors such as planting date and control of insect vectors such as *Apion radiolus*, *Hypera variabilis*, *Pachytychius strumarius*, and *Spodoptera exgua* may greatly influence BBMV infection in a broad bean field (Ahmed and Elsa, 1992). The use of resistant cultivars such as 'Assiut-12 and resistant broad bean species such as *Vicia sativus* in a breeding program is a viable option. The genetic mechanism(s) within broad bean cultivar Assiut-12~ for reduced virus titer is unknown.

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