ASSOCIATION BETWEEN, AND SOME ENVIRONMENTAL EFFECTS ON, LESION SIZE AND STUNTING OF ALFALFA BY XANTHOMONAS CAMPESTRIS PV. ALFALFAE

by

JOHN ISAAC EDMUNDS

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Approved by:

Donald L. Gottlieb
Major Professor

Bacterial leaf spot (BLS) of alfalfa (Medicago sativa L.), caused by Xanthomonas campestris pv. alfalfae (Riker, Jones and Davis) Dye, was first described in Wisconsin in 1935 (13). The disease is widespread in the central and southern United States (6) and in several other countries (2) and may become severe at high temperatures. In Kansas, X. c. pv. alfalfae caused postemergent damping-off and marked stunting of alfalfa seedlings in the laboratory and in naturally infected fall-seeded stands in the field (16). Under controlled conditions, most 'Cody' alfalfa seedlings grown in soil infested with X. c. pv. alfalfae were stunted (16). KS76 alfalfa germplasm, derived from 'Kanza' by four cycles of recurrent phenotypic selection for resistance to BLS, has more than 95% resistant plants compared to 0.1% for Kanza (14). However, in unpublished work, a large proportion of infected KS76 seedlings exhibited various degrees of stunting, indicating that resistance, based on reduced lesion size, may not be closely associated with stunting caused by X. c. pv. alfalfae.

This study documents environmental influences on seedling stunting by X. c. pv. alfalfae and on BLS stem lesion development and shows relationships between seedling stunting and lesion size over a range of temperatures. Preliminary results were reported (4,5).

Materials and Methods

Bacterial isolates. Unless otherwise noted, isolate KX-1 of X. c. pv. alfalfae, isolated in 1964 from a diseased plant in Kansas (11), was used. A separate experiment included isolate Ona 1-1 of X. c. pv. alfalfae, isolated in 1985 from a

diseased alfalfa plant collected near Ona, FL; strain 5757 of X. c. pv. vasculorum; obtained from L. E. Claflin (Dept. of Plant Pathology, Kansas State University, Manhattan, KS); and strains F-1 and F-6 of X. c. pv. citrumelo (7), which have high coefficients of similarity to X. c. pv. alfalfae based on RFLP analysis of genomic DNA (8), obtained from E. L. Civerolo (USDA, ARS, Plant Science Institute, Fruit Laboratory, Beltsville, MD). Cultures for immediate use were maintained on petri plates of nutrient broth (Difco Laboratories, Detroit, MI) plus 1.8% agar (Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, NJ) (NBA).

Inocula preparation. Suspensions for inoculations were prepared by seeding 50 ml sterile nutrient broth (Difco Laboratories) in 300-ml side-arm flasks (Bellco Glass, Inc., Vineland, NJ) with 1 ml of nutrient broth with 16-hr-old bacterial growth and incubating 4-7 hr on a rotary shaker (120 rpm) at 30 C. Bacterial concentration was monitered spectrophotometrically (590 nm) during incubation, and samples were taken from the suspension at readings which gave desired concentrations for inocula preparations. Cell concentration of all final inocula was confirmed by plating 10-fold serial dilutions onto NBA. Plates were incubated at 25 C, and colonies were counted within 3-5 days.

In tests to study seedling stunting, cell suspensions ($A_{590nm}=0.13$) were centrifuged at 4000 rpm for 20 min at 22 C. The pellets were washed twice and resuspended in 12.5 mM phosphate buffer (pH=7.1). Inoculum concentration was adjusted ($A_{590nm}=0.13$) with buffer to 2.5-3.4 x 10^8 colony-forming units (cfu) per milliliter.

To study BLS lesion length, stems were injected with cell suspensions in nutrient broth ($A_{590nm}=0.13$) diluted 20-fold with sterile distilled water (4 x 10^6-

2×10^7 cfu/ml). Control stems were injected with comparably diluted sterile nutrient broth.

Plant inoculation procedures. Seedling stunting. Alfalfa (Medicago sativa L.) was seeded 1.5 cm deep in rows 14 cm long and 2.4 cm apart in autoclaved fine masonry sand in aluminum bread pans (24.5 x 14 x 7 cm) and kept at 20 C with continuous fluorescent lighting (100 $\mu E\, m^{-2} sec^{-1}$) in a growth chamber. Five days later, stands were thinned to 10 seedlings per row, and a plug was removed from one cotyledon of the 80 seedlings in a pan using a 26-gauge hypodermic needle modified to be analogous to a cork borer. To inoculate, a 0.5 μl aliquot containing 1.6-2.8×10^5 cfu of bacteria was applied with a P20 Pipetman (Rainin Instrument Co., Inc., Woburn, MA) to each hole in the treated seedlings. Sterile buffer was applied similarly to control seedlings. Pipet tips were discarded after each aliquot application.

Stem lesion size. Following their evaluation for stunting, seedlings were potted in a 2:1 soil:sand mixture in 7.5-cm-sq pots. The basal 2-3 internodes of stems were injected (26-gauge needle) with an aliquot of X. c. pv. alfalfae suspension (4×10^6-2×10^7 cfu/ml). Plants were watered with 10% Hoagland's solution (9) as needed throughout the experiments.

Plant incubation. Effect of temperature on seedling stunting. Four pans of inoculated Kanza and KS76 seedlings were placed in growth chambers at 20, 25, 30, and 35 C under continuous cool-white fluorescent light of 100 $\mu E\, m^{-2} sec^{-1}$. Plant heights were recorded 7, 9, and 14 days after inoculation. Measurements were made from the sand surface to the uppermost growth of each plant. The test was repeated three times.

Relationship between stunting and stem lesion size. Inoculated seedlings of both populations from the stunting experiment were grown and tested for BLS lesion length on their regrowth stems. The plants were kept at 22 C under continuous cool-white fluorescent light of 100 μE $m^{-2}sec^{-1}$ throughout the experiment, and growth was removed at the flowering stage until tested. Stems 20-30 cm tall were inoculated. The length of the longest stem lesion on each plant was recorded 28-30 days after inoculation. The test was repeated on second regrowth. Both measurements for each plant were correlated with that plant's seedling height at 7, 9, and 14 days after cotyledon inoculation and incubation at 20, 25, 30, and 35 C.

Heritability of stunting. Following testing for stem lesion length, those plants that as seedlings in each temperature group (Table 1) comprised the tallest ca. 15% and the most stunted ca. 15% of the treated seedlings of Kanza and KS76 were selected. Each of the four groups of about 45 plants were intercrossed by pollen transfer with toothpicks. Within each group, 15 seeds from each plant were bulked and used to evaluate the Syn 1 populations. Seeds were scarified and planted in rows for tests of seedling stunting as described; however, testing was performed at 30 C only. Kanza and KS76 also were included. Heights of treated and control seedlings were recorded 4, 7, 9, and 14 days after inoculation. The test was repeated three times.

Influence of temperature and photoperiod on stem lesion size, internode length, and stem length. Two alfalfa plants, Kanza 120-4 and Kanza 220-16, which produced relatively long stem lesions in both tests to evaluate lesion length, were asexually reproduced by rooting stem cuttings in sand. Sixteen plants each of clone Kanza 120-4 and Kanza 220-16 were used for the experiment.

Two growth chambers (20 and 30 C, 24-hr photoperiod of 100 $\mu E\, m^{-2} sec^{-1}$, 60±5% RH) were used. To establish a 10-hr photoperiod inside each chamber, a wood frame covered on sides and top with black cloth was placed over half of the plants for 14 hr daily. Shoots were removed at the flowering stage, and four plants of each clone were assigned randomly to each environmental regime.

Eight days after shoot removal, the second and third internodes subtending the apex of the tallest stem of two plants of each clone in each regime were inoculated. Comparably diluted aliquots of sterile nutrient broth were applied similarly to the other two plants of each clone in each regime. The lengths of the first, second, third, and fourth internodes of all test stems, as well as the lengths of the test stems, were recorded just prior to inoculation and 4, 8, 12, and 16 days after inoculation. The lengths of the two lesions produced on each test stem were recorded 8, 12, and 16 days after inoculation. The test was repeated three times with the same plants. Before each test the plants were held to flowering, the shoots were harvested, and plants were reassigned randomly to the regimes.

Effect of inoculum concentration and temperature on stem lesion size. Two plants each of susceptible clones Kanza 120-4, Kanza 220-16, and Kanza 435-8 were placed into two growth chambers (20 and 30 C, 24-hr photoperiod, 60±5% RH). Aliquots of X. c. pv. alfalfae suspension (5.2×10^6 cfu/ml) were used to inoculate the test stem of one plant of each clone, and a more concentrated suspension (6.6×10^8 cfu/ml) was applied similarly to the test stem of the other plant of each clone. Lesion size, internode length, and test stem length were recorded as described in the previous experiment. The test was repeated three times.

Other pathovars. Strains of other Xanthomonas campestris pathovars were

tested for possible stunting of alfalfa seedlings at 30 C and included those isolates and strains previously described. Seeding of Kanza and KS76, preparation of inoculum, and inoculation were as described for KX-1. Additional testing of the F-1 strain of X. c. pv. citrumelo was performed using more concentrated aliquots containing 2-3 x 10^6 cfu. Plants were incubated at 30 C throughout the experiment, and heights of treated and control seedlings were recorded 7, 9, and 14 days after inoculation. The test was repeated once for each isolate.

Limited tests of all strains for pathogenicity were made on pans of 14-day-old seedlings of Kanza and KS76 in a growth chamber (30 C, 24-hr photoperiod, 60±5% RH). Suspensions (2.5 x 10^7-3.0 x 10^8 cfu/ml) in buffer and containing 1-2 drops Tween-20 wetting agent were sprayed to run-off onto seedlings in half of each pan. Sterile buffer with wetting agent was applied similarly to seedlings in the other half of each pan. The plants were placed under transparent covers (100% RH) for 48 hr at 30 C, and 12 days later were observed for foliar symptoms.

Statistical methods. Statistical analyses software was provided by Statistical Analysis Systems (release 5.16) (SAS Institute Inc., Cary, NC) (15). The SAS ANOVA procedure was used to perform analysis of variance (ANOVA) on mean heights of treated and control seedlings in tests to determine the effect of temperature on stunting of alfalfa by X. c. pv. alfalfae, in tests to assess the heritability of stunting, and in tests of seedling stunting by other Xanthomonas pathovars. Mean comparisons were made with Fisher's least significant difference (LSD) test. This test controls Type I but not Type II experimentwise error rates. The SAS CORR procedure was used to test for a relationship between seedling stunting by X. c. pv. alfalfae and BLS lesion size over a range of incubation

temperatures.

Experiments to determine the effect of temperature on stunting and to determine the effect of inoculum concentration of X. c. pv. alfalfae and temperature on BLS lesion size were split-split plot designs with temperature as the whole plot, population and clone as subplots, and inoculation (treated or control) and concentration as sub-subplots, respectively. The experiment to determine the effect of temperature and photoperiod on lesion size was a split-split-split plot design with temperature as the whole plot, photoperiod as subplot, clone as sub-subplot, and inoculation as sub-sub-subplot. Experiments to assess the heritability of stunting and to examine seedling stunting by other Xanthomonas pathovars were split-plot designs with population as the whole plot and inoculation as the subplot.

RESULTS

Effect of temperature on seedling stunting. Control seedlings were noticeably taller than treated seedlings, and variation in height among the treated seedlings of both populations was apparent by 2 days after cotyledon inoculation. When first measured at day 7, the mean height of control plants was significantly ($P=0.01$) greater than the mean height of inoculated plants within the same population (Table 1). These height differences (control-treated) increased with time at all four temperatures and increased more rapidly in Kanza than in KS76. Kanza controls were significantly ($P=0.05$) taller than KS76 controls, except at 20 C at day 7 (Table 1). However, heights of treated Kanza and KS76 plants did not differ significantly at day 7 or 9, but at day 14 at 25 and 30 C treated KS76 plants were taller than the

TABLE 1. Effect of temperature on height of Kanza and KS76 alfalfa seedlings 7, 9, and 14 days after a 0.5 μl aliquot containing either buffer (control) or 1.6-2.8 x 10^5 cfu of Xanthomonas campestris pv. alfalfae (isolate KX-1) was applied to a 26-gauge needle hole through a cotyledon of 5-day-old plants

			Mean height (mm)[z]			
			Incubation temperature (C)			
Day	Population	Treatment	20	25	30	35
7	Kanza	Control	36.6	45.3	52.1	42.2
		Treated	25.1	22.6	19.9	16.3
	KS76	Control	35.8	40.0	46.9	36.2
		Treated	24.3	20.9	21.0	15.9
9	Kanza	Control	61.6	82.9	96.8	66.0
		Treated	37.3	34.7	34.0	25.0
	KS76	Control	56.5	75.7	83.1	54.6
		Treated	37.1	35.6	38.2	25.4
14	Kanza	Control	102.5	133.9	139.1	84.8
		Treated	57.1	58.5	53.4	30.3
	KS76	Control	87.4	116.4	117.8	68.6
		Treated	62.4	70.5	66.5	35.2

TABLE 1. Continued

[z]Fisher's least significant differences (P=0.05) for day 7, 9, and 14, respectively: between treatments for same temperature and population, 8, 7, and 12; between populations for same temperature and treatment, 4, 5, and 8; and between temperatures for same population and treatment, 8, 8, and 14.

Kanza plants. Treatment x temperature interactions were highly significant ($P=0.001$) for all 3 days. Population x treatment interaction was highly significant ($P=0.001$) for day 9 and 14. At 14 days postinoculation at 30 C, the range in height of treated and control seedlings, respectively, was 14-132 mm and 30-198 mm for Kanza and 8-132 mm and 49-176 mm for KS76. Stunted seedlings had shorter internodes and usually much smaller leaves than normal plants. No BLS lesions developed during this experiment.

Relationship between stunting and lesion size. Seedling height and lesion length on regrowth stems at either 22 or 30 C were not related. At 22 C, lesion lengths on stems ranged from 0-30 mm (mean 7.2 mm) for Kanza and 0-25 mm (mean 3.1 mm) for KS76. Correlations of seedling height 7, 9, and 14 days after cotyledon inoculation and incubation at 20, 25, 30, or 35 C with longest lesion length and with the average lesion length yielded correlation coefficients from -0.26 to +0.17 with most coefficients having values near 0.00. No P values were less than 0.05, and most were between 0.6 and 0.9.

The experiment was repeated with a second group of 320 plants of Kanza and KS76, except that tests for seedling height and lesion size on their regrowth were conducted at 30 C. About 80 seedlings of each population were treated. Again, seedling stunting and subsequent stem lesion size on the same plant were unrelated. Correlation coefficients ranged from -0.19 to +0.15, with values being near 0.00. No values had probabilities less than 0.05 with most being 0.4-0.6.

Numerous dark, necrotic flecks developed repeatedly over entire lengths of inoculated stems of several plants, particularly those of KS76, and were observed occasionally on uninoculated stems of the same plant (Fig. 1 A). Many plants of

Fig 1. Reaction of two selections of alfalfa germplasm KS76 to stem injections of Xanthomonas campestris pv. alfalfae (isolate KX-1). **A,** stems developing numerous necrotic flecks in internodes above site of stem inoculation. **B,** stem sections each with a small non-spreading lesion at the inoculation site (center) and water-soaked blisters.

A.

B.

both Kanza and KS76 developed raised blisters along the surface of inoculated stems (Fig. 1 B). With one exception, plants which developed necrotic flecks had stem lesions less than 4.5 mm long.

To test whether these necrotic flecks and blisters contained X. c. pv. alfalfae, 1-cm-long sections of inoculated and uninoculated stems from 8 plants with flecks and/or blisters were excised with sterile single-edged razor blades and plated on MXP semi-selective medium (1). Plates were incubated at 25 C, and were observed every 24 hr for 5 days.

X. c. pv. alfalfae grew only on stem sections containing the site of inoculum injection.

Heritability of stunting. Treated Kanza Tall Syn 1 plants were taller ($P=0.05$) than the treated Kanza Stunted Syn 1 plants at all four days (Table 2). Treated KS76 Tall Syn 1 plants were significantly ($P=0.05$) taller than treated KS76 Stunted Syn 1 plants at day 4 and 7, but this difference was significant only at the $P=0.10$ level at day 9 and 14. Syn 1 Tall control plants tended to be taller than the parent control plants in both populations but these differences were not significant at $P=0.05$.

Effects of temperature and photoperiod on lesion size. Stem lesions on plants of both clones were significantly ($P=0.05$) longer at 30 C than at 20 C at both photoperiods (Table 3). At 30 C, lesions on clone Kanza 220-16 were longest at the 10-hr photoperiod, whereas on clone Kanza 120-4 they were longest at the 24-hr photoperiod. The photoperiod x clone interaction and the temperature x photoperiod x clone interaction were highly significant ($P=0.01$).

To assess interrelationships between X. c. pv. alfalfae and environment on

TABLE 2. Effect of one cycle of selection for tallness and stunting within Kanza and KS76 populations following inoculation with Xanthomonas campestris pv. alfalfae (isolate KX-1) on the heights of the resulting Syn 1 population

		Mean height (mm)[x,y,z]					
		Population					
			Kanza Syn 1			KS76 Syn 1	
Day	Treatment	Kanza	Tall	Stunted	KS76	Tall	Stunted
4	Control	44.7 a	46.6 a	41.8 ab	41.7 ab	41.6 ab	37.9 b
	Treated	27.8 a	30.6 a	21.9 b	28.2 a	27.6 a	22.3 b
7	Control	66.9 ab	71.8 a	62.7 b	59.9 b	64.0 ab	57.7 b
	Treated	35.3 a	41.3 a	26.8 b	36.2 a	34.9 a	27.8 b
9	Control	88.2 ab	94.6 a	81.2 b	79.7 b	83.4 ab	77.5 b
	Treated	42.1 abc	53.6 a	30.9 c	44.8 ab	48.8 ab	36.5 bc
14	Control	139.6 ab	151.2 a	132.8 ab	125.9 b	135.8 ab	131.1 ab
	Treated	72.2 abc	92.0 a	50.2 c	76.9 ab	90.8 a	71.6 abc

[x]Values shown are the mean heights of alfalfa seedlings 4, 7, 9, and 14 days after application of a 0.5 µl aliquot containing either buffer (control) or 2-3 x 10^5 cfu of bacteria to a 26-gauge hole through a cotyledon of 5-day-old plants and incubation at 30 C.

TABLE 2. Continued

[y]Mean heights of treated and control seedlings of a population are significantly different (P=0.001) 4, 7, 9, and 14 days after inoculation.

[z]Values within rows followed by the same letter do not differ significantly (P=0.05) according to Fisher's least significant difference.

TABLE 3. Effect of temperature and photoperiod on lesion length 8, 12, and 16 days after stems of plants of two Kanza alfalfa clones were injected (26-gauge needle) with a suspension (about 2 x 10^8 cfu/ml) of Xanthomonas campestris pv. alfalfae (isolate KX-1)

			Mean lesion length (mm)[y,z]	
			Clone	
Day	Temperature (C)	Photoperiod (hr)	Kanza 120-4	Kanza 220-16
8	20	10	1.4	3.4
		24	1.5	2.8
	30	10	8.2	13.9
		24	8.6	8.0
12	20	10	2.1	5.0
		24	2.2	3.9
	30	10	8.6	18.1
		24	12.0	8.6
16	20	10	2.3	5.1
		24	2.8	4.8
	30	10	9.2	20.2
		24	14.4	8.8

TABLE 3. Continued

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[y]Each value is the mean of 16 lesions produced from artificial inoculation of the second and third internodes subtending the apex of a stem of two plants of a specified clone (four replications).

[z]Fisher's least significant differences (P=0.05) for day 8, 12, and 16, respectively: between clones for same temperature and photoperiod, 1.7, 2.5, and 3.6; between photoperiods for same temperature and clone, 1.7, 2.5, and 4.1; and between temperatures for same photoperiod and clone, 3.2, 3.6, and 4.8.

stem development, the lengths of stem internodes and stems of treated and control test stems were compared in the different regimes.

Length of the second internode of test stems on all plants did not increase beyond 8 days after inoculation, and elongation of the third internode had ceased by the time of inoculation (Table 4). Lengths of treated second and third internodes and their corresponding control internodes were not significantly different for either clone, and both clones reacted similarly in all regimes. Thus, the normal elongation of the second internode was unaffected after inoculation with X. c. pv. alfalfae. Lesions tended to be longer in the second internode than in the third internode of test stems of plants of both clones, but the differences were not significant (Table 5).

Control stems were significantly ($P=0.05$) longer than treated stems at 30 C, and the differences were greater at the 10-hr photoperiod than at the 24-hr photoperiod (Table 6). However, stems were longer at the 24-hr photoperiod than at the 10-hr photoperiod. At 20 C, stem elongation was not affected by X. c. pv. alfalfae. Plants grown under the 10-hr photoperiod produced no buds or flowers, whereas those grown under continuous light were flowering by 12 days after inoculation.

Effect of inoculum concentration and temperature on stem lesion size. Lesion length was increased more by temperature than by the inoculum concentration used (Table 7). On all three clones at all 3 days, lesions were longest at 30 C and the highest inoculum concentration, and were next longest at 30 C and the lowest concentration; however, these differences were significant ($P=0.05$) only for clone Kanza 120-4. There was a significant ($P=0.01$-0.04) temperature x clone interaction at all 3 days. Lesion size continued to increase with time at both temperatures.

TABLE 4. Effect of temperature and photoperiod on the length of the first, second, and third internodes subtending the apex of stems of plants of two alfalfa clones injected (26-gauge needle, second and third internodes) with sterile dilute nutrient broth (control) or Xanthomonas campestris pv. alfalfae (isolate KX-1) suspension (about 2 x 10^8 cfu/ml)

			Mean internode length (mm)[z]											
			Temperature (C) / Photoperiod (hr)											
			20/10			20/24			30/10			30/24		
			Days after inoculation											
Clone	Treatment	Internode	0	8	12	0	8	12	0	8	12	0	8	12
Kanza	Treated	1	4			6			13			15		
120-4		2	15	30	30	24	49	49	38	44	45	39	57	57
		3	23	24	24	30	33	33	45	45	45	53	54	54
	Control	1	5			6			15			17		
		2	16	35	35	21	49	49	42	48	48	37	58	58
		3	21	22	22	32	36	36	38	38	38	52	56	56
Kanza	Treated	1	6			8			9			12		
220-16		2	18	42	42	20	46	46	32	47	47	34	56	56
		3	36	41	41	37	40	40	53	55	55	58	61	61
	Control	1	4			8			15			17		
		2	16	43	43	22	46	45	36	48	48	40	61	61
		3	21	21	21	38	39	39	49	52	52	53	54	54

TABLE 4. Continued

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[z]Each value is the mean of eight internodes from stems of plants of the specified clone (four replications).

TABLE 5. Effect of temperature and photoperiod on lesion development within the second and third internodes subtending the apex of stems of plants of two alfalfa clones injected with Xanthomonas campestris pv. alfalfae (isolate KX-1) suspension (about 2 x 10^8 cfu/ml)

		Mean lesion length (mm)[z]							
		Temperature (C) / Photoperiod (hr)							
		20/10		20/24		30/10		30/24	
		Internode							
Clone	Day	2	3	2	3	2	3	2	3
Kanza	8	2.0	0.8	1.6	1.4	9.0	7.5	9.1	8.0
120-4	12	2.6	1.5	2.4	2.0	9.4	7.9	13.4	10.6
	16	2.8	1.9	2.9	2.8	10.1	8.4	16.2	12.5
Kanza	8	4.5	2.2	3.2	2.2	15.4	12.4	7.9	8.1
220-16	12	5.8	4.2	4.6	3.2	20.5	15.6	8.5	8.6
	16	5.9	4.4	5.2	4.4	21.6	18.9	8.6	8.9

[z]Each value is the mean of eight lesions produced from artificial inoculation of stems of two plants of the specified clone (four replications).

TABLE 6. Effect of temperature and photoperiod on the length of test stems of plants of two alfalfa clones 8, 12, and 16 days after injection (26-gauge needle) with sterile dilute nutrient broth (control) or Xanthomonas campestris pv. alfalfae (isolate KX-1) suspension (about 2 x 10^8 cfu/ml)

			Mean stem length[z] (mm)		
			Days after inoculation		
Temperature (C)	Photoperiod (hr)	Treatment	8	12	16
20	10	Control	185	240	287
		Treated	188	230	266
	24	Control	235	301	359
		Treated	236	298	360
30	10	Control	304	362	399
		Treated	242	254	264
	24	Control	423	514	579
		Treated	379	458	523

[z]Each value is the mean of 16 test stems from four plants of each of clone Kanza 120-4 and Kanza 220-16 (four replications). Fisher's least significant difference (P=0.05) for day 8, 12, and 16, respectively: between treated and control means of stems of plants grown at same temperature and photoperiod 28, 34, and 39.

TABLE 7. Effect of inoculum concentration on stem lesion development at 20 and 30 C at 8, 12, and 16 days after stems of three Kanza alfalfa clones were each injected with 5.2 x 10^6- or 6.6 x 10^8 cfu/ml of Xanthomonas campestris pv. alfalfae (isolate KX-1) suspension

Days after inoc.	Inoculum conc. (cfu/ml)	Temperature (C)	Mean lesion length (mm)[y,z] Clone Kanza 120-4	Kanza 220-16	Kanza 435-8
8	5.2x10^6	20	0.8	0.9	1.9
		30	5.6	5.5	6.6
	6.6x10^8	20	2.0	4.4	2.6
		30	10.1	6.1	7.9
12	5.2x10^6	20	0.9	2.5	2.4
		30	7.8	5.8	6.6
	6.6x10^8	20	3.0	5.0	3.5
		30	14.5	6.1	8.0
16	5.2x10^6	20	1.0	3.1	2.5
		30	9.5	5.9	6.8
	6.6x10^8	20	3.4	5.6	3.5
		30	17.1	6.2	8.2

TABLE 7. Continued

[y]Each value is the mean of eight lesions produced from artificial inoculation of the second and third internodes subtending the apex of a stem of one plant of a specified clone (four replications).

[z]Fisher's least significant differences (P=0.05) for day 8, 12, and 16, respectively: between concentrations for same temperature and clone, 2.2, 3.6, and 4.7; between clones for same temperature and concentration, 1.7, 2.8, and 4.0; and between temperatures for same clone and concentration, 1.8, 3.5, and 5.5.

Stunting and pathogenicity tests including other Xanthomonas campestris pathovars. Only isolates Ona 1-1 and KX-1 of X. c. pv. alfalfae caused stunting of Kanza and KS76 alfalfa seedlings (Table 8). In subsequent tests for stunting with the F-1 strain at 10-fold higher inoculum concentration than that used in tests with isolates KX-1 and Ona 1-1, it still did not cause stunting (data not shown). In pathogenicity tests, isolate Ona 1-1 and the F-1 strain of X. c. pv. citrumelo produced typical BLS water-soaked lesions on leaves and stems of Kanza and KS76 plants, with more and larger lesions occurring on Kanza than on KS76. Strain F-6 of X. c. pv. citrumelo and strain 5757 of X. c. pv. vasculorum produced no symptoms.

DISCUSSION

My work has demonstrated resistance to X. c. pv. alfalfae-induced stunting in Kanza and KS76 alfalfa seedlings not associated with resistance to BLS lesion size. This lack of association was soon evident because KS76, which is highly resistant to BLS, was stunted severely (Table 1) and was verified by the lack of correlation between seedling height of treated seedlings and length of stem lesions on regrowth of the same plants. The inability of strain F-1 of X. c. pv. citrumelo to stunt alfalfa and yet be highly pathogenic also suggests the independence of these characters.

The ability to select for resistance to X. c. pv. alfalfae-induced seedling stunting, perhaps as early as 4 days after cotyledon inoculation, appears promising. By intercrossing the tallest 15% of treated seedlings significant increases in resistance to stunting was expressed in the Syn 1 generation (Table 2). Intercrossing a smaller percentage of the taller plants from larger populations should further improve the

TABLE 8. Mean heights of alfalfa seedlings 7, 9, and 14 days after application of a 0.5 μl aliquot containing either buffer (control) or 2-3 x 10^5 cfu of a specified strain of Xanthomonas campestris pathovar to a 26-gauge needle hole through a cotyledon of 5-day-old plants and incubation at 30 C

			Mean height (mm)[x]		
			Days after inoculation		
Strain[y]	Population	Treatment	7	9	14
5757	Kanza	Control	64.6 a	85.8 a	141.8 a
		Treated	61.7 a	82.6 a	135.5 ab
	KS76	Control	52.0 b	73.1 a	124.0 bc
		Treated	55.0 ab	73.8 a	119.5 c
F-1	Kanza	Control	67.6 a	92.6 a	155.8 a
		Treated	68.4 a	92.9 a	149.1 ab
	KS76	Control	65.4 a	81.8 a	145.1 ab
		Treated	62.7 a	84.7 a	140.3 b
F-6	Kanza	Control	62.2 a	81.8 a	131.9 a
		Treated	61.7 a	81.0 a	128.9 a
	KS76	Control	56.7 b	74.2 b	119.2 b
		Treated	57.8 b	74.5 b	123.1 b

TABLE 8. Continued

Strain[y]	Population	Treatment	Mean height (mm)[x] Days after inoculation 7	9	14
Ona 1-1	Kanza	Control	73.0 a	97.5 a	155.6 a
		Treated	35.3 b	41.4 b	77.1 b
	KS76	Control	70.4 a	94.7 a	144.2 a
		Treated	32.6 b	43.9 b	77.4 b
KX-1[z]	Kanza	Control	52.1 a	96.8 a	139.1 a
		Treated	19.9 b	34.0 c	53.4 d
	KS76	Control	46.9 a	83.1 b	117.8 b
		Treated	21.0 b	38.2 c	66.5 c

[x]Values within the same day for a bacterial strain followed by the same letter are not significantly different at P=0.05 according to Fisher's least significant difference.

[y]Strain 5757 of X. c. pv. vasculorum; strains F-1 and F-6 of X. c. pv. citrumelo; isolates Ona 1-1 and KX-1 of X. c. pv. alfalfae.

[z]Data obtained from Table 1 (30 C).

efficiency of a selection program in addition to the expected progress from increased cycles of recurrent phenotypic selection.

My research results did not define an optimum set of environmental conditions for evaluating stunting and BLS lesion size in alfalfa, except that 30 C appears to be the optimal temperature. Maximum stunting and lesion length both occurred at 30 C (Tables 1,3), which also is optimum for X. c pv. alfalfae growth (12). However, the diverse reaction of the two Kanza clones to 10- and 24-hr photoperiods (Table 3) indicates a need for more work on environmental factors affecting lesion development, although adult plant stunting (Table 6) was greater at the 10- than at the 24-hr photoperiod for both clones.

Stage of internode elongation at time of inoculation did not significantly affect lesion length (Table 5), although increasing inoculum concentration from 5.2×10^6 to 6.6×10^8 cfu/ml did increase lesion size on one of three clones tested.

I was unable to isolate X. c. pv. alfalfae from uninoculated internodes having the numerous necrotic flecks or raised water-soaked blisters. As these systemic-like responses were observed mostly on the inoculated stems of the most resistant KS76 plants, they may be initiated by X. c. pv. alfalfae and should be addressed by physiological studies. X. c. pv. alfalfae does not colonize the vascular tissues of alfalfa plants (3).

The F-1 and F-6 strains of X. c. pv. citrumelo were earlier identified as Florida Citrus Nursery strains of Xanthomonas campestris with undetermined pathovar status (8). Both strains have a high coefficient of similarity to X. c. pv. alfalfae based on RFLP analysis of genomic DNA (8). Some Florida Citrus Nursery strains have been reported to cause disease symptoms on alfalfa (10).

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ASSOCIATION BETWEEN, AND SOME ENVIRONMENTAL EFFECTS ON, LESION SIZE AND STUNTING OF ALFALFA BY XANTHOMONAS CAMPESTRIS PV. ALFALFAE

by

JOHN ISAAC EDMUNDS

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Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
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Stunting is common among alfalfa (Medicago sativa L.) plants with bacterial leaf spot (BLS) caused by Xanthomonas campestris pv. alfalfae (Riker, Jones and Davis) Dye. This study reports the effects of environmental factors on seedling stunting by X. c. pv. alfalfae and on BLS stem lesion development and the relationship between seedling stunting and lesion size as influenced by temperature.

To evaluate stunting, five-day-old seedlings of 'Kanza' (susceptible) and KS76 alfalfa germplasm (resistant) were inoculated on one cotyledon with either 1.6 - 2.8 x 10^5 cfu of X. c. pv. alfalfae or buffer (control) and their heights compared under various conditions. Height differences between control and treated plants of both populations appeared by 2 days after inoculation, and were greatest at 30 C. No seedlings developed BLS lesions during these tests.

Height of treated seedings was unrelated (r^2=-0.26 to +0.17) to subsequent stem lesion size on regrowth of the same plant. Also, strain F-1 of X. c. pv. citrumelo did not stunt alfalfa seedlings, yet induced typical BLS lesions on them.

One cycle of selection for tallness or stunting within both Kanza and KS76 seedlings inoculated with X. c. pv. alfalfae produced the expected shifts toward tallness or stunting among the treated seedlings in the resulting Syn 1 populations. This finding, and the fact that mean heights of control seedlings from the Syn 1 populations did not differ significantly from those of control seedlings of the parent populations, demonstrated that resistance to stunting in alfalfa caused by X. c. pv. alfalfae is heritable, and can be overcome by selecting for tall seedlings, perhaps as early as 4 days after cotyledon inoculation.

Both susceptible alfalfa clones tested developed longer stem lesions at 30 C than at 20 C at 10- and 24-hr photoperiods. At 30 C, lesions on clone Kanza 220-

16 were longest at the 10-hr photoperiod, whereas on clone Kanza 120-4 they were longest at the 24-hr photoperiod. Stem elongation was inhibited above the inoculation site at 30 C in both clones, whereas lengths of their inoculated internodes were unaffected. Lesions resulting from injecting stems with 5.2×10^6 or 6.6×10^8 viable cells per ml of inoculum varied according to alfalfa clone and growing temperature, but were longest at the higher concentration and at 30 C.