

## ALFALFA MOSAIC ALFOMOVIRUS IN ALFALFA FLORAL PARTS, PODS AND SEEDS AT DIFFERENT STAGES OF DEVELOPMENT.

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### ABSTRACT

Infectivity test and indirect ELISA were used for detection of alfalfa mosaic alfamovirus (AMV) in flowers, pods, intact seeds and seed parts of alfalfa cv. El-Wadi El-Gadid during maturation. Infective virus incidence determined by infectivity test or viral antigen incidence determined by indirect ELISA decreased with maturation. ELISA was more sensitive than infectivity test for detection AMV in flowers, pods, seeds, seed coats and embryos. Higher percentage of AMV infection was detected in male sex organ (androecium) as compared with the female one (gynoecium). Infectivity test did not detect infective virus in the separated seed coats. Indirect ELISA showed that higher proportion of embryos contained AMV antigen as compared with seed coats.

Results showed that alfalfa seeds contain virus inhibitors. Seed extracts diluted at 1:20 (w/v) significantly reduced number of local lesions produced by AMV, whereas those diluted at 1:100 and 1:250 did not affect lesion counts.

### INTRODUCTION

Alfalfa mosaic alfamovirus (AMV) is one of the most important viruses affecting alfalfa (forster *et al.*, 1985; Dall *et al.*, 1986; Miczynski 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 Fegla *et al.*, 2000) and pepper (Fegla and Younes, 1999).

AMV often reaches high incidence in alfalfa seed (Frosheiser, 1974) but levels may be considerably influenced by virus strain, host cultivar, environmental conditions and method of virus detection (Frosheiser, 1974; Hiruki and Miczynski, 1987 and Fegla *et al.*, 2000).

Infected seed is of major importance in the epidemiology of AMV. Randomly distributed primary infection result from such seed (Frosheiser, 1970 and Hemmati and McLean, 1977) and subsequent secondary spread by several aphid species (Crill *et al.*, 1970) often leads to decreases in forage yield (Gibbs, 1962; Tu and Holmes, 1980; Bailiss and Ollennu, 1986 and Fath-Alla, 1999).

The extent of seed transmission of any virus depends on many factors but survival of virus during seed maturation has received relatively little attention (Bailiss and Offei, 1990).

Infectivity and ELISA tests (Frosheiser, 1974; Pesic and Hiruki, 1986; Bailiss and Offei, 1990 and Fegla *et al.*, 2000) are the most practical means of indexing alfalfa seed for infective AMV and AMV antigen, respectively. The major aim of such tests is to determine the proportion of seeds which will produce virus – infected plants.

The aim of the present work is to use infectivity and ELISA assays to follow changes in infective virus and viral antigen associated with alfalfa floral parts, pod and seed at different stages of maturation.

## **MATERIALS AND METHODS**

Isolate No. 1 of AMV used in this study was originally obtained from a naturally infected alfalfa cv. El-Wadi El-Gadid grown at Desert Development Center Farm (Fath-Allah, 1999). The isolated AMV was maintained on *Nicotiana glutinosa* which served as a source of the virus for the subsequent studies.

### **Antiserum:**

AMV antiserum used in this study was locally prepared (Fegla *et al.*, 2000). The antiserum has a dilution end point (Titer) of 1:256 as determined by microprecipitin test.

### **Experimental plants:**

AMV – free plants of alfalfa cv. El-Wadi El-Gadid kept in the green house were sap-inoculated with AMV from infected *N. glutinosa* leaves ground in SB buffer (0.07M Sorensen's phosphate buffer pH 7.5), with tissue buffer ratio of 1:100 (w/v). Plants to be inoculated were kept in dark for 48 hrs then their leaves were first dusted with carborundum (600 mesh) before inoculation. Inoculated plants were observed for symptoms development and infected plants were labeled and kept for flowering and seed production. Flowers, pods and seeds were collected periodically from labeled plants producing abundant inflorescences and pods. AMV-free plants grown at the same time on a separate part in the green house provided control material.

### **Extraction for infectivity tests and ELISA:**

Flower parts were dissected from flowers and rinsed after each dissection. The flowers and their parts were washed under running tap- water for 30 min to remove any contaminating virus, then divided to 20 groups, each of five. Each group of flowers and flower organs were separately ground in 1.5 ml and 1 ml SB buffer, respectively and assayed by indirect ELISA and infectivity test.

After allocation on the appropriate maturation category (Table 1), pods were dissected to remove the seeds. Seeds and pods were divided to 20 groups, each of five, then separately washed as above for 30 min. Each group was separately group in 1 ml SB buffer and assayed by infectivity test and ELISA. Harvested seeds stored for 3 and 6 months were scarified, then placed on moist filter paper in Petri dishes and allowed to imbibe water for 24 h at 22°C. Each seed was cleanly dissected into seed coat and embryo and these were separately decontaminated by rinsing as before. Each 5 seeds, 5 seed coats and 5 embryos were separately ground in 1.5, 1 and 1 ml SB buffer, respectively and assayed by indirect ELISA and infectivity test.

**Table 1: Seed maturation and storage categories of alfalfa cv. El-Wadi El-Gadid**

Stage	Category	Features
I	Days after pod set 0-20	Seeds green
II	21-30	Seeds yellow and hard
III	31-40	Seeds mature and brown
IV	Storage of mature seeds 3 months	Seeds mature and brown
V	6 months	Seeds mature and brown

**Infectivity tests and ELISA:**

Extracts in SB were inoculated to primary leaves of 12 days old *phaseolus vulgaris* L. cv. Contender and the resulting necrotic local lesions were counted 7 days after inoculation. Indirect ELISA was carried out as described by Fegla et al., (1997). In indirect ELISA and infectivity tests, percentage of 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 flower parts and pods in each group.

**Inhibitions:**

Endogenous inhibitors which may effect lesion counts on bean and /or possibly play an *in vivo* role in alfalfa tissue were extracted from seed gathered from plants previously indexed and shown to be AMV-free. Dry seeds were ground in a ball mill, whereas immature seeds were ground in a pestle and mortar. Distilled water to give a weight/volume ratio of 1:10 and the mixture stirred for 10 min. After recovering the supernatant following centrifugation at 3000g for 15 min, the extract was diluted further with distilled water so that after mixing with an equal volume of either SB (control extract) or sap from AMV-Infected alfalfa leaves (test extract) the desired final weight / volume dilution specified in the results was obtained.

Leaf sap preparation were made from systemically infected alfalfa leaves ground in SB (tissue:buffer ratio 1:50 w/v) then centrifuged at 3000g/15 min. Inoculations were made to primary leaves of 12 days old *phaseolus vulgaris* for comparisons between test extracts and controls. Mean lesion counts, were compared using T-test after lesion counts had been transformed (Kleckowski, 1955). There were at least three leaves comparison per treatment in all experiments.

**RESULTS**

**AMV incidence in alfalfa flower and flower parts:**

Results presented in Table (2) indicate that AMV could be detected by infectivity test and ELISA in flowers and sexual organs of infected alfalfa plants cv. El-Wadi El-Gadid. Howere ELISA was found to be more sensitive

than infectivity test. ELISA detected the viral antigen in the flower organs whereas infectivity test failed to detect AMV in calyx and corolla floral parts. Both tests recorded higher percentage of infection in male sex-organ (androecium) as compared with the female (gynoecium).

The incidence of AMV in flower, androecium and gynoecium of infected plants decreased as flowers matured.

**Table 2: Incidence of AMV as infective virus or antigen in the flowers of infected alfalfa plants cv. El-Wadi El-Gadid**

test	stage ****	AMV detected in									
		flower		calyx		corolla		androecium		gynoecium	
		No infect. group*	Infect. %**	No infect. group*	Infect. %**	No infect. group*	Infect. %**	No infect. group*	Infect. %**	No infect. group*	Infect. %**
Infectivity Test ***	1	7	8.30	0	0	0	0	8	9.72	5	5.60
	2	2	2.09	0	0	0	0	4	4.32	2	2.09
ELISA***	1	14	20.39	4	4.37	5	5.60	18	39.91	10	13.45
	2	11	14.8	2	2.09	2	2.09	14	20.39	7	8.30

\* : Number of infected groups ( each of 5) , out of 20 groups

\*\* : determined by formula of Maury et al., (1985)

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

\*\*\*\*: Flowers were collected at bud stage (1) and 15 days later (2).

**AMV incidence during pod maturation of alfalfa:**

The same trend was observed with pods (Table 3). The incidence of AMV recoverable from pod tissue progressively decreased as pods matured, with a marked decline in the proportion of infected pods from stage I (8.26, 39.91%) to stage II (3.20, 18.94%) and stage III (1.03, 2.09%) when indexed by infectivity test and ELISA, respectively.

Pericarp has the lower percentage of virus infection. Infectivity test failed to detect the infective virus in the pericarp at the second and third stages of pod development while ELISA failed to detect the viral antigen at the third stage of pod development (Table 3).

**Table 3: Incidence of AMV as infective virus or antigen in pods of infected alfalfa plants cv. El-Wadi El-Gadid.**

stage	Incidence of AMV							
	Infectivity test****				ELISA ***			
	Pod		Pericarp		Pod		pericarp	
	No infect. Group*	Infect. %**	No infect. group*	Infect. %**	No infect. group*	Infect. %**	No infect. group*	Infect. %**
I	7	8.26	1	1.03	18	39.91	3	3.20
II	3	3.20	0	0	13	18.94	1	1.03
III	1	1.03	0	0	2	2.09	0	0

\* : Number of infected groups (each of 5 pods) , out of 20 groups

\*\* : determined by formula of Maury et al., (1985)

\*\*\* : Extract of groups each of 5 were used for ELISA and infectivity tests

**AMV incidence during seed maturation:**

Results presented in Table (4) indicate that infective virus-inducing local lesions within 7 days of inoculation were recovered from 11 groups out of 20 groups tested (14.77%). There was a similar trend in the incidence of AMV in seed, where incidence progressively decreased during the green seed (stage 1) and during ripening and dehydration (stages II and III).

Infective virus incidence in mature seeds was 8.26% compared with 14.77% in immature seed. Further tests after storage for 3 and 6 months gave incidence values of 4.37 and 2.09% respectively.

Higher percentage of viral antigen was detected by ELISA in all seed maturation stages I, II and III, as compared with infectivity test. However, viral antigen decreased as seeds matured and reached 8.26% and 6.89% in matured seeds after storage for 3 and 6 months, respectively (Table 4).

**Table 4: Incidence of AMV during maturation of alfalfa seeds, seed coats and embryos of seed groups determined by infectivity test and ELISA.**

Test	stage	AMV detected in					
		Seed		Seed coat		embryo	
		No inf * groups	Infec **%	No inf * groups	Infec **%	No inf * groups	Infec **%
Infectivity test***	1	11	14.77	N.T	N.T	N.T	N.T
	11	8	9.72	N.T	N.T	N.T	N.T
	111	7	8.26	0	0	5	6.89
	1V	4	4.37	0	0	3	3.20
	V	2	2.09	0	0	2	2.09
ELISA test ***	1	17	31.58	N.T	N.T	N.T	N.T
	11	15	24.22	N.T	N.T	N.T	N.T
	111	12	16.75	4	4.37	9	11.72
	1V	7	8.26	2	2.09	5	5.60
	V	6	6.89	1	1.03	4	4.37

\*: Number of infected groups ( each of 5 ) , out of 20 groups

\*\* : determined by formula of Maury et al., (1985)

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

N.T: Not tested.

**AMV incidence in seed coat and embryo of alfalfa:**

The incidence of AMV as infective virus or antigen in seed coats and embryos was examined. Results in Table (4) showed that AMV remaining on the seed coat was not detected by the Infectivity assay. However AMV was recovered from some embryos with percentages 6.89, 3.20 and 2.09 at stages III and after storage for 3 months (1V) and 6 months (V) respectively. ELISA showed that higher proportion of embryos (11.72, 5.60 and 4.37) contained AMV antigen as compared with seed coats (4.37, 2.09 and 1.03) at stage III and after storage for 3 and 6 months respectively.

**Seed inhibitors:**

Results with seed extracts made from seed at different maturation stages and after storage for 3 and 6 months showed that extracts diluted at 1:20 (w/v) from such seeds significantly reduced lesion counts (Table 5). The degree of inhibition increased as seeds matured. Inhibition was abolished when extracts were diluted at 1:100 (w/v) or 1:250 (w/v).

**Table 5: Effect of extracts of alfalfa (cv. El-Wadi El-Gadid) seed at five maturation stages on lesion numbers produced by AMV on *Phaseolus vulgaris***

Seed maturation stage	Seed extract dilution (w/v)	Mean lesion counts / leaf <sup>a</sup>		
		Treatment	Control <sup>c</sup>	Decrease (%) <sup>d</sup>
I	1:20	73 <sup>b</sup>	123	40.60
	1:100	119	127	6.29
	1:250	117	124	5.64
II	1:20	57	116	50.86
	1:100	109	128	14.84
	1:250	108	112	3.57
III	1:20	45	120	62.50
	1:100	106	124	14.51
	1:250	114	120	5.00
IV	1:20	31	123	74.79
	1:100	107	126	15.07
	1:250	110	120	8.33
V	1:20	23	125	81.60
	1:100	111	127	12.59
	1:250	109	122	10.65

a. Mean of three treatment

b. Counts significantly less than control: \* p=0.05

c. AMV- infected alfalfa leaf sap mixed with distilled water to give a final sap dilution of 1:100 (w/v).

d. Percentage decrease in lesion counts caused by treatment.

## DISCUSSION

This study has shown that marked changes in incidence of both infective AMV and antigen occur in alfalfa floral parts, pods and seeds as they mature.. Infective virus and antigen were recovered from androecium and gynoecium of infected alfalfa plants, showing that the virus had systemically invaded the male and female reproductive organs of some alfalfa flowers.

The virus was recovered with higher percentages from male sex organs than from female one. Such findings are in line with those reported by Frosheiser (1974) and Hemmati and Mclean (1977) who found that the virus enters the seed both through the ovule and in pollen grain, with pollen grain transmission being more frequent than ovule transmission, the transmission frequency through pollen grains ranged from 0.5 to 26.5% and transmission through the ovules ranged from 0 to 9.5%. On the other hand Bailiss and Offei, (1990) suggested that ovule transmission was more frequent than pollen grain transmission.

The incidence of AMV antigen or infective virus decreased as pod matured, with only 1.03% and 2.09% of pods containing infective virus and AMV antigen respectively at maturation stage 5. Bailiss and Offei, (1990) likewise found the incidence of virus decreased to 3-6% after alfalfa pods became dry.

AMV incidence in seeds during maturation followed a similar pattern to that in pods. Presence of the virus in immature seed showed that virus had successfully invaded such seed either in the maternally-derived testa and /or embryo via an infected megaspore mother cell or infected pollen grain.

Absence of AMV antigen in embryo sac noted by Pesic *et al.*, (1988) suggests that embryo infection might occur solely or predominantly via infected male gametes during fertilization

The virus was detected in intact infected alfalfa seeds using infectivity test and ELISA. Infective AMV was recovered from some embryos but never from testas by infectivity test. ELISA test detected the viral antigen in seed coats and embryos. However relatively higher proportion of embryos was found to contain AMV antigen as compared with testas. Bailiss 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.

Our results showed that infective virus was declined during seed maturation or after storage of harvested seeds. Decreases in the incidence of infective virus in mature seed, compared with immature one may be attributed in a part to the presence of inhibitors in seeds. Such inhibitors were detected and their amount and /or potency increased as alfalfa seed matured when extracts were diluted at 1:20 (w/v). Such results agree with those reported by Bailiss and Offei, (1990).

Non- infective AMV antigen in seed coats makes the estimation of virus incidence in seeds by ELISA unreliable. The occurrence of such false positive when individual mature seeds were tested could cause the rejection of a seed lot which was in fact free from infective AMV and would not give rise to infected seedling. Reliable results could be obtained when seedlings were used for virus detection. Bailiss and Offei, (1990) used seedlings at 17 or more days old for AMV detection by either ELISA or infectivity tests. Such tests would give the best measure of the proportion of seed giving rise to infected plants. ELISA often produced higher incidence values than infectivity tests (Bailiss and Offei, 1990). Fegla, *et al.*, (2000) used tissue blot immunoassay (TBIA), indirect ELISA and infectivity test for detection of AMV in seedlings and found that TBIA revealed higher proportion of infected seedlings as compared with indirect ELISA or infectivity test.

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تواجد فيروس موزايك البرسيم الحجازي في الأجزاء الزهرية والقرون والبذور في نباتات البرسيم الحجازي خلال مراحل الكشف المختلفة.  
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تم الكشف عن تواجد فيروس موزايك البرسيم الحجازي في الأزهار والقرون والبذور الكاملة و أجزاء من بذور البرسيم الحجازي صنف الرادي الجديد وذلك خلال مراحل النضج المختلفة باستخدام اختبارات القدرة المعدية infectivity test وطريقة الأليزا غير المباشرة indirect ELISA وأوضحت النتائج إن نسبة الفيروس المعدية المقدرة باختبار القدرة المعدية أو نسبة الانتجين الفيروسي المقدرة باختبار الأليزا غير المباشرة تتخفف مع النضج وإن اختبار الأليزا غير المباشرة كان أكثر حساسية من اختبار القدرة المعدية في الكشف عن الفيروس في الأزهار والقرون والبذور الكاملة و القصرة والجنين. إن القدرة المعدية لم تكشف عن وجود الفيروس في القصرة وظهر اختبار الأليزا احتواء نسبة عالية من الأجنة على انتجين فيروس موزايك البرسيم الحجازي مقارنة بقصره البذور ووجدت نسبة أعلى من الإصابة بالفيروس في الأعضاء الجنسية المذكورة للأزهار (الطلع) مقارنة بالأعضاء المؤنثة (المتاع)

وقد أظهرت النتائج المتحصل عليها احتواء بذور البرسيم الحجازي على مثبطات لفيروس موزايك البرسيم الحجازي إذ أدت مستخلصات بذور البرسيم المخففة بنسبة ٢٠:١ (وزن/جم) إلى خفض معنوي في عدد البقع الموضعية المنتجة بواسطة الفيروس، بينما لم يكن لتخفيفات مستخلصات البذور ١٠٠:١ و ٢٥٠:١ (وزن/جم) أي تأثير معنوي على عدد البقع الموضعية.