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DETECTION OF ALFALFA MOSAIC ALFAMOVIRUS IN SEEDS, SEED PARTS AND SEEDLINGS OF TWO ALFALFA CULTIVARS

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ABSTRACT

Infectivity test, indirect ELISA and tissue blot immunoassay (TBIA) were used to detect alfalfa mosaic alfamovirus (AMV) in intact seeds, seed parts and seedlings of two alfalfa cultivars. ELISA was more sensitive than infectivity test for detection AMV in seeds, seed coats, seed embryos and seedlings. Percentage of seed infection detected by ELISA was 5.6% in cv. Siriver and 4.4% in cv. El-Wadi El-Gadid, while it was 2.1% and 1.55% in cvs Siriver and El-Wadi El-Gadid, respectively when indexed with infectivity test. Infectivity test did not detect the virus antigen in the separated seed coats of the two tested cultivars. Indirect ELISA showed that higher proportion of embryos contained AMV as compared with seed coats.

Data concerning detection of virus antigen in 5 and 21 days old seedlings revealed that the proportion of infected plants was higher in 21 day old seedlings than 5 days old ones, when ELISA or infectivity test was used in detection.

When TBIA was used for testing 21 day old seedlings, higher proportion of infected seedlings was detected (21% in cv. Siriver and 17% in cv. El-Wadi El-Gadid) as compared with indirect ELISA or infectivity test. TBIA proved to be simpler and more practical than ELISA.

INTRODUCTION

Alfalfa mosaic alfamovirus (AMV) is one of the most important viruses affecting alfalfa (Forster et al., 1985; Dall et al., 1986; Micznyski and Hiruki, 1987; Avgelis and Katis, 1989 and Fath-Alla, 1999). The virus was isolated in Egypt from naturally infected plants of alfalfa (El-Kady et al., 1985; Gamal El-Din *et al*., 1985 and Fath-Alla, 1999), potato (Gamal El-Din *et al*., 1994) and pepper (Fegla and Younes, 1999).

AMV is transmitted through seeds of infected plants of alfalfa (Frosheiser, 1964) and pepper (Fegla and Younes, 1999). Hemmati and Mclean (1977) found that seed transmission of AMV ranged from 0.6 to 10.3% in commercial seed production of seven different alfalfa cultivars. According to the results obtained by ELISA, the average incidence of AMV in seeds of alfalfa cv. Beaver was 20.6%, while the average incidence of the virus in seedlings was 7.3% (Pesic and Hiruki, 1986).

The aim of the present work was to compare between indirect ELISA, tissue blot immunoassay (TBIA) and infectivity test for detection of AMV in seeds and seedlings of two alfalfa cuultivars.

MATERIALS AND METHODS

Virus source: AMV isolate 1 isolated from naturally infected alfalfa plants (Fath-Alla,1999) was used and maintained on *Nicotiana glutinosa*.

Virus Purification: Virus was extracted and purified by a modification of the method described by Kaiser and Robertson (1976). One hundred grams of AMV isolate-1 systemically infected *Nicotiana glutinosa* were collected 20-25 days after inoculation and homogenized (1g/1ml) with freshly prepared 0.1 M phosphate buffer pH 7.5 containing 1% 2 mercaptoethanol. The extract was stirred for 10 min with equal volume of a 1:1 cold mixture of n-butanol and chloroform . The emulsion was broken by low speed centrifugation, 8000 rpm at 4°C, using Beckman centrifuge Model J. TB-024F. The clarified extract was left at 4°C for 16 hr, then centrifuged at 8000 rpm for 10 min. the virus was concentrated by two cycles of differential centrifugation, using Beckman L7-65 ultracentrifuge (Rotor type 70-1 T_1).

Virus yield was approximately 15 mg/100g tissue of *N. glutinosa* based on ultraviolet absorption measurements using extinction coefficient E_{260nm} = 5.2 (Noordam, 1973).

Antiserum production: Antiserum was prepared by injecting rabbits five times, each with 2 mg of purified virus. The first two injections were intravenously and the followed three ones were intramuscularly. Injections were done one week apart. In intramuscular injection the virus suspension was emulsified with an equal volume of Freund's complete adjuvant. The microprecipitin test under paraffin oil was used to determine the titer of obtained antiserum (Van Slogteren, 1954).

Preparation of seeds and seedlings for virus detection:

Experiments were carried out on seeds of two cultivars, Siriver and El-Wadi El-Gadid collected from artificially infected plants at 2-3 leaf stage kept in the greenhouse. The harvested seeds were stored in water proof backets at room temperature (20°C) and used for AMV detection in seeds, seed parts (coats and embryo) and seedlings.

For seed tests, two groups each of 200 randomly selected seeds from each cultivar were scarified , plated on wet filter paper in dish and incubated for 48 h at room temperature. Each 5 seeds in the first group were extracted in 1.5 ml PBST (0.01 M phosphate-bufer saline, pH 7.4 containing 0.05% (v/v) tween, 10 g/L PVP and 1 g/L egg albumin) and assayed by indirect ELISA and infectivity test. Seeds of the second group were dissected with a sterile blade under stereomicroscope into seed coat and embryo. Each 5 seed coats/ml buffer and 5 embryos/ml buffer were separately ground in PBST and assayed by indirect ELISA and infectivity test. For seedling tests, harvested seeds from infected plants were scarified and sown in seed trays in the green house at 20°C. Obtained seedlings were indexed at 5 and 21 days after sowing using the aforementioned assays. 21 days old seedlings were indexed also by tissue blot immunoassay (TBIA). Two hundred 5-days

old seedlings of each cultivar were washed and each 5 seedlings was ground in 2 ml PBST and used for virus detection by ELISA and infectivity tests.

Assays used for virus detection:

Infectivity test:

Materials either seeds, seed parts or seedlings were extracted in PBST (0.01 M phosphate-buffer saline, pH 7.4 containing 0.05% (v/v) tween) and inoculated to primary leaves of 12 days-old *Phaseolus vulgaris* L. cv. Contender and resulted necrotic local lesions were counted 5 days after inoculation.

Indirect ELISA:

Indirect ELISA was carried out as described by Fegla *et. al.* (1997).

TBIA:

TBIA was performed as described by Hsu and Lawson (1991). Alfalfa seedlings, 21 days old were divided to 40 groups, each of five. Seedlings of each group were collected together in one bundle by a parafilm membrane and sharply cut with a new razor blade in a steady motion with other hand to obtain a single plane cut surface. The fresh cut surface was pressed, firmly and gently on to nitrocellulose membranes (NSM). (0.45µm Bio. Rod Laboratories, Richmond.

After achieving TBIA, each five seedlings was ground in 2.5 ml PBST for AMV detection by indirect ELISA and infectivity test.

In both tests, indirect ELISA and TBIA antiserum of AMV was used at a dilution of 1:500 and conjugate at dilution of 1:1000. To reduce nonspecific reaction, virus antiserum was diluted 1:500 with filtered extract from healthy plant tissues diluted 1:20 in serum buffer, PBST containing 2% PVP and 0.2% BSA and incubated for 45 min at 37°C, precipitate which had formed was removed by centrifugation for 10 min at 5000 rpm. Percentage of seed infection in case of TBIA was determined directly by counting the number of infected seedlings out of the total tested seedlings, whereas in indirect ELISA and infectivity tests, percentage of seed infection was calculated using the formula of Maury *et al.* (1985).

$$P = [\, 1 - (H/N)^{1/n} \,] \times 100$$

P = The percentage of infection
H = Number of healthy groups
N = Total number of tested groups
n = Number of seeds or seedlings in each group

RESULTS

Characterization of the antiserum:

Antiserum produced and used in this study has dilution end point (titer) 1:256 as determined by microprecipitin test. No reaction was detected with extracts of healthy plants when diluted at 1:16.

Detection of AMV in seeds, seed coats and embryos of alfalfa by ELISA and infectivity tests:

Scarified seeds of each cultivar Siriver and El-Wadi El-Gadid had been allowed to imbibe water for 24 hrs to facilitate seed coat removal.

Results of Table 1 show that ELISA test is more sensitive than infectivity test for detection of AMV in intact seeds, seed coats and embryos of the two cultivars. Percentage of seed infection with AMV detected by indirect ELISA was 5.6% in cv. Siriver and 4.4% in cv. El-Wadi El-Gadid while the percentage of infected seeds were 2.1% and 1.55% in cv Siriver and cv. El-Wadi El-Gadid, respectively when indexed with infectivity test.

The same trend was observed with seed coats and embryos. In contrast to the ELISA test, infectivity test did not detect the virus antigen in the separated seed coats of the two tested cultivars. ELISA test showed that higher proportion of embryos (3.2% and 2.1%) contained AMV as compared with seed coats (1.55% and 1%). Generally the virus was detected with more frequencies in seeds than in embryos or seed coats.

Detection of AMV in seedlings by TBIA, ELISA and infectivity tests :

The incidence of AMV was determined in 5 and 21 days- old seedlings raised from seeds collected from AMV infected plants for comparing with data obtained of seed extract tests as well as to determine the effect of seedling age on virus transmission, estimated by infectivity, ELISA and TBIA tests.

Results are presented in Tables 2 and 3 and Figs 1 and 2. Data concerning detection of virus antigen in 5 and 21 days old seedlings revealed that the proportion of infected plants was higher in 21 day old seedlings than in 5 days old one, when ELISA or infectivity test was used in detection. However, ELISA proved again to be more sensitive than infectivity test (Table 2).

Infection percentage was 6.23% and 9% as determined by ELISA and 3.2% and 6.23% as determined by infectivity test, in 5 and 21 days old seedlings of cv. Siriver, respectively, while it was 4.4% and 9% with ELISA and 2.1% and 4.4% with infectivity test in 5 and 21 days old seedlings of cv. El-Wadi El-Gadid, respectively (Table 2).

When TBIA was used for testing 21 day old seedlings, higher proportion of infected seedlings was detected as compared with ELISA or infectivity test. Infection percentage reached 21% in cv. Siriver and 17% in cv. El-Wadi El-Gadid (Table 2).

Comparison among infectivity test, ELISA and TBIA (Table 3 and Figs 1 and 2) revealed that not all infected samples detected by TBIA were

Fig. 1: Detection of AMV in 21 day old seedling groups of cv Siriver by TBIA. A positive reaction (infected seedlings) was indicated by the development of purple colour on the blots. A negative reaction (uninfected seedlings) was indicated by development of no or green colour.

Fig. 2: Detection of AMV in 21 day old seedling groups of cv El-Wadi El-Gadid by TBIA. A positive reaction (infected seedlings) was indicated by the development of purple colour on the blots. A negative reaction (uninfected seedlings) was indicated by development of no or green colour.

detected with ELISA or infectivity test. For example group No 39 of cv Sirvier was found to include one infected seedling in TBIA while ELISA and infectivity tests showed negative results with this group. Groups of cv El-Wadi El-Gadid No 8, 14, 17, 23, 27 and 31 gave negative results when tested with infectivity test and positive results when tested with ELISA and TBIA. The same trend was observed with samples of cv Siriver. Groups No 12, 24, 25 and 38 showing negative reaction with infectivity test, gave positive reactions with ELISA and TBIA.

Table 1: Detection of AMV isolate 1 in seeds, seed coats and embryos of seed groups of alfalfa, by infectivity and ELISA tests

Test & replicate*	No of AMV infected seed-groups of cultivars					
	Siriver			El-Wadi EL-Gadid		
	Seed	Seed coat	Embryo	Seed	Seed coat	Embryo
Infectivity:						
Replicate-1	1	0	0	1	0	1
Replicate-2	1	0	1	0	0	1
Replicate-3	1	0	1	1	0	0
Replicate-4	1	0	1	1	0	1
Total	4	0	3	3	0	3
**AMV incidence%	2.1	0.0	1.55	1.55	0.0	1.55
ELISA:						
Replicate-1	4	3	3	1	1	2
Replicate-2	2	0	1	3	1	1
Replicate-3	1	0	1	2	0	1
Replicate-4	3	0	1	2	0	0
Total	10	3	6	8	2	4
**AMV incidence%	5.6	1.55	3.2	4.4	1	2.1

*** Each replicate consists of 10 groups each of 5 seeds : (10x5=50 seeds)**
**** AMV incidence percentages were calculated using the formula of Maury *et al*. (1985)**

Table 2: Detection of AMV, at different ages of seedling grown from seeds of infected alfalfa, by TBIA, ELISA and infectivity tests.

Cultivar	Seedling age (Day)	Methods used for AMV detection					
		TBIA		ELISA		Infectivity	
		* No inf. seedings	Infec. %	**No inf groups	*** Infec. %	** No inf groups	*** Infec. %
Siriver	5	N.T.	——	11	6.23%	6	3.2
	21	42	21%	15	9%	11	6.23
El-wadi	5	N.T.	____	8	4.4%	4	2.1%
El-Gadid	21	34	17%	15	9%	8	4.4

N.T.: Not tested
*** : Number of infected seedlings, out of 200 seedling examined.**
**** : Number of infected groups (each of 5 seedlings), out of 40 groups.**
***** : determined by formula of Maury *et. al.* (1985).**

Table 3: Incidence of AMV, isolate-1 in 21-day old seedlings of two alfalfa cultivars detected by three methods.

Group No	Indexing values of infected seedlings					
	El- wadi El- Gadid			Siriver		
	TBIA*	ELISA**	L.L./Leaf***	TBIA*	ELISA**	L.L./Leaf***
C	-	0.063	-	-	0.103	-
1	-	0.080	-	-	0.106	-
2	-	0.083	-	-	0.118	-
3	-	0.081	-	-	0.127	-
4	-	0.071	-	-	0.116	-
5	2	0.577	1	-	0.124	-
6	5	0.885	3	4	0.742	6
7	-	0.098	-	-	0.132	-
8	1	0.471	-	-	0.116	-
9	-	0.095	-	-	0.142	-
10	-	0.077	-	3	0.823	2
11	-	0.112	-	-	0.121	-
12	4	0.651	4	1	0.472	-
13	-	0.107	-	2	0.554	1
14	1	0.495	-	-	0.115	-
15	-	0.111	-	3	0.745	1
16	-	0.112	-	4	0.824	5
17	2	0.452	-	-	0.132	-
18	-	0.097	-	-	0.136	-
19	-	0.090	-	3	0.747	3
20	-	0.101	-	5	0.777	2
21	-	0.063	-	-	0.134	-
22	2	0.557	1	-	0.146	-
23	2	0.548	-	3	0.590	3
24	-	0.094	-	1	0.287	-
25	-	0.084	-	1	0.255	-
26	2	0.550	1	-	0.138	-
27	1	0.496	-	-	0.124	-
28	5	0.740	4	-	0.104	-
29	-	0.074	-	3	0.556	2
30	2	0.550	1	3	0.670	2
31	1	0.374	-	-	0.147	-
32	-	0.063	-	-	0.106	-
33	-	0.102	-	4	0.563	1
34	2	0.576	1	-	0.136	-
35	-	0.081	-	-	0.127	-
36	-	0.091	-	-	0.130	-
37	-	0.118	-	-	0.118	-
38	2	0.579	2	1	0.306	-
39	-	0.090	-	1	0.110	-
40	-	0.100	-	-	0.119	-
Total	34	15	8	42	15	11
Infection%	17	9	6	21	9	6.23

*** :No of infected seedlings out of 5 tested**

**** :Extracts of groups each of 5 were used for ELISA and infectivity tests.**

*****: Primary leaves of *Phaseoslus vulgaris* cv. Contender.**

DISCUSSION

The studied AMV was shown to be seed borne and seed transmissible. Such results were reached by other investigators (Avgelis and katis, 1989; Bailiss and Offei , 1990 and Frosheiser, 1974). The virus incidence was obviously detected in intact infected alfalfa seeds as well as in seed portions, i.e., embryo and seed coat, using infectivity and ELISA tests. The rate of AMV occurrence was 0.0 -1.55%, 1.55 - 3.2% and 1.55-5.6% in seed coat, embryo and seed respectively, with ELISA to be more sensitive in virus detection and cv. Siriver to had more rate of seed trans mission, in addition, infective AMV was recovered from some embryoes but never from testas by infectivity test. ELISA test detected the viral antigen in seed coats and embryoes. However, relatively higher proportion of embryos was found to contain AMV antigen (2.1-3.2%) as compared with testas (1-1.55%). Baillis and Offei (1990) detected also infective AMV in some embryos but never in testas. On the other hand, They showed by ELISA, that higher proportion of testas (17.1%) contained AMV as compared with embryos (4.5%) and both testa and embryo infection was detected in 13.5% of the seed tested.

Infectivity test, ELISA and TBIA were used to detect the virus in the seedlings raised from seeds formed on AMV infected plants. Results showed that ELISA, again, was the more sensitive test for virus detection in 5 days old seedlings as compared with infectivity test. The same conclusion was reached by Bailiss and Offei (1990). They found that the proportion of infected seedlings detected by ELISA was higher than that detected by infectivity test for 5 day old seedlings. They added, that these differences progressively decreased in 11 and 17 days old seedlings until both indexing methods identified the same infected seedlings after 23 and 29 days. However our results showed that proportion of infected seedlings detected by ELISA and infectivity test increased by increasing seedling age from 5 to 21 days, but ELISA was still more sensitive than infectivity test.

TBIA is the most recent serological method. It was first used by Lin *et al.*(1990) for detection of some plant viruses and mycoplasma like organism and by Hsu and Lawson (1991) for detection of tomato spotted wilt virus. This test was also used for detecting barley yellow dwarf virus in cereals (Makkouk and Comeau, 1994) and faba bean necrotic yellows virus in faba bean (Abdel – Salam *et al.* 1997) and AMV in pepper (Fegla and Younes,1999).

When TBIA was used in addition to ELISA and infectivity test for AMV detection in 21 days old seedlings, results showed that percentage of infected seedlings calculated for TBIA was higher (17-21%) than that calculated for ELISA (9%), although the groups containing infected seedling (s), as detected by TBIA, are nearly the same groups giving positive reaction in indirect ELISA. This could be attributed to the fact that in TBIA, the infection percentage was calculated directly from the number of infected seedlings, observed in the blots, out of total tested seedlings, while in ELISA, it was estimated by the equation of Maury *et al.*(1985). TBIA proved to be simpler and more practical than ELISA. The same conclusion was reached by Makkouk and Kumari (1996)

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الكشف عن فيروس موزيك البرسيم الحجازى فى بذور وأجزاء بذور وبادرات صنفين من البرسيم الحجازى

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تم استخدام اختبارات القدرة المعدية infectivity test ، الاليزا غير المباشرة indirect ELISA والبصمة النسيجية tissue blot immunoassay (TBIA) للكشف عن فيروس موزيك البرسيم الحجازى فى بذور كاملة وأجزاء بذور وبادرات صنفين من البرسيم الحجازى. وكانت الاليزا غير المباشرة أكثر حساسية من اختبار القدرة المعدية فى الكشف عن الفيروس فى البذور وأجزائها (قصرة البذرة، الجنين) وكذلك فى البادرات.

وصلت نسبة البذور المصابة الملاحظة بالاليزا الى ٥,٦% فى صنف سيريفير و٤,٤% فى صنف الوادى الجديد. بينما كانت ٢,١% و ١,٥٥% فى صنفى سيريفير والوادى الجديد على التوالى عندما قدرت بإختبار القدرة المعدية. هذا ولم يلاحظ الانتيجن الفيروسى بواسطة أختبار القدرة المعدية فى قصرة البذور المفصولة لصنفى البرسيم الحجازى. أظهر أختبار الاليزا أحتواء نسبة أعلى من الاجنة على فيروس موزيك البرسيم الحجازى مقارنة بقصرة البذور.

أظهرت النتائج المتعلقة بالكشف عن الفيروس فى البادرات أن نسبة النباتات المصابة كانت أعلى فى البادرات ذات عمر ٢١ يوم عن البادرات ذات عمر ٥ أيام عندما أستخدمت الاليزا أو أختبار القدرة المعدية فى التقدير. كما بين اختبار البصمة النسيجية عند الكشف عن الفيروس فى البادرات ذات عمر ٢١ يوم عن وجود نسبة اعلى من البادرات المصابة (٢١% فى صنف سيريفير و١٧% فى صنف الوادى الجديد) مقارنة بالاليزا او اختبار القدرة المعدية. ولقد اتضح أن اختبار البصمة النسيجية اكثر بساطة وذات ميزات عملية اكثر من اختبار الاليزا.