Growth and Flowering of Resistant Alfalfa Infected by *Verticillium albo-atrum*

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ABSTRACT

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Eight cultivars of alfalfa (Vertus, NAPB 110, NAPB 108, CW 8015, WL 316, Apollo II, Cimmaron, and Saranac AR) were evaluated following inoculation with *Verticillium albo-atrum* to determine whether the growth of plants lacking foliar symptoms was affected by the pathogen. All infected plants showed significant ($P = 0.05$) reductions in height, dry weight, and flowering. Plants free from foliar symptoms of Verticillium wilt also had significant ($P = 0.05$) reduction in height and flowering. Dry weight and height showed a significant inverse relation with the number of hours that the host/pathogen system experienced temperatures between 19 and 25 C, a temperature range favorable to growth of *V. albo-atrum*. Flowering was not significantly affected by pathogen-favorable temperatures. The effect of *V. albo-atrum* on flowering appears to be influenced by day length. No consistent differences were noted in cultivar response to *V. albo-atrum*.

Additional key words: lucerne, *Medicago sativa*

Verticillium wilt of alfalfa (*Medicago sativa* L.), caused by *Verticillium albo-atrum* Reinke & Berth., has the potential to cause large reductions in alfalfa yields and crop stands. Although the alfalfa strain of *V. albo-atrum* is a relatively recent introduction into the United States (4), it has been known in Europe since 1918 (22). Efforts to develop *Verticillium*-resistant alfalfa cultivars have followed two approaches. The classical breeding method involving crosses between wild resistant alfalfas, notably *M. hemicycla* (26), and commercially desirable cultivars culminated in England in the release in 1970 of the cultivar Maris Kabul (8). The alternate procedure of recurrent selection from commercial high-yielding material challenged with *V. albo-atrum* resulted in the development of the cultivar Sabilt in Wales in 1972 (1) and the cultivar Vertus in Sweden in 1975 (12). The majority of existing resistant cultivars have been developed by the second method.

Resistance to *V. albo-atrum* in alfalfa was examined by Panton (15,16), who determined that it was a multigenic system with either additive or multiplicative effects. Nielsen and Andreasen (13) confirmed the additive nature of the gene effect. Viands (26), examining the inheritance of resistance to Verticillium wilt in Maris Kabul and Vertus, concluded that a dominant resistance gene was present in Maris Kabul and an additive gene system was operative in Vertus. In 1982, Latunde-Dada and Lucas (11) used Maris Kabul, Vertus, Sabilt, and two other cultivars of alfalfa in a study of resistance to *V. albo-atrum* and concluded that resistance was heterogeneous and further complicated by the autotetraploid nature of alfalfa. Consequently, all resistant cultivars have a percentage of susceptible plants.

Although the pathogen is considered to be soilborne (14), Isaac (9) showed that *V. albo-atrum* sporulated externally on the stem bases of alfalfa and readily infected the plant through wounds in the stem. Consequently, mowing and haymaking are effective mechanisms for secondary spread of the pathogen through a stand of alfalfa (9,22). As a result, once a primary infection site is established in a field, most of the alfalfa plants in that field probably will be exposed to the pathogen.

Isolations (17,20) from infected alfalfa plants lacking foliar symptoms, and thus classified as resistant, showed that *V. albo-atrum* can persist in resistant plants and retain pathogenicity. Resistance is the primary method of controlling this disease in alfalfa. Therefore, we were interested in examining the effect of *V. albo-atrum* on the growth of resistant alfalfa cultivars and on the growth of infected symptomless plants. A portion of this study was previously reported (19,21).

MATERIALS AND METHODS

Experiment 1. Eight alfalfa cultivars—Apollo II, CW 8015 (Dekalb-135), NAPB 108 (Endure), NAPB 110 (Admiral), Vertus, WL 316, Cimmaron, and Saranac AR—were grown from seed in greenhouse carts (90 × 60 × 9 cm) filled with Terra-Lite Reddi Earth Peat-Lite mix (W. R. Grace & Co., Cambridge, MA) as previously described (20). The seedlings were thinned to 10 per 60-cm row and were cut back after 4 wk of growth to stimulate production of multiple stems. A split-plot design was used with eight replicates of one row of 10 plants of each cultivar per treatment and two treatments. Inoculation with *V. albo-atrum* and no inoculation were the main plot treatments, and cultivars were the subplot treatments.

At 3 mo of age, one-half of the plants were inoculated with *V. albo-atrum* by misting freshly cut, 10-cm-tall stubble with a spore suspension. The *V. albo-atrum* isolate used in these studies was obtained from a field-grown alfalfa plant and was maintained on prune-yeast extract agar (24) in an incubator at 22 C. The spore suspension, which was prepared by washing spores from 3-wk-old cultures of *V. albo-atrum* grown on prune-yeast extract agar into sterile distilled water, had a concentration of 3.5×10^6 spores per milliliter. Each replicate received 125 ml of the spore suspension and was kept in a mist chamber for 24 hr before being returned to the greenhouse. The remaining plants were cut back to 10 cm, sprayed with distilled water, and used as the noninoculated treatment. A vertically suspended plastic sheet prevented pathogen spread to the noninoculated control plants. Natural light in the greenhouse was supplemented between 8 a.m. and 8 p.m. with 400W metal-halide lamps.

After inoculation, the plants were allowed to grow for 42 days, after which the top growth was cut back, leaving approximately 10 cm of stubble. The plants underwent four such growing periods. Plant height, number of stems, and disease rating were assessed before

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harvest at 11, 17, and 23 wk after inoculation. The aerial biomass from plants in each row was bulked, and dry weight was determined after drying the specimens for 48 hr in a drying oven at 75 C. Presence or absence of flowering was recorded for each plant before the final harvest. The disease rating was based on a three-point scale, with 1 = no symptoms, 2 = any symptoms of Verticillium wilt, and 3 = dead plant (20). Disease rating was subsequently used to select symptomless plants for statistical analyses.

After the final harvest, the inoculated plants were allowed to regrow for 6 wk, after which stem sections were placed on water agar to verify the presence of *V. albo-atrum*.

Experiment 2. The study was repeated using cultivars WL 316, NAPB 110, Vertus, and Saranac AR. The experiment was conducted as in experiment 1 but with an inoculum concentration of 2.95×10^6 spores per milliliter. Previously described growth parameters were assessed before harvest at 12 and 18 wk after inoculation. In addition, each plant was classified as either flowering or not flowering after 18, 25, and 40 days of growth during the first growing period and after 30 and 42 days during the second growing period. The disease rating scale was also modified to reflect the varying degrees of host response to the pathogen. The scale corresponded to that used by Graham et al (4), with 1 = no symptoms, 2 = one or two chlorotic leaflets, 3 = leaflets on more than one shoot chlorotic, 4 = most of the leaflets chlorotic, and 5 = dead plant. As in experiment 1, the disease rating was used to identify symptomless plants for further statistical analyses.

Environmental conditions. Greenhouse temperatures were recorded by hygrothermograph and varied between the two experiments. Plants in experiment 1 experienced temperatures between 19 and 25 C for 33% (336 of 1,008 hr) of the first growing period during which data was collected and 36% (358 of 984 hr) and 39% (412 of 1,056 hr) of the second and third growing periods, respectively. During experiment 2, temperatures between 19 and 25 C existed for 67% (658 of 984 hr) of the first growing period and 44% (442 of 1,008 hr) of the second growing period.

The effect of the temperature variations on the growth of plants infected with *V. albo-atrum* was examined through regression analysis of the percentage of each growing period between 19 and 25 C vs. the respective growth parameter index. The growth parameter index values for each growing period were obtained by dividing the mean of the inoculated population by the mean of the noninoculated population.

RESULTS AND DISCUSSION

Alfalfa cultivars used in these studies were previously shown (20) to include the following percentages of plants lacking foliar symptoms after inoculations with *V. albo-atrum*: Vertus, 54%; NAPB 108, 54%; NAPB 110, 47.5%; Apollo II, 36%; CW 8015, 36%; WL 316, 35%; Cimmaron, 22.5%; and Saranac AR, 10%. In addition, isolation studies indicated that all symptomless inoculated plants in experiment 1 were infected with *V. albo-atrum* (20). The success of the stubble inoculation technique made it possible to assume in succeeding tests that all inoculated plants were subsequently infected by the pathogen.

Results of the analysis of variance comparing the mean growth parameter values of the inoculated population with those of the noninoculated population are presented in Table 1. Plant height and incidence of flowering showed a consistent response to the presence of the pathogen. Because cultivar × treatment interactions were inconsistent and were significant in only 17% of the analyses, cultivar differences will not be discussed. Significant differences between inoculated and noninoculated means ($P = 0.05$) were present at all sampling times, and in all cases the inoculated plants were shorter and had a lower incidence of flowering. The reduction in the height of inoculated plants agrees with Petersen's (22) observation that alfalfa plants infected with *V. albo-atrum* were shorter than noninfected plants and corresponds to similar findings in potato infected with this pathogen (7).

Plants free from foliar symptoms were identified, by means of their disease rating, from within the inoculated population, and the respective growth parameter means were analyzed and compared with the means from a noninoculated population having the same disease rating. Dry weight of plants free from foliar symptoms was not analyzed because all plants within a replication were bulked before being weighed. Saranac AR was excluded from these analyses because of the small number of plants lacking foliar symptoms in this cultivar. Results of the statistical analysis (Table 2) showed that the significant differences in plant height and incidence of flowering between the two populations persisted when only plants

Table 1. Comparison of growth parameter means of a population of alfalfa plants inoculated with *Verticillium albo-atrum* with those of a population of noninoculated alfalfa plants

	Harvests				
	1984			1985	
Parameter	1	2	3	1	2
Height (cm)					
Inoculated	21.0	24.9	25.3	21.8	22.2
Noninoculated	23.5*[a]	28.3*	30.2*	35.1*	30.1*
Number of stems/plant					
Inoculated	3.1	3.8	4.2	3.7	4.1
Noninoculated	3.3	4.6*	4.4	4.6*	4.2
Dry weight (gm/10 plants)					
Inoculated	5.3	5.1	5.6	5.8	7.2
Noninoculated	5.3	6.4	6.6*	10.6*	9.8*
Percentage of plants flowering					
Inoculated	NT[b]	NT	6.6	15.6	30.6
Noninoculated	NT	NT	27.2*	33.0*	46.1*

[a] * = Significant difference between inoculated plants and noninoculated plants at $P = 0.05$.
[b] NT = not tested.

Table 2. Comparison of alfalfa plants inoculated with *Verticillium albo-atrum* but lacking foliar symptoms with the noninoculated population of alfalfa plants

	Harvests				
	1984			1985	
Parameter	1	2	3	1	2
Height (cm)					
Inoculated	21.2	22.8	24.6	24.1	24.9
Noninoculated	23.8	27.7*[a]	29.9*	35.2*	31.5*
Number of stems/plant					
Inoculated	3.1	3.3	3.9	3.6	4.5
Noninoculated	3.3	4.2*	4.4	4.5*	4.4
Percentage of plants flowering					
Inoculated	NT[b]	NT	5.4	21.0	33.4
Noninoculated	NT	NT	22.3*	34.0*	46.4*

[a] * = Significant difference between inoculated plants and noninoculated plants at $P = 0.05$.
[b] NT = not tested.

without foliar symptoms were considered.

The number of stems per plant was inconsistent in reflecting a significant response to the treatment (Tables 1 and 2) and was therefore considered to be of questionable value in assessing the effect of *V. albo-atrum* on the growth of alfalfa. A similar situation was reported in potato by Harrison and Isaac (6), who noted little or no change in stem number when plants were infected with *V. albo-atrum*. On one occasion in each experiment, we detected a significant reduction in stem number in the inoculated population, which may indicate that this parameter is sensitive to the pathogen under specific environmental conditions.

Dry weight (Table 1) of the inoculated population was significantly less than that of the noninoculated population at the final harvest of experiment 1 and during all harvests in experiment 2. Dry weight was not consistently affected in experiment 1, which may reflect the influence of environment on the host/pathogen interaction. Latunde-Dada and Lucas (11) noted that the response of alfalfa to *V. albo-atrum* was sensitive to temperature and that the expression of resistance was favored by high temperatures. A similar host/pathogen response to temperature was reported in tomato infected with *V. albo-atrum* (5).

Considerable variation in temperature occurred between the two experiments. Because *V. albo-atrum* is active between 19 and 25 C (2), we subjected the percentage of each growing period at these temperatures and the growth parameter indices to regression analyses. Results (Table 3) indicated that the negative response of alfalfa to *V. albo-atrum*, as reflected by plant height and dry weight, was significantly increased by the number of hours that the host/pathogen system experienced temperatures favorable to the pathogen. Number of stems per plant and incidence of flowering were not influenced by the number of hours of pathogen-favorable temperatures (Table 3). In the case of stem number, these results were expected, because the pathogen had no significant effect on this parameter (Tables 1 and 2). The lack of a significant effect on the incidence of flowering was unexpected and may indicate that day length, which varied between growing periods, had more impact on flowering than did temperature favorable to the pathogan. In all cases, however, the inoculated plants had a significantly lower incidence of flowering (Tables 1 and 2).

Flowering was examined more extensively in experiment 2. Data on incidence of flowering at three times during growing period 1 were subjected to regression analysis. Results (Fig. 1) indicate that incidence of flowering in the noninoculated population increased over time. In the inoculated population, however, flowering failed to increase over time, and in the population of inoculated plants without foliar symptoms, flowering decreased over time (Fig. 1). Although the slopes of the two lines appear very similar, the amount of variation prevented us from concluding that flowering in the total inoculated population decreased over time.

Data on incidence of flowering were collected twice during the second growing period in experiment 2, and analysis of variance indicated that there was a significant increase in flowering over time in the inoculated population as well as in the inoculated population that lacked foliar symptoms (Fig. 2). In both cases, however, the inoculated plants had a significantly lower ($P = 0.05$) incidence of flowering than did the noninoculated population.

The day length in growing period 1 was approximately 1 hr shorter (13 hr lengthening to 14 hr) than that during growing period 2 (14 hr lengthening to 14.5 hr), and this day length difference could account for the increased incidence of flowering noted during growing period 2 (Fig. 2). The overall reduction in flowering, however, indicates that the pathogen interferes with the reproductive process regardless of day length, although day length appears to influence the intensity of the expression of that interference.

Water stress was implicated in the delayed flowering of five species of annual *Medicago* (3), and water stress

Table 3. Growth parameter indices[a] and results of regression analysis of these indices vs. percentage of each growth period during which temperatures remained in the *Verticillium albo-atrum* favorable range of 19–25 C[b]

	Growth period						
	Experiment 1			Experiment 2			
Growth parameter	**1**	**2**	**3**	**1**	**2**	**Regression coefficient**	**R^2[d] (%)**
Dry weight	1.0	0.80	0.85	0.55	0.74	−0.76*[e]	85
Height	0.89	0.88	0.84	0.62	0.74	−1.15**	93
Height, NFS[c]	0.89	0.82	0.82	0.68	0.79	−1.71**	93
Number of stems/plant	0.94	0.83	0.95	0.80	0.98	−0.96	31
Number of stems/plant, NFS	0.94	0.78	0.87	0.80	1.02	−0.41	9
Percentage of plants flowering	NT[f]	NT	0.24	0.47	0.66	+0.16	5
Percentage of plants flowering, NFS	NT	NT	0.24	0.61	0.72	+0.27	21

[a] Mean parameter value of inoculated population/mean parameter value of noninoculated population.
[b] Experiment 1: period 1 = 33%, period 2 = 36%, period 3 = 39%. Experiment 2: period 1 = 67%, period 2 = 44%.
[c] NFS = no foliar symptoms.
[d] Coefficient of determination.
[e] * = Significant at $P = 0.05$, ** = significant at $P = 0.01$.
[f] NT = not tested.

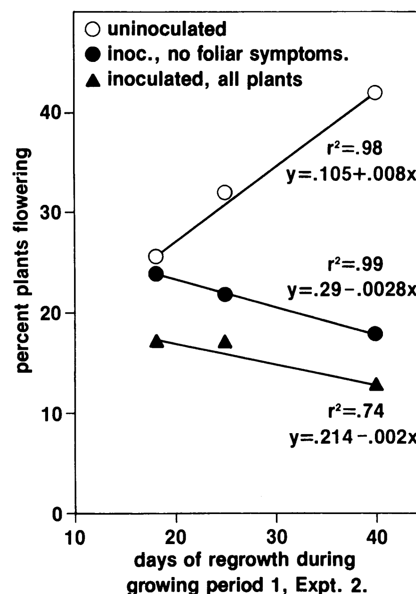

Fig. 1. Effect of *Verticillium albo-atrum* on the relationship between flowering and time in four alfalfa cultivars during growing period 1, experiment 2.

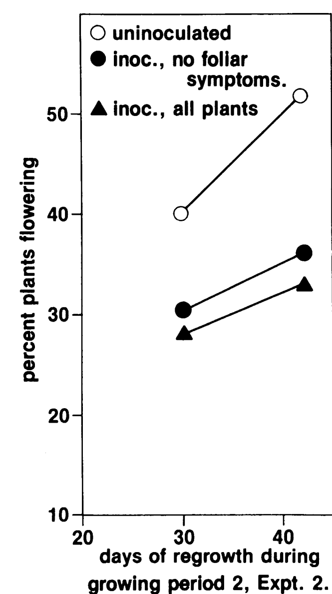

Fig. 2. Effect of *Verticillium albo-atrum* on the relationship between flowering and time in four alfalfa cultivars during growing period 2, experiment 2.

during floral induction reduced flowering in *Lolium temulentum* (10) and soybean (23). Plant water stress has been associated with Verticillium wilt in susceptible plants (25), and Pennypacker and Leath (18) have documented anatomical changes in *V. albo-atrum*-infected susceptible alfalfa plants that could contribute to impaired water translocation. It is possible that the reductions in flowering noted in the plants lacking foliar symptoms may be due to subtle disruptions in water availability during floral induction. Because of their genetic heterogeneity, alfalfa plants do not flower simultaneously. Therefore, not all plants would be in as susceptible a stage of growth when the water stress occurred. Thus, not all plants would be affected—a situation compatible with our observations.

Because of the use of seed plants rather than clonal plants, it was not possible to select a noninoculated population that was genetically identical to the population of inoculated plants lacking foliar symptoms. Consequently, the possibility exists that the observed reduction in flowering may have been due to selection pressure rather than to a host/pathogen interaction. Additional experiments using clonal plants are being conducted to resolve this point.

Regardless of the cause, reduction in flowering in populations of plants infected with *V. albo-atrum* has the potential to interfere with alfalfa breeding programs by removing some genotypes from the breeding population, thus altering the genetic makeup of the resultant seed crop.

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