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COMPARISON BETWEEN DIFFERENT ELISA PROCEDURES FOR DETECTION OF SOME *VICIA FABA* VIRUSES

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ABSTRACT

In this study three different procedures of enzyme-liked immunosorbent assay (ELISA) were evaluated for detecting the most important viruses affecting faba bean. These viruses are Faba bean necrotic yellows virus (FBNYV), Bean yellow mosaic virus (BYMV), Broad bean mottle virus (BBMV), and Broad bean stain virus (BBSV). In each test six dilutions, i.e., 1/20, 1/100, 1/500, 1/1000, 1/2500 and 1/5000 were used. Among the results, the enzyme amplification-ELISA (EA-ELISA) was the most sensitive, where the BBMV, BYMV, BBSV and FBNYV were detected in sap dilution of 1/2500, 1/2500, 1/1000 and 1/1000, respectively. While in the case of pencillinase-based ELSA (PNC-ELISA) BBSV and FBNYV were detected in sap dilutions of 1/500. The double antibody sandwich-ELISA (DAS-ELISA) was the least sensitive except for BBMV that detected by DAS-ELISA in sap dilution of 1/2500. On the other hand the ELISA values were higher in the case of EA-ELISA followed by DAS-ELISA and PNC-ELISA.

Key words: Faba bean, FBNYV, BBMV, BBSV, BYMV, ELISA

INTRODUCTION

Faba bean (*Vicia faba* L.) is considered the main protein source for a large part of the population (**Bos *et al* 1988**). Its productivity is affected by a number of viruses in some Arab countries, i.e., Egypt (**Allam *et al* 1979; Makkouk *et al* 1988; Makkouk *et al* 1994 and Salama *et al* 1997**), Morocco (**Fisher, 1979**), Jordan (**Al-Musa and Mansour, 1984**) and Syria, Sudan, Tunisia and Lebanon (**Makkouk *et al* 1988**).

The most important and widely used methods for detection of plant viruses are those depend on the surface properties of the coat protein in the intact virus (**Abdel-Ghaffar *et al* 1998**) or the protein subunits from disrupted virus (**Hill, 1984**).. Serological techniques are fre-

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quently favored for plant virus detection because of their specificity and speed (**Baunoch *et al* 1992**).

The microtiter plate method of enzyme-linked immunosorbent assay (ELISA) is a sensitive technique (**Clark and Adams, 1977** and **Makkouk *et al* 1994**). In double antibody sandwich-ELISA (DAS-ELISA) technique, the same type of specific antibodies used for coating, are usually labeled with the alkaline phosphatase (AP). **Clark and Adams (1977)** reported that the microplate method of DAS-ELISA could be very effectively applied to the detection of plant viruses. Since that time the method has come to be more and more widely used.

Enzymes other than AP can be used effectively in ELISA tests for the detection of plant viruses (**Kumari and Makkouk, 1993** and **Salama *et al* 1997**). In **1989, Sudarshana and Reddy** found that pencillinase-based ELISA (PNC-ELISA) is as sensitive as AP-based ELISA. However, PNC-ELISA was found to be more convenient for visual scoring and less expensive that AP-based ELISA. However, based on ELISA values, incubation time required for visual result and cost, the PNC-ELISA was more convenient than DAS-ELISA and also less expensive.

Torrance (1987) described the EA-ELISA procedure for increasing the sensitivity of ELISA assays, in which the enzyme bound to the antibody catalyzes the conversion of NADP to NAD, which then takes part in a second enzyme-mediated cyclic reaction to produce a red-colored end product. The method could be used for rapid diagnosis of the phloem-limited viruses occurring in very low concentrations in plants as luteoviruses, and also for detecting the virus in individual aphid.

Makkouk *et al* (1988) reported that Bean leaf roll virus (BLRV), Bean yellow mosaic virus (BYMV), Broad bean mottle virus (BBMV) and to a lesser extent Broad bean stain virus (BBSV) were the most common viruses on faba bean in the six Arab countries surveyed. Faba bean necrotic yellows virus (FBNYV) is a recently detected associating with virus diseases affecting several important food and fodder legumes (**Katul *et al* 1993**).

One of the most important steps in controlling such viruses is the early detection particularly in the case of symptomless plants using a sensitive technique(s) (**Salama *et al* 1997**). Therefore, this study was aimed to compare between three different ELISA techniques for rapid and sensitive detection of FBNYV, BYMV, BBMV and BBSV affecting faba bean in Egypt.

MATERIAL AND METHODS

Source of plant materials

Leaves of faba bean plants artificially and separately inoculated with Bean yellow mosaic potyvirus (BYMV), Broad bean mottle virus (BBMV), Broad bean stain virus (BBSV), and Faba bean necrotic yellows nanovirus (FBNYV) were used as a sources for virus-infected samples. These plants were grown in the greenhouse at Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC). Also healthy faba bean plants were also used to serve as control.

Sample preparation

For evaluation of the sensitivity of the three ELISA techniques under investigation in detecting some *Vicia faba* viruses, five dilutions, i.e., 1/20, 1/100, 1/500, 1/1000, 1/2500 and 1/5000 were applied. These dilutions were prepared in the coating buffer, pH 9.6 **(Clark and Adams, 1977)** for PNC-ELISA, and in 0.2 M phosphate buffer, pH 6.0 **(Makkouk *et al* 1988)** in the case of DAS-ELISA and EA-ELISA.

Source of antisera

Antibodies specific for BYMV, BBMV, BBSV and FBNYV, kindly provided by Prof. Dr. K. Makkouk, Virology Laboratory, ICARDA, Aleppo, Syria. were used.

DAS-ELISA

This experiment was carried out as described by **Clark and Adams (1977).** The absorbence was measured at 405 nm.

PNC-ELISA

Two hundred µl of virus-infected samples (dilutions) prepared in coating buffer, pH 9.6 were added well^{-1} (two wells were used for each dilution). In the following the plate was incubated for 4 hr at 37°C and washed with phosphate buffered saline-Tween (PBS-T, **Clark and Adams, 1977**) three times, five min for each. The wells were then filled with 2% bovine serum albumin (BSA) in PBS-T and incubated for 1 hr at 37°C and washed as mentioned before. Virus-specific antibodies (VSAb) were added (200 µl well^{-1}) in the suitable dilution and incubated for 4 hr at 37°C and washed as mentioned above. Goat-antirabbit (GAR) antibodies conjugated to pencillinase at dilution 1/1500 in conjugate buffer **(Clark and Adams, 1977)** were added (200 µl well^{-1}) and incubated for 4 hr at 37°C. 200 µl aliquots of freshly prepared substrate (Bromocresol purple (BCP) + Bromothymol blue (BTB)) (5.8 mg of BCP and 2.9 mg of BTB in 0.2 M NaOH and neutralized by HCl, and the volume was completed up to 100 ml with distilled water (d.H_2O), and then Pencillin-G at 1.0 mg ml^{-1} was added and the pH adjusted to 7.2. At room temperature (RT), the plate was incubated for 30-60 min or as long as necessary to observe reaction and the absorbance was measured at 450 nm (**Sudarshana and Reddy, 1989**).

EA-ELISA

In this experiment, 200 µl of purified VSAb prepared in coating buffer, pH 9.6 (1µg/ml) were added well^{-1} (two wells were used for each dilution). This was followed by incubation for 4 hr at 37°C and washing with PBS-T as mentioned before. 200 µl of prepared sample (dilution) were added and the plate was then incubated for 4 hr at 37°C. After washing 200 µl VSAb conjugated to AP in conjugate buffer (1µl ml^{-1}) **(Clark and Adams, 1977)** were added well^{-1}, incubated and washed. 100 µl of 0.2 mM β-nicotinamide-adenine dinucleotide phosphate monosodium salt (β-NADP) in 0.5 M diethanolamine buffer, pH 9.5 was added well^{-1}. On incubation for 30 min at RT, 15 µl 0.05 M 4-nitrophenyl disodium orthophosphate (o-NPP) in 0.025 M phosphate buffer, pH 7.0 were added well^{-1}. This was followed by adding 150 µl well^{-1} of stock amplification mixture (700 units alchohol dehydrogenase, 100

units lipoamide dehydrogenase, 3% ethanol and 1 mM p-iodonitrotetrazolium violet in 15 ml of 0.025 M phosphate buffer, pH 7.0, **Van den Heuvel and Peters, 1989**). The reaction was observed after 5-30 min incubation at RT, or as long as necessary and the ELISA values were determined at 492 nm. Adding 15 µl H_2SO_4 to each well stopped the reaction.

Blank, control and ELISA judging

It is important to mention that in the three ELISA techniques, 200 µl of coating buffer were added to the A1 well to serve as blank while sample from healthy faba bean leaf, at dilution of 1/20, was also used for judging the positive samples. In ELISA tests, positive result was indicated by absorbance readings greater than two times the value of negative control (**Clark and Adams, 1977** and **Hsu *et al* 1992**).

RESULTS AND DISCUSSIONS

Most serological assays in plant virology have dealt with the detection of virus-infected plants and seeds (**Lommel *et al* 1982 and Nolan and Campbell, 1984**) or strain differences within virus groups (**Van Regenmortel and Burckard, 1980 and Devergne *et al* 1981**). These assays have all been based on the relatively abundant viral capsid protein as the antigen.

Stanley *et al* (1985) reported that EA-ELISA is one of the most sensitive ELISA techniques and can detect very low concentrations of virus, because, the AP-enzyme can amplify through cyclic reactions which produce many times of the final colored product (**Figure 1, Van den Heuvel and Peters, 1989**). Such procedure was more expensive to conduct, but it recommended only when very high sensitivity of detection is required and cost is not a limiting factor.

In this study three different procedures of ELISA were tested for detecting the FBNYV, BYMV, BBMV and BBSV, which reported to be the most significant viruses affecting faba bean as reported by **Makkouk *et al* (1988)** in six Arab countries. In this study dilutions of 1/20, 1/100, 1/500, 1/1000, 1/2500 and 1/5000, prepared from virus-infected sap of faba bean leaf were used.

Among the results in Tables (1), (2) and (3), the EA-ELISA was the most sensitive, where the BBMV and BYMV were detected in sap dilution of 1/2500, while, BBSV and FBNYV were detected in sap dilution of 1/1000. In the case of PNC-ELISA, BBSV and FBNYV were detected in sap dilutions from 1/20 to 1/500; this could be due to the low concentration of these viruses. The DAS-ELISA was the least sensitive except for BBMV that detected by it up to dilution of 1/2500. On the other hand the ELISA values were higher in the case of EA-ELISA followed by DAS-ELISA and PNC-ELISA as shown in Figures 2, 3 and 4. In the case of EA-ELISA, these values ranging from 1.315 to 2.873, while PNC-ELISA gave values ranging from 0.664 to 1.315 and DAS-ELISA gave values from 1.112 to 1.827.

Kumari and Makkouk (1993) in Syria reported that, the most sensitive procedure for BBSV and Pea seed-borne mosaic potyvirus (PSbMV) detection in lentil was EA-ELISA followed by PNC-ELISA, indirect-ELISA and DAS-ELISA. On the basis of serological detection using DAS-ELISA technique, **Makkouk *et al* (1994)** detected five

Table 1. Detection of four viruses affecting faba bean using DAS-ELISA

Dilutions	BBMV	BBSV	BYMV	FBNYV
1/20	1.827*	1.112	1.217	1.192
	+	+	+	+
1/100	1.777	0.778	0.473	0.847
	+	+	+	+
1/500	1.384	0.529	0.348	0.575
	+	+	+	+
1/1000	0.687	0.184	0.178	0.173
	+	-	-	-
1/2500	0.475	0.098	0.166	0110
	+	-	-	-
1/5000	0.195	0.000	0.057	0.000
	-	-	-	-

+ : Positive. - : Negative. *: ELISA values at A_{405} nm.

Table 2. Detection of four viruses affecting faba bean using PNC-ELISA

Dilutions	BBMV	BBSV	BYMV	FBNYV
1/20	1.320*	0.925	1.077	0.664
	+	+	+	+
1/100	1.298	0.751	1.072	0.610
	+	+	+	+
1/500	1.191	0.585	1.049	0.437
	+	+	+	+
1/1000	0.850	0.220	0.743	0.166
	+	-	+	-
1/2500	0.538	0.093	0.302	0.120
	+	-	+	-
1/5000	0.132	0.000	0.112	0.099
	-	-	-	-

+ : Positive. - : Negative. *: ELISA values at A_{450} nm.

Table 3. Detection of four viruses affecting faba bean using EA-ELISA

Dilutions	BBMV	BBSV	BYMV	FBNYV
1/20	2.111*	1.315	1.667	2.873
	+	+	+	+
1/100	1.869	1.108	1.229	2.593
	+	+	+	+
1/500	1.398	0.892	0.846	1.913
	+	+	+	+
1/1000	0.725	0.358	0.460	0.879
	+	+	+	+
1/2500	0.486	0.160	0.358	0.210
	+	-	+	-
1/5000	0.093	0.000	0.102	0.130
	-	-	-	-

+ : Positive. - : Negative. *: ELISA values at A_{492} nm.

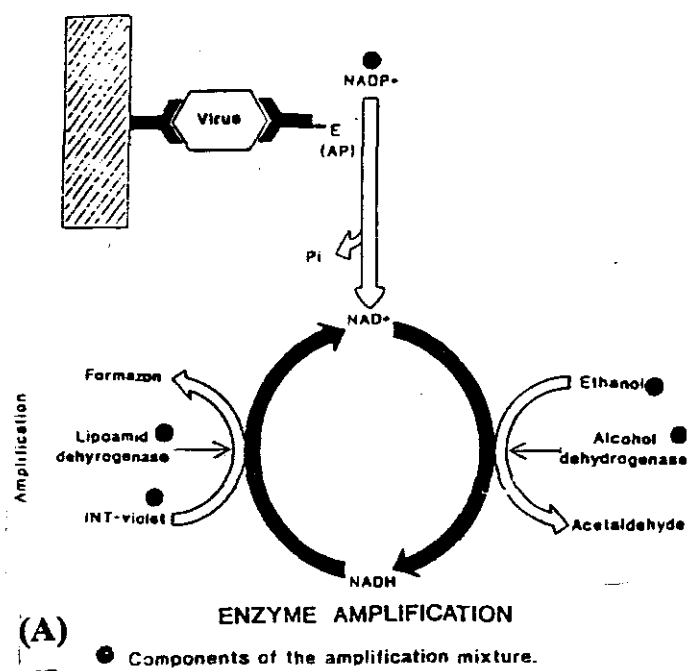

Figure 1. A Diagram shows the principal of EA-ELISA (**Stanley *et al* 1985 and van den Heuvel and Peters, 1989**) used for detection of some viruses affecting *Vicia faba* .

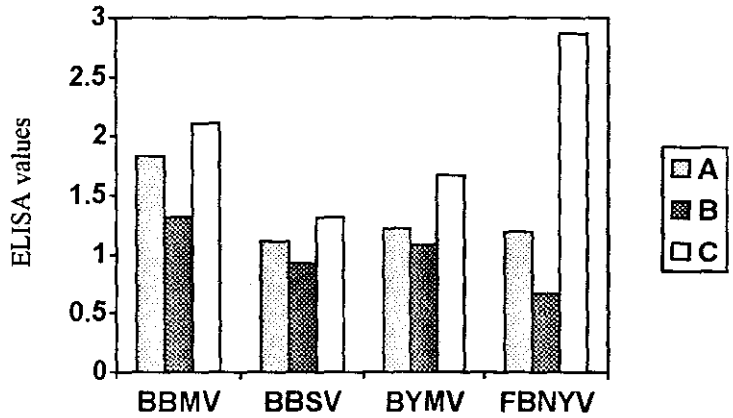

Figure 2. Detection of BBMV, BBSV, BYMV and FBYYV using different ELISA techniques (A, DAS-ELISA, B, PNC-ELISA and C, EAELISA) in sap dilution of 1-20 of virus-infected faba bean leaf.

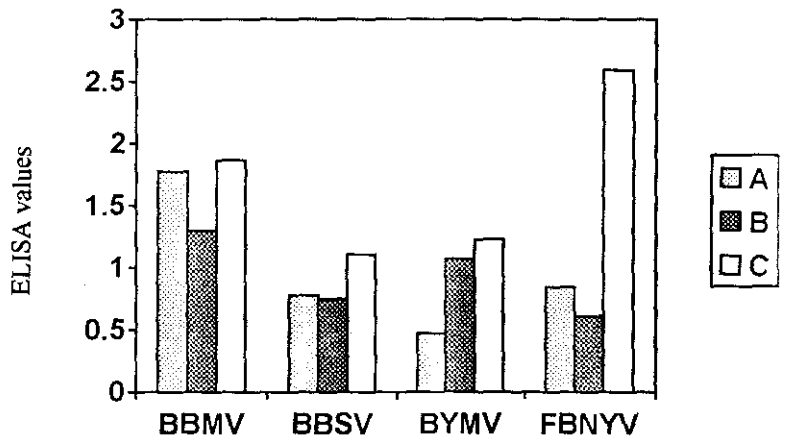

Figure 3. Detection of BBMV, BBSV, BYMV and FBYYV using different ELISA techniques (A, DAS-ELISA, B, PNC-ELISA and C, EA-ELISA) in sap dilution of 1/100 of virus-infected faba bean leaf.

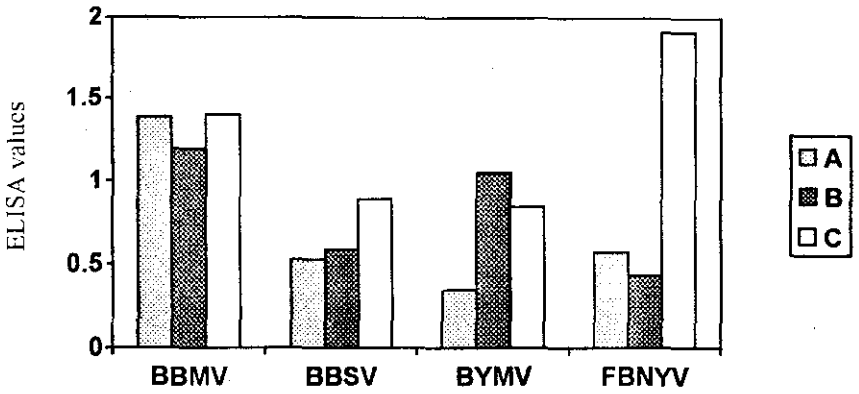

Figure 4. Detection of BBMV, BBSV, BYMV and FBYYV using different ELISA techniques (A, DAS-ELISA, B, PNC-ELISA and C, EA-ELISA) in sap dilution of 1/500 of virus-infected faba bean leaf.

viruses (AMV, CMV, BLRV, PSbMV and FBNYV) in faba bean samples collected from Delta, Middle Egypt and Fayoum.

Salama *et al* (1997) compared the sensitivity of four different ELISA techniques for detection of PSbMV in diluted extracts from faba bean infected-plants. Among the serological tests used, dot blot-ELISA was the most sensitive one; as it detected the virus in faba bean tissue extract diluted 1/31250. Other serological techniques, such as DAS-ELISA, PNC-ELISA and EA-ELISA were equally sensitive and they detected the virus in tissue extracts diluted 1/6250 but not 1/31250.

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مجلة حوليات العلوم الزراعية ، كلية الزراعة ، جامعة عين شمس ، القاهرة ، م٤٨ ، ع(١) ، ٥٧-٦٧ ، ٢٠٠٣

مقارنة بين طرق مختلفة من الأليـزا للكشف عن بعض الفيروسات التي تصيب نبات الفول البلدى

[٤]

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في هذه الدراسة تم تقييم ثلاثة طرق مختلفة من الأليزا للكشف عن أهم الفيروسات التي تصيب نبات الفول البلدى. وهذة الفيروسات كانت فيروس النيكروزسيس الأصفر في الفول (FBNYV) وفيروس الموزيك الأصفر في الفول (BYMV) وفيروس التبقع في الفول (BBMV)وفيروس الصبغ في الفول (BBSV). وفي كل طريقة تم استخدام ست تخفيفات هي : ١/٢٠، ١/١٠٠، ١/٥٠٠، ١/١٠٠٠، ١/٢٥٠٠، ١/٥٠٠٠ . ومن خلال النتائج اتضح أن طريقة الأكثار الأنزيمي-الأليزا (enzyme amplification-ELISA) هي الأكثر حساسية حيث تم الكشف عن فيروسات الـ BBMV, BYMV, BBSV, FBNYV في العصير المخفف حتي ١/٢٥٠٠، ١/٢٥٠٠، ١/١٠٠٠، ١/١٠٠٠ علي التوالي. بينما في حالة الأليزا المعتمده علي أنزيم البنسلينيز (pencillinase-based-ELISA) تم

الكشف عن فيروسات الـ BBSV, FBNYV في العصير المخفف حتي ١/٥٠٠ . وكانت طريقة الأليزا الذي يستخدم فيها ساندوتش الجسم المضاد (DAS-ELISA) أقل حساسية فيما عدا امكانية الكشف عن فيروس الـ BBMV بهذه الطريقة حتي تخفيف ١/٢٥٠٠. وقد لوحظ أن قيم الأليزاكانت أعلي في حالة طريقة الأكثارالأنزيمي-الأليزا تليها طريقة الأليزا المعتمده علي أنزيم البنسلينيز ثم الأليزا الذي يستخدم فيها ساندوتش الجسم المضاد.

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