Comparison of Methods to Evaluate Alfalfa Cultivars for Reaction to *Verticillium albo-atrum*

C. R. GRAU, Professor, Department of Plant Pathology, University of Wisconsin-Madison 53706, S. L. NYGAARD, Research Pathologist, W-L Research, Inc., Evansville, WI 53536, D. C. ARNY, Emeritus Professor, Department of Plant Pathology, University of Wisconsin-Madison 53706, and P. A. DELWICHE, Plant Pathologist, L. D. Maffaei Seed Co., Newman, CA 95360

ABSTRACT

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The evaluation of 34 alfalfa (*Medicago sativa* L.) cultivars for reaction to *Verticillium albo-atrum* was conducted in controlled and field environments. In controlled environments, the results of two different methods of host inoculation were compared and found to be similar. Inoculation with conidia of *V. albo-atrum* was accomplished by either uprooting 6-wk-old plants, clipping off their stems, and soaking the entire plant in a conidial suspension or by clipping the stems off with an infested scissors and spraying the stubble with a conidial suspension. In the field, Verticillium wilt symptom frequency and stand density were recorded for 4 yr at one location. Maximum symptom expression occurred during the spring regrowth in the third and fourth years of the stand. Maximum field disease severity was significantly correlated with results from either of the inoculation methods in controlled environments. Several cultivars expressed less disease in the field test than was predicted based on evaluations conducted in controlled environments. These cultivars may possess a type of field resistance that is not readily identified by artificial inoculation and incubation in controlled environments.

Verticillium wilt of alfalfa (*Medicago sativa* L.), caused by *Verticillium albo-atrum* Reinke & Berthier, was first reported in Sweden in 1918 (8) and subsequently has been identified elsewhere in Europe (10,17), North America (1,7), and New Zealand (24). Although certain cultural practices aid in control of this destructive vascular disease, the use of alfalfa cultivars resistant to Verticillium wilt can also substantially reduce its severity and prolong stand life (1,3,5,18,21).

The success of a disease resistance breeding program is dependent upon the methods employed for inoculation, evaluation, and selection within the target host population. Roots are a common site for artificial inoculation of alfalfa with *V. albo-atrum*. Inoculum may be introduced into the root zone by amending the soil with colonized wheat seed (19,22) or by soaking wounded roots in a conidial suspension followed by transplantation into steamed soil (2–4,13). Stems are an alternate but less frequently used infection court. Conidia of *V. albo-atrum* can be introduced by cutting stems with an infested cutting device (15,22) or by spraying conidia onto freshly cut stems (15,21). Alfalfa germ plasm can also be assayed for its reaction to *V. albo-atrum* by inoculating callus tissue (14), by subjecting cut stems to propagule-free culture filtrates (9), and by monitoring symptom development in populations of infected plants in the field (20).

Methods for evaluating germ plasm for disease resistance must minimize the number of susceptible plants that escape detection and be indicative of population performance in natural epidemics. The objectives of this study were to compare the reactions of alfalfa cultivars to *V. albo-atrum* using two different artificial inoculation methods in a controlled environment and to compare this data with that derived from evaluations from field environments. Additionally, this study is intended to help estimate the amount of cultivar resistance needed to

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suppress the incidence of Verticillium wilt of alfalfa in the field.

MATERIALS AND METHODS

Inoculum. Six isolates of *V. albo-atrum*, recovered from alfalfa stems collected in Wisconsin, were used in all experiments. Isolates were individually cultured on potato-dextrose agar at 21 C. Five mycelial plugs (5 mm in diameter) cut from the margin of a colony were transferred into 80 ml of Czapek's-Dox broth in a 500-ml, baffle-sided Erlenmeyer flask. Flasks were placed on a rotary shaker (120 rpm) at 20–23 C for 5–7 days. The suspension of conidia was passed through three layers of cheesecloth and centrifuged at 2,700 × G for 15 min. The supernatant was discarded and the pellet of conidia resuspended in distilled water. Inoculum was quantified using a hemacytometer and adjusted to a concentration of 8×10^6 conidia per milliliter. A composite inoculum was made that consisted of equal numbers of conidia from each isolate.

Alfalfa cultivars. Thirty-four cultivars (Table 1) were chosen on the basis of prior selection for resistance to *V. albo-atrum*. The cultivars Saranac and Vertus were included as susceptible and resistant checks, respectively.

Controlled environment, transplant inoculation method. A method of inoculation whereby the whole plant was soaked in inoculum and then transplanted, hereafter referred to as the transplant method, was used to evaluate 30 alfalfa cultivars for reaction to *V. albo-atrum*. In this method, eight plants were grown from seed in plastic pots 10 cm in diameter, filled with steamed planting medium (1:1, v/v, sand:muck soil). Each pot constituted a replicate and each cultivar was replicated four times. Pots were placed in saucers that were filled with water daily. Plants were maintained in a controlled environment room (21 C day/16 C night, 14 hr photoperiod, light intensity = 200 $\mu Em^{-2}sec^{-1}$) for the duration of each experiment. Eight weeks after planting, plants were lifted and washed free of excess soil, the roots trimmed to a length of ca. 8 cm, and the stems clipped to a height of 3 cm. Plants were completely immersed in a conidial suspension of *V. albo-atrum* for 20 min, then transplanted into fresh steamed soil mix. Plants were evaluated for severity of foliar symptoms 5–6 wk after inoculation. This experiment was repeated.

Controlled environment, stem inoculation method. A method referred to as the stem inoculation method was used to evaluate all 34 cultivars for reaction to *V. albo-atrum* in the same controlled environment room described above. Cone-shaped pots (165 cc), held upright by means of specially designed racks (both by Ray Leach Cone-Tainer Nursery, Canaby, OR 97013), were used. A 2.5-cm layer of coarse vermiculite was placed in the bottom of each pot and overlain with the steamed soil mix described above. The racks suspended the pots in 53- × 27-cm plastic pans (T. O. Plastics Inc., Minneapolis, MN). Water was added to each pan to a level that covered the opening at the bottom of each pot. Ten alfalfa seeds were sown into each pot and covered with 5 mm of coarse vermiculite. The seeded pots were watered from above to assure a firmly established, continuous column of water throughout the pot. After seedling emergence, water was supplied solely via uptake from the pan below. Seedlings were thinned to five per pot 7–9 days after planting. Five pots (25 plants) were used per replicate and each cultivar was replicated four times. Beginning 4 wk after planting, plants were fertilized every 14 days by adding Hoagland's nutrient solution to the existing water in the reservoir.

Six-week-old plants were inoculated by clipping the stems to 3 cm (in height) with scissors dipped into the conidial suspension (8×10^6 conidia/ml). The scissor was redipped before each clip. The freshly cut stems were then sprayed with the inoculum suspension amended with a wetting agent (polyoxyethene 20 sorbitan monolaurate, 1 ml/L). Stems were sprayed to run-off four times using a compressed air hand sprayer. This experiment was repeated.

Disease severity index. In the controlled environment evaluations, the severity of foliar symptoms on individual plants was rated 6 wk after inoculation (bud-stage) on a scale of 1–5: 1 = none to minimal chlorosis of lower leaves; 2 = chlorosis of lower and middle leaves but no chlorosis or necrosis of terminal leaves; 3 = well-developed symptoms of chlorotic, necrotic, and twisted terminal leaflets on at least one, but not all, mainstems; 4 = severe symptoms of chlorosis, necrosis, and twisting of all leaflets on all mainstems; and 5 = killed plant. Cultivars were characterized for their

Table 1. Reaction of alfalfa cultivars to *Verticillium albo-atrum* when evaluated in controlled and field environments

	Controlled environment[a]						
	Resistant plants[c] (%)		Disease severity index[c]		Field environment[b] (Verticillium wilt field index)		
Cultivar	Stem	Trans.	Stem	Trans.	Year 2	Year 3	Year 4
Excalibur	44	25	3.08	3.49	1	18	37
DK 135	39	26	3.15	3.03	1	19	26
Endure	39	···	3.13	···	0	2	14
Vertus	38	55	3.15	2.57	1	2	15
Admiral	37	···	3.31	···	1	7	16
Emerald	29	31	3.39	2.97	1	4	22
Apollo II	29	26	3.64	3.47	1	1	24
Wrangler	24	11	3.67	3.50	1	13	40
Trumpetor	24	25	3.97	3.30	1	24	29
Chippewa	24	15	3.80	3.40	2	21	39
WL 316	20	27	3.64	3.27	0	11	26
Decathalon	19	14	3.65	3.40	2	30	54
Valor	19	24	3.83	3.30	1	12	35
Baker	19	19	4.00	3.40	1	9	38
Armor	17	3	3.76	3.97	2	29	93
Blazer	17	8	3.83	3.63	1	29	28
Oneida	17	4	3.91	4.10	1	19	41
Vancor	17	3	3.92	4.03	2	43	129
Magnum	17	14	4.12	3.57	2	27	37
Perry	15	4	4.43	3.83	2	17	38
Atlas	15	···	4.23	···	3	35	52
Duke	13	2	4.11	3.73	2	22	57
DK 120	12	4	4.27	3.83	2	15	35
DK 130	11	16	4.21	3.57	1	26	71
Vernal	12	5	4.12	3.97	2	36	48
Olympic	12	0	4.33	3.90	3	48	104
Arc	7	2	4.14	3.77	2	31	54
532	7	2	4.35	4.23	2	44	78
Iroquois	7	···	4.41	···	2	30	80
Vista	6	0	4.44	4.23	3	39	208
Phytor	5	2	4.35	4.00	2	29	86
Weevilchek	3	7	4.71	3.90	2	36	184
Saranac	1	2	4.50	4.23	1	51	76
Thor	0	0	4.80	4.20	3	61	226
LSD ($P = 0.05$)	10	16	0.42	0.53	ns	16	105
Mean	18	12	3.96	3.67	2	25	63

[a] Stem inoculation method (stem) and transplant method (trans.); data presented are from the first repetition of each experiment.

[b] Verticillium wilt field index was calculated by dividing the number of symptomatic stems per plot 2 days before harvest by the estimated percent of stand for the plot. Stand density estimates were determined after 8 cm of regrowth following winter dormancy.

[c] Reaction of cultivars to Verticillium wilt expressed as percent resistant plants (percent of classes 1 and 2 combined) and disease severity index (DSI) based on a scale of 1–5.

reaction to *V. albo-atrum* by calculating their mean disease severity index (DSI). Additionally, the percentage of resistant plants (i.e., the sum of the percentages of plants rated as classes 1 and 2) was used to characterize the cultivars.

Field environment evaluation. Thirty-four cultivars were evaluated for reaction to Verticillium wilt at a site on the University of Wisconsin Agricultural Research Station at Arlington, WI on Plano silt loam (fine-silty, mixed, mesic Typic Argiudoll). Verticillium wilt had been diagnosed the previous year in a 3-yr-old stand of alfalfa. The plot area was plowed the preceding fall and prepared for planting on 10 May. The herbicide EPTC (formulated as Eptam 4EC, Stauffer Chemical Co.) was incorporated before planting at a rate of 3.36 kg a.i./ha. Cultivars were seeded at a rate of 5 g of seed per 4.6 m row, and rows were spaced 0.76 m apart. Cultivars were replicated four times in a randomized complete block design. Plots were harvested three times per year when 10% of the stems expressed flowers.

V. albo-atrum was artificially introduced into the plots at the second harvest by spraying the freshly cut alfalfa stubble with a suspension of conidia (8×10^6 conidia per ml) on 10 August of the establishment year. Thereafter, plots were monitored for the onset of Verticillium wilt symptoms. At each harvest, in each year, plots were evaluated by counting individual stems that expressed symptoms of Verticillium wilt. Plant stand density was determined by estimating the percent of ground cover for each plot after ca. 8 cm of regrowth had occurred. A field index for Verticillium wilt was calculated by dividing the number of symptomatic stems per plot (determined 1–2 days before harvest) by the estimated percent stand (recorded at the beginning of each harvest period). This conversion was implemented as a means to more accurately assess the proportion of plot area with symptoms of Verticillium wilt.

Statistical tests. Analysis of variance, Fischer's least significant difference test for comparison of means, regression analysis, and Spearman's rank correlation test were used to determine differences among cultivars and relationships among treatment means (23).

RESULTS AND DISCUSSION

Comparison of data derived from different inoculation methods. Percent resistant plants and DSI were used to characterize the reaction of alfalfa cultivars to *V. albo-atrum* and to compare the stem and transplant inoculation methods to one another. Both methods of inoculation resulted in a high level of disease severity and a differential reaction of the alfalfa cultivars to *V. albo-atrum*. Saranac and Vertus were rated as susceptible and resistant, respectively, by both methods (Table 1). Cultivar reactions, measured by either DSI or percent resistant plants, were significantly correlated between inoculation methods ($P = 0.01$). Spearman's rank correlation coefficient (r_s) was 0.84 for percent resistant plants and 0.70 for DSI. A smaller percentage of resistant plants was attained using the transplant method, and the stem method resulted in an overall greater mean DSI. The stem method tended to separate plants into both extremes of the disease severity scale and, consequently, resulted in fewer intermediate severity (class 3) plants (data not shown).

Evaluation in a field environment. Symptoms of Verticillium wilt were first observed at the first harvest of the second year. The incidence of foliar symptoms progressively increased through the fourth year of the stand; within each year, symptoms were most prevalent during the first harvest period. Thus, data presented on reactions of cultivars is from the first harvest of each year (Table 1).

Cultivars that had undergone previous intentional selection for Verticillium wilt resistance (i.e., Excalibur, DK 135, Endure, Vertus, Admiral, Emerald, Apollo II, Wrangler, Trumpetor, WL 316, and Decathalon) generally expressed a lower incidence of Verticillium wilt in the field, compared with cultivars that had not undergone intentional selection for resistance (Table 1). However, several of the unselected cultivars (eg. Valor, Baker, Oneida, and Perry) expressed a lower than anticipated Verticillium wilt index (Table 1). It was not determined whether this phenotypic response was due to a generalized and nonspecific reaction to *V. albo-atrum* or whether these alfalfa cultivars were unintentionally subjected to selection pressure for resistance to *V. albo-atrum* in breeding nurseries and/or seed production fields.

Relationship between results from controlled and field environments. The reactions of alfalfa cultivars determined by both methods of inoculation in controlled environments were significantly correlated with transformed (ln(x+1)) Verticillium wilt field indices recorded at the first harvest period for years 3 and 4 ($P = 0.01$) (Fig. 1).

We conclude, based on the similarity of intercepts and slopes (Fig. 1) ($P = 0.01$), that either method of artificial inoculation is satisfactory for predicting the reaction of alfalfa cultivars to *V. albo-atrum* in field environments. However, there are several advantages for using the stem inoculation method. It requires less material, less space, and far less labor; transplant shock is eliminated as a complicating factor; and it more closely approximates inoculum spread in the field (11,12). We have repeated both the transplant and stem methods of inoculation at least three times using

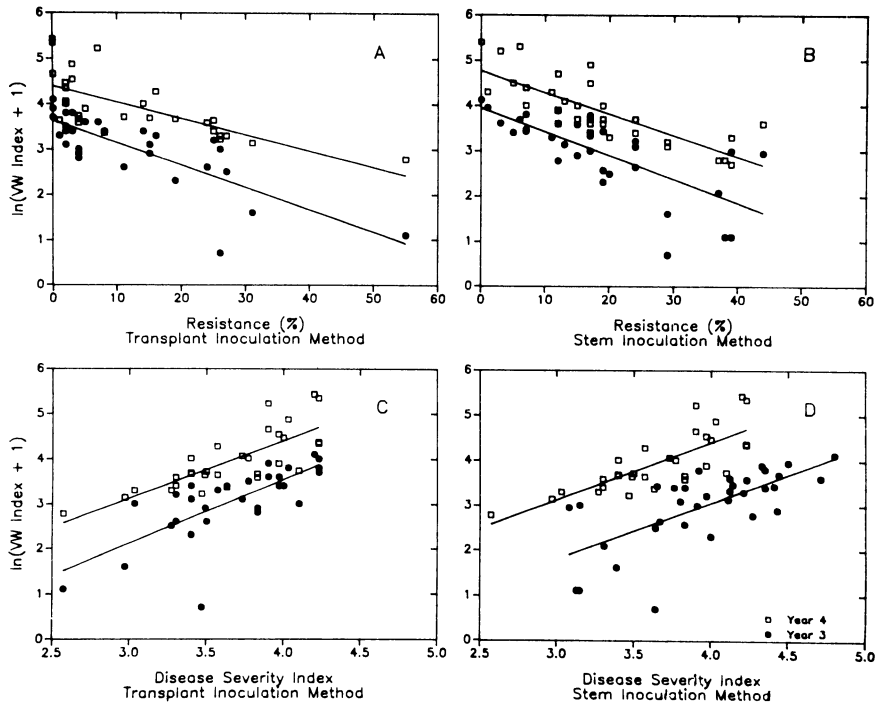

Fig. 1. Relationship between Verticillium wilt field index (ln(x+1)) and percent resistant plants or disease severity index as determined by the transplant and stem methods of plant inoculation in a controlled environment. All linear regressions were significant ($P = 0.01$). Regression equations for years three (■) and four (□), respectively, were: **(A)** $y = 3.63 - 0.48x$ ($r^2 = 0.61$) and $y = 4.40 - 0.38x$ ($r^2 = 0.49$); **(B)** $y = 3.95 - 0.53x$ ($r^2 = 0.52$) and $y = 4.76 - 0.48x$ ($r^2 = 0.63$); **(C)** $y = -2.15 + 1.42x$ ($r^2 = 0.53$) and $y = -0.68 + 1.25x$ ($r^2 = 0.61$); **(D)** $y = -2.04 + 1.27x$ ($r^2 = 0.46$) and $y = -0.74 + 1.16x$ ($r^2 = 0.61$).

Vertus, Saranac, and subsets of the 34 cultivars presented here. Results (percent resistant plants and DSI) were repeatable with each trial.

Verticillium wilt can be suppressed to a low incidence by planting cultivars with 50–60% resistant plants (Fig. 1A and B). Busch and Christie (3) suggested that alfalfa cultivars with 60% resistant plants would provide maximum protection against Verticillium wilt. More recently, Papadopoulous, et al (20) concluded that alfalfa cultivars should possess levels of resistance comparable to that of Vertus to avoid yield losses caused by Verticillium wilt. However, in our tests, many cultivars with 10–15% resistant plants reacted similarly in the field to cultivars with 25–30% resistant plants (Table 1). For example, the cultivars Blazer, Oneida, Magnum, Perry, and DK 120 had field indices approximately equal to the cultivars Emerald, Apollo II, Wrangler, Trumpetor, Chippewa, and WL 316 (Table 1). These results may be the consequence of differing genetic systems that govern the expression of resistance to *V. albo-atrum* (25) and, thus, merit further investigation.

Alfalfa populations have been improved for resistance to Verticillium wilt by phenotypic recurrent selection (19) and by mass selection (16). Resistance in Maris Kabul, a cultivar developed by phenotypic recurrent selection, is conferred by a single dominant gene derived from *M. hemicycla* L. (6) and restricts the colonization of stems by *V. albo-atrum* (8). In contrast, multiple gene action is operative in Vertus, a cultivar developed by mass selection (25) and colonization of stems by *V. albo-atrum* is only partially restricted (8,21). Resistance in Vertus was likely derived from *M. sativa*. The difference in expression of disease resistance due to single and multiple gene systems could potentially result in reactions that differ based on evaluations conducted in controlled versus field environments. However, no cases have been reported of the genetic system in the target population being influenced by the inoculation technique per se.

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