Virulence of North American and European Isolates of *Verticillium albo-atrum* on Alfalfa Cultivars

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ABSTRACT

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Four North American and three European geographical isolates of *Verticillium albo-atrum* from *Medicago sativa* were used to inoculate two U.S. and four European cultivars of *M. sativa*. Tests at two temperatures were conducted at London, U.K., and at Prosser, WA. Incubation temperatures at London were fluctuating, 17–30 C (night–day) and 12–21 C, and at Prosser were 27 ± 2 C and 20 ± 1 C, respectively. Disease severity was significantly greater ($P = 0.01$ and 0.05, respectively) at the higher temperatures at both locations. Differences in percent resistant plants between each of the cultivars, Maris Kabul, Vertus, Europe, Sabilt, Agate, and Apalachee, were significant ($P = 0.01$) except that Agate and Apalachee were not different at London, and Sabilt was not included in the Prosser test. Relative rank order of the cultivars, based on mean percent resistant plants, was the same at both locations with a high of 80% for Maris Kabul and a low of 1% for Apalachee. The temperature × isolate interaction was not statistically significant, but the temperature × cultivar interaction was significant ($P = 0.01$). A significant ($P = 0.05$) mean difference of 1.6% resistant plants following inoculation of alfalfa with European and North American isolates at Prosser was not supported by the data obtained at London. This difference is smaller than is usually obtained in tests of a single isolate in repeated tests with the same cultivars. We concluded that there is no difference among isolates as measured by virulence on highly susceptible and highly resistant alfalfa cultivars and that the North American outbreaks of Verticillium wilt very likely originated from introduction of a European strain of the pathogen.

Verticillium wilt of alfalfa (*Medicago sativa* L.) was first recorded from Europe in Sweden during 1918 and from about 1950 onwards throughout northern Europe (11), but was not found in the United States until 1976 (7). The alfalfa strain of *Verticillium albo-atrum* Reinke and Berth. has a limited host range (1,3,8,10). Some investigators have reported possible, though not conclusive, differences among geographical isolates (5,6,12,13), while others have found none (1,9). Two U.S. isolates were apparently more virulent towards Maris Kabul and Sabilt than two European isolates (one French, one British) in a field test in the United Kingdom in 1976 (5). This was a drought year with higher than normal temperatures.

This study was initiated to test the hypothesis that the same strain of *V. albo-atrum* exists in Europe and North America. This information would also be useful in making comparisons of research results and in selecting germ plasm for sources of resistance in alfalfa breeding programs.

MATERIALS AND METHODS

Geographical isolates. *V. albo-atrum* isolates 40-3, 43-2, 80A-1 from Washington State, and 137-1 from British Columbia, Canada, were selected as representative of 38 previously tested geographical isolates, single-spored, and stored on silica gel at 5 C (1) at Prosser, WA. Isolates NI and E.7 from Norfolk and Cambridge, U.K. (respectively, previously used as representative virulent isolates to evaluate seedling wilt resistance in trials at London), and V.31.2, kindly supplied as a representative virulent isolate by J. Gondran from Toulouse, France, were single-spored and stored on potato-dextrose agar (PDA) at 5 C at London, U.K. Isolates from the two storage locations were exchanged prior to virulence tests.

Alfalfa cultivars. Seed of cultivars Agate and Apalachee from seed lots in Prosser, and cultivars Europe, Maris Kabul, Sabilt, and Vertus seed lots from the National Institute of Agricultural Botany (NIAB) in Cambridge were exchanged to provide common seed sources for tests in both locations. Seeds were treated with chlorine gas (generated by adding 3.3 ml of hydrochloric acid to 100 ml of 5.25% NaOCl) (14) in a laboratory dessicator prior to exchange. Seeds of cultivars Europe, Maris Kabul, Sabilt, and Vertus apparently were treated with carboxin by customs officials before delivery to the United States.

Prosser, WA. Seeds of all cultivars were planted in metal flats (35 × 50 × 10 cm) that contained a steamed (4 hr at 20.7 kg·cm^{-2}) mixture of Ritzville silt loam, sand, and peat (2:2:1, v/v). Plants were grown in a greenhouse under natural lighting (24,000–53,000 lux) at 15–21 C for 12 wk with 5 g of 15-15-15 (N-P-K) fertilizer applied to each flat at monthly intervals beginning the fourth day after planting.

Inoculum of each of the isolates consisted of a suspension (8×10^6 conidia per milliliter) of washed conidia produced in Difco-Czapek Dox broth as described earlier (1). Plants were dug and the roots were rinsed and then soaked in the inoculum suspension for 20 min. Plants (39 per treatment per replication) were transplanted into flats of newly prepared soil mix. Isolate 43-2 from the U.S. was not used, owing to lack of space in growth chambers. Two consecutive tests were conducted in growth chambers under daylight-type fluorescent lighting at 7,600 lux for 14 hr each day.

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Temperatures during the respective tests were 20 ± 1 and 27 ± 2 C. Percent resistant plants and mean disease severity index (SI) for each isolate-cultivar combination within each temperature were recorded at 6 and 5 wk, respectively. A rating scale of 1 to 5 was used to rate each plant with 1 = no symptoms, 2 = one or two leaflets chlorotic, 3 = trifoliolates on one shoot chlorotic, 4 = trifoliolates on more than one shoot chlorotic, and 5 = plant dead. Severity index was calculated as $\Sigma[(N_1 \cdot 1) + (N_2 \cdot 2) + (N_3 \cdot 3) + (N_4 \cdot 4) + (N_5 \cdot 5)]/\Sigma\ N_{1\cdots5}$ in which $N_{1\cdots5}$ = the number of plants in each respective rating category. Plants rated at severity index 1 or 2 were considered resistant and the sum of the numbers of plants in the two categories was used to calculate percent resistant plants. There were not enough plants of Sabilt from the common seed source to plant all four replications, so data from Sabilt was not used in the statistical analysis. Data were analyzed on a three-way analysis with four replications and with orthogonal subdivision of degrees of freedom for cultivars, isolates, and temperature × cultivar interactions.

London, U.K. Isolates were transferred to PDA plates and incubated in the dark at 20–21 C for 10 days. Conidia were washed from each plate with 10 ml of sterile distilled water and the concentration was adjusted to 1×10^6 conidia per milliliter. Aliquots (0.2 ml) were spread over PDA plates and incubated for 3 days at 20 C. Conidia again were washed off the agar plates with gentle agitation of 10 ml of sterile distilled water. Dilution was made to obtain a working inoculum concentration of 5×10^6 conidia per milliliter. Seeds of each alfalfa cultivar were pregerminated for 36–48 hr on moistened sterile filter paper and transferred to 7.5-cm-diameter plastic pots when emergent radicles were 1.0–1.5 cm long. Each pot contained a mixture of John Innes' Compost No. 1 (loam), Levington's Potting Compost (peat), and acid-washed sand (1:1:1, v/v). Seedlings were grown for 2 wk in glasshouse conditions with a supplemental 16-hr daylength provided by 500W mercury vapor lamps (giving overall 37,000 to 55,000 lux at plant height) and at a temperature range of 12–21 C (daylight hours at 18–21 C). A second similar test was made at 17–30 C (daylight hours, 23–30 C).

Fifty 2-wk-old seedlings (10 seedlings in each of five pots) of each cultivar were inoculated with each isolate by immersing the roots in 30–50 ml of the inoculum for 10 min. Roots of 20 control seedlings of each cultivar were dipped for 10 min in sterile distilled water. Inoculated and control seedlings were transferred to fresh potting mixture, 10 seedlings per 12.7-cm-diameter plastic pot, and placed in the glasshouse with the environmental parameters as before. Symptoms on the 16–20 leaflets developing on the seedlings were assessed 21 days after inoculation using the following key,

TABLE 1. Analysis of variance for five alfalfa cultivars inoculated at Prosser, WA, with six isolates of *Verticillium albo-atrum* and incubated at 20 and 27 C

Source	df	Mean square: Resistant plants (%)[a]		Mean square: Severity index	
Replicates	3	0.049	*	0.333	*
Temperatures	1	0.002	NS	0.569	*
Isolates	5	0.015	NS	0.241	NS
NA vs European	1	0.047	*	0.898	**
137-1 vs 40-3 + 80A-1[b]	1	0.000	NS	0.005	NS
40-3 vs 80A-1	1	0.001	NS	0.004	NS
V.31.2 vs E.7 + NI	1	0.000	NS	0.006	NS
E.7 vs NI	1	0.026	NS	0.293	NS
Cultivars	4	5.70	**	46.99	**
NA vs European	1	15.675	**	119.33	**
Agate vs Apalachee	1	1.261	**	7.111	**
M. Kabul vs Europe + Vertus	1	5.132	**	55.594	**
Europe vs Vertus	1	0.731	**	5.912	**
Temperatures × isolates	5	0.008	NS	0.035	NS
Temperatures × cultivars	4	0.042	**	0.341	*
(NA vs European) × T	1	0.058	*	0.126	NS
(Agate vs Apalachee) × T	1	0.044	*	0.229	NS
(M. Kabul vs Europe + Vertus) × T	1	0.009	NS	0.338	*
(Europe vs Vertus) × T	1	0.055	*	0.670	**
Isolates × cultivars	20	0.011	NS	0.098	NS
Temperatures × cultivars × isolates	20	0.010	NS	0.071	NS
Error	177	0.009		0.084	

[a] Percent resistant plants was based on the sum of plants rated at severity 1 or 2 divided by the total number of plants observed. Percentage values were transformed to arc sin ($\sqrt{[x]}$) before analysis. NS, not significant; * and **, indicate statistical significance at $P = 0.05$ and $P = 0.01$, respectively.
[b] A "+" between isolates or cultivars indicates the comparison is with the mean of the indicated components.

TABLE 2. Analysis of variance for six alfalfa cultivars inoculated at London, U.K., with seven isolates of *Verticillium albo-atrum* and incubated at fluctuating 12–21 and 17–30 C

Source	df	Mean square: Resistant (%)[a]		Mean square: Severity index	
Temperatures	1	3.699	**	87.047	**
Isolates	6	0.010	NS	0.081	NS
NA vs European	1	0.026	NS	0.04	NS
137-1 vs 40-3 + 80A-1 + 43-2[b]	1	0.005	NS	0.228	NS
40-3 vs 80A-1 + 43-2	1	0.000	NS	0.073	NS
80A-1 vs 43-2	1	0.000	NS	0.065	NS
V.31.2 vs E.7 + NI	1	0.015	NS	0.075	NS
E.7 vs NI	1	0.017	NS	0.004	NS
Cultivars	5	0.958	**	16.232	**
NA vs European	1	2.512	**	47.20	**
Agate vs Apalachee	1	0.004	NS	0.096	NS
M. Kabul vs Vertus + Sabilt + Europe	1	1.769	**	28.036	**
Vertus vs Sabilt + Europe	1	0.325	**	3.725	**
Sabilt vs Europe	1	0.179	**	2.101	**
Temperatures × isolates	6	0.011	NS	0.136	NS
Temperatures × cultivars	5	0.847	**	1.777	**
(NA vs European) × T	1	0.148	**	2.837	**
(Agate vs Apalachee) × T	1	0.052	*	0.624	**
(M. Kabul vs Vertus + Sabilt + Europe) × T	1	0.216	**	4.791	**
(Vertus vs Sabilt + Europe) × T	1	0.001	NS	0.026	NS
(Sabilt vs Europe) × T	1	0.007	NS	0.609	**
Isolates × cultivars	30	0.008	NS	0.113	NS
Error (Temperatures × cultivars × isolates)	30	0.008		0.113	

[a] Percent resistant plants was based on the sum of plants rated 0 or 0.5 in severity. Percentage values were transformed to arc sine ($\sqrt{[x]}$) before analysis. NS, not significant; * and **, significant at $P = 0.05$ and 0.01, respectively.
[b] A "+" between isolates or cultivars indicates the comparison is with the mean of the indicated components.

TABLE 3. Mean disease reaction, across all isolates, of 12-wk-old plants of five alfalfa cultivars inoculated with *Verticillium albo-atrum* and incubated at two temperatures at Prosser, WA

Incubation temperature (c)	Cultivars: Maris Kabul	Vertus	Europe	Agate	Apalachee	Means[w]
Resistant plants (%)[x]						
21	67	41	21	10	0.0	28
27	67	33	21	8	1	26
Means[y]	67 a	37 b	21 c	9 d	0.5 e	
Severity index[x,z]						
21	2.3	3.1	3.8	4.3	4.9	3.7 *
27	2.3	3.5	3.9	4.4	4.8	3.8 *
Means[y]	2.3 a	3.3 b	3.85 c	4.35 d	4.85 e	

[w] Temperature means significantly different ($P = 0.05$) for severity index only.
[x] LSDs ($P = 0.05$) for differences between the cultivar × temperature means under resistant plants and severity index are 5.5 and 0.23, respectively.
[y] Cultivar means significantly different from each other ($P = 0.01$) as determined by orthogonal comparisons.
[z] Severity index ratings based on a scale of 1 to 5 with 1 = no symptoms and 5 = dead; classes 1 and 2 are considered resistant.

TABLE 4. Mean disease reaction, across all isolates, of six alfalfa cultivars inoculated at London, U.K., at 2 wk plant age with *Verticillium albo-atrum* and incubated at two temperatures

Incubation temperature range (c)	Cultivars						Row means (%)[w]
	Maris Kabul	Vertus	Europe	Sabilt	Agate	Apalachee	
Resistant plants (%)[x]							
12–21	80	67	59	41	32	39	53 **
17–30	68	27	17	8	3	1	21 **
Means[y]	74 a	47 b	38 c	29 d	17 e	20 e	
Severity index[x,z]							
12–21	0.61	1.18	1.34	2.18	2.62	2.44	7.73 **
17–30	1.37	3.23	3.79	4.04	4.88	5.29	3.77 **
Means[y]	0.99 a	2.20 b	2.56 c	3.11 d	3.75 e	3.86 e	

[w] Temperature-range row means were significantly different ($P = 0.01$).
[x] LSDs ($P = 0.05$) for differences between the cultivar × temperature means under resistant plants and severity index are 12.2 and 0.50, respectively.
[y] Cultivar means followed by the same letter were not statistically different ($P = 0.01$) as determined by orthogonal comparisons.
[z] Severity index ratings based on a scale of 0, 0.5, 1, 2, 3, 4, 5, 6. Classes 0 and 0.5 are considered resistant. Class 6 = plant dead.

employed at the NIAB, Cambridge (4): 0 = no symptoms; 0.5 = slight chlorosis of basal leaves; 1 = chlorosis or necrosis of basal leaves, 2 = 10% of trifoliolate leaves showing symptoms; 3 = 25% of trifoliolate leaves showing symptoms; 4 = 50% of trifoliolate leaves showing symptoms; 5 = 75% of trifoliolate leaves showing symptoms; and 6 = plant dead. Mean wilt or SI scores were calculated for the 50 plants in each isolate × cultivar combination and number of plants in categories 0 and 0.5 were used to calculate the percent resistant plants. A three-way analysis of variance was performed on percent resistant plants and SI data. Subdivision of degrees of freedom for isolates, cultivars, and the temperature × cultivar interaction was made by using orthogonal comparisons.

RESULTS AND DISCUSSION

Analyses of variance (Tables 1 and 2) show a significant temperature effect ($P = 0.01$) for percent resistant plants and SI in the London data and for SI ($P = 0.05$) only in the Prosser data. The trend at both locations was toward increased severity of disease reaction at the higher temperature. The fluctuating temperature at the lower level in the London test (12–21 C) was below the published optimum level for *V. albo-atrum* (21–22 C) (1); therefore, a longer incubation period might have been required for maximum development of the disease in susceptible plants. However, there is evidence that plants inoculated 2 wk after seeding, and incubated at 20 C were less susceptible than older plants (2). Apalachee was very susceptible and has shown <5% resistant plants in numerous tests (A. A. Christen and R. N. Peaden, *unpublished*).

Differences between cultivars were significant ($P = 0.01$) (Tables 1 and 2). Ranking of the cultivars was similar at both temperatures at each location (Tables 3 and 4).

There is no clear pattern for the significant temperature × cultivar interaction. Most differences are in the magnitude of difference between incubation temperatures when two cultivars are compared. The difference in number of resistant Maris Kabul plants at the two temperatures in London was 12% while it was 40% for Vertus (Table 4). These differences in the Prosser test were negligible for Maris Kabul and only 8% for Vertus. We propose that a longer incubation period at the lower temperature might produce results similar to those at the higher temperature. There is not sufficient evidence in these experiments to show whether age of plants at the time of inoculation would affect disease development at the lower temperature.

Differences in overall mean effect of isolates were not significant at either location. However, subdivision of degrees of freedom into logical components of North American versus European, Canadian versus U.S., and French versus U.K. isolates revealed a significant ($P = 0.05$) NA versus European comparison in the Prosser data that was not supported by the London data (Tables 1 and 2). Mean percentage (26.2) of resistant plants at Prosser with NA isolates was significantly less than 27.8 ($P = 0.05$) with European isolates (Table 5). The difference (1.6), however, is much smaller than normal for repeated tests of resistance with a single isolate. For example, percent resistance in Vertus in several tests (A. A. Christen and R. N. Peaden, *unpublished*) has ranged from 36 to 50 when the susceptible checks were less than 7%.

TABLE 5. Mean disease reaction of alfalfa cultivars inoculated with geographical isolates of *Verticillium albo-atrum* from North America and Europe

Isolate	London, U.K.[w]		Prosser, WA, USA[x]	
	Resistant plants (%)	Severity index	Resistant plants (%)[y]	Severity index[z]
40-3 (USA)	36.5	2.8	25.6 a	3.8 a
43-2 (USA)	36.0	2.7	...	...
80A-1 (USA)	35.5	2.8	26.3 a	3.8 a
137-1 (Canada)	36.0	2.6	26.8 a	3.8 a
NI (U.K.)	34.5	2.8	29.0 b	3.6 b
E.7 (U.K.)	38.5	2.8	26.6 b	3.7 b
V.31.2 (France)	41.0	2.6	27.8 b	3.7 b

[w] Means are the average of two incubation temperatures and six alfalfa cultivars and are not statistically different ($P = 0.05$).
[x] Means are the average of four replications of two temperatures and five alfalfa cultivars.
[y] Differences between the averages for the North American and for the European isolates are significant ($P = 0.05$) according to orthogonal comparisons.
[z] Differences between the averages for the North American and for the European isolates are significant ($P = 0.01$) according to orthogonal comparisons.

Neither the temperature × isolate interaction nor any of the single-degree-of-freedom components of this interaction were significant. We concluded that there was no consistent difference in the virulence of the European and NA isolates as measured by plant resistance or severity index. From these results, it seems possible that the North American outbreak of Verticillium wilt could have resulted from introduction of a European strain of the pathogen. This does not rule out differences that might be measured by other means such as growth on selective media and optimum temperature for growth, even though virulence might not be affected.

The alfalfa cultivars in these tests represent a wide range of resistance levels, but do not represent all germ plasm sources of *Medicago sativa*. It is possible that geographical isolate × cultivar interactions might be shown as additional cultivars are tested.

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