

Analysis of Photosynthesis in Resistant and Susceptible Alfalfa Clones Infected with *Verticillium albo-atrum*

B. W. Pennypacker, D. P. Kniewel, K. T. Leath, E. J. Pell, and R. R. Hill, Jr.

Doctoral candidate, Department of Plant Pathology, The Pennsylvania State University; associate professor of crop physiology, The Pennsylvania State University; research plant pathologist, USDA-ARS, The U.S. Regional Pasture Research Laboratory; professor of plant pathology, The Pennsylvania State University; and research agronomist, USDA-ARS, The U.S. Regional Pasture Research Laboratory, University Park, PA 16802.

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ABSTRACT

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The effect of *Verticillium albo-atrum* on net photosynthesis, stomatal conductance, stomatal limitation of photosynthesis, and the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was determined for a resistant and a susceptible alfalfa clone through *A/Ci* (net photosynthesis vs. internal CO₂) response curve analysis. In addition, leaf protein was extracted, and the activity and amount of totally activated Rubisco were determined by ¹⁴CO₂ assimilation and gel electrophoresis. No significant reductions in net photosynthesis, Rubisco activity, or stomatal conductance, or increases in stomatal limitation of photosynthesis were detected in the infected resistant clone by either in

vivo or in vitro methods. Net photosynthesis, Rubisco activity, and stomatal conductance were reduced significantly in the infected susceptible clone, but stomatal limitation of net photosynthesis was not affected. There was a significant reduction in both the total activity and amount of Rubisco in the infected susceptible clone. The lack of effect of reduced stomatal conductance on net photosynthesis coupled with the reduction in amount and activity of Rubisco support the conclusion that the reduction in net photosynthesis in the infected susceptible clone was due to the reduction in Rubisco activity.

Additional keywords: lucerne, *Medicago sativa*, *Verticillium* wilt.

Vascular wilt fungi have evolved to occupy a selective ecological niche, the xylem vessels of their hosts. They exist in an environment that is low in nutrients and oxygen and subject to diurnal fluxes of varying strength depending on the transpiration rate (19). Wilt-inducing *Verticillium* spp. cause stunting, wilting, chlorosis, epinasty, foliar desiccation, and premature defoliation (28,30), symptoms that are similar to those caused by water stress of abiotic origin. Water stress, induced by biotic rather than abiotic factors, has been implicated in many of the diseases caused by these fungi (7,9,14,28). Douglas and MacHardy (6), working with *Verticillium* wilt of chrysanthemum, found a strong correlation between occlusion of xylem vessel lumens and the development of wilt symptoms. Similarly, Scheffer et al (24) noted that transpiration was inhibited in tomato infected with *Verticillium albo-atrum* Reinke & Berth., and Duniway (7,8) documented increased hydraulic resistance in stems and petioles of tomato infected with *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & Hansen.

Extensive studies on the water relations of cotton infected with *V. dahliae* Kleb. (29) and tomato infected with *F. o. lycopersici* (8) have shown reductions in stomatal conductance, leaf water potential, and relative water content in infected plants. Reduced stomatal conductance reduces the concentration of CO₂ within the leaf, and it is tempting to assume that photosynthesis likewise would be affected. However, studies on abiotically droughted plants have shown that the reduction in photosynthesis that is

associated with drought stress is due primarily to increased mesophyll resistance to CO₂ diffusion (3,18,31), rather than to stomatal limitation of CO₂ movement into the leaf. Mesophyll resistance involves the movement of CO₂ through the cytoplasm to the chloroplast, the site of the carboxylation reaction (13) catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (32). Reduction in the activity of Rubisco and in the regeneration of its sugar substrate, ribulose-1,5-bisphosphate, are responsible for the reduced net photosynthesis noted in abiotically drought-stressed plants (10,18,26,31).

Although water stress has been implicated as a major factor in diseases caused by vascular wilt fungi, little is known about the effect of pathogen-induced water stress on photosynthesis. Duniway and Slayter (9) determined that photosynthesis was reduced in tomato infected with *F. o. lycopersici*, but similar evidence is lacking for other host-vascular pathogen systems.

Ideally, determination of the effect of vascular wilt fungi on photosynthesis should be assessed in vivo to eliminate the possibility of disrupting the host-pathogen interaction, thus altering the dynamic relationship. Development of *A/Ci* (assimilation vs. internal CO₂) response curve analysis by Farquhar and Sharkey (10) has provided the necessary in vivo technique for assessing the effect of both stomatal and mesophyll factors on photosynthesis. Previous work on resistant alfalfa infected with *V. albo-atrum* (21,22) documented reductions in both growth and stomatal conductance in infected plants. The growth reduction may represent a redirection of photosynthate into defense-related compounds; however, it might also be a consequence of reduced net photosynthesis. The current study examined stomatal and mesophyll factors involved in CO₂ assimilation in both susceptible and resistant alfalfa plants infected

with *V. albo-atrum*. In addition, we hoped to determine whether the reduced growth noted in resistant alfalfa infected with *V. albo-atrum* was the result of a reduction in net photosynthesis.

MATERIALS AND METHODS

Plant material. Clonal plants were used in this study to minimize the variation associated with the genetic heterogeneity of alfalfa plants grown from seed. The clones were supplied by R. N. Peadar USDA-ARS, Irrigated Agriculture Research and Extension Center, Prosser, WA). Clone 774, susceptible to *V. albo-atrum*, was selected from the cultivar Vertus, and resistant clone 1079 was selected from the cultivar Agate. The clones were used in previous studies (20,22).

Rooted cuttings from each clone were planted in 15-cm clay pots filled with commercial potting mix (Terra-Lite, Reddi-Earth, Peat-Lite Mix, W. R. Grace & Co. Cambridge, MA) and placed in the greenhouse. Plants were fertilized with 13% N-13% P-13% K slow-release fertilizer (Osmocote, Sierra Chemical Co., Milpitas, CA) and were not nodulated with *Rhizobium meliloti*. Plants in experiment 1 were grown for 3 wk before being inoculated with the pathogen. In contrast, plants in experiment 2 were grown for 3 mo and were cut back to 4-cm height twice before inoculation. Experiment 1 was conducted from October to December 1988, and experiment 2 was conducted from August to October 1989. In both experiments, daylight was supplemented with 400-W, metal-halide lamps. The lamps supplied an additional $109 \mu\text{mol m}^{-2}\text{s}^{-1}$ of photosynthetically active radiation (PAR) at soil level and $2,820 \mu\text{mol m}^{-2}\text{s}^{-1}$ of PAR at 75 cm above soil level. Day length was 15 hr during both experiments.

Inoculation method. Plants were inoculated by cutting the stems at a 4-cm height and placing a 20- μl drop of a spore suspension of *V. albo-atrum* on the end of each freshly cut stub. The inoculum was prepared from a 2-wk-old, potato-dextrose agar culture of the isolate of *V. albo-atrum* used in previous studies (21,22). The inoculum, which had a concentration of 10^6 spores/ml in experiment 1 and 3.1×10^6 spores/ml in experiment 2, was dispensed with a micropipette. Two plants were inoculated per clone in experiment 1, and three plants were inoculated per clone in experiment 2. An additional 25 ml of inoculum was poured onto the surface of the potting mix of each inoculated plant in experiment 1. In both experiments, the newly inoculated plants were kept in a mist chamber for 24 hr and then were returned to the greenhouse. Control plants were treated similarly with sterile water but were not placed in the mist chamber to minimize the possibility of cross contamination. Plants were grown for 6 wk, then were cut to a 4-cm height and allowed to regrow for 3 wk before photosynthesis was measured.

Photosynthesis measurements. Measurements were taken in a laboratory at 25 C, under $1,050 \pm 25 \mu\text{mol m}^{-2}\text{s}^{-1}$ of incandescent light that was filtered through 10 cm of running tap water to remove infrared radiation produced by the lamps. A closed infrared gas analyzer system (IRGA) (LI-6200 Portable Photosynthesis System, LI-COR, Lincoln, NE) with a 0.25-L leaf chamber (LI-6000-11) was used for all measurements.

Sampling was conducted on the youngest fully expanded leaf on a stem. Although only symptomless leaves were measured, in many cases symptoms were present on older leaves on the same stem. The leaf, while still attached to the plant, was inserted into the leaf chamber and allowed to acclimate for approximately 5 min to 340–350 $\mu\text{g}/\text{ml}$ CO_2 at the light intensity previously mentioned. After acclimation, CO_2 was injected into the closed circulating gas-stream loop of the IRGA with a syringe, until the atmospheric CO_2 concentration in the sample chamber reached approximately 600 $\mu\text{g}/\text{ml}$. Seventy-five sequential measurements of net photosynthesis were taken over an interval of 20–25 min, as photosynthesis depleted the sample-chamber CO_2 concentration to near the CO_2 compensation point. Sudden large increases in CO_2 concentration cause stomatal closure, thus making it difficult to increase the level of CO_2 within the leaf. Consequently, the CO_2 -enhancing procedure was modified slightly in experiment 2 by waiting until net photosynthesis approached

0 before injecting CO_2 into the IRGA. The procedural modification simplified the process of increasing the leaf's internal CO_2 concentration above ambient levels because the stomates were at maximum aperture in response to the low CO_2 level in the chamber.

When the sequential measurements were completed, leaf area was measured with a portable area meter (LI-3000A, LI-COR) fitted with a conveyor belt assembly (LI-3050A). Net photosynthesis, internal CO_2 concentration, and stomatal conductance were calculated with the leaf area value. All leaves measured in experiment 2 were surface sterilized in 0.525% sodium hypochlorite for 5 min and then aseptically placed on 2% water agar in petri dishes. The leaves were incubated at 25 C for 10 days and then examined for conidiophores of *V. albo-atrum*.

A/Ci response curve analysis. Net photosynthesis (A) values were plotted against the respective internal CO_2 concentrations (Ci) to produce an A/Ci response curve for each leaf measured. As net photosynthesis approached 0, CO_2 became the factor limiting photosynthesis. Therefore, the slope of the A/Ci line as net photosynthesis approaches 0 (Fig. 1) represents the activity of Rubisco (10). Net photosynthesis and corresponding internal CO_2 values for the linear portion of the response curve were subjected to linear regression analysis with the Minitab statistical program (Minitab, State College, PA) to determine the slope.

In addition to determination of the activity of Rubisco, analysis of the A/Ci response curve permitted determination of the effect of stomatal limitation on net photosynthesis (Fig. 1). The effect of stomates on photosynthesis can be seen when net photosynthesis at the ambient external CO_2 concentration of 340 $\mu\text{g}/\text{ml}$ is subtracted from net photosynthesis when the internal CO_2 concentration of the leaf is raised to the ambient level (340 $\mu\text{g}/\text{ml}$). In the latter case, net photosynthesis is at the rate it would be if no stomates were present to limit access to ambient CO_2 . The resulting value then must be divided by the rate of net photosynthesis when internal CO_2 is at 340 $\mu\text{g}/\text{ml}$. This step removes the problem of assuming a linear response to CO_2 when, in fact, a curvilinear response is just as possible (10). The two other parameters obtained from the A/Ci response curve were the actual stomatal conductance at ambient (340 $\mu\text{g}/\text{ml}$) CO_2 and the corresponding rate of net photosynthesis.

The in vivo experiment was a 2×2 factorial arranged in a completely randomized design. The treatments were clone (resistant and susceptible) and *V. albo-atrum* (inoculated and

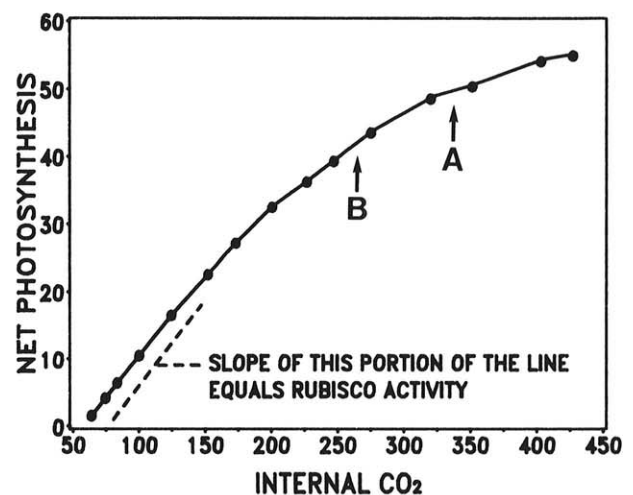


Fig. 1. Diagram of an A/Ci (net photosynthesis vs. internal CO_2) response curve showing the portion of the curve used to determine the activity of Rubisco. Availability of CO_2 is the only factor limiting photosynthesis in this region of the response curve. Therefore, the slope of this region of the curve is directly correlated with the activity of the carboxylase enzyme. A represents the rate of net photosynthesis when internal CO_2 is at 340 $\mu\text{g}/\text{ml}$, and B is the rate of net photosynthesis when the external CO_2 concentration is at 340 $\mu\text{g}/\text{ml}$. The limitation of photosynthesis imposed by the stomates can be estimated by the equation $(A - B)/A$.

control). Ten leaves per treatment were measured in each experiment. The Rubisco activity values, stomatal limitation of photosynthesis values, and values for net photosynthesis and stomatal conductance at ambient CO₂ were combined for the two experiments and subjected to a one-way analysis of variance with the General Linear Models (GLM) procedure of SAS Institute Inc., Cary, NC. The analysis included testing for the significance of experiment-by-treatment interactions and the significance of pathogen-by-clone interactions.

Rubisco extractions. After photosynthesis determinations, a 0.4-g sample of asymptomatic leaves of the same age as those measured in the IRGA was collected from each treatment and immediately frozen in liquid nitrogen. Five samples per treatment were collected in the first experiment and two samples per treatment were collected in the second experiment. The leaf samples were stored in liquid nitrogen until the leaf protein was extracted, and the total activity of Rubisco was determined by the modified protocol of Dann and Pell (5). Initial activity of the extracted Rubisco was not investigated; therefore, the extraction buffer was modified by the addition of 0.04 M MgCl₂·6H₂O and 0.05 M NaHCO₃. The modification made it possible to precipitate the protein in a 500- μ l aliquot of the centrifuged leaf-homogenate supernatant directly in 50% trichloroacetic acid without the additional incubation described by Dann and Pell (5). The precipitated leaf protein was washed in acetone (5) and stored at -10 C for further analysis.

A 900- μ l aliquot of centrifuged leaf-homogenate supernatant was incubated in 0.1 M NaHCO₃ and 0.25 M MgCl₂·H₂O for 10 min to totally activate the extracted Rubisco. The totally activated Rubisco sample was assayed for activity by adding a 50- μ l aliquot of the sample to assay buffer (5) to which was added 25 μ l of ribulose biphosphate and 25 μ L of ¹⁴C-labeled NaHCO₃. The CO₂ fixing reaction proceeded for 30 sec before being stopped with 2 N HCl, a process that released all unfixed ¹⁴CO₂. The amount of ¹⁴CO₂ fixed by the extracted Rubisco was determined in a Beckman LS7000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA), and the resulting values were converted to μ mol CO₂ fixed min⁻¹g⁻¹ dry weight. Results also are reported as percent of the control to allow comparisons with the Rubisco activity values obtained through A/Ci response curve analysis.

The data from the two in vitro experiments determining total Rubisco activity were transformed with the natural log transformation to correct for nonhomogeneous error variances between the two experiments. The transformed data were combined over experiments and subjected to a one-way analysis of variance using GLM. Leaf protein was extracted and assayed for total activity of Rubisco over a period of several days. The transformed data were analyzed with day of activity assay as replications. There were, consequently, four replications in experiment 1 and one in experiment 2.

Gel electrophoresis. The frozen leaf protein pellets were thawed and reconstituted in 400- μ l of extraction buffer to which was added 100 μ l of sodium dodecyl sulfate (SDS) reducing buffer, a mixture of 0.5 M Tris-HCl (pH 6.8), 10% SDS, 0.71 M 2-mercaptoethanol, 0.05% bromophenol blue, and 10% glycerol. The pellets were boiled for 10 min with stirring and then cooled before electrophoresis. SDS-polyacrylamide gel electrophoresis

(PAGE) mini gels were prepared according to the method of Laemmli (12) as modified by Dann and Pell (5), and 2.6- μ l aliquots of each leaf protein sample were loaded into wells in the gel. Two additional wells were loaded with a low-molecular-weight standard (14,400-94,000 Da) (Pharmacia, Piscataway, NJ). Electrophoresis was conducted at 200 mV for 45 min or until the dye front reached the bottom of the gel. Gels were stained in 0.1% Coomassie blue stain for 30 min and destained overnight in a solution of 40% methanol and 10% glacial acetic acid.

The stained gels were scanned with a Quick-Scan, Jr., densitometer (Helena Laboratories, Beaumont, TX). Results were recorded in densitometer integration units, and the amount of Rubisco was calculated by adding the integration values for the large and small subunits.

Data on actual amount of Rubisco expressed in integration units were transformed using the natural log to correct for non-homogeneous error variances and were combined over experiments and subjected to the same analysis as the protein extraction and activity assay data, with two replications. The leaf protein samples were run on duplicate gels, which then were analyzed as replications.

RESULTS

A/Ci response curve analysis. *V. albo-atrum* was isolated from 60% of the petioles and 50% of the leaves measured on the inoculated, susceptible clone and from all of the petioles and 70% of the leaves measured on the inoculated, resistant clone. No symptoms of Verticillium wilt were present on the measured leaves from either clone. The combined analysis of the A/Ci response curve data revealed that plants infected with *V. albo-atrum* had significantly lower net photosynthesis ($P = 0.0001$), reduced Rubisco activity ($P = 0.0001$), and reduced stomatal conductance ($P = 0.007$) (Table 1). The limiting effect of the stomates on net photosynthesis was not altered by *V. albo-atrum*. In addition to the significant main effects, pathogen-by-clone interactions were detected for net photosynthesis ($P = 0.0001$), Rubisco activity ($P = 0.0001$), and stomatal conductance ($P = 0.006$) (Fig. 2A-C). In every case, the interactions were the consequence of the significant negative effect of *V. albo-atrum* on the susceptible clone. Net photosynthesis, in vivo Rubisco activity, and stomatal conductance were not significantly affected by *V. albo-atrum* in the resistant clone, despite the presence of the pathogen in the leaves.

Data from the analysis of the A/Ci response curves also were analyzed over the two experiments. Experiment-by-pathogen interactions were detected for stomatal conductance and for net photosynthesis (Fig. 3A and B). The stomatal conductance of leaves on inoculated plants was not different between the experiments. However, the stomatal conductance of the control leaves was significantly ($P = 0.002$) higher during experiment 2. The significant interaction indicates that the pathogen was limiting stomatal conductance of the inoculated plants during experiment 2 and not during experiment 1 (Fig. 3A). Similarly, net photosynthesis of leaves on the plants infected with *V. albo-atrum* was not different between experiments. Net photosynthesis, however, was significantly ($P = 0.001$) higher in the control leaves of

TABLE 1. Main effects from the analysis of variance data on the effect of *Verticillium albo-atrum* on resistant and susceptible alfalfa^a

Main effects	Photosynthesis (μ mol m ⁻² s ⁻¹)	Rubisco (in vivo) (μ mol min ⁻¹ g ⁻¹)	Conductance (mol m ⁻² s ⁻¹)	Stomatal limitation (%)
No fungus	22.76	0.18	0.40	28.0
Fungus	18.31** ^b	0.14**	0.32**	28.0
Clone 1079	23.03	0.17	0.43	27.0
Clone 774	18.03**	0.15**	0.30**	30.0
Experiment 1	20.35	0.16	0.29	32.0
Experiment 2	20.71	0.16	0.43**	25.0**

^a Means represent the average of 40 values.

^b ** = significant at $P = 0.01$.

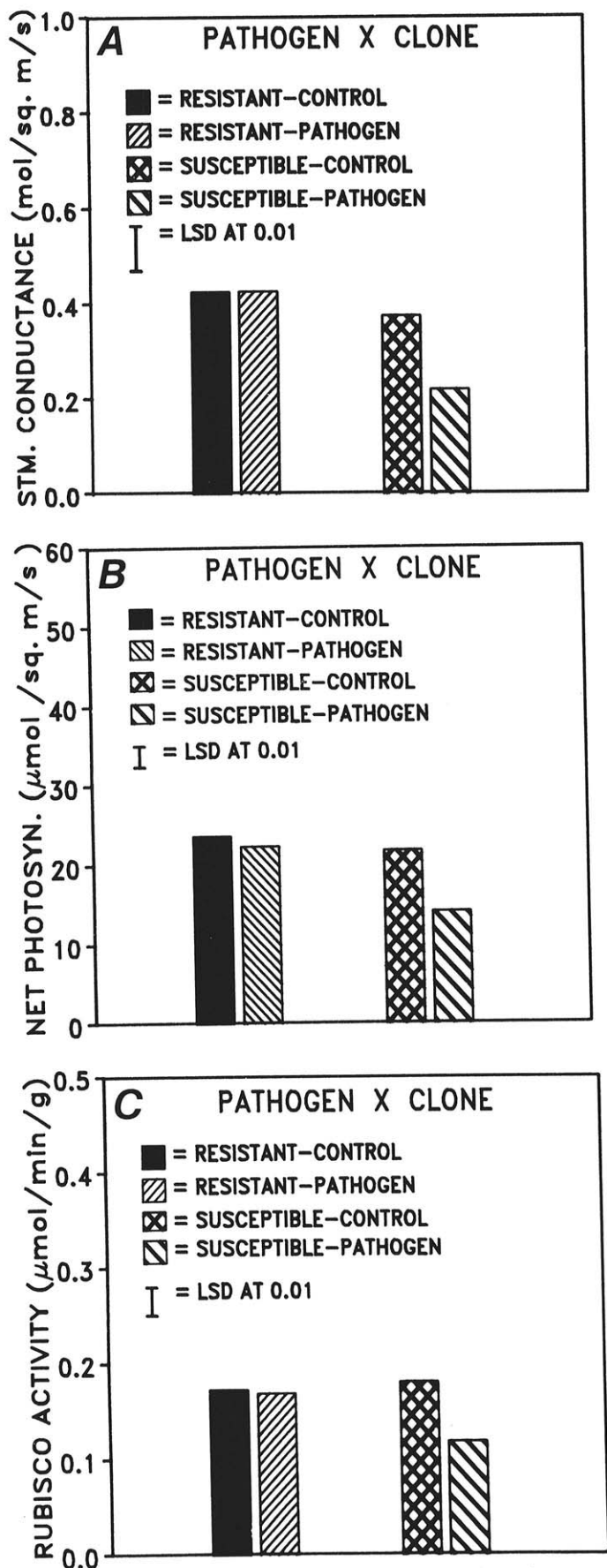


Fig. 2. Analysis of pathogen-by-clone interactions in resistant and susceptible alfalfa infected with *Verticillium albo-atrum* as seen in: A, stomatal conductance, B, net photosynthesis, and C, activity of Rubisco. In all cases, the significant interaction was caused by the negative effect of the pathogen on the susceptible clone. Means represent the average of 20 values. LSD = least significant difference.

experiment 2, indicating that the pathogen significantly reduced net photosynthesis in that experiment (Fig. 3B).

Stomatal limitation of photosynthesis was not altered by *V. albo-atrum*, and there was no pathogen-by-clone or pathogen-by-experiment interaction. The stomates had a significantly greater limiting effect on photosynthesis during experiment 1 (Table 1), but this was probably due to the differential response of the stomates to vapor pressure deficit. The lack of any effect of the pathogen on stomatal limitation of photosynthesis indicates that reduced net photosynthesis in the infected susceptible clone was not caused by reduced CO_2 availability resulting from reduced stomatal conductance in these plants. The reduced Rubisco activity detected in the leaves of the infected susceptible clone, however, would limit net photosynthesis by reducing the amount of CO_2 fixed.

Rubisco extractions and analysis. Total in vitro activity of Rubisco was determined by extraction of the leaf protein and subsequent evaluation of $^{14}\text{CO}_2$ fixation by the extracted protein.

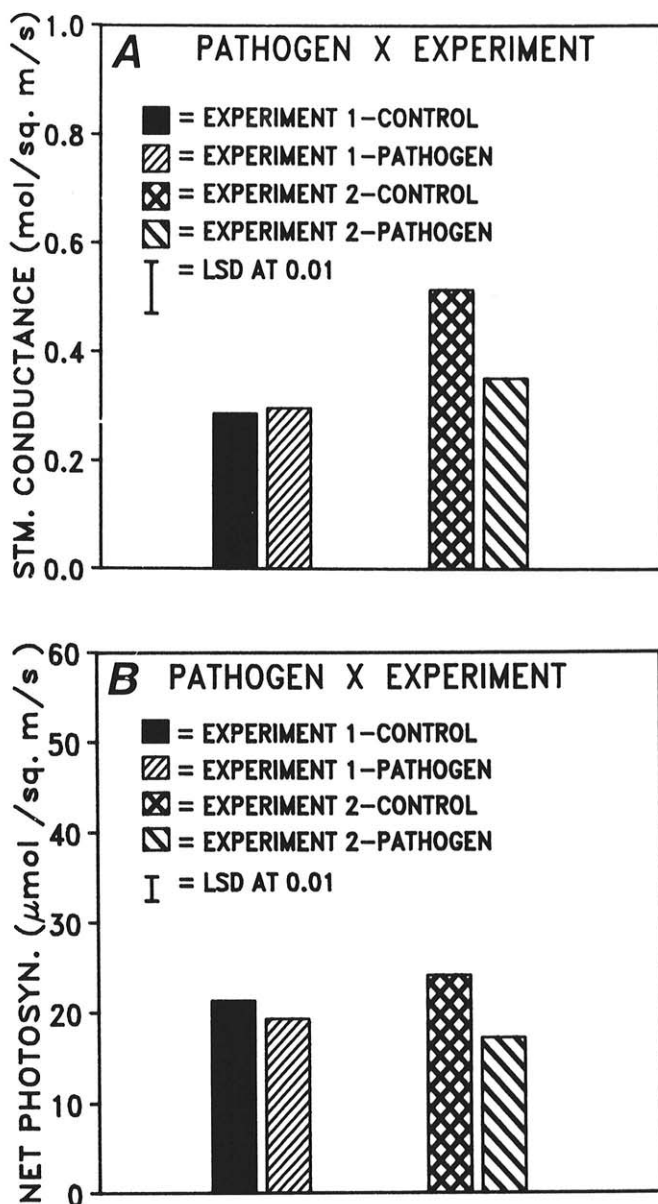


Fig. 3. Analysis of pathogen-by-experiment interaction in resistant and susceptible alfalfa infected with *Verticillium albo-atrum* as reflected in: A, stomatal conductance, and B, net photosynthesis. Note the lack of response in the stomates of the infected plants to the reduced vapor pressure deficit in experiment 2 as compared with the control plants. Means represent the average of 20 values. LSD = least significant difference.

TABLE 2. Main effects of analysis of variance of natural log transformed data from the in vitro Rubisco activity assay and the gel electrophoresis determination of the amount of Rubisco^a

Main effect	Rubisco activity ^b (in vitro assay)	Gel electrophoresis ^b
No fungus	3.29	4.47
Fungus	3.08**	4.40
Clone 1079	3.26	4.56
Clone 774	3.10	4.30*
Experiment 1	3.90	4.83
Experiment 2	1.40***	4.03***

^aLeaves of the same age as those subjected to *A/Ci* (net photosynthesis vs. internal CO₂) response curve analysis were sampled from the uninfected resistant (1079) and susceptible (774) clones of alfalfa and the resistant and susceptible clones infected with *Verticillium albo-atrum*. Rubisco activity data are means of 14 values for both fungus and clone, 20 values for experiment 1, and eight values for experiment 2. Electrophoresis data are means of eight analyses.

^b* = significant at $P = 0.04$; ** = significant at $P = 0.02$; and *** = significant at $P = 0.0001$.

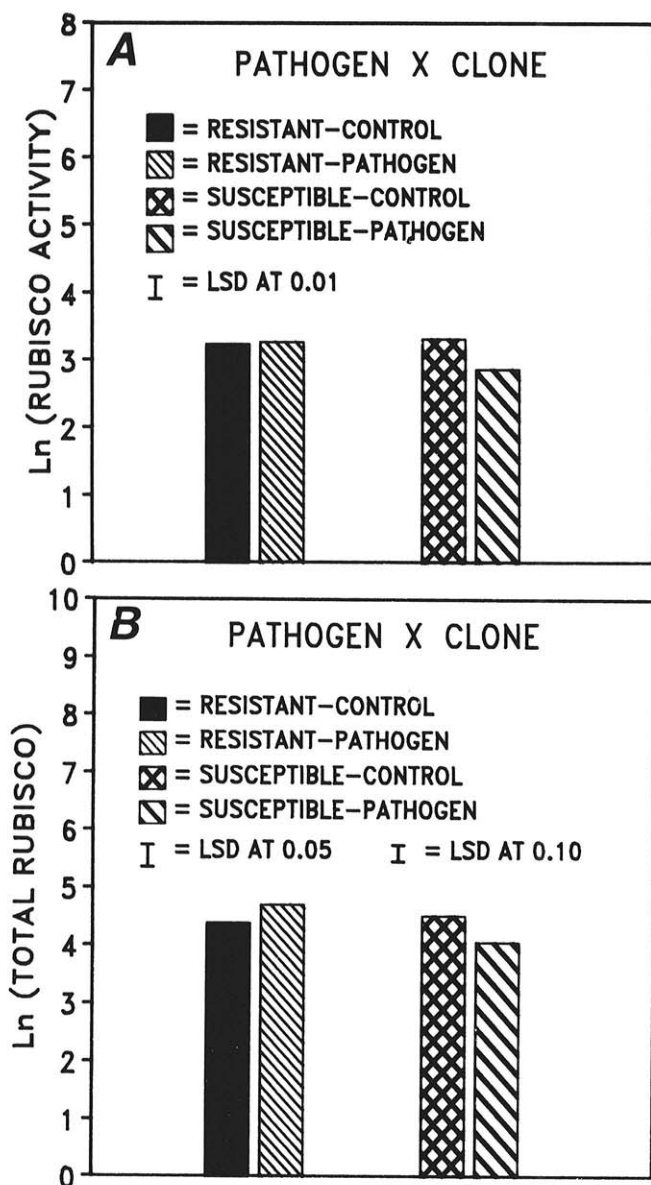


Fig. 4. Analysis of pathogen-to-clone interaction in resistant and susceptible alfalfa infected with *Verticillium albo-atrum* as reflected in the in vitro activity of Rubisco (A) and in the total amount of Rubisco (B), as determined by gel electrophoresis. Data were subjected to natural log transformation and the means represent the mean of seven values for A and four values for B. LSD = least significant difference.

There was a significant ($P = 0.02$) reduction in total activity of Rubisco extracted from leaves infected with *V. albo-atrum* and a significant ($P = 0.0001$) difference between experiments (Table 2). In addition, there was a significant ($P = 0.01$) pathogen-by-clone interaction (Fig. 4A), which indicated that the two clones did not respond similarly to the pathogen. There were no significant pathogen-by-experiment interactions, indicating that *V. albo-atrum* had the same effect on in vitro Rubisco activity during both experiments. Examination of the untransformed results, expressed as percent of the control to allow comparisons between in vivo and in vitro results, indicated that the activity of Rubisco from the infected susceptible clone was only 64.6% of the control (Table 3). The in vivo Rubisco activity was 66% of the control, which compares favorably with the in vitro values. The in vitro results for Rubisco activity of the infected resistant clone showed no effect of the pathogen (Table 3). Results from the *A/Ci* response curve analysis, when expressed as percent of the control, indicated that the Rubisco activity of the infected resistant clone was 98.5%, a value that compared closely with the in vitro finding (Table 3).

The leaf protein was disassociated and separated into its components by gel electrophoresis. Although the total amount of protein in the sample was unknown, the identical sample weight and careful application of the technique along with standardized scanning on the densitometer allowed comparison of the amount of Rubisco among treatments. In all cases, the values represent integration units and are not convertible into metric weights. Statistical analysis of transformed data detected no effect of pathogen on the amount of Rubisco (Table 2); however, there was a significant ($P = 0.007$) pathogen-by-clone interaction (Fig. 4B). There was no significant experiment-by-pathogen interaction although there was a significant ($P = 0.05$) experiment-by-clone interaction. Untransformed, in vitro results from gel electrophoresis, when expressed as percent of the control, indicate that the infected susceptible leaves had only 65% of the Rubisco of the control leaves, a value that agrees with the previously mentioned in vivo and in vitro values (Table 3). Conversely, gel electrophoresis using the leaf protein from infected resistant plants yielded a percent-of-the-control Rubisco value of 132%, a value that is 34% higher than that determined using in vivo techniques. The analysis of variance at the $P = 0.05$ level, however, indicates that this value is not different from the control.

DISCUSSION

The rate of photosynthesis is affected by environmental conditions such as drought, temperature extremes, and light intensity (2,10,26). In all cases, altered photosynthesis is ultimately the consequence of factors that affect the carboxylation of ribulose-1,5-bisphosphate (16): namely, the concentration of CO₂ within the leaf, the rate of regeneration of the sugar substrate, and the amount or activity of the carboxylase enzyme (16,26). Development of *A/Ci* response curve analysis by Farquhar and Sharkey in 1982 (10) provided a reliable, noninvasive means of evaluating the factors involved in the carboxylation reaction. The

TABLE 3. Comparison of in vitro and in vivo analysis of Rubisco in resistant and susceptible alfalfa infected with *Verticillium albo-atrum*^a

Method	Rubisco content ^b	
	Clone 1079	Clone 774
In vitro		
Total activation	104.6 ± 17.9	64.6 ± 10.2
Electrophoresis	132.0 ± 34.8	65.0 ± 16.7
In vivo		
<i>A/Ci</i> curve	98.5	66.0

^aThe in vitro total activation and electrophoresis data are means of seven and five values, respectively, for resistant clone 1079 and four values for susceptible clone 774. The *A/Ci* (net photosynthesis vs. internal CO₂) data are means of 20 values.

^bPercent of control ± standard deviation.

in vivo technique is ideally suited to studying the effect of biotic interactions on the three factors affecting CO₂ assimilation.

We noted a significant experiment-by-pathogen interaction that was reflected in stomatal conductance and net photosynthesis. Vapor pressure during measurements to determine the *A/Ci* response curves was the primary environmental difference between the two experiments. In experiment 1, the vapor pressure was approximately 1.0 kPa, and in experiment 2, it was between 2.0 and 2.4 kPa during the photosynthesis measurements. Stomates have a feed-forward response to vapor pressure deficit that reduces their aperture when the deficit is large (13,15,25). This response is independent of leaf water status and allows the plant to adjust its transpiration rate, thereby conserving water. The other consequence of the feed-forward response is a change in the internal CO₂ to external CO₂ ratio. When the vapor pressure deficit is large, the concentration of CO₂ within the leaf falls in direct response to the reduction in stomatal conductance (15). Analysis of the effect of stomatal limitation on net photosynthesis showed a large effect during experiment 1 and a smaller effect in experiment 2, indicating that the stomates were limiting photosynthesis more during the first experiment. The differences in vapor pressure deficit and stomatal conductance between the two experiments are consistent with the feed-forward hypothesis of stomatal response to vapor pressure deficit and may explain the pathogen-by-experiment interaction observed in this study.

Stomates of the susceptible plants infected with *V. albo-atrum* failed to respond to the more favorable vapor pressure in experiment 2. Ayres (1) noted that the stomates of infected plants lose the ability to respond to environmental variation, and our results for the susceptible, infected plants are in agreement with that observation.

A/Ci response curve analysis also was used to examine the effect of the pathogen on net photosynthesis and Rubisco activity. The significant interaction between pathogen and clone indicated that the two clones responded differently to the pathogen. Stomatal conductance, net photosynthesis, and the activity of Rubisco were significantly ($P = 0.01$) reduced in the susceptible clone infected with *V. albo-atrum*. Analysis of the effect of stomatal limitation on net photosynthesis indicated, however, that there was no significant difference between inoculated and uninoculated treatments in the susceptible clone. The reduction in CO₂ concentration that resulted from reduced stomatal conductance in the infected susceptible plant did not limit photosynthesis. The results indicate, rather, that the reduction in carboxylase enzyme activity was the factor responsible for reduced net photosynthesis. The in vitro results showed reductions in total Rubisco activity and in the amount of carboxylase enzyme that closely corresponded to the reduced Rubisco activity detected by *A/Ci* response curve analysis of infected susceptible leaves. Bunce (3) noted the possibility of underestimating the effect of stomatal limitation on photosynthesis when the internal CO₂ concentration is not homogeneous across the leaf. The activity of Rubisco ultimately controls the rate of net photosynthesis (11,31); therefore, the similarity of results from the in vivo and in vitro methods indicates that we have not underestimated the effect of the stomates on net photosynthesis.

Cralle and Heichel (4) demonstrated that photoassimilate partitioning in alfalfa was not constant throughout the plant growth cycle. After 21 days of regrowth, the uppermost, fully expanded leaf began exporting photosynthate to the unexpanded leaves and the shoot apex (4). The reduction in net photosynthesis that we noted in the youngest, fully expanded leaves of the susceptible clone would reduce the photosynthate available for partitioning to the apical meristematic regions and thus could contribute to the stunting and reduced leaf size noted in the inoculated plants.

In contrast to the response of susceptible plants to *V. albo-atrum*, net photosynthesis and the activity of Rubisco were not significantly affected by the pathogen in resistant plants, even though *V. albo-atrum* was isolated from 70% of the leaves of infected resistant plants and only 50% of the inoculated susceptible leaves. Extraction of Rubisco and total activation of the enzyme

confirmed the conclusion that the in vivo activity of the carboxylase enzyme was not affected in the resistant plant. Examination of the amount of Rubisco via gel electrophoresis also indicated no change in the amount of carboxylase enzyme in infected resistant plants at the $P = 0.05$ level of significance. However, when the least significant difference was computed at $P = 0.10$, as Steel and Torrie (27) suggest is acceptable for small experiments, there was significantly more Rubisco in the infected resistant leaves (Fig. 4B). This finding suggests some interesting possibilities. Roby et al (23), working with *Colletotrichum lagenarium* (Pass.) Ell. & Halsted in melon (*Cucumis melo* L. 'Cantaloup Charentais') found an increased level of mRNAs coding for the two subunits of Rubisco early in the infection process; however this level dropped rapidly. They speculated that Rubisco synthesis was turned off to allow the synthesis of defense-related compounds. Similarly, Mouly et al (17) found increased levels of mRNAs coding for the small subunit of Rubisco in the early stages of infection of sunflower (*Helianthus annuus* L.) with *Sclerotinia sclerotiorum* (Lib.) de Bary. The levels declined after 2 days in the susceptible host and after 8 days in the more tolerant host. In both cases, the pathogen eventually killed the host. The delay in reduction of mRNAs in the more tolerant sunflower and the possible increase in Rubisco in our resistant alfalfa infected with *V. albo-atrum* seem to indicate that maintenance rather than reduction of Rubisco levels (23) is involved in resistance.

The present analysis of net photosynthesis in resistant alfalfa clones infected with *V. albo-atrum* indicates that the growth reductions observed in resistant plants (21,22) were not due to a reduction in the photosynthetic ability of the youngest, fully expanded leaves. We further conclude that the reduction in net photosynthesis detected in susceptible, infected plants was due to reduction in the amount and activity of Rubisco rather than to reduced internal CO₂ concentrations caused by reduced stomatal conductance.

LITERATURE CITED

1. Ayres, P. G. 1981. Responses of stomata to pathogenic microorganisms. Pages 205-221 in: Stomatal Physiology. P. G. Jarvis and T. A. Mansfield, eds. Cambridge University Press, Cambridge, England.
2. Bradford, K. J., and Hsiao, T. C. 1982. Physiological responses to moderate water stress. Pages 264-324 in: Physiological Plant Ecology. II. Water Relations and Carbon Assimilation. O. L. Lange, P. S. Nobel, C. B. Osmond, and H. Ziegler, eds. Springer-Verlag, New York.
3. Bunce, J. A. 1988. Nonstomatal inhibition of photosynthesis by water stress. Reduction in photosynthesis at high transpiration rate without stomatal closure in field-grown tomato. Photosynth. Res. 18:357-362.
4. Cralle, H. T., and Heichel, G. H. 1988. Photosynthate partitioning in alfalfa before harvest and during regrowth. Crop Sci. 28:948-953.
5. Dann, M. S., and Pell, E. J. 1989. Decline of activity and quantity of ribulose biphosphate carboxylase/oxygenase and net photosynthesis in ozone-treated potato foliage. Plant Physiol. 91:427-432.
6. Douglas, S. M., and MacHardy, W. E. 1981. The relationship between vascular alterations and symptom development in Verticillium wilt of chrysanthemum. Physiol. Plant Pathol. 19:31-39.
7. Duniway, J. M. 1971. Resistance to water movement in tomato plants infected with *Fusarium*. Nature 230:252-253.
8. Duniway, J. M. 1971. Water relations of *Fusarium* wilt in tomato. Physiol. Plant Pathol. 1:537-546.
9. Duniway, J. M., and Slayter, R. O. 1971. Gas exchange studies on the transpiration and photosynthesis of tomato leaves affected by *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathology 61:1377-1381.
10. Farquhar, G. D., and Sharkey, T. D. 1982. Stomatal conductance and photosynthesis. Annu. Rev. Plant Physiol. 33:317-345.
11. Farquhar, G. D., Von Caemmerer, S., and Berry, J. A. 1980. A biochemical model of photosynthetic CO₂ assimilation in leaves of C₃ species. Planta 149:78-90.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680-685.
13. Long, S. P. 1985. Leaf gas exchange. Pages 453-499 in: Photosynthetic Mechanisms and the Environment. J. Barber and N. R. Baker, eds. Elsevier Science Publishing Co., New York.
14. MacHardy, W. E., Busch, L. V., and Hall, R. 1976. Verticillium

- wilt of chrysanthemum: Quantitative relationship between increased stomatal resistance and local vascular dysfunction preceding wilt. *Can. J. Bot.* 54:1023-1034.
15. Morison, J. I. L., and Gifford, R. M. 1983. Stomatal sensitivity to carbon dioxide and humidity. *Plant Physiol.* 71:789-796.
 16. Mott, K. A., Jensen, R. G., and Berry, J. A. 1986. Limitation of photosynthesis by RuBp regeneration rate. Pages 33-43 in: *Biological Control of Photosynthesis*. R. Marcelle, H. Clijsters, and M. VanPoucke, eds. Martinus Nijhoff Publishers, Boston, MA.
 17. Mouly, A., Roby, D., and Esquerre-Tugaye, M-T. 1988. Ribulose 1,5-bisphosphate carboxylase-oxygenase small subunit transcripts as a susceptibility reflecting molecular marker in sunflower infected with *Sclerotinia sclerotiorum*. *Plant Sci.* 56:219-225.
 18. Nicolodi, C., Massacci, A., and DiMarco, G. 1988. Water status effects on net photosynthesis in field-grown alfalfa. *Crop Sci.* 28:944-948.
 19. Pegg, G. 1985. Life in a black hole—The micro-environment of the vascular pathogen. *Trans. Br. Mycol. Soc.* 85:1-20.
 20. Pennypacker, B. W., and Leath, K. T. 1986. Anatomical response of a susceptible alfalfa clone infected with *Verticillium albo-atrum*. *Phytopathology* 76:522-527.
 21. Pennypacker, B. W., Leath, K. T., and Hill, R. R., Jr. 1988. Growth and flowering of resistant alfalfa infected by *Verticillium albo-atrum*. *Plant Dis.* 72:397-400.
 22. Pennypacker, B. W., Leath, K. T., and Hill, R. R., Jr. 1990. Growth and physiological response of resistant alfalfa clones infected with *Verticillium albo-atrum*. *Phytopathology* 80:1247-1253.
 23. Roby, D., Marco, Y., and Esquerre-Tugaye, M-T. 1988. Ribulose 1,5-bisphosphate carboxylase/oxygenase gene expression in melon plants infected with *Colletotrichum lagenarium*. Activity level and rate of synthesis of mRNAs coding for the large and small subunits. *Physiol. Mol. Plant Pathol.* 32:411-424.
 24. Scheffer, R. P., Gothoskar, S. S., Pierson, C. F., and Collins, R. P. 1956. Physiological aspects of *Verticillium* wilt. *Phytopathology* 46:83-87.
 25. Schulze, E.-D., Turner, N. C., Gollan, T., and Shackel, K. A. 1987. Stomatal responses to air humidity and to soil drought. Pages 311-321 in: *Stomatal Function*. E. Zeiger, G. D. Farquhar, and I. R. Cowan, eds. Stanford University Press, Stanford, CA.
 26. Seeman, J. R. 1986. Mechanisms for the regulation of CO₂ fixation by ribulose-1,5-bisphosphate carboxylase. Pages 71-82 in: *Biological Control of Photosynthesis*. R. Marcelle, H. Clijsters, and M. VanPoucke, eds. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
 27. Steel, R. G. D., and Torrie, J. H. 1980. Page 91 in: *Principles and Procedures of Statistics*. McGraw-Hill, New York.
 28. Talboys, P. W. 1968. Water deficits in vascular disease. Pages 255-311 in: *Plant Water Consumption and Response*. Vol III. T. T. Kozlowski, ed. Academic Press, New York.
 29. Tzeng, D. D., and DeVay, J. E. 1985. Physiological responses of *Gossypium hirsutum* L. to infection by defoliating and nondefoliation pathotypes of *Verticillium dahliae* Kleb. *Physiol. Plant Pathol.* 26:57-72.
 30. Tzeng, D. D., Wakeman, R. J., and DeVay, J. E. 1985. Relationships among *Verticillium* wilt development, leaf water potential, phenology and lint yield in cotton. *Physiol. Plant Pathol.* 26:73-81.
 31. Vu, J., Cu, V., Allen, L. H., Jr., and Bowes, G. 1987. Drought stress and elevated CO₂ effects on soybean ribulose bisphosphate carboxylase activity and canopy photosynthetic rates. *Plant Physiol.* 83:573-578.
 32. Woodrow, I. E., and Berry, J. A. 1988. Enzymatic regulation of photosynthetic CO₂ fixation in C₃ plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39:533-594.