Impact of *Verticillium albo-atrum* and Photosynthetic Photon Flux Density on Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in Resistant Alfalfa

B. W. Pennypacker, D. P. Knievel, M. L. Risius, and K. T. Leath

First, second, and third authors: Department of Agronomy, The Pennsylvania State University, University Park 16802; and fourth author: USDA-ARS, Pasture Systems and Watershed Management Unit, University Park, PA 16802.

We thank N. L. Eckhardt, Department of Plant Pathology, The Pennsylvania State University, for sharing her expertise in Rubisco; K. L. Campbell, Department of Agronomy, The Pennsylvania State University, for technical assistance; W. L. Stout, USDA-ARS, Pasture Systems and Watershed Management Unit, for use of the Campbell micrologger, thermistors, and LI-6200 Photosynthesis System; E. J. Pell and H. Flores, Department of Plant Pathology, The Pennsylvania State University, for use of the Beckman LS 1701 scintillation counter and Pharmacia LKB laser densitometer; and R. A. Haldeman, USDA-ARS, Pasture Systems and Watershed Management Unit, for photographing the gel.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA, nor does it imply approval to the exclusion of other products that may also be suitable.

This research was supported by a USDA NRICGP postdoctoral fellowship grant to B. W. Pennypacker under agreement 91-37303-6434 from the Cooperative State Research Service, USDA.

Accepted for publication 21 October 1994.

ABSTRACT

Pennypacker, B. W., Knievel, D. P., Risius, M. L., and Leath, K. T. 1995. Impact of *Verticillium albo-atrum* and photosynthetic photon flux density on ribulose-1,5-bisphosphate carboxylase/oxygenase in resistant alfalfa. Phytopathology 85:132-138.

Resistance of an alfalfa clone to *Verticillium albo-atrum* was attenuated by manipulating photosynthetic photon flux density (PPFD). Effects of PPFD and *V. albo-atrum* on dark respiration, in vivo ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity, in vitro total Rubisco activity, quantity of Rubisco, molar activity of Rubisco, and ribulose-1,5-bisphosphate (RuBP) regeneration rate were examined in a factorial experiment with pathogen (inoculated or noninoculated) and PPFD (40, 70, and 100% PPFD) as the treatments. Pathogen × PPFD interactions were detected in dark respiration, total protein, and amount of Rubisco. Dark respiration was suppressed by *V. albo-atrum* under 40% PPFD, increased under 70% PPFD, and unaffected by the pathogen under 100% PPFD. Total protein and Rubisco were reduced in inoculated plants only under 40% PPFD. In vitro total Rubisco activity was increased and regeneration rate of RuBP was reduced in all inoculated plants. The photosynthetic properties of the inoculated, resistant plants under 40% PPFD mimicked those reported for susceptible plants, but only dark respiration and amount of Rubisco were reduced by the presence of *V. albo-atrum;* the other properties were reduced by the PPFD treatment. The increase in in vitro total Rubisco activity and decrease in RuBP regeneration rate in inoculated, resistant alfalfa suggest that photosynthetic acclimation may be optimizing carbon assimilation in alfalfa resistant to *V. albo-atrum.*

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

Net photosynthesis is sensitive to abiotic stress associated with detrimental environmental changes (9) and is also adversely affected by the biotic stress induced by systemic, vascular wilt fungi. Net photosynthesis is suppressed in susceptible tomato by *Fusarium oxysporum* f. sp. *lycopersici* (3) and in susceptible potato, cotton, and alfalfa (*Medicago sativa* L.) by *Verticillium* spp. (2,5,6,16). *Verticillium albo-atrum* Reinke & Berthier does not affect the photosynthetic rate of resistant alfalfa (16,17). However, when resistant alfalfa plants infected with *V. albo-atrum* are grown under shade cloth, which concomitantly reduces net photosynthesis, resistance to *V. albo-atrum* is attenuated and plants are phenotypically susceptible (17). Height and leaf, stem, and aerial biomass of infected, resistant alfalfa plants grown under 40% photosynthetic photon flux density (PPFD) are suppressed while disease rating is increased compared with similar plants grown under 100% PPFD (17). Loss of resistance when environmental conditions reduce net photosynthesis suggests that carbon assimilation is a critical factor in resistance.

The role of carbon assimilation in resistance appears to vary depending upon the host-pathogen system. Berghaus and Reisener (1), working with *Puccinia graminis* f. sp. *tritici,* found greater suppression of net photosynthesis in moderately resistant, infected wheat cultivars than in susceptible cultivars. The drop in net photosynthesis correlated with a decline in chlorophyll in a restricted area around the infection site. The authors hypothesized that photosynthate was reduced in the immediate vicinity of the parasite, which limited mycelial growth (1). In contrast, vascular wilt fungi derive energy from carbon skeletons released from enzymatically degraded cell walls rather than by direct consumption of photosynthate (15). *Verticillium* spp. elicit host defense responses that utilize products of the phenylpropanoid pathway (8,13,18) and, as a consequence, require increased carbon flow through that secondary metabolic pathway. Net photosynthesis in broad bean (*Vicia faba*) leaves infected with *Uromyces viciae-fabae* was reduced significantly but was increased in noninfected leaves on the same plant (12). The leaves with increased rates of photosynthesis were resistant to rust infection but lost that resistance when the photosynthetic rate was reduced by shading, again suggesting a critical role for carbon assimilation in resistance (12).

The rate of net photosynthesis ultimately is controlled by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme responsible for capturing CO_2 and catalyzing its incorporation into sugar phosphate (4,20). Environmental factors that alter the concentration of CO_2 and/or affect the availability of ribulose-1,5-bisphosphate (RuBP), the sugar substrate, will affect net photosynthesis through their effect on the activity of Rubisco (4,11,20). Resistant alfalfa plants infected with *V. albo-atrum* respond phenotypically as susceptible plants when grown under

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1995.

40% PPFD (17), but their physiological response is less clear. The objectives of this study were to examine carbon assimilation in *V. albo-atrum*-infected, resistant alfalfa plants exposed to photosynthetic stress and to determine 1) whether the susceptible response of resistant plants grown under 40% PPFD was physiologically similar to the previously reported physiological response of a genetically susceptible alfalfa clone (16) and 2) whether there is a mechanism that maintains or enhances carbon assimilation in *V. albo-atrum*-infected, resistant alfalfa.

MATERIALS AND METHODS

Experimental design. The effect of *V. albo-atrum* on the physiological properties associated with carbon assimilation was determined by altering the PPFD under which resistant alfalfa plants were grown. The experiment was a split-plot in a randomized complete block design with three replicates. The main plot treatment was PPFD (40, 70, and 100% ambient), and the subplot treatments were a 2×3 factorial of pathogen (*V. albo-atrum* or no *V. albo-atrum*) and sampling time (3 wk). Plants were destructively sampled weekly during weeks 3, 4, and 5 of the 5-wk experiment. The experiment was conducted during October and November 1991 and was repeated during March and April 1992.

Plant material, inoculation method, PPFD treatments, and environmental conditions. A portion of this study was previously reported, and the plant material, inoculation method, PPFD treatments, and environmental conditions were described in detail (17). Alfalfa grown from seed is genetically heterogeneous; therefore, resistant alfalfa clone 1079 was used to minimize this variation and allow definitive conclusions. The clones were grown in 15-cm clay pots for 6 wk before being cut to 4-cm height and either inoculated with *V. albo-atrum* or treated with sterile water. A 20-μl drop of a spore suspension (3×10^6 spores per milliliter) was placed on each freshly cut stem stub, and the inoculated plants were kept in a dark mist chamber for 24 h before being returned to the greenhouse. Noninoculated plants were not placed in the mist chamber to minimize the possibility of cross contamination. Inoculated and noninoculated plants were grown on separate benches under high-intensity discharge, metal-halide lamps (400 W) for an additional 6 wk before being used in the PPFD experiment.

After the 6-wk pathogen-establishment period, plants were cut to a 4-cm height, and the basal portion of each excised stem was cultured on 2% water agar to confirm the presence of *V. albo-atrum.* The three PPFD treatments were initiated by placing the freshly cut plants either in shade-cloth covered cages (three cages per PPFD treatment) that provided PPFD environments of 40 or 70% ambient PPFD or on the unshaded bench for the 100% ambient PPFD treatment. Plants were supported with bamboo stakes to eliminate interplant contact and minimize the possibility of cross contamination. The actual PPFD measurements and the temperature regime during both experiments were previously reported (17).

Sampling procedures. One plant per treatment per replicate was removed for growth analysis (17) and physiological measurements each week during weeks 3, 4, and 5 of the experiment. The plants were taken to the laboratory and placed in a mist chamber overnight to protect them from the stress associated with low vapor pressure deficits common in climate-controlled laboratories. The following day, plants were acclimated for a minimum of 1 h at PPFD levels similar to those under which they were grown before determination of CO_2 response (A/Ci) curves, net photosynthesis, stomatal conductance, and dark respiration. After the physiological measurements were conducted, a 0.4-g (fresh weight) sample of tissue from the youngest, fully expanded leaves was removed from the apical portions of the stems and frozen in liquid nitrogen within 45 s of harvest. The frozen leaf tissue was stored at −80 C until used for quantification of Rubisco and determination of in vitro total Rubisco activity and total protein. An additional, similarly aged, 0.4-g (fresh weight) sample of leaves was collected, dried at 70 C for 48 h, and then weighed.

Net photosynthesis and dark respiration protocols. The physiological measurements were made in situ on the youngest fully expanded leaf on one stem per plant. PPFD levels were 1,800, 1,200, or 700 μmol m^{-2} s^{-1} during the measurements and were selected to represent PPFD levels on a sunny day under the respective greenhouse-imposed PPFD treatments. Plants were measured under the PPFD level corresponding to their growing conditions. The three PPFD levels were obtained by varying the plant's distance from the water-cooled, incandescent light source, a procedure that maintained uniform spectral quality among treatments. The selected leaf was enclosed in the 0.25-L sample chamber of the LI-6200 Photosynthesis System (LICOR, Lincoln, NE) and allowed to acclimate to a humidified ambient gas mixture composed of 21% O_2 and 0.035% CO_2 in N_2 for 5 min at the selected PPFD level. After acclimation, the LI-6200 was placed in the closed-circulation mode, and the leaf was allowed to draw CO_2 down to the CO_2 compensation point, the point where net photosynthesis was zero, while sequential measurements of net photosynthesis were recorded. CO_2 then was injected into the gas stream loop of the LI-6200, and the CO_2 concentration within the sample chamber was increased to ~750 μmol mol^{-1}. Additional sequential net photosynthesis readings were taken as CO_2 was depleted to <300 μmol mol^{-1}. Alfalfa stomates close rapidly with sudden increases in CO_2, which complicates efforts to increase internal CO_2 levels within the leaf. This problem was alleviated by injecting CO_2 when stomates were at maximum aperture in response to the low CO_2 levels associated with the CO_2 compensation point.

After the net photosynthesis measurements were completed, all lights were extinguished; the sample chamber was covered with aluminum foil; the LI-6200 was returned to the open gas circulation mode; and the leaf was allowed to acclimate to 350 μmol mol^{-1} of CO_2 for 2 min. After the acclimation period, the LI-6200 was placed in the closed gas circulation mode, and dark respiration was measured for 2 min. When all gas exchange measurements were completed, the leaf was removed from the sample chamber, and its area was measured with a LI-3000A portable area meter (LICOR). All net photosynthesis and internal CO_2 concentrations were recalculated by using the actual leaf area. All leaves used for net photosynthesis measurements were removed from the plant, surface sterilized in 10% bleach (5.25% sodium hypochlorite) for 2 min, drained on paper towels, and aseptically placed on 2% water agar for isolation of *V. albo-atrum.* Plates were incubated at 25 C for 2 wk and examined microscopically for the presence of verticilliate conidiophores of the pathogen.

***A/Ci* protocol.** A/Ci curve analysis was used to determine both the in vivo activity of the carboxylase enzyme, Rubisco, and the rate of regeneration of the sugar substrate, RuBP (4,16,31). Net photosynthesis values (A) obtained from the CO_2 drawdown measurements were plotted against the corresponding internal CO_2 concentrations (Ci) to produce a curve of A versus Ci for each leaf measured. The slope of the line as net photosynthesis approached zero was determined through regression analysis with the general linear models program (GLM) of SAS (SAS Institute Inc., Cary, NC). The carboxylase enzyme follows Michaelis-Menten kinetics for an enzyme with a single substrate (CO_2) with a competitive inhibitor (O_2) (25); therefore, the slope of the curve as net photosynthesis approaches zero represents the in vivo activity of Rubisco (4). The plateau of the curve represents J_{max}, the rate of RuBP regeneration, which reflects photosynthetic electron transport (28).

In vitro total Rubisco activity. The frozen leaf tissue was ground in a frozen mortar in liquid N_2 and then placed into an ice-cold Tenbroeck tissue grinder (Fisher Scientific, Pittsburgh, PA) (16). The leaf tissue was homogenized for 30 s in 4 ml of extraction buffer (50 mM bicine, pH 8.0, 20 mM $NaHCO_3$, 20 mM $MgCl_2 \cdot H_2O$, 0.1 mM EDTA, 2 mM dithioerythritol, 10 mM leupeptin sulfate, and 1 mM phenylmethylsulfonyl fluoride), filtered, microfuged for 15 s at 15,850 *g*, and separated into aliquots for determination of total Rubisco activity, total protein, and quantity

of Rubisco.

Aliquot 1 was brought to 10 mM $NaHCO_3$ and incubated on ice for 10 min to fully activate the available sites on the carboxylase enzyme (21). Sawada et al (24) found that both initial and total Rubisco activation levels remained steady for 2 h when the enzyme preparations were held on ice rather than at room temperature, and Sharkey et al (26) noted that incubation on ice reduced variation between replicates. After activation, aliquot 1 was assayed for total Rubisco activity by determining the amount of $^{14}CO_2$ incorporated into acid-stable products in 30 s. The reaction was carried out in microfuge tubes containing 400 μl of assay buffer (50 mM bicine, pH 8.0, 20 mM $NaHCO_3$, 20 mM $MgCl_2 \cdot H_2O$, 0.1 mM EDTA), 25 μl of $NaH^{14}CO_3$ solution (2 μCi per assay), and 25 μl of 10 mM RuBP. The RuBP was prepared from ribose 5-phosphate according to the method of Sharkey et al (26) to avoid the inhibitors associated with commercial RuBP. The reaction was started by adding 50 μl of aliquot 1 to the reaction mixture and was stopped after 30 s by adding 100 μl of 2 N HCl and allowing unfixed $^{14}CO_2$ to escape. The amount of $^{14}CO_2$ fixed by Rubisco was determined in a Beckman LS7000 scintillation counter (Beckman Instruments Inc., Fullerton, CA) and expressed on the basis of the dry weight of the leaf sample.

Total protein protocol. Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) was used to determine the total protein content of aliquot 2. Twenty microliters of leaf extract was added to 80 μl of extraction buffer and 3 ml of the Coomassie assay reagent and incubated for 10 min at room temperature. The amount of total protein was determined spectrophotometrically by absorbance at 595 nm. Absorbance values were compared with those of a bovine serum albumin (Pierce) standard curve, and the amount of total protein was determined mathematically by using the regression equation for the standard curve.

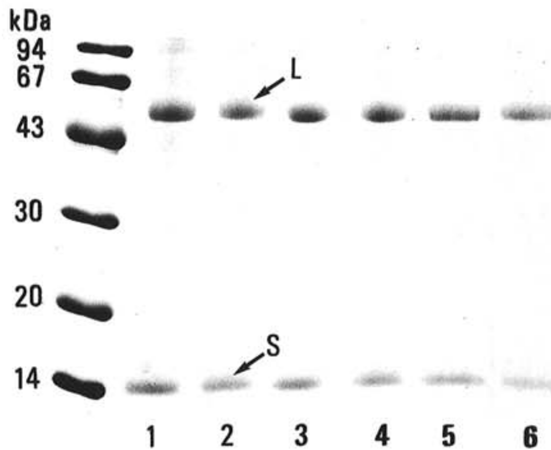

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of leaf extract from resistant alfalfa plants in experiment 2 at the end of week 5. Amount of leaf extract applied to each lane was adjusted on the basis of the Coomassie total protein assay to standardize the amount of total protein in each lane. The two dark bands in each lane represent the large (L) and small (S) subunits of Rubisco. Lane 1, 100% photosynthetic photon flux density (PPFD), noninoculated; lane 2, 100% PPFD, *Verticillium albo-atrum;* lane 3, 70% PPFD, noninoculated; lane 4, 70% PPFD, *V. albo-atrum;* lane 5, 40% PPFD, noninoculated; and lane 6, 40% PPFD, *V. albo-atrum.*

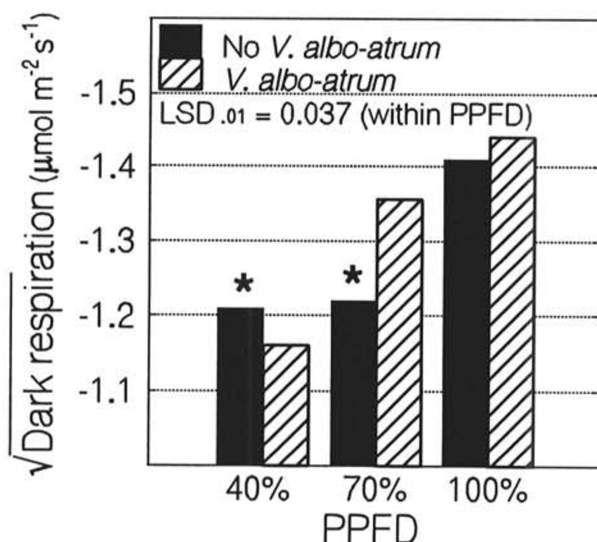

Fig. 2. *Verticillium albo-atrum* × photosynthetic photon flux density (PPFD) interaction in dark respiration of a resistant alfalfa clone. Note that dark respiration is significantly suppressed in inoculated plants compared with noninoculated plants under 40% PPFD and is significantly increased in inoculated plants compared with noninoculated plants under 70% PPFD. * = Significant difference at $P = 0.01$. Bars represent the pooled data from two experiments.

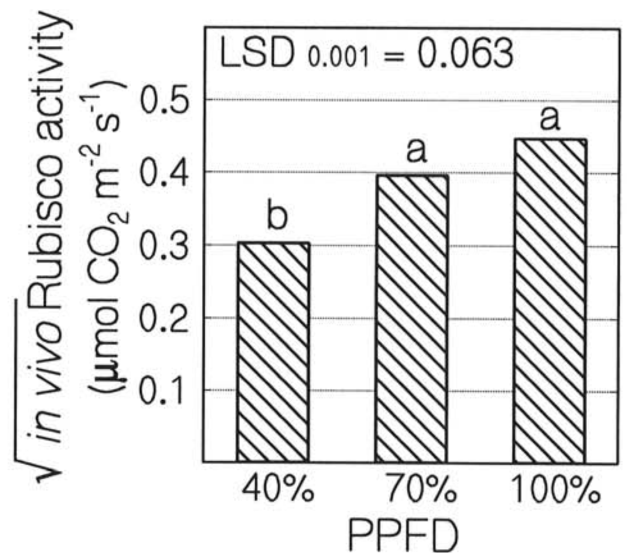

Fig. 3. Main effect of photosynthetic photon flux density (PPFD) on in vivo Rubisco activity of resistant alfalfa determined with A/Ci curve analysis. In vivo Rubisco activity was suppressed by the 40% PPFD treatment. Data were pooled from two experiments. Bars with the same letter are not significantly different at $P = 0.001$.

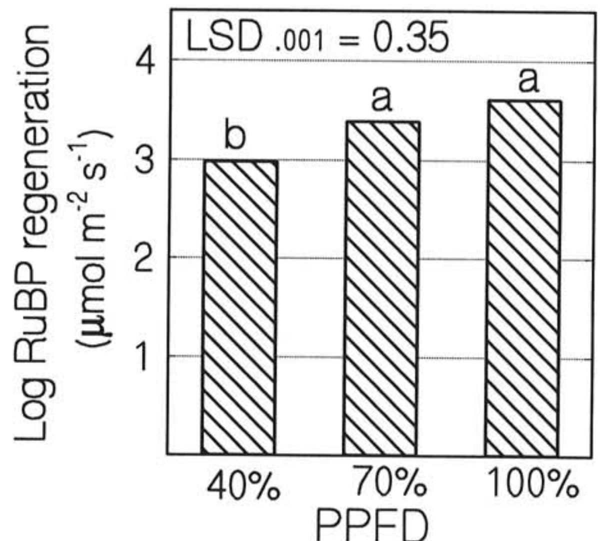

Fig. 4. Main effect of photosynthetic photon flux density (PPFD) on the rate of ribulose-1,5-bisphosphate (RuBP) regeneration in resistant alfalfa. Data were pooled from two experiments. Bars with the same letter are not significantly different at $P = 0.001$.

Quantification of Rubisco. The total amount of Rubisco in the leaf extract was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, a process that involved adding 200 μl of aliquot 3 to 100 μl of SDS reducing buffer (a mixture of 0.5 M tris-HCl, pH 6.8, 10% SDS, 0.71 M 2-mercaptoethanol, 0.05% bromphenol blue, and 10% glycerol). The mixture was boiled for 1 min to denature the proteins and loaded onto a 14% SDS polyacrylamide minigel with a 4.5% stacking gel (Jule Inc., New Haven, CT) in a Mini-Protean II electrophoresis cell (Bio-Rad, Melville, NY). Two wells per gel 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. After electrophoresis, gels were stained with 0.1% Coomassie blue for 30 min and destained overnight in a solution of 25% methanol and 8% glacial acetic acid. Protein bands were quantified with an LKB Bromma-Ultroscan XL laser densitometer (Pharmacia), and Rubisco large and small subunits were identified by migration compared with the low molecular weight standards (Fig. 1). The percentage of the total protein that was Rubisco was determined by integration of the area under the densitometer curves. The actual amount of Rubisco was determined mathematically by combining the results of the Coomassie total protein assay and the gel electrophoresis data.

Statistical analyses. The laboratory processing of all in vitro leaf samples was conducted according to the previously described experimental design so that variation caused by any procedural differences between assay dates would be incorporated into the replication error mean square. Data from the two experiments were combined because the error variances were homogeneous as determined by an F test. 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) by using the general linear models (Proc GLM) of SAS, and single-degree orthogonal polynomials were examined to determine the significance of the linear and quadratic components of all interactions (27). Residuals were examined and data were transformed by the natural log or square root transformation as necessary. When the ANOVA indicated a significant interaction, a predicted response curve (qualitative variables) or a predicted response surface (quantitative variables) was generated by least squares means and a multiple regression procedure (Proc Stepwise) of SAS. In all cases, the components of the predicted equations were those indicated as significant by the orthogonal polynomials in ANOVA. When significant interactions involved only qualitative variables, Fisher's protected least significant difference test was used within treatment levels.

RESULTS

Isolation of pathogen. *V. albo-atrum* was isolated from the basal portion of the stems of 96% (26 of 27) of the inoculated plants in experiment 1 and 70% (19 of 27) of the inoculated plants in experiment 2. In experiment 1, the pathogen was isolated from two noninoculated plants, which were subsequently removed from the study, and was not recovered from any noninoculated plants in experiment 2. Isolations were also conducted on the leaves used for photosynthesis and dark respiration measurements. *V. albo-atrum* was isolated from 30% of those leaves in experiment 1 and from 7% of those leaves in experiment 2 and was not recovered from any leaves on the noninoculated plants in either experiment.

In vivo properties. A significant pathogen × PPFD interaction was detected in dark respiration (Fig. 2) and was caused by a reversal in the response of inoculated plants grown under 40% PPFD compared with similar plants grown under 70% PPFD. There was no significant difference in dark respiration between inoculated and noninoculated plants under 100% PPFD. Results of the net photosynthesis and stomatal conductance measurements were previously reported (17), and no pathogen × PPFD interactions or overall effects of *V. albo-atrum* were detected for either parameter. Similarly, in vivo Rubisco activity was not affected by *V. albo-atrum* regardless of PPFD growth environment but was significantly reduced by the 40% PPFD treatment (Fig. 3). There were no experiment × pathogen or experiment × pathogen × PPFD interactions for any in vivo parameters.

A significant main effect of pathogen was noted in the rate of regeneration of RuBP, the sugar substrate required by Rubisco. Inoculated, resistant plants, regardless of PPFD growth conditions, had a significant ($P = 0.10$) reduction in the RuBP regeneration rate compared with noninoculated plants (log transformed values were 3.4 and 3.2 μmol m^{-2} s^{-1} for the noninoculated and inoculated plants, respectively). RuBP regeneration was also significantly suppressed by the 40% PPFD treatment (Fig. 4) but was not significantly different under the 70 and 100% PPFD environments.

In vitro properties. Significant pathogen × PPFD interactions were detected in total protein (Fig. 5) determined with the Coomassie assay and in quantity of Rubisco (Fig. 6). There were no differences in the amount of total protein or Rubisco under

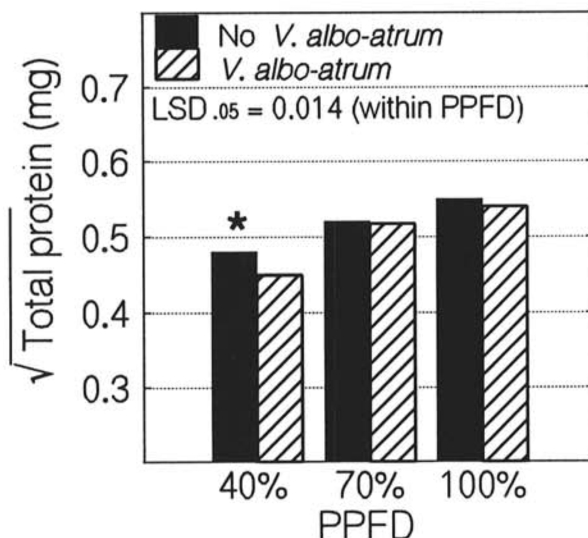

Fig. 5. *Verticillium albo-atrum* × photosynthetic photon flux density (PPFD) interaction in total protein determined with the Coomassie total protein assay. Total protein was reduced in inoculated, resistant alfalfa plants grown under 40% PPFD compared with noninoculated plants. Data were pooled from two experiments. * = Significant difference at $P = 0.05$.

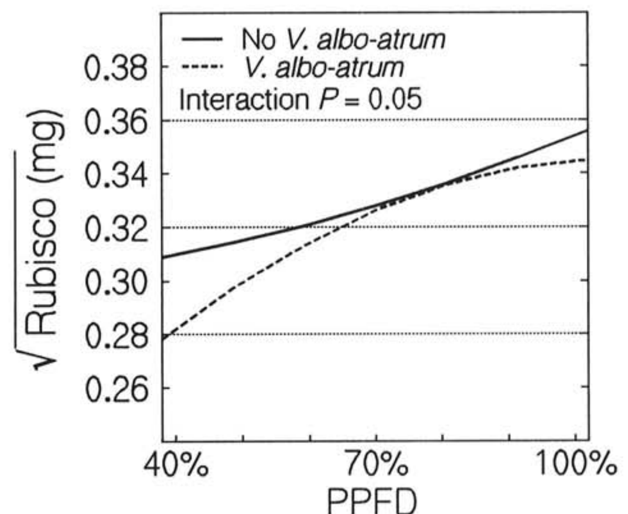

Fig. 6. Predicted response curve showing a significant *Verticillium albo-atrum* × photosynthetic photon flux density (PPFD) interaction in the amount of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in resistant alfalfa. Note that the quantity of Rubisco is reduced in inoculated plants grown under 40% PPFD. The predicted curve is based on analysis of variance and orthogonal contrast analysis of pooled data from two experiments. The P value indicates the significance of the interaction.

100 and 70% PPFD; but under 40% PPFD, resistant plants infected with *V. albo-atrum* had significantly less total protein and less Rubisco in the youngest, fully expanded leaves than noninoculated plants had. A pathogen × week interaction was significant for in vitro total Rubisco activity (Fig. 7) determined by uptake of $^{14}CO_2$. The slight differences in in vitro total Rubisco activity from week to week caused the significant interaction; but in all cases, the in vitro total activity of Rubisco was significantly greater in the inoculated, resistant plants than in the noninoculated plants. A significant PPFD × week interaction was detected in in vitro total Rubisco activity (Fig. 8) and indicated that carboxylase activity was increased under 40% PPFD and that the increase was stable over time compared with the decline in in vitro total Rubisco activity over time under the 100% PPFD treatment. No other significant interactions were detected.

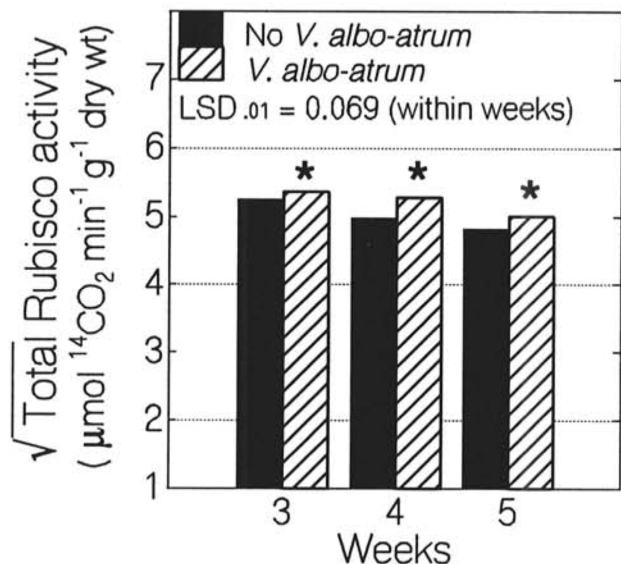

Fig. 7. *Verticillium albo-atrum* × week interaction in in vitro total ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity of resistant alfalfa plants determined by $^{14}CO_2$. * = Significant difference at $P = 0.01$ within weeks. Data were pooled from two experiments.

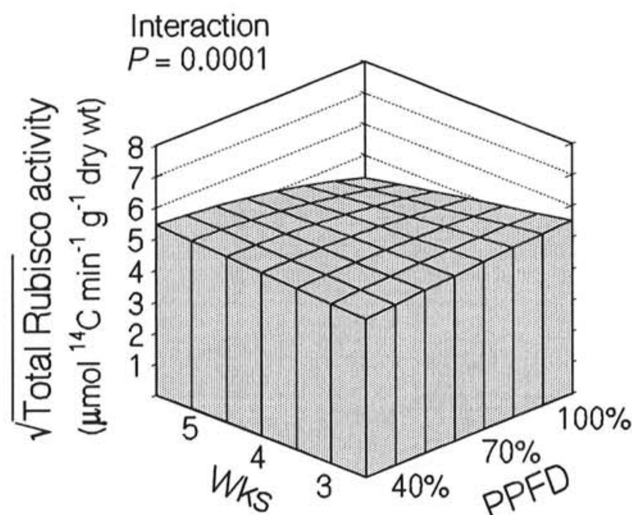

Fig. 8. Predictive response surface showing a photosynthetic photon flux density (PPFD) × week interaction in in vitro total ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity of resistant alfalfa. In vitro total Rubisco activity is higher in resistant alfalfa grown under 40% PPFD, and the increase is stable over time compared with resistant plants under 100% PPFD. Predicted response surface is based on analysis of variance and orthogonal contrast analysis of pooled data from two experiments. The *P* value indicates the significance of the interaction.

The total molar activity of Rubisco was determined by dividing the in vitro total activity of Rubisco by the amount of Rubisco (14,25). Total molar activity of Rubisco, which reflects the concentration of tight-binding inhibitors of Rubisco activity in the leaf, was significantly increased under the 40% PPFD treatment compared with the 100% PPFD treatment (Fig. 9), indicating less inhibition of Rubisco activity under low PPFD. *V. albo-atrum* had no detectable effect on total molar activity of Rubisco in resistant alfalfa plants.

DISCUSSION

Net photosynthesis is sensitive to many abiotic factors, including temperature, light, water stress, mineral nutrients, and salinity (11), and that sensitivity is mediated, in part, by Rubisco, the enzyme controlling the environmental-biochemical interface in the leaf (20,31). Net photosynthesis in potato, cotton, and alfalfa also is sensitive to biotic stress imposed by *Verticillium* spp. when the host is susceptible (2,5,6,16). Mechanisms responsible for reduced net photosynthesis caused by *Verticillium* spp. vary with host species and include lowered Rubisco activity in susceptible alfalfa (16), a combination of reduced Rubisco activity and reduced stomatal conductance in cotton (5), and reduced stomatal conductance in potato (2). In contrast, neither net photosynthesis nor in vivo Rubisco activity was affected by *V. albo-atrum* in a resistant alfalfa clone (16). However, imposition of PPFD-induced reduced net photosynthesis attenuated the resistant response of the resistant alfalfa clone, and disease ratings and growth measurements verified the loss of resistance under 40% PPFD (17).

Comparisons between inoculated and noninoculated, resistant alfalfa plants subjected to PPFD-induced reduced net photosynthesis in this study documented significant reductions in dark respiration and the amount of Rubisco in inoculated, resistant alfalfa under 40% PPFD. These results mirror those reported for several *Verticillium*-infected susceptible hosts. Rubisco concentrations were lower in susceptible alfalfa infected with *V. albo-atrum* (16), and dark respiration was reduced in susceptible field-grown cotton infected with *V. dahliae* (5) but not in susceptible, growth chamber-grown potato (2). The apparent lack of a dark respiration response in *Verticillium*-infected, susceptible potato is perplexing but may reflect the small sample size of that study.

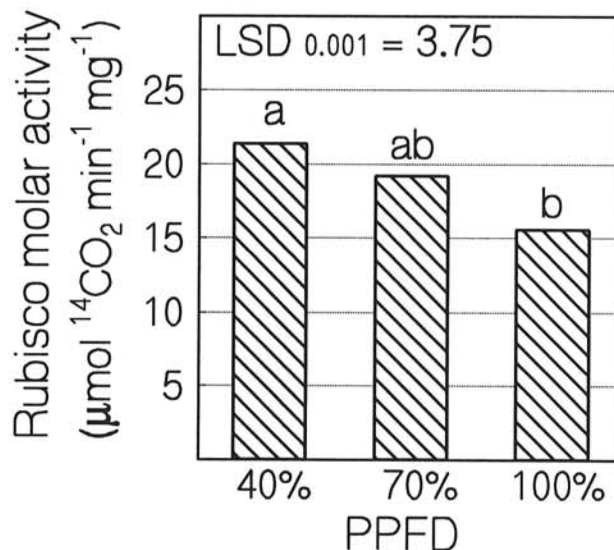

Fig. 9. Main effect of photosynthetic photon flux density (PPFD) on the molar activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in resistant alfalfa. Molar activity is the ratio of in vitro total Rubisco activity to the amount of Rubisco and is an indication of the concentration of tight-binding inhibitors of Rubisco activity. The higher molar activity under 40% PPFD indicates less inhibition of Rubisco at that PPFD level. Data from two experiments were pooled. Bars with the same letter are not significantly different at $P = 0.001$.

Not all physiological properties related to carbon assimilation in the resistant, infected alfalfa plants under 40% PPFD in our study mimicked the responses of susceptible plants. As previously reported, net photosynthesis and stomatal conductance were not affected by *V. albo-atrum* in the resistant alfalfa clone; however, the photosynthetic stress caused by the 40% PPFD treatment reduced net photosynthesis and stomatal conductance (17). The reductions in net photosynthesis and stomatal conductance, although not caused by the pathogen, were similar to those reported in susceptible alfalfa infected with *V. albo-atrum* (16). Thus, the susceptible response noted in resistant alfalfa grown under 40% PPFD was both phenotypically and physiologically similar to that of genetically susceptible plants.

The observation that net photosynthesis was not affected by the pathogen in resistant alfalfa regardless of PPFD treatment (17) is intriguing and implies that net photosynthesis may be intimately involved in resistance. Ultimately, the carboxylase enzyme, Rubisco, controls net photosynthesis (4,11,20); therefore, it may play a key role in regulating resistance to *V. albo-atrum* in alfalfa. Rubisco, a holoenzyme located in the chloroplast stroma, is composed of eight large and eight small subunits and has eight active sites, none of which is catalytically competent until activated by the binding of activator CO_2 and Mg^{++} (7,20,31). When carbamylation and Mg^{++} binding are complete, the activated enzyme is capable of binding RuBP, the sugar substrate. Once RuBP is bound to an activated catalytic site, CO_2 can react with the sugar to initiate the chemical reactions of carbon fixation.

Light modulates activation of Rubisco in vivo through two mechanisms, one that primarily controls light and dark activation changes and a second that implements the response of Rubisco to alterations in PPFD. The tight-binding inhibitor CA-1-P (2-carboxyarabinitol 1-phosphate), which is found in many plants, exerts direct control over the carboxylase enzyme (8). CA-1-P inactivates Rubisco in the dark by binding to catalytic sites on the holoenzyme and then frees those sites by its rapid degradation in the light. In addition to light and dark regulation by CA-1-P, Rubisco activity responds to variations in PPFD. Rubisco activity is inhibited in the light by the tight binding of RuBP to nonactivated catalytic sites (10,20,29). Most of the intermediate sugar phosphates of the Calvin cycle also can act as inhibitors of Rubisco activity by preventing carbamylation of the catalytic sites (10). Changes in Rubisco activation in response to fluctuations in PPFD are caused by the action of the small chloroplast protein, Rubisco activase (22). Rubisco activase, which requires ATP from the photochemical reactions of photosynthesis, catalyzes removal of tight-binding inhibitors such as RuBP and CA-1-P, thus freeing those catalytic sites for carbamylation and subsequent binding of RuBP and CO_2 to the now activated catalytic sites (7,10,20,29).

The increased in vitro total Rubisco activity that we noted in the inoculated, resistant plants in the absence of a corresponding increase in the amount of carboxylase enzyme may have been the result of 1) increased amounts of Rubisco activase, which would reduce the number of active sites blocked by tight-binding inhibitors (18) and/or 2) reductions in the regeneration rate of RuBP (10,20,25). Reduced RuBP regeneration would lower concentrations of the sugar and therefore lower the concentration of tight-binding inhibitors in leaf tissue (19,23,29). The rate of regeneration of RuBP was significantly reduced under 40% PPFD in all plants in this study and also was reduced under all PPFD treatments by the presence of *V. albo-atrum*. The 40% PPFD treatment reduced the photons available for the ATP production necessary for RuBP regeneration (19,23,30). Consequently, there would have been less RuBP inhibition of catalytic sites on Rubisco (29), resulting in the increased in vitro total Rubisco activity detected under 40% PPFD in all plants regardless of the presence of the pathogen.

In vitro total Rubisco activity was increased in plants inoculated with *V. albo-atrum* in this study, a phenomenon that also may have been caused by reduced enzyme inhibition. However, unlike the first situation, a simple reduction in the number of incoming photons will not explain why all the inoculated plants had increased in vitro total Rubisco activity. We did not measure chlorophyll content in the youngest, fully expanded leaves, but the increase in in vitro total activity coupled with the lack of a concomitant increase in Rubisco concentration and the small but significant reduction in RuBP regeneration rate that we detected in inoculated plants suggest that the Rubisco-chlorophyll ratio may have changed in *V. albo-atrum*-infected plants. The Rubisco-chlorophyll ratio reflects the number of photon-capturing chlorophyll molecules per unit of carboxylase enzyme (25). An increase in that ratio, in the absence of any change in Rubisco concentration, would indicate a reduction in the light-harvesting components of photosynthesis and a reduction in ATP generation. Plants acclimate to different PPFD levels through changes in their photosynthetic machinery (25), and a change in the Rubisco-chlorophyll ratio could be an indication of photosynthetic acclimation to the biotic stress imposed by *V. albo-atrum*.

An alternative explanation for the increased total in vitro activity of inoculated plants that we noted would be an increased concentration of Rubisco activase. We did not measure concentrations of Rubisco activase, but the molar activity of Rubisco was calculated and provides an indication of the concentration of tight-binding inhibitors (25). The higher the molar activity, the fewer the tight-binding inhibitors of Rubisco activity. Although all parameters in this study were tested with the random linear additive model of ANOVA, which permits extrapolation to a broader population of plants (24), because the experiment × pathogen mean square was not significantly different from the residual mean square ($P = 0.29$), we also tested molar activity of Rubisco with the residual mean square. The molar activity of Rubisco was significantly higher ($P = 0.02$) in inoculated, resistant plants compared with noninoculated, resistant plants when the residual mean square was the error term. On this basis, we speculate that there may indeed be either fewer tight-binding inhibitors of Rubisco or a higher concentration of Rubisco activase in *V. albo-atrum*-inoculated, resistant plants. Resolution of this point will require further investigation.

Although in vitro total Rubisco activity was significantly higher in all inoculated plants in this study, the difference was not reflected in in vivo Rubisco activity. In vivo Rubisco activity, which is analogous to in vitro initial Rubisco activity, reflects the actual carbamylation level or catalytic competency of the enzyme, whereas in vitro total activity reflects the enzyme's potential carbamylation level (29). In vivo Rubisco activity was determined through A/Ci curve analysis and therefore was subject to the confounding problem of stomatal response to varying vapor pressure deficit during measurement. Efforts were made to control vapor pressure deficit during the measurements by removing the heat from the light system with circulating cold water. These efforts were successful for noninoculated leaves; however, the intrinsic limitations of the LI-6200 became a factor in the measurement of inoculated leaves because of their smaller size. As a consequence of that small leaf size, we were occasionally unable to adequately reduce the flow rate through the desiccant, a procedure necessary for control of vapor pressure deficit in the leaf chamber, and that may have compromised our ability to detect subtle differences in in vivo Rubisco activity.

The significantly greater dark respiration in inoculated, resistant plants under 70% PPFD that we documented, coupled with no corresponding increase in net photosynthesis in the same plants (17), suggests that total photosynthesis (net photosynthesis + dark respiration + photorespiration) may be greater in those plants. Such a possibility stands in stark contrast to the significant reductions in net photosynthesis previously reported for susceptible potato, cotton, and alfalfa infected with *Verticillium* spp. (2,5,6,16) and provides indirect evidence that resistant alfalfa plants challenged with *V. albo-atrum* may have a mechanism that protects and/or enhances photosynthetic capabilities. Similarly, the increase in in vitro total Rubisco activity and the decrease in RuBP regeneration rate in inoculated, resistant alfalfa that we detected contrast with the decrease in in vitro total Rubisco activity reported for infected, susceptible alfalfa plants (16) and suggest

that photosynthetic acclimation may be optimizing carbon assimilation in alfalfa resistant to *V. albo-atrum*.

LITERATURE CITED

1. Berghaus, R., and Reisener, H. J. 1985. Changes in photosynthesis of wheat plants infected with wheat stem rust (*Puccinia graminis* f. sp. *tritici*). Phytopathol. Z. 112:165-172.
2. Bowden, R. L., Rouse, D. I., and Sharkey, T. D. 1990. Mechanism of photosynthesis decrease by *Verticillium dahliae* in potato. Plant Physiol. 94:1048-1055.
3. Duniway, J. M., and Slatyer, 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.
4. Farquhar, G. D., von Caemmerer, S., and Berry, J. A. 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta 149:78-90.
5. Hampton, R. E., Wullschleger, S. D., and Oosterhuis, D. M. 1990. Impact of Verticillium wilt on net photosynthesis, respiration and photorespiration in field-grown cotton (*Gossypium hirsutum* L.). Physiol. Mol. Plant Pathol. 37:271-280.
6. Haverkort, A. J., Rouse, D. I., and Turkensteen, L. J. 1990. The influence of *Verticillium dahliae* and drought on potato crop growth. 1. Effects on gas exchange and stomatal behavior of individual leaves and crop canopies. Neth. J. Plant Pathol. 96:273-289.
7. Jensen, R. G., and Zhu, G. 1992. Rubisco fallover and negative cooperativity of substrate binding. Pages 617-620 in: Research in Photosynthesis, vol. 2. N. Murata, ed. Kluwer Academic Publishers, Dordrecht, the Netherlands.
8. Kobza, J., and Seemann, J. R. 1989. Mechanisms for light-dependent regulation of ribulose-1,5-bisphosphate carboxylase activity and photosynthesis in intact leaves. Proc. Natl. Acad. Sci. USA 85:3815-3819.
9. Lee, S., Nazar, R. N., Powell, D. A., and Robb, J. 1992. Reduced PAL gene suppression in *Verticillium*-infected resistant tomatoes. Plant Mol. Biol. 18:345-352.
10. Lilley, R. McC., and Portis, A. R., Jr. 1990. Activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) by Rubisco activase—Effects of some sugar phosphates. Plant Physiol. 94:245-250.
11. Long, S. P. 1985. Leaf gas exchange. Pages 453-499 in: Photosynthetic Mechanisms and the Environment. J. Barber and N. R. Baker, eds. Elsevier, New York.
12. Murray, D. C., and Walters, D. R. 1992. Increased photosynthesis and resistance to rust infection in upper, uninfected leaves of rusted broad bean (*Vicia faba* L.) New Phytol. 120:235-242.
13. Newcombe, G., and Robb, J. 1988. The function and relative importance of the vascular coating response in highly resistant, moderately resistant and susceptible alfalfa infected by *Verticillium albo-atrum*. Physiol. Mol. Plant Pathol. 33:47-58.
14. Pearcy, R. W., and Seeman, J. R. 1990. Photosynthetic induction state of leaves in a soybean canopy in relation to light regulation of Ribulose-1-5-bisphosphate carboxylase and stomatal conductance. Plant Physiol. 94:628-633.
15. Pegg, G. F. 1985. Life in a black hole—The micro-environment of the vascular pathogen. Trans. Br. Mycol. Soc. 85:1-20.
16. Pennypacker, B. W., Knievel, D. P., Leath, K. T., Pell, E. J., and Hill, R. R., Jr. 1990. Analysis of photosynthesis in resistant and susceptible alfalfa clones infected with *Verticillium albo-atrum*. Phytopathology 80:1300-1306.
17. Pennypacker, B. W., Knievel, D. P., Risius, M. L., and Leath, K. T. 1994. Photosynthetic photon flux density × pathogen interaction in growth of alfalfa infected with *Verticillium albo-atrum*. Phytopathology 84:1350-1358.
18. Pennypacker, B. W., and Leath, K. T. 1993. Anatomical response of resistant alfalfa infected with *Verticillium albo-atrum*. Phytopathology 83:80-85.
19. Perchorowicz, J. T., Raynes, D. A., and Jensen, R. G. 1981. Light limitation of photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings. Proc. Natl. Acad. Sci. USA 78:2985-2989.
20. Portis, A. R., Jr. 1992. Regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:415-437.
21. Sage, R. F., Sharkey, T. D., and Seemann, J. R. 1988. The in-vivo response of the ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated CO_2 in *Phaseolus vulgaris* L. Planta 174:164-171.
22. Salvucci, M. E. 1989. Regulation of Rubisco activity in vivo. Physiol. Plant. 77:164-171.
23. Sassenrath-Cole, G. F., and Pearcy, R. W., 1992. The role of ribulose-1,5-bisphosphate regeneration in the induction requirement of photosynthetic CO_2 exchange under transient light conditions. Plant Physiol. 99:227-234.
24. Sawada, S., Usuda, H., Hasegawa, Y., and Tsukui, T. 1990. Regulation of ribulose-1,5-bisphosphate carboxylase activity in response to changes in the source/sink balance in single-rooted soybean leaves: The role of inorganic orthophosphate in activation of the enzyme. Plant Cell Physiol. 31:697-704.
25. Seeman, J. R., 1989. Light adaptation/acclimation of photosynthesis and the regulation of ribulose-1,5-bisphosphate carboxylase activity in sun and shade plants. Plant Physiol. 91:379-386.
26. Sharkey, T. D., Savitch, L. V., and Butz, N. D. 1991. Photometric method for routine determination of kcat and carbamylation of Rubisco. Photosynth. Res. 28:41-48.
27. Steel, R. G. D., and Torrie, J. H. 1980. Analysis of variance I: The one-way classification. Pages 149-153 in: Principles and Procedures of Statistics. McGraw-Hill, New York.
28. von Caemmerer, S., and Farquhar, G. D. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153:376-387.
29. Wang, Z. Y., and Portis, A. R., Jr. 1992. Dissociation of ribulose-1,5-bisphosphate bound to ribulose-1,5-bisphosphate carboxylase/oxygenase and its enhancement by ribulose-1,5-bisphosphate carboxylase/oxygenase activase-mediated hydrolysis of ATP. Plant Physiol. 99:1348-1353.
30. Woodrow, I. E., and Berry, J. A. 1988. Enzymatic regulation of photosynthetic CO_2 fixation in C_3 plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39:533-594.
31. Wullschleger, S. D. 1993. Biochemical limitations to carbon assimilation in C3 plants—A retrospective analysis of the A/Ci curves from 109 species. J. Exp. Bot. 44:907-920.