Photosynthetic Photon Flux Density × Pathogen Interaction in Growth of Alfalfa Infected with *Verticillium albo-atrum*

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ABSTRACT

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Photosynthetic photon flux density (PPFD) was manipulated in greenhouse experiments to determine whether carbon assimilation regulated expression of resistance to *Verticillium albo-atrum*. Treatments were pathogen (*V. albo-atrum* or no *V. albo-atrum*), clone (resistant or susceptible), PPFD (100, 70, or 40% of ambient), and time (3 wk). Treatment effects on disease ratings, dry weight of plant parts, net photosynthesis, and stomatal conductance were evaluated weekly. Significant pathogen × PPFD × week interactions were detected in disease rating, plant height, stem dry weight, and aerial biomass, and a pathogen × PPFD interaction was noted in leaf dry weight when data from the resistant clone were analyzed. In all cases, the interactions were caused by the loss of host resistance under 40% PPFD. *V. albo-atrum* did not affect net photosynthesis or stomatal conductance of the resistant clone, but these parameters were reduced by the 40 and 70% PPFD treatments. Carbon assimilation, therefore, was critical for expression of resistance. The susceptible clone failed to respond to PPFD levels when treated identically to the resistant clone. The inability of the susceptible clone to alter its response to *V. albo-atrum* is evidence that the defense mechanism under investigation is not simply a constitutive part of all alfalfa plants but is unique to the resistant clone.

Additional keywords: *Medicago sativa*, photosynthetic stress, Verticillium wilt.

Host resistance is the primary means of controlling Verticillium wilt of alfalfa (*Medicago sativa* L.), a disease that occurs throughout the northern alfalfa growing region of the United States. Resistant alfalfa cultivars provide conservative yield advantages of 1.16 Mg/ha in the second production year and 2.87 Mg/ha by the third year compared to susceptible cultivars when infected by the vascular wilt fungus, *Verticillium albo-atrum* Rke. et Berth. (40). Resistant alfalfa plants are not immune to the pathogen (31). *V. albo-atrum* spreads through alfalfa fields on hay-making equipment, enters the plant through harvest-wounded stems (15), and can infect and suppress the growth of resistant plants in the absence of typical foliar symptoms (33). The growth suppression in infected resistant alfalfa clones is thought to be a conse-

quence of the energy demands of the host-pathogen interaction (33). Anatomical examination of the resistant clones has documented defense mechanisms that involve the timely deposition of suberin and lignin on both vessel element walls and on the vessel-inhabiting hyphae of *V. albo-atrum* (30). Newcombe and Robb (26), working with detached alfalfa stems infused with conidia, correlated early xylem vessel wall coating with suberin to disease resistance. Lignin and suberin are products of the phenylpropanoid pathway, and increased activity of key pathway enzymes in response to infection by *V. albo-atrum* has been demonstrated in alfalfa and tomato (20,22).

Physiological studies of susceptible and resistant alfalfa clones infected with *V. albo-atrum* have documented a 35% reduction in net photosynthesis in infected susceptible plants and no significant change in the rate of net photosynthesis of infected resistant plants (28). These contradictory responses of net photosynthesis to the presence of *V. albo-atrum* and the involvement of carbon- and energy-intensive secondary metabolites in defense (14,17) give rise to the hypothesis that net photosynthesis has a regulatory role in the expression of resistance in alfalfa.

The objectives of our study were to determine: 1) whether carbon assimilation regulates the response of resistant alfalfa to *V. albo-atrum*, and 2) whether *V. albo-atrum*-infected resistant and susceptible alfalfa clones respond similarly to alterations in the rate of net photosynthesis. These objectives were tested by applying photosynthetic stress to plants by manipulating the photosynthetic photon flux density (PPFD) under which inoculated and noninoculated alfalfa plants were grown. A portion of this study was reported previously (28).

MATERIALS AND METHODS

Experimental design. The PPFD experiment was conducted as a split-plot in a randomized-complete-block design with three replications. The main plot treatment was PPFD (40, 70, and 100% of ambient), and the subplot treatments were a $2 \times 2 \times 3$ factorial of pathogen (inoculated and noninoculated), clone (resistant and susceptible), and time (3 wk). There were a total of 108 plants in the experiment, which ran for 5 wk with plants sampled during weeks 3, 4, and 5. The experiment was conducted during October and November 1991 and was repeated during March and April 1992.

PPFD treatments and environmental conditions. Two levels of PPFD (40 and 70% of ambient PPFD) were created by covering wooden cages (82 cm wide $\times$ 89 cm high $\times$ 122 cm long) with shade cloth rated at 30 or 60% shade (Weathashade, E. C. Geiger, Inc., Harleysville, PA). The third PPFD level was 100% ambient PPFD. Ambient light was supplemented with one high-intensity discharge (HID) metal-halide lamp (400 W) positioned 100 cm above the bench top over each PPFD treatment. The photoperiod was 16 h throughout the experiment. Two lamps were used over the 100% PPFD treatment during experiment 2 to compensate for the lower light levels associated with central Pennsylvania winters. A glass heat barrier was installed 2 cm below those lamps, and a fan was used to dissipate the heat above the glass barrier. A heat barrier was not necessary over the other PPFD treatments, which had the same supplemental lighting in both experiments. PPFD (Fig. 1) was monitored with LICOR quantum sensors (LICOR, Lincoln, NE) located 20 cm below the supplemental lamps and was recorded with a Campbell 21X micrologger (Campbell Scientific, Inc., Logan, UT). Temperatures within the three PPFD treatments were measured with thermistors and the hourly average was recorded. The number of hours during the 5-wk experiment that temperatures were between 19–25 C, optimum temperatures for *V. albo-atrum* activity (5,32), are shown in Figure 2.

Plant material and inoculation method. Alfalfa plants grown from seed are genetically heterogeneous for resistance to *V. albo-atrum*, making clonal plants necessary for definitive studies. The resistant (clone 1079) and susceptible (clone 774) clones used in this and previous studies (27,30,33,34) were selected from the cultivars Agate and Vertus, respectively, and were provided by R. N. Peaden (USDA-ARS, Irrigated Agriculture Research and Extension Center, Prosser, WA).

Rooted cuttings were planted in a commercial potting mix (Terra-Lite, reddi-Earth, Peat-Lite Mix, W. R. Grace & Co, Cambridge, MA) in 15-cm clay pots in the greenhouse. Plants were fertilized with 13% N-P-K slow-release fertilizer (Osmocote, Mallinckrodt, Inc., St. Louis) rather than being nodulated by *Rhizobium meliloti* to ensure a uniform level of nitrogen fertility among experimental units. Plants were cut to a 4-cm height after 6 wk of growth and either inoculated with *V. albo-atrum* or treated with sterile water. The isolate of *V. albo-atrum* (PL-1546), which was stored on silica gel beads at -25 C and used in previous studies (27,30,33,34), was activated as needed by placing several silica gel beads on potato-dextrose agar in petri dishes and incubating them at 25 C. Conidia from 2-wk-old cultures were suspended in sterile water, counted with a hemacytometer, and the spore concentration was adjusted to 3×10^6 spores per milliliter. Plants were inoculated by placing a 20-μL drop of spore suspension on the end of each freshly cut stem stub. Inoculated plants were kept in a dark mist chamber under saturated relative humidity for 24 h before being placed in the greenhouse. Noninoculated plants were treated similarly with sterile water but were not placed in the mist chamber to minimize the possibility of cross-contamination.

The inoculated and noninoculated plants were grown on separate greenhouse benches under HID metal-halide lamps (400 W) with a photoperiod of 16 h for 6 wk preceding the PPFD experiment. At the end of the pathogen establishment period, plants were cut to 4-cm height and used in the PPFD study. Inoculation timing was identical for the two clones in the first experiment but varied by 6 wk in the second experiment. In experiment 2, the resistant clone was inoculated as described for experiment 1, and the susceptible clone was inoculated 6 wk later when the PPFD treatments were initiated. Plants were supported with

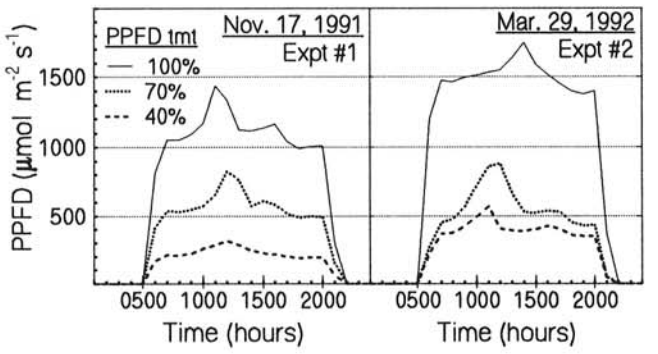

Fig. 1. Representative diurnal photosynthetic photon flux density (PPFD) curves for three light environments. Values for each hour represent the average of 60 readings. In experiment 1, a single high-intensity discharge (HID) metal-halide lamp was used to supplement the ambient light over each light treatment, whereas in experiment 2 the 100% ambient treatment was supplemented with two HID metal-halide lamps.

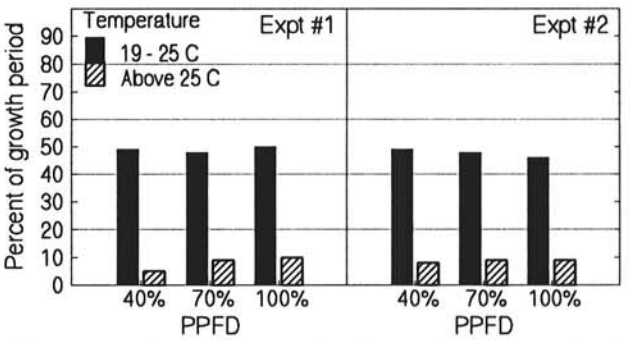

Fig. 2. Summary of the temperature data from each experiment showing the percentage of the growth period during which temperatures were between 19 and 25 C, the temperature range favorable for growth of *Verticillium albo-atrum*.

bamboo stakes during both experiments to eliminate interplant contact and minimize the risk of cross-contamination due to the presence of both inoculated and noninoculated plants in each PPFD treatment.

Pathogen isolations and plant measurements. At the end of the pathogen establishment period and immediately preceding initiation of the PPFD treatments, plants were cut to 4-cm height, and the basal 3-cm of all excised stems was cultured for *V. albo-atrum*. Stem tissue was surface-sterilized in 10% bleach (5.25% sodium hypochlorite) for 5 min, drained on paper towels, aseptically placed on 2% water agar, and incubated for 2 wk at room temperature (~25 C). The presence of *V. albo-atrum* was determined microscopically by the presence of typical verticillate conidiophores. Due to the delayed inoculation of the susceptible clone during experiment 2, isolations were not conducted on the inoculated, susceptible plants in that experiment.

One plant from each subplot treatment was removed from each PPFD treatment and replication weekly during weeks 3, 4 and 5 of the experimental period. The selected plants were taken to a laboratory and placed in a mist chamber overnight. Net photosynthesis and stomatal conductance were measured on the following day. The mist chamber was necessary to protect the plants from photosynthetic stress caused by the low relative humidity common in laboratories during the winter. Physiological parameters were measured in the laboratory rather than the greenhouse to standardize environmental conditions during measurements and thereby reduce experimental error. Plants were acclimated prior to the physiological measurements for a minimum of 1 h under light levels similar to those of the PPFD treatments (12). Net photosynthesis and stomatal conductance were measured on the youngest fully expanded leaf of the resistant plants, with a LI-6200 photosynthesis system (LICOR) as previously reported (27). PPFD levels for the photosynthesis measurements were set at 1,800, 1,200, and 700 μmol m^{-2} s^{-1}, equivalent to a sunny day, and corresponded to the 100, 70, and 40% PPFD treatments, respectively. Plants were measured at the PPFD level corresponding to the PPFD under which they were grown. Physiological parameters were not measured on the susceptible plants, but those plants otherwise were treated identically to the resistant plants.

All plants were given a disease rating (1 = no symptoms, 2 = one or two chlorotic leaves, 3 = leaflets on more than one shoot chlorotic, 4 = most of the leaflets chlorotic, and 5 = dead) (11), the length of the longest stem was measured, and the plants were harvested. Stems and leaves were separated, dried for 72 h at 70 C, and weighed.

Statistical analyses. A combined analysis over experiments was conducted when the error variances were homogeneous as determined with an F test. When the data from the two experiments were combined for statistical analyses, experiment was treated as a random effect, and all interactions were tested with the appropriate experiment × treatment interaction.

Data were subjected to an analysis of variance (ANOVA) using the general linear model Proc GLM of SAS (SAS Institute, Inc., Cary, NC) and single-degree of freedom orthogonal polynomials were examined to determine the significance of the linear and quadratic components of all interactions (37). Residuals were examined, and data were transformed with the natural log or square-root transformation as necessary. When the ANOVA indicated a significant interaction, a predicted response surface (two quantitative variables) or a predicted line (one quantitative variable) were generated with least squares means and a multiple regression procedure (Proc Stepwise) in SAS. In all cases, the components of the predictive equations were those indicated as significant by the ANOVA. When significant interactions involved only qualitative variables, Fisher's protected least significant difference test was used within treatment levels.

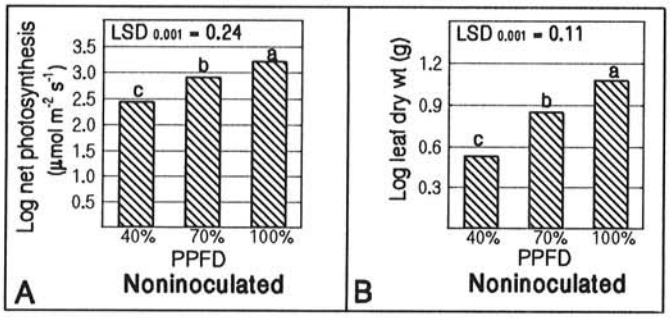

Fig. 3. The effect of the photosynthetic photon flux density (PPFD) environments on **A,** rate of net photosynthesis of noninoculated resistant alfalfa plants and **B,** leaf dry weight of noninoculated resistant and susceptible alfalfa plants, documenting the existence of a photosynthetic stress under the 70 and 40% PPFD treatments. Data were combined over experiments for statistical analysis and subjected to the natural log transformation. Bars with the same letters are not significantly different.

TABLE 1. Summary of the analysis of variance of disease rating, plant height, and leaf dry weight of the resistant alfalfa clone, showing mean squares (MS) and significance levels for main effects of photosynthetic photon flux density (PPFD), *Verticillium albo-atrum*, time, and all interactions involving *V. albo-atrum*

		Disease rating		Height		Leaf dry weight	
Source[a]	df	MS	P	MS	P	MS	P
PPFD[b]	2	0.1830	0.019	1.0305	0.211	2.8383	0.001
Vaa[c]	1	1.5507	0.024	0.3612	0.819	0.0001	0.001
Wk[d]	2	0.5730	0.000	13.7460	0.000	1.9429	0.000
Vaa × PPFD	2	0.1680	0.028	0.1513	0.364	0.0327	0.076
Vaa × wk	2	0.5803	0.004	1.1681	0.523	0.0355	0.521
Vaa × PPFD × wk	4	0.0880	0.019	0.3788	0.092	0.0491	0.296
Orthogonal Contrasts[e]							
Vaa × PPFD-L	1	0.0669	0.065	0.0031	0.866	0.0120	0.168
Vaa × PPFD-Q	1	0.2690	0.017	0.2995	0.204	0.0533	0.046
Vaa × wk-L	1	0.8010	0.003	1.7194	0.366	0.0446	0.395
Vaa × wk-Q	1	0.3747	0.006	0.6457	0.551	0.0274	0.488
Vaa × PPFD-L × wk-L	1	0.1905	0.008	0.0160	0.690	0.0106	0.569
Vaa × PPFD-L × wk-Q	1	0.0335	0.111	0.1390	0.276	0.0931	0.141
Vaa × PPFD-Q × wk-L	1	0.1278	0.016	1.2905	0.018	0.0616	0.210
Vaa × PPFD-Q × wk-Q	1	0.0000	0.972	0.0868	0.375	0.0337	0.332

[a]Data from experiments 1 and 2 were combined for this analysis. Experiment was treated as a random variable and all interactions were tested with the appropriate experiment × interaction mean square.
[b]PPFD at 40, 70, or 100% of ambient. Error term for PPFD was the replication × PPFD (experiment) mean square.
[c]*V. albo-atrum* and no *V. albo-atrum*.
[d]Wk = week of sampling.
[e]L = linear contrast; Q = quadratic contrast.

RESULTS

Efficacy of inoculation. The stem bases of all plants in experiment 1 were cultured for *V. albo-atrum* at the initiation of the PPFD treatments. *V. albo-atrum* was isolated from 98% (53/54 plants) of the inoculated plants and from three noninoculated plants that were removed from the study. Due to the difference in the timing of inoculation of the susceptible and resistant clones in experiment 2, isolations were not carried out on the inoculated, susceptible plants. The pathogen was isolated from 70% (19/27 plants) of the resistant, inoculated plants and was not recovered from noninoculated plants of either the susceptible or the resistant clone.

Efficacy of PPFD treatments. Net photosynthetic rate of the noninoculated, resistant plants was reduced significantly under both 40 and 70% PPFD (Fig. 3A), verifying the photosynthetic stress imposed by the light regimes. There was no significant experiment × PPFD interaction, indicating that the PPFD treatments affected net photosynthesis similarly in both experiments.

Leaf dry weight, stem dry weight, plant height, and aerial biomass (leaf plus stem dry weight) were determined weekly on noninoculated plants of both resistant and susceptible clones. No clone × PPFD or experiment × clone × PPFD interactions were detected, indicating that the two clones responded similarly to the various PPFD levels. The significant PPFD effect is shown for leaf dry weight (Fig. 3B). Aerial biomass, stem dry weight, and plant height responded similarly (data not shown), documenting the suppressive effect of the 40 and 70% PPFD treatments on plant growth.

Growth analysis of the resistant clone. Our first objective was to determine whether PPFD had a regulatory effect on resistance to *V. albo-atrum*; therefore, only data from the resistant clone were used in this portion of the study (Table 1). Significant pathogen × PPFD × week interactions were detected in disease rating (Fig. 4A) and in plant height (Fig. 4B). The effect of *V. albo-atrum* was significantly more severe in disease rating and plant height when the PPFD level was reduced to 40% of ambient,

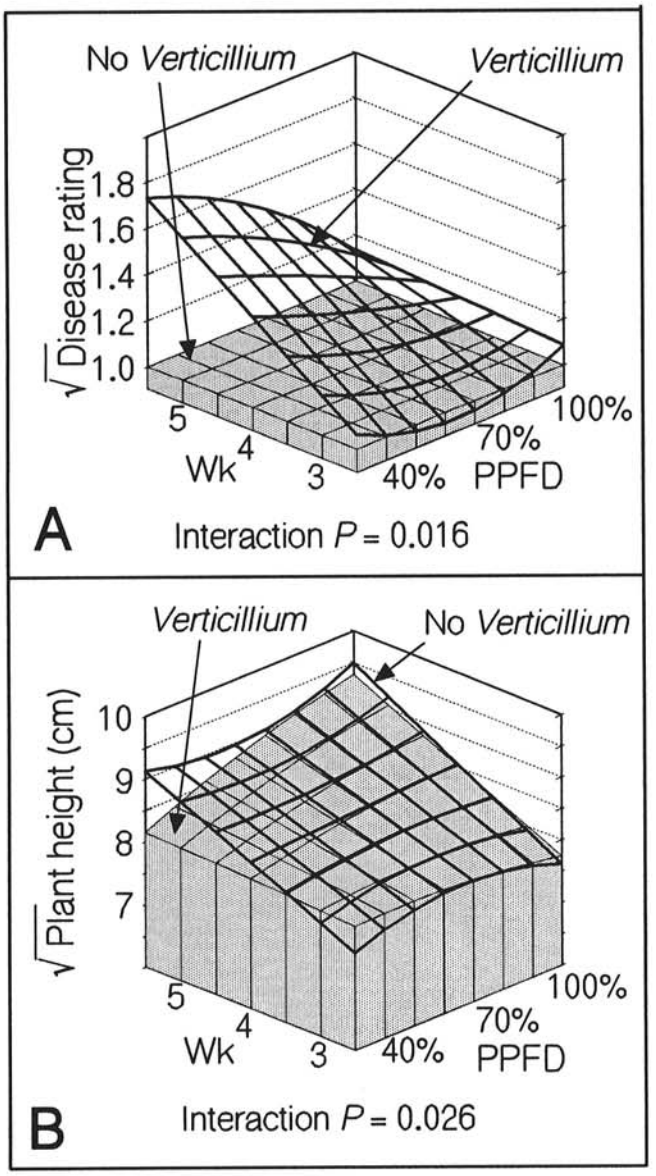

Fig. 4. Predictive response surfaces illustrating the *Verticillium albo-atrum* × photosynthetic photon flux density (PPFD) × week interactions in **A,** disease rating and **B,** height of resistant alfalfa plants. The response surfaces are based on analysis of variance and orthogonal contrast analysis. The *P* value represents the significance of the interaction. In **A**, the upper response surface represents the inoculated resistant plants, whereas in **B** the upper response surface corresponds to the noninoculated resistant plants. Disease was more severe over time at 40% PPFD, as seen in the increase in disease rating (**A**) and the decrease in plant height (**B**) relative to the noninoculated plants. Response surfaces represent the pooled data from experiments 1 and 2.

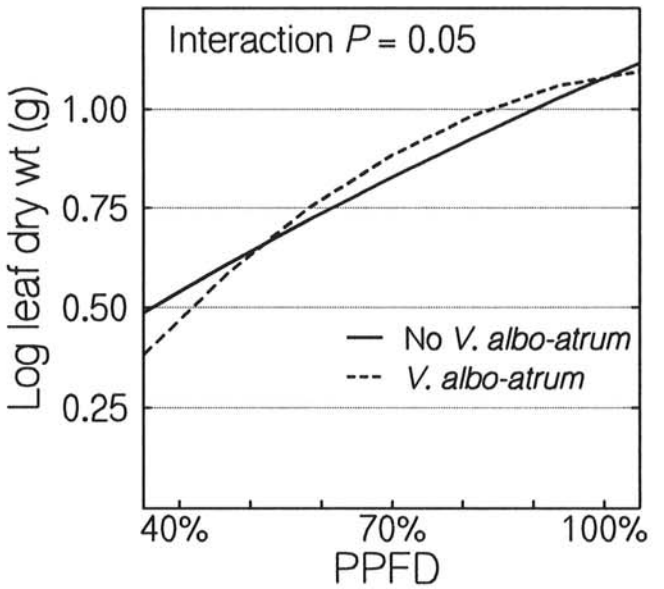

Fig. 5. Predictive curves of pooled data from two experiments representing the *Verticillium albo-atrum* × photosynthetic photon flux density (PPFD) interaction in leaf dry weight of resistant alfalfa. Data were subjected to the natural log transformation. The effect of the pathogen was uniform over time and was more suppressive under the 40% PPFD treatment. The *P* value represents the significance of the interaction.

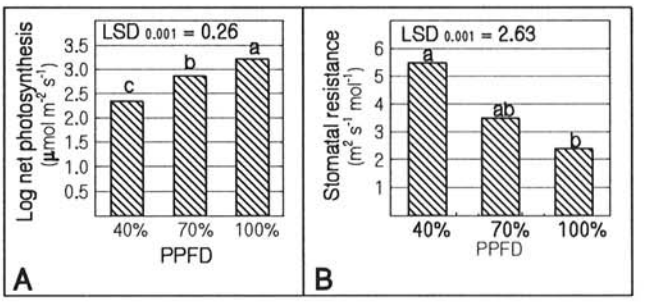

Fig. 6. Main effect of photosynthetic photon flux density (PPFD) on **A,** rate of net photosynthesis and **B,** stomatal resistance of resistant alfalfa plants noninoculated and inoculated with *Verticillium albo-atrum*. Bars represent the means of the pooled data from both experiments. There was a significant reduction in net photosynthesis and increase in stomatal resistance under the 40% PPFD treatment. Stomatal resistance is the inverse transformation of stomatal conductance, which was the measured parameter. The net photosynthesis data was subjected to the natural log transformation. Bars with the same letters are not significantly different.

and the effect intensified over time. A significant pathogen × PPFD interaction occurred in leaf dry weight (Fig. 5), and again the pathogen caused growth suppression under 40% PPFD. There was neither a significant pathogen × PPFD interaction nor a significant effect of pathogen on net photosynthesis or stomatal conductance, but there was a significant effect of PPFD on both physiological processes (Fig. 6A and B). No experiment × pathogen × PPFD interactions were detected, indicating that the pathogen × PPFD interactions were similar during both experiments.

Heterogeneous error variances prevented the combining of data

TABLE 2. Summary of analysis of variance of stem dry weight and aerial biomass of the resistant alfalfa clone, showing the mean squares (MS) and significance levels for main effects of photosynthetic photon flux density (PPFD), *Verticillium albo-atrum*, time, and all interactions involving *V. albo-atrum*

		Experiment 1				Experiment 2			
		Stem dry weight		Aerial biomass		Stem dry weight		Aerial biomass	
Source[a]	df	MS	*P*	MS	*P*	MS	*P*	MS	*P*
PPFD[b]	2	1.6854	0.000	2.9240	0.000	4.1458	0.000	6.3482	0.000
Vaa[c]	1	0.6615	0.000	1.1263	0.000	0.8083	0.002	1.2826	0.001
Wk[d]	2	1.4678	0.000	2.1349	0.000	4.5230	0.000	6.0247	0.000
Vaa × PPFD	2	0.0020	0.907	0.0084	0.762	0.0719	0.356	0.1015	0.333
Vaa × wk	2	0.1426	0.003	0.1945	0.005	0.0441	0.526	0.0608	0.512
Vaa × PPFD × wk	4	0.0319	0.213	0.0654	0.104	0.0899	0.279	0.1343	0.225
Orthogonal contrasts[e]									
Vaa × PPFD-L	1	0.0019	0.761	0.0047	0.698	0.0176	0.612	0.0233	0.612
Vaa × PPFD-Q	1	0.0021	0.752	0.0122	0.535	0.1261	0.181	0.1797	0.165
Vaa × wk-L	1	0.2851	0.001	0.3889	0.001	0.0544	0.375	0.0496	0.461
Vaa × wk-Q	1	0.0004	0.880	0.0006	0.883	0.0338	0.483	0.0719	0.376
Vaa × PPFD-L × wk-L	1	0.0049	0.627	0.0212	0.414	0.0125	0.669	0.0115	0.721
Vaa × PPFD-Q × wk-L	1	0.1048	0.031	0.2062	0.015	0.0492	0.399	0.0440	0.487
Vaa × PPFD-L × wk-Q	1	0.0177	0.360	0.0333	0.308	0.1139	0.203	0.2194	0.127
Vaa × PPFD-Q × wk-Q	1	0.0000	0.985	0.0000	0.972	0.1841	0.109	0.2621	0.096

[a] Data from experiments 1 and 2 were not combined due to heterogeneous error variances.
[b] PPFD at 40, 70, or 100% of ambient. Error term for PPFD was the replication × PPFD mean square.
[c] *V. albo-atrum* and no *V. albo-atrum*.
[d] Wk = week of sampling.
[e] L = linear contrast; Q = quadratic contrast.

TABLE 3. Summary of analysis of variance of disease rating, leaf and stem dry weight, and aerial biomass of the resistant and susceptible alfalfa clones in experiment 1, showing mean squares (MS) and significance levels for main effects of photosynthetic photon flux density (PPFD), clone, *Verticillium albo-atrum*, time, and all interactions involving *V. albo-atrum*

		Disease rating		Leaf dry weight		Stem dry weight		Aerial biomass	
Source[a]	df	MS	*P*	MS	*P*	MS	*P*	MS	*P*
PPFD[b]	2	0.2071	0.001	1.8806	0.000	2.9048	0.000	4.9543	0.000
Vaa[c]	1	13.0388	0.000	3.5734	0.000	6.2426	0.000	10.1437	0.000
Clone[d]	1	5.3497	0.000	0.4128	0.000	2.5650	0.000	2.7185	0.000
Wk[e]	2	0.2373	0.000	0.7872	0.000	1.8057	0.000	2.6036	0.000
Vaa × PPFD	2	0.0999	0.001	0.0522	0.028	0.1418	0.007	0.1867	0.014
Vaa × clone	1	5.3370	0.000	0.9353	0.000	1.8260	0.000	2.8630	0.000
Vaa × wk	2	0.2315	0.000	0.2676	0.000	0.5715	0.000	0.8808	0.000
Vaa × clone × wk	2	0.0948	0.001	0.0466	0.041	0.0514	0.153	0.1066	0.083
Vaa × PPFD × wk	4	0.0225	0.160	0.0181	0.276	0.0186	0.594	0.0346	0.504
Vaa × PPFD × clone	2	0.0309	0.104	0.1073	0.001	0.1670	0.003	0.2686	0.003
Vaa × PPFD × clone × wk	4	0.0468	0.011	0.0389	0.032	0.0803	0.024	0.1221	0.026
Orthogonal contrasts[f]									
Vaa × PPFD-L	1	0.0209	0.212	0.0088	0.428	0.0095	0.553	0.0186	0.504
Vaa × PPFD-Q	1	0.1789	0.000	0.0955	0.011	0.2741	0.002	0.3549	0.005
Vaa × wk-L	1	0.3553	0.000	0.5322	0.000	1.1413	0.000	1.7545	0.000
Vaa × wk-Q	1	0.1174	0.004	0.0053	0.537	0.0046	0.678	0.0136	0.568
Vaa × clone × PPFD-L	1	0.0401	0.086	0.0004	0.862	0.0012	0.831	0.0015	0.847
Vaa × clone × PPFD-Q	1	0.0218	0.203	0.2142	0.000	0.3329	0.000	0.5356	0.001
Vaa × clone × wk-L	1	0.0751	0.020	0.0902	0.013	0.1017	0.055	0.2073	0.028
Vaa × clone × wk-Q	1	0.1194	0.004	0.0039	0.596	0.0018	0.796	0.0079	0.662
Vaa × PPFD-L × wk-L	1	0.0446	0.071	0.0153	0.296	0.0013	0.825	0.0124	0.585
Vaa × PPFD-L × wk-Q	1	0.0260	0.165	0.0495	0.063	0.0460	0.196	0.1031	0.118
Vaa × PPFD-Q × wk-L	1	0.0182	0.244	0.0036	0.611	0.0084	0.576	0.0015	0.849
Vaa × PPFD-Q × wk-Q	1	0.0014	0.747	0.0042	0.583	0.0176	0.418	0.0209	0.479
Vaa × clone × PPFD-L × wk-L	1	0.0446	0.070	0.0061	0.509	0.0039	0.699	0.0090	0.642
Vaa × clone × PPFD-L × wk-Q	1	0.0068	0.474	0.0021	0.696	0.0007	0.872	0.0039	0.758
Vaa × clone × PPFD-Q × wk-L	1	0.1351	0.002	0.1352	0.003	0.2895	0.002	0.4349	0.002
Vaa × clone × PPFD-Q × wk-Q	1	0.0017	0.719	0.0083	0.439	0.0185	0.407	0.0278	0.414

[a] Data from experiment 1 only.
[b] PPFD at 40, 70, or 100% of ambient. Error term for PPFD was the replication × PPFD mean square.
[c] *V. albo-atrum* and no *V. albo-atrum*.
[d] Resistant and susceptible.
[e] Wk = week of sampling.
[f] L = linear contrast; Q = quadratic contrast.

over experiments for stem dry weight and aerial biomass (Table 2). Significant pathogen × PPFD × week interactions were detected in both stem dry weight (Fig. 7A) and aerial biomass (Fig. 7B) in experiment 1. In every case, the effect of *V. albo-atrum* was more severe under 40% PPFD, and the effect was intensified over time. No pathogen × PPFD or pathogen × PPFD × week interactions were noted for these parameters in experiment 2.

Growth analysis of both clones. The second objective of this study was to determine whether PPFD affected the response of susceptible alfalfa to *V. albo-atrum*. Data from both susceptible and resistant clones were analyzed to answer this question (Tables 3 and 4). The data were not combined over experiments, due to heterogeneous error variances caused by the different inoculation times for the susceptible clone in each experiment. Significant pathogen × PPFD × clone × week interactions were detected in experiment 1 in disease rating, leaf dry weight, stem dry weight, and aerial biomass (Fig. 8A–D). In every case, the interaction was caused by the failure of the susceptible clone to respond similarly to the resistant clone. Unlike the response of the resistant clone to *V. albo-atrum* that varied with PPFD level and intensified over time, the effect of the pathogen on the susceptible clone was constant over PPFD levels and time, indicating that PPFD did not alter the susceptible response.

No pathogen × clone × PPFD interactions were detected in experiment 2; however, significant pathogen × clone interactions were noted in disease rating, stem dry weight, leaf dry weight, and aerial biomass (Fig. 9A–D). Growth was significantly suppressed by the presence of *V. albo-atrum* in the susceptible clone but not in the resistant clone. The resistant clone either was not affected by *V. albo-atrum* (Fig. 9B) or growth was significantly enhanced by the presence of the pathogen (Fig. 9C and D). Significant pathogen × clone interactions also were detected in experiment 1 (data not shown), and the response of the susceptible clone to *V. albo-atrum* was similar to that found in experiment 2. The growth enhancement noted in the infected, resistant clone in experiment 2 was not found in experiment 1, where no significant difference was detected in aerial biomass, stem dry weight or plant height between inoculated and noninoculated resistant plants. Leaf dry weight of the inoculated resistant clone, however, was significantly less than that of the noninoculated resistant clone.

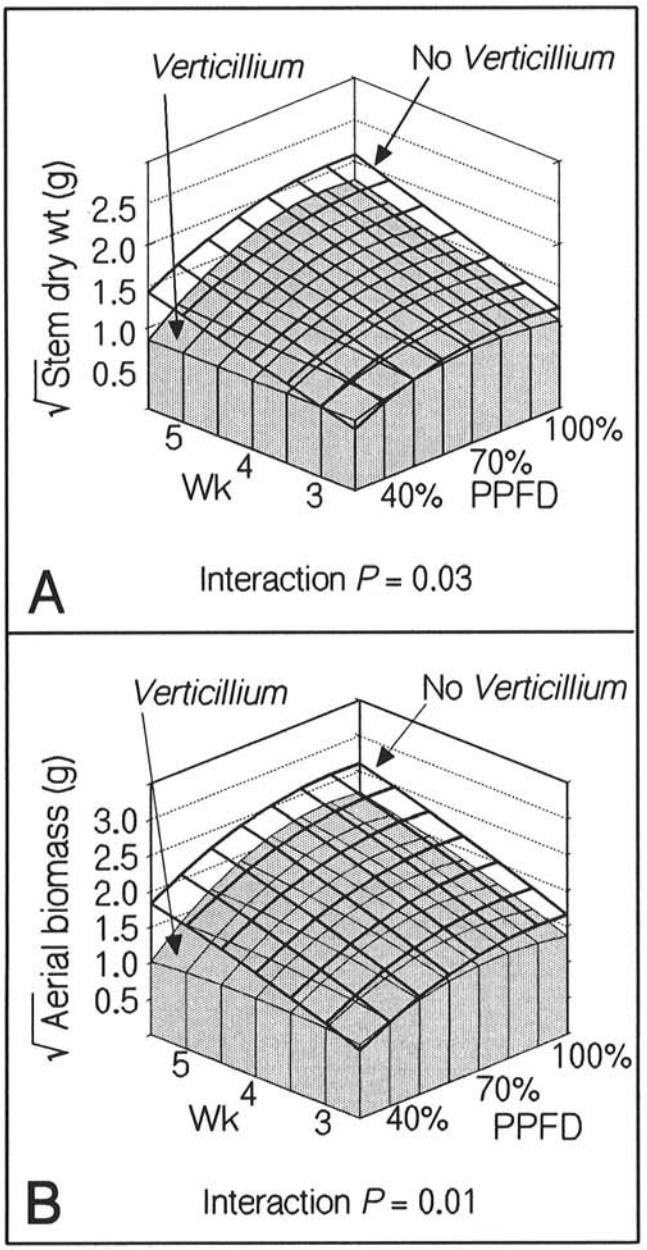

Fig. 7. Predictive response surfaces showing the *Verticillium albo-atrum* × photosynthetic photon flux density (PPFD) × week interaction in **A,** stem dry weight and **B,** aerial biomass of resistant alfalfa in experiment 1. The response surfaces are based on analysis of variance and orthogonal contrast analysis. The *P* value represents the significance of the interaction. In both graphs, the upper response surface represents the noninoculated resistant plants, and the lower response surface represents the inoculated resistant plants. The pathogen caused more growth suppression over time under 40% PPFD.

DISCUSSION

PPFD was manipulated to provide three light environments that would affect the rate of net photosynthesis but have a minimum of direct impact on other physiological processes. The physical presence of the PPFD environments was documented by quantum sensors, whereas the physiological effect of the different light environments was reflected in the net photosynthesis and growth data from noninoculated plants. Temperature has a direct effect on the development of Verticillium wilt symptoms (5,21,32) and on the partitioning of carbon in alfalfa (1). The precautions taken during these experiments to minimize temperature differences resulted in differences of less than 1 C among the PPFD environments, effectively eliminating temperature as a potential confounding factor within experiments. Temperatures differed between experiments, however, and the greatest difference occurred under the 100% PPFD treatment. During experiment 2, plants in the 100% PPFD treatment spent 4% more time at temperatures below 19 C and 4% less time under pathogen-favorable temperatures (19–25 C) than in experiment 1. Alfalfa stem growth rates are significantly reduced at temperatures below 18 C (8), and the suppressive effect of low temperature on stem elongation of the noninoculated plants may account for the lack of a pathogen × PPFD interaction in stem dry weight during experiment 2.

Light, a primary component of photosynthesis, affects the rate of net photosynthesis early in the carbon assimilation process by altering the rate of regeneration of ribulose bisphosphate, the sugar substrate required by the carboxylase enzyme, ribulose bisphosphate carboxylase/oxygenase (7). Stomates, which are the major source of variable resistance in leaf gas exchange, also are affected by light (6). Data from inoculated and noninoculated resistant plants documented a significant reduction in stomatal conductance under 40% PPFD as compared to 100% PPFD. Reduction in stomatal aperture reduces transpiration of H_2O but does not always reduce CO_2 entry into the leaf to the same degree due to the vastly different sizes and subsequent diffusion rates of the two molecules (6). A reduction in stomatal aperture, therefore, does not always correlate with a reduction in the rate of net photosynthesis. *V. albo-atrum* spreads through the plant via spore translocation in the xylem (29), and it is tempting to assume that a reduction in stomatal conductance would increase host resistance to *V. albo-atrum*. Translocation of *V. albo-atrum* spores in the xylem was suppressed when transpiration was reduced by

drought stress (34), and, as expected, resistant alfalfa plants remained symptomless. In contrast, the reduction in stomatal conductance noted in the present study was not correlated with increased host resistance, and infected resistant plants were severely affected by the pathogen when grown under 40% PPFD.

An alteration in the response of resistant plants to *V. albo-atrum* under the various PPFD regimes would be indicated statistically as a pathogen × PPFD interaction. Several pathogen × PPFD and pathogen × PPFD × week interactions were detected and, in all cases, were due to the resistant plants being susceptible when grown under 40% PPFD. The reduction in host resistance under 40% PPFD, despite the correlation between reduced transpiration and spore movement (34) that should favor resistance, suggests that the corresponding reduction in net photosynthesis was critically involved in the loss of resistance. Plant defense responses that involve phenylpropanoid products, such as lignin and suberin (30), may require a high rate of photosynthate production throughout the daily photosynthetic period to combat a pathogen whose dissemination within the host is aided by transpiration. In addition to reducing the rate of net photosynthesis, the 40% ambient PPFD treatment also reduced the amount of photosynthate accumulated by the infected plants. Plants grown under 40% PPFD rather than 100% PPFD intercepted proportionally fewer photons in the 16-h photosynthetic period and consequently assimilated less carbon during the photoperiod.

Lee et al (22) working with tomato isogenic for the *Ve* gene for Verticillium wilt resistance, found that mRNAs for phenylalanine ammonia-lyase, a key enzyme in the phenylpropanoid pathway, were suppressed in the susceptible tomato and not affected in the resistant tomato. They theorize that *V. albo-atrum* actively suppresses enzyme activity in the susceptible tomato, whereas tomato with the *Ve* gene may actively resist such suppression (22). In addition to being used for the production of defense compounds, photosynthate accumulated by polygenically resistant alfalfa may be necessary to combat possible pathogen-induced enzyme suppression.

The onset of symptoms of Verticillium wilt has been associated with flowering in chrysanthemum, tobacco, and potato (3,4,25). Similarly, field observations noted increased symptom expression in *Verticillium*-infected sunflower at flowering, but subsequent greenhouse experiments did not confirm those observations (35). The greenhouse results, however, may have been confounded by additional plant stress caused by the restricted root zone volume of the plant containers (18). Expression of Verticillium wilt symptoms is photoperiod sensitive in alfalfa and tomato (16,19); however, sensitivity is not tied to flower initiation. Tomato was photoperiod insensitive with regard to flowering, and alfalfa, a partially photoperiod-sensitive, long-day-flowering plant, expressed no symptoms under a 16-h photoperiod but was symptomatic under an 8-h photoperiod (19).

The photoperiod sensitivity of symptom expression in plants infected with *Verticillium* is not universal, and this lack of uniformity may be due to differences in the genetics of resistance. Potato and alfalfa are polygenically resistant to *Verticillium* spp., whereas tomato varieties may have either polygenic or race-specific resistance to this pathogen (10,24,39). Polygenically resistant potato, tomato, and alfalfa plants grown under 16-h photoperiods expressed resistance to *Verticillium* but became symptomatic when grown under 8-h photoperiods (3,16,19,38). In contrast, tomato with race-specific resistance to *V. dahliae* remained symptomless under a 4-h photoperiod despite the presence of the pathogen (16). Polygenic resistance is characterized genetically by additive gene activity, continuous distributions in populations, and the apparent lack of the recognition and response reaction

TABLE 4. Summary of analysis of variance of disease rating, leaf and stem dry weight, and aerial biomass of the resistant and susceptible alfalfa clones in experiment 2, showing mean squares (MS) and significance levels for main effects of photosynthetic photon flux density (PPFD), clone, *Verticillium albo-atrum*, time, and all interactions involving *V. albo-atrum*

		Disease rating		Leaf dry weight		Stem dry weight		Aerial biomass	
Source[a]	df	MS	*P*	MS	*P*	MS	*P*	MS	*P*
PPFD[b]	2	0.2685	0.002	3.4295	0.000	7.0502	0.000	11.0228	0.000
Vaa[c]	1	4.3377	0.000	0.0016	0.795	0.0040	0.819	0.0058	0.809
Clone[d]	1	0.8957	0.000	1.4160	0.000	5.9005	0.000	7.3559	0.000
Wk[e]	2	0.2907	0.002	2.1623	0.000	6.4753	0.000	8.8052	0.000
Vaa × PPFD	2	0.1868	0.014	0.0709	0.057	0.1552	0.139	0.2443	0.093
Vaa × clone	1	0.7894	0.000	0.8776	0.000	1.7691	0.000	2.7880	0.000
Vaa × wk	2	0.3381	0.001	0.0304	0.285	0.0337	0.645	0.0597	0.551
Vaa × clone × wk	2	0.0803	0.147	0.0033	0.871	0.0210	0.761	0.0243	0.784
Vaa × PPFD × wk	4	0.0551	0.258	0.0943	0.006	0.2385	0.021	0.3418	0.013
Vaa × PPFD × clone	2	0.0060	0.862	0.0012	0.950	0.0013	0.982	0.0032	0.968
Vaa × PPFD × clone × wk	4	0.0243	0.664	0.0364	0.204	0.0549	0.582	0.0851	0.494
Orthogonal contrasts[f]									
Vaa × PPFD-L	1	0.0633	0.217	0.0322	0.248	0.0555	0.397	0.0795	0.374
Vaa × PPFD-Q	1	0.3034	0.008	0.1125	0.033	0.2605	0.069	0.4175	0.044
Vaa × wk-L	1	0.6576	0.000	0.0009	0.842	0.0157	0.652	0.0095	0.758
Vaa × wk-Q	1	0.0218	0.466	0.0601	0.117	0.0509	0.417	0.1090	0.298
Vaa × clone × PPFD-L	1	0.0068	0.683	0.0013	0.814	0.0027	0.852	0.0049	0.823
Vaa × clone × PPFD-Q	1	0.0049	0.727	0.0011	0.827	0.0000	0.984	0.0016	0.898
Vaa × clone × wk-L	1	0.0109	0.605	0.0028	0.733	0.0404	0.469	0.0459	0.498
Vaa × clone × wk-Q	1	0.1507	0.058	0.0037	0.694	0.0013	0.895	0.0023	0.878
Vaa × PPFD-L × wk-L	1	0.0725	0.186	0.0289	0.274	0.0952	0.268	0.1283	0.259
Vaa × PPFD-L × wk-Q	1	0.0087	0.645	0.2015	0.005	0.4229	0.022	0.6354	0.014
Vaa × PPFD-Q × wk-L	1	0.1336	0.074	0.1048	0.039	0.3195	0.045	0.4381	0.039
Vaa × PPFD-Q × wk-Q	1	0.0015	0.849	0.0341	0.235	0.0956	0.267	0.1359	0.246
Vaa × clone × PPFD-L × wk-L	1	0.0301	0.392	0.0277	0.284	0.0233	0.583	0.0437	0.509
Vaa × clone × PPFD-L × wk-Q	1	0.0449	0.297	0.0007	0.859	0.0347	0.503	0.0235	0.628
Vaa × clone × PPFD-Q × wk-L	1	0.0241	0.443	0.0780	0.074	0.0642	0.363	0.1345	0.249
Vaa × clone × PPFD-Q × wk-Q	1	0.0022	0.815	0.0376	0.213	0.0998	0.257	0.1391	0.241

[a] Data from experiment 2 only.
[b] PPFD at 40, 70, or 100% of ambient. Error term for PPFD was the replication × PPFD mean square.
[c] *V. albo-atrum* and no *V. albo-atrum*.
[d] Resistant and susceptible.
[e] Wk = week of sampling.
[f] L = linear contrast; Q = quadratic contrast.

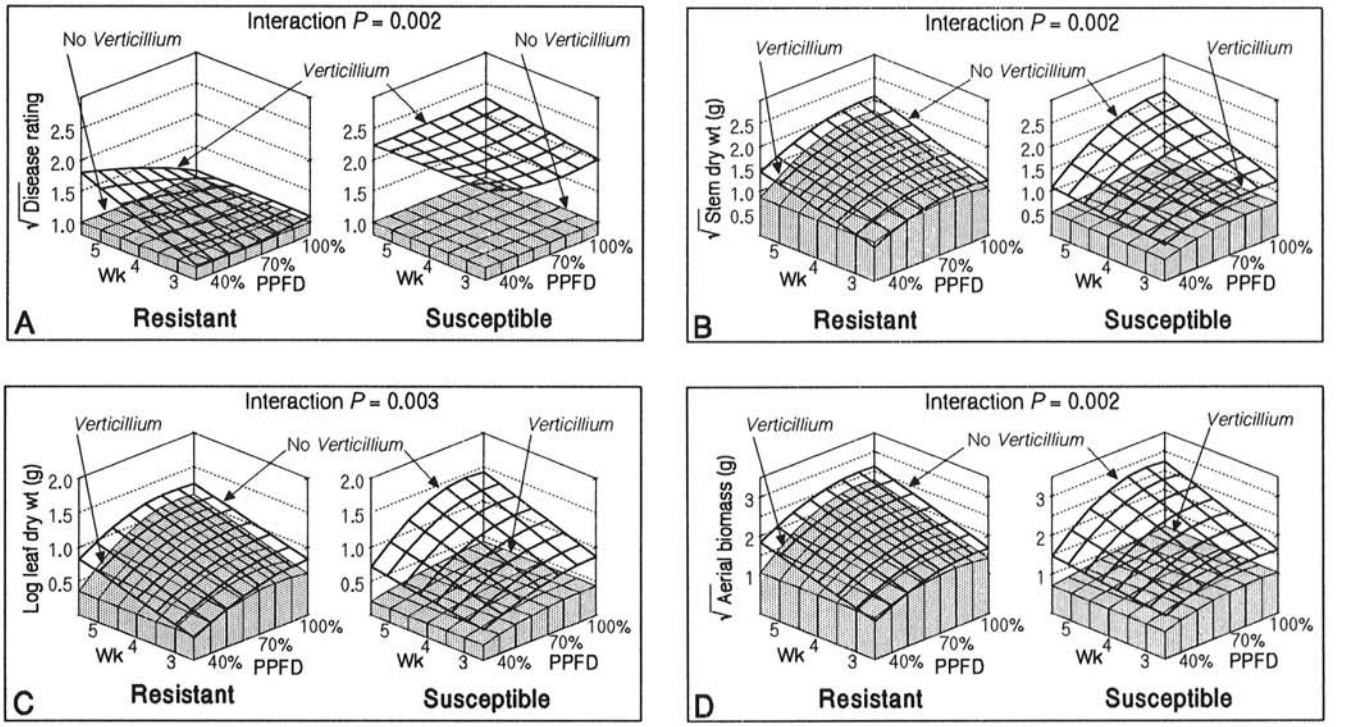

Fig. 8. Predictive response surfaces illustrating the *Verticillium albo-atrum* × clone × photosynthetic photon flux density (PPFD) × week interactions in **A,** leaf dry weight, **B,** stem dry weight, **C,** disease rating, and **D,** aerial biomass of resistant and susceptible alfalfa clones. Data are from experiment 1, in which the two clones were inoculated at the same time. The response surfaces are based on analysis of variance and orthogonal contrast analysis. The resistant and susceptible response surface graphs must be considered simultaneously for each parameter to understand the four-way interaction. In all cases, the interaction was caused by the failure of the inoculated susceptible plant to respond to the different PPFD levels. This is most evident under 100% PPFD, where the response surface for the infected susceptible clone remains flat, while that of the inoculated resistant clone mirrors the noninoculated resistant clone's response. In **B**, **C**, and **D**, the upper response surface represents the noninoculated plants, whereas in **A** the upper response surface represents the inoculated plants. The *P* values indicate the significance of the four-way interaction.

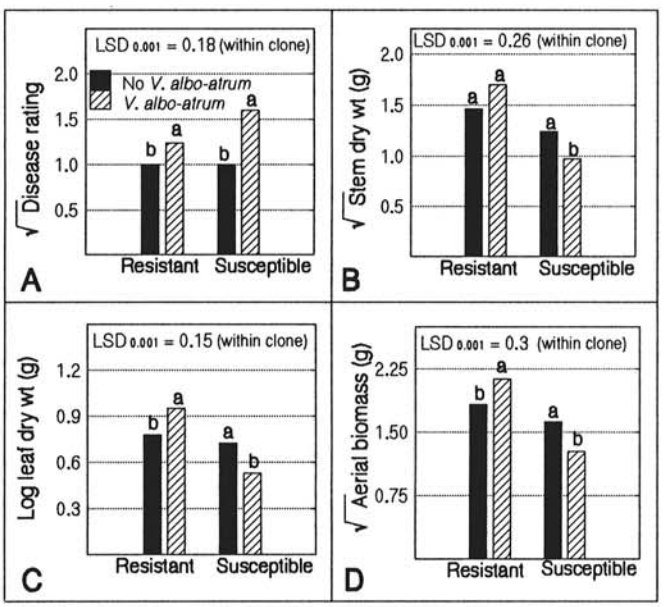

Fig. 9. *Verticillium albo-atrum* × clone interactions in **A,** disease rating, **B,** stem dry weight, **C,** leaf dry weight, and **D,** aerial biomass of resistant and susceptible alfalfa clones. Data are from experiment 2, and the resistant clone was infected 6 wk before the susceptible clone. Interactions were due to the consistently suppressive effect of the pathogen on the growth of the susceptible clone, despite the fact that the susceptible clone was infected for a shorter period of time than the resistant clone. Bars with the same letters within clones are not significantly different.

common to race-specific resistance (36). The multiple genes controlling polygenic resistance frequently have additional, nondefense-related roles in the host (13). The mode of action of polygenic resistance is not fully understood; however, the involvement of carbon- and energy-intensive defense reactions (17) mediated by products of the phenylpropanoid pathway was noted in several polygenically resistant hosts (20,22,23,26,30).

The mechanistic basis for the sensitivity of polygenic resistance to photoperiod is unclear. In every case in which resistance to *Verticillium* was sensitive to photoperiod or flowering, the resistant plant became susceptible under short photoperiods. Polygenically resistant tomato was susceptible to *V. dahliae* under an 8-h photoperiod and resistant under 16-h photoperiods (16). Resistant alfalfa was symptomatic both under an 8-h photoperiod (19) and, in our study, under a 16-h photoperiod with 40% PPFD. Evidence suggests that it is the amount and rate of carbon assimilation that is critical for the expression of resistance in alfalfa. A phytochrome response, however, also may be involved in plants with floral sensitivity to photoperiod due to physiologic changes that occur when plants become reproductive. Floral initiation is accompanied by a shift in carbon partitioning away from the vegetative organs and toward the developing seed, which becomes a strong sink for photosynthate (9). Beverage et al (2) working with a sweet pea mutant that was day neutral for floral initiation found that the shift in carbon partitioning actually preceded floral initiation and was controlled by a single photoperiod gene. Similarly, Wallace et al (41) argued that a single photoperiod gene controls carbon partitioning in bean. The onset of symptoms of Verticillium wilt in potato has been associated with tuber formation, a process that occurs under short photoperiods and also provides a strong sink for photosynthate (3,38). The link between photoperiod sensitivity and symptom expression in plants

with polygenic resistance to *Verticillium* spp. may be the reduction in carbon available for secondary-phenylpropanoid metabolism and the resultant production of defense compounds.

Our second objective was to determine whether susceptible and resistant clones would respond similarly to PPFD when infected with *V. albo-atrum*. The significant pathogen × PPFD × clone × week interactions in experiment 1 indicated that, unlike the resistant clone, the susceptible clone was symptomatic at all light levels. When the susceptible clone was infected only 45% as long (5 wk rather than 11 wk) as the resistant clone, no pathogen × clone × light interactions were detected. The two clones responded similarly to PPFD only when the susceptible clone was given the advantage of a delayed inoculation with *V. albo-atrum*. Despite the shortened time of infection during experiment 2, a significant pathogen × clone interaction was noted in all parameters. In every case, growth of the susceptible clone was significantly suppressed by *V. albo-atrum*, whereas growth of the resistant clone either was not affected or was stimulated by the presence of the pathogen. The inability of the susceptible alfalfa clone to respond to the various PPFD levels when all treatments were applied identically suggests that the defense mechanism under investigation is not a constitutive part of all alfalfa plants but is unique to the resistant clone.

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