Techniques for Assaying Alfalfa Susceptible to Alfalfa Mosaic Virus

Pat Crill, D. J. Hagedorn, and E. W. Hanson

Department of Plant Pathology, University of Wisconsin, Madison 53706. Present address of senior author: University of Florida, Gulf Coast Experiment Station, Bradenton 33505.

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ABSTRACT

When the relative sensitivity of three local lesion assays for alfalfa mosaic virus (AMV) was studied, the assay involving number of lesions per bean leaf was superior to those concerned with number of lesions per cowpea leaf or maximum number of lesions per square centimeter of cowpea leaf. Ten-day-old bean plants grown at 24 C developed the most discrete and readable lesions upon inoculation with AMV, as compared to beans grown longer and at other temperatures. Studies on the effect of time of day of inoculation indicated most lesions developed after beans had been exposed to at least 12 hr of light. Although alfalfa cultivars Ranger and Vernal reacted in an opposite manner, light intensities in the range 100-1,400 ft-c had no significant effect on the development of AMV. More virus developed in Glacier alfalfa under a 6-hr photoperiod than under 12 or 18 hr. There was a positive relationship between increased AMV development and increasing temperature up to 28 C. Phytopathology 60:1517-1520.

Additional key words: alfalfa mosaic virus, assaying alfalfa for AMV, bean local lesion assay for AMV.

The very real importance of the alfalfa mosaic virus (AMV) in Wisconsin alfalfa (*Medicago sativus* L.) was recently pointed out by Crill et al. (3). The most promising control measure for this disease appeared to be development of resistant varieties. The isolation of alfalfa genetic material conditioning resistance to AMV is dependent upon the investigator recognizing such material. Previous reports (1, 4, 5, 6, 8, 9, 10) indicated that considerable difficulty would be encountered. The investigations reported herein were conducted to determine optimum environmental conditions for recognition of alfalfa plants susceptible to AMV. Methods of assaying for AMV, effects of environment on assays, and on AMV development in alfalfa were studied.

When Weimer (10) first described alfalfa mosaic, he noted that the disease was most obvious during the cooler seasons, especially in spring before the first cutting. Other researchers (1, 5, 7, 11) reported that AMV symptoms in alfalfa are masked during the hot summer. However, Diachun & Henson (4) found symptoms suppressed at low temp but prominent in the summer. Panzer (9) studied the effect of temp on the bean (*Phaseolus vulgaris* L.) AMV assay host, and found that this plant was most susceptible to AMV at moderate temp. These and other studies which did not complement one another prompted the experiments described below.

METHODS AND RESULTS.—*Relative sensitivity of three local lesion assays for AMV.*—Two hosts, Bountiful bean and cowpea (*Vigna sinensis* 'California No. 5 Blackeye') are local lesion hosts for AMV and adapted for measuring relative infectivity of the virus. The relative sensitivity of each of these hosts was compared to obtain the most sensitive and presumably the most precise measure of AMV relative infectivity.

Primary leaves of bean and cowpea were dusted with 400-mesh Carborundum and inoculated with plant sap obtained from alfalfa infected with AMV-isolate 514. Three dilutions 1:2, 1:10, and 1:100 (w/v) were rubbed onto the leaves with a cheesecloth pad. Four different sources of inocula were used; alfalfa leaves from plants grown at 6, 12, and 18 hr of daylight, and flowers from alfalfa plants grown at 18 hr of daylight. All source plants were grown at 16 C, 2,000 ft-c light, and 70% relative humidity (RH). Bean and cowpea plants were grown under the same light and RH conditions, but at 24 C and a 12-hr day length.

The means reported in Fig. 1 are the averages of 28 observations from three experiments, and are all from the 1:10 (w/v) dilution. (The 1:2 dilution resulted in so many lesions that an accurate count was not possible.) Lesions were counted by three different methods; number of lesions per bean leaf, number of lesions per cowpea leaf, and max number of lesions per cm^2 of cowpea leaf. (Lesions per cm^2 bean leaf were too few to constitute a valid measurement.) Slopes of the 3 curves (Fig. 1) indicated that number of lesions per bean leaf was a more sensitive method of measuring amounts of AMV than was total number of lesions per cowpea leaf or max number of lesions per cm^2 of cowpea leaf.

Factors affecting susceptibility of Bountiful bean to AMV.—Effect of plant age and postinoculation temp.—The effect of postinoculation temp and age of Bountiful bean plant on the symptom development of AMV was determined with three separate strains of virus. Primary leaves of 10-, 12-, 14-, and 16-day-old bean plants which were grown at 24 C were inoculated with AMV at a 1:2 dilution (w/v) and immediately placed at temp of 16, 20, 24, and 28 C. Plants were observed daily for the development of symptoms. Trials with AMV strains 515 and 657 were replicated three times; those with strain 514, four times. Plant age was the number of days from planting to inoculation.

These data were recorded as the number of days

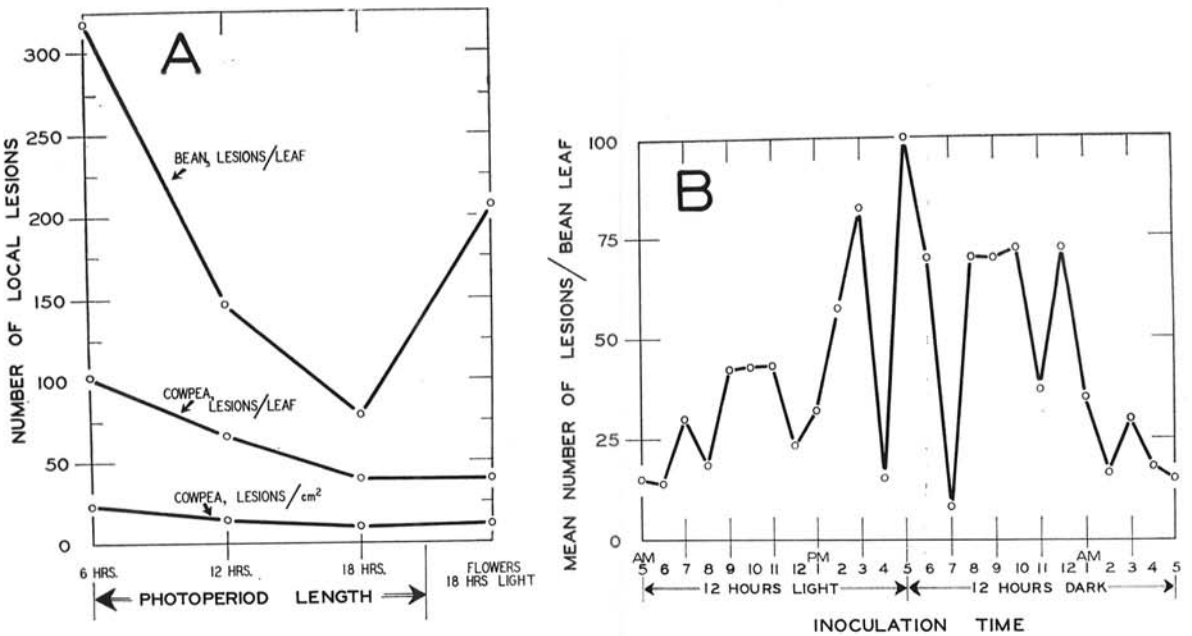

Fig. 1. A) Relative sensitivity of three local lesion assays for alfalfa mosaic virus (AMV) in alfalfa leaves grown under three photoperiods and in alfalfa flowers produced under an 18-hr light regime. **B)** The effect of time of day of inoculation upon the sensitivity of Bountiful bean leaves in local lesion assays for AMV.

from time of inoculation until symptoms developed (Table 1). They were summarized as the total number of treatments which contained infected plants. At 16 C, the number of plants infected was about half that at 20, 24, or 28 C. Severity of symptoms was also much reduced at 16 (Table 1). Likewise, there was a substantial decrease in susceptibility of Bountiful bean as age of plant increased. When grand sums of plant ages were compared there were 11 susceptible at 10 days, 6 at 12 days, 7 at 14 days, and only 3 at 16 days. It appeared that 10-day-old bean plants at 20, 24, or 28 C were the most susceptible, but plants grown at 24 C developed the most discrete and readable lesions.

Effect of time of day of inoculation.—Desiccated pea (*Pisum sativum* L.) tissue containing AMV strain 514 was triturated with a mortar and pestle in distilled water, and the resulting sap rubbed onto the primary bean leaves which had been previously dusted with 400-mesh Carborundum. Uniformity was emphasized both in the application of Carborundum and the rubbing of infective sap. The experimental design was a completely randomized design with eight replications of one plant each. Inoculations were made every hr for 24 hr. Environmental conditions in which the beans were grown from time of planting were 24 C, 70% RH, and 2,000 ft-c of light with a day-length of 12 hr. The light period began at 5:10 AM. Lesions were first visible 2 days after inoculation, and were counted 3 days later.

Results (Fig. 2) were similar to those reported earlier (9), in that considerable variation occurs. There was no straight-line relationship between photoperiod and number of lesions per leaf, but rather a series of high and low peaks. The over-all trend was for numbers of lesions to increase with increased hr of light and to decrease with increased hr of dark. Very highly significant differences (.005 level) existed among times of inoculation. The greatest number of lesions occurred when bean plants were inoculated after exposure to 12 hr of light.

Effect of environment on development of AMV in alfalfa.—Light intensity.—Certified alfalfa seeds of Ranger and Vernal alfalfa cultivars were germinated in saucers of vermiculite and transplanted (5 plants/pot) into 6-inch pots containing a 3:1 mixture of compost soil to sand. The plants were grown at 21 C for 2 weeks, at which time they were mechanically inoculated with AMV. Immediately after inoculation, the plants were moved to growth chambers at light intensities of 100, 550, 950, and 1,400 ft-c of light as measured 6 inches above the upper foliage. The photoperiod was 14 hr of light and 10 hr of dark. Pots were arranged in the chambers in a split-plot design with varieties as mainplots and light intensities as subplots with four replications. Plants were kept at these conditions for 37 days, at which time virus development was determined by measuring relative infectivity using Bountiful bean as a local lesion assay host. Beans were grown in 4-inch pots, and were 12 days old when the primary leaves were dusted with 400-mesh Carborundum and inoculated with expressed sap from the alfalfa plants. To obtain readable assays, the inoculum was applied at dilutions of 1:2, 1:10, and 1:100 (w/v) in distilled water. The most useful counts were obtained with the 1:10 dilution, and all counts reported are from this dilution. Lesions were counted 3 days after they first appeared.

Differences between cultivars as well as differences

TABLE 1. Number of days from time of inoculation of Bountiful bean to development of symptoms with three isolates of alfalfa mosaic virus at four plant ages and four temp

Temp	Plant age	Isolate no. 515	514	657	Sum of treatments containing infected plants	Grand sum temp	Grand sum plant age
C	*days*						
16	10	4	5	0	2		11
	12	4	—[a]	0	1	4	6
	14	6	0	0	1		7
	16	0	0	0	0		3
20	10	3	3	3	3		
	12	3	—	3	2	8	
	14	4	4	0	2		
	16	3	0	0	1		
24	10	3	3	3	3		
	12	4	—	3	2	8	
	14	4	3	0	2		
	16	4	0	0	1		
28	10	3	4	3	3		
	12	3	—	0	1	7	
	14	3	4	0	2		
	16	4	0	0	1		

[a] — = data not available; 0 = no symptoms developed. All readings based on three or more replications.

among light intensities appeared to be considerable, but were not significant (Table 2). There was, however, a strikingly opposite reaction of the two alfalfa cultivars to the light conditions studied. Less AMV developed in Ranger as light intensity increased; more developed in Vernal.

Photoperiod.—Four-week-old rooted cuttings of AMV-infected Glacier-4 alfalfa clone were transplanted into compost soil in 4-inch pots, 1 plant/pot. Plants were maintained in growth chambers for 2 weeks in a completely randomized design with four replications and three treatments of 6, 12, and 18 hr of daylight. The temp was 16 C; light intensity, 2,000 ft-c; and RH, 70%. Bountiful bean plants for the assay were grown at the same light intensity and RH, but at 24 C.

TABLE 2. Effect of light intensity, photoperiod, and temp on the development of alfalfa mosaic virus in alfalfa as indicated by local lesion assay on Bountiful bean

Alfalfa strain	Light intensity in ft-c 100	550	950	1,400
Ranger	110[a]	41	51	44
Vernal	48	64	75	121
	Photoperiod in hr			
	6	12	18	
Clone G 4	194[b]	18	19	
	Temp, C			
	16	20	24	28
Clone V 8	16	74	89	116

[a] Data indicate mean number local lesions per bean leaf. Light intensity differences were not significant. Coefficient of variability:light intensity 85.8%; varieties 99.9%.

[b] Photoperiod data coefficient of variability = 40.5%. LSD .01 = 143.5; HSD .01 = 169.4.

The upper foliage of the alfalfa plants was removed and triturated, and the 1:2 diluted sap rubbed onto Carborundum-dusted primary leaves of 10-day-old Bountiful beans. Each alfalfa plant was assayed on two bean leaves, and number of lesions per leaf was counted 7 days after inoculation.

These data are presented in Table 2 as mean number of lesions per bean leaf. The difference in development of AMV in alfalfa plants grown at 12 and 18 hr of daylight was not significant. The differences between 6 and 12 hr and 6 and 18 hr were, however, highly significant.

Temperature.—To study the effect of temp, stem cuttings of alfalfa clone Vernal 8 were rooted in sand for 1 month, then transplanted into compost soil in 4-inch pots. The plants were grown at 16 C for 1 week before being randomly assigned to four treatment temp of 16, 20, 24, and 28 C. Other environmental conditions consisted of a 12-hr light intensity of 2,000 ft-c at the plant surface and RH of 70%. Plants were maintained at treatment temp for 2 weeks, then inoculated with AMV. Noninoculated control plants were included. Two weeks after inoculation, the upper foliage was removed, triturated in a mortar with a pestle at 1:4 (w/v) dilution in distilled water, and the resulting sap rubbed onto Carborundum-dusted primary leaves of Bountiful bean which had been grown for 10 days at 24 C with a 12-hr day-length at 750 ft-c of light and 70% RH. In each of two trials, at least five alfalfa plants were studied at each temp, and 28 to 36 bean leaves were used for assaying test plants from each treatment.

Chlorotic rings as well as interveinal, semirectangular chlorotic blotches were evident on alfalfa leaves growing at the three lower temp, but were most evident at 20 and 24 C. No symptoms were observed at 28 C. Local lesions on bean first appeared 2 days after inoculation and were counted 3 days later. There was a positive relationship between increased AMV development as measured by numbers of local lesions on Bountiful bean and increasing temp (Table 2). About seven times more lesions were produced by sap from plants growing at 28 than from plants at 16 C, even though symptoms never developed in alfalfa at 28 C. It appeared that concn of AMV was greatest in plants grown under warmer temp.

DISCUSSION.—The elucidation of the environmental factors affecting development of the disease in alfalfa caused by AMV provides background necessary for development of a screening program. Determination of environmental effects on assay hosts and establishment of local lesion assays on bean primary leaves as more sensitive than either assay with cowpea should result in more reliable results from future screening tests. Thus, the ideal screening program for determining the presence of AMV in alfalfa would entail the use of Bountiful bean leaves grown at 24 C for 10 days with a 12-hr photoperiod.

Panzer (9) concluded that beans were more susceptible to AMV when conditioned at moderate temp with a marked decrease occurring below 15 C. Results of the postinoculation temp studies reported herein were

similar to Panzer's (9) preinoculation temp results. Results of the time of day of inoculation experiments are comparable to those obtained by Matthews (8) using tobacco.

Results of the light intensity effects on AMV development were striking and informative in that the two cultivars, Ranger and Vernal, reacted in an opposite manner. It is, however, doubtful that either cultivar would be more susceptible than the other under field conditions, as other studies (2) have shown both react similarly upon inoculation with several AMV isolates.

The marked development of AMV in alfalfa grown with a 6-hr photoperiod was a new and unexpected discovery. There are few reports on the advantageous effects of short day-lengths on AMV development, although Hagedorn & Hanson (6) found that AMV would infect the largest percentage of red clover plants under postinoculation conditions of 4 hr of light compared to 8 and 16 hr of light.

Results concerning the effects of temp on AMV development in alfalfa agree with observations reported by Diachun & Henson (4), who noted more symptoms under warm summer conditions and a suppression of symptoms at low temp. Other workers disagree (1, 5, 10); it should be emphasized, however, that symptom expression may not always be associated with high relative infectivity.

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