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Nitrogen Stress and Apparent Photosynthesis in Symbiotically Grown *Pisum sativum* L.[1]

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ABSTRACT

Pea plants (*Pisum sativum* L. cv. Alaska) were inoculated individually with one of 15 *Rhizobium leguminosarum* strains and grown under uniform environmental conditions in the absence of combined N. Differences in effectiveness of the *Rhizobium* strains produced plants with differing rates of whole plant apparent N_2 fixation and total N content at the same morphological stage of development. Plants were analyzed to determine interactions between N_2 fixation, N allocation, apparent photosynthesis, and growth. Total leaf N increased linearly with total N_2 fixation (R^2 = 0.994). The proportion of total N allocated to leaves, the per cent N content of individual leaves, and the photosynthetic efficiency of individual leaves showed a curvilinear response with increasing plant N content. Differences in allocation patterns of leaf N between plants with low and high N content resulted in differences in the relationship between total N content and plant dry weight. Results from this study show that N_2 fixation interacts with leaf photosynthetic efficiency and plant growth in a manner that is dependent upon the allocation of symbiotically fixed N.

The interdependence of N_2 fixation, photosynthesis, and growth in legumes has been substantiated by several investigators (*e.g.* 9, 12, 19, 21). However, detailed relationships between those three processes are not well understood. In some cases, environmental changes that can increase photosynthesis promoted N_2 fixation and plant growth (4, 9). In other experiments different rates of apparent photosynthesis were measured in peas when N_2 inputs were varied by providing separate strains of *Rhizobium leguminosarum* (2).

When developing legumes obtain reduced N only from the cotyledons or from N_2 through the *Rhizobium* symbiosis, seedlings go through a period of N stress (15, 21) which occurs as seed N reserves are depleted and before N_2 fixation is sufficient to supply the N demands for growth. Although there have been many studies concerned with the growth responses of legumes to combined N (*e.g.* 15, 18), little is known about the allocation of symbiotically fixed N and the functional responses to fixed N during this period. Most research on N allocation in legumes has been concerned with the allocation of seed N either during germination (8, 13) or during the later stages of plant growth (18).

The simple question of how a symbiotically dependent legume allocates N between the conflicting demands for constructing a photosynthetic apparatus and the requirement for building a root system where nodules can be formed is unanswered. The more complex question of how plant growth rates change at different levels of N sufficiency in the root and the leaf has not been considered. In this study, N allocation patterns were examined in Alaska peas (*Pisum sativum* L.) inoculated individually with one of 15 *R. leguminosarum* strains that differed in symbiotic effectiveness. The purpose was to determine the pattern of N allocation in plants that had differing rates of symbiotic N_2 fixation and to understand how those patterns of N allocation relate to photosynthesis and growth.

MATERIALS AND METHODS

Plants. Alaska peas (*P. sativum* L.) were grown in a controlled environment chamber under a 14/10 h light/dark cycle at 21/20 C, 50% RH, and a photosynthetic photon flux density of 700 $\mu E \cdot m^{-2} \cdot s^{-1}$. Plants were grown individually in modified "Leonard jar" assemblies (14) consisting of bottomless, 750-ml wine bottles inverted into 1-liter glass jars. The necks of the bottles were plugged with two-holed rubber stoppers and filled with vermiculite and a 1-cm-thick surface layer of dry perlite. The jar acted as a nutrient solution reservoir and initially contained 750 ml of N-free nutrient solution. The nutrient solution contained 2 mM $CaSO_4$, 1 mM K_2SO_4, 1 mM K_2HPO_4, 2 mM $MgSO_4$, 4 μM $CoCl_2$, 1 ml micronutrient solution/l after Johnson *et al.* (11), and 18.7 mg/l Sequestrene 138Fe iron chelate (courtesy of Ciba-Geigy). The solution was adjusted to pH 7.0 and changed less than 0.5 pH unit during the course of each experiment. The complete Leonard jar assemblies were autoclaved before planting. Pea seeds weighing 0.21 to 0.23 g and containing 7.72 ± 0.14 mg N were surface-sterilized with 70% ethanol, rinsed, and germinated on sterile paper towels with distilled H_2O. Three days after seed imbibition seedlings were selected for uniformity, planted in the Leonard jar assemblies, and inoculated with specific *Rhizobium* strains.

Bacteria. Eight plants were inoculated with each strain of *R. leguminosarum.* Strains 92F1 and TA101 from previous studies (2) and 13 strains (248, 300, 3622, 3711, 3713, 3718, 3737, 3738, 3740, 3745, 3747, 3758, and 16015) obtained from Dr. N. J. Brewin (John Innes Institute, Colney Lane, Norwich, NR4 7UH, United Kingdom) were used. The symbiotic characteristics of 12 of those strains have been described elsewhere (7). One additional strain, 3758, used in this study is genetically similar to strain 3740 and also was obtained from N. J. Brewin.

Photosynthetic Measurements. Gas exchange measurements were made on an open system gas analysis apparatus (1). CO_2 exchange was measured with a Beckman model 315A IR gas analyzer. Water vapor concentrations were measured with a RH sensor (Vaisala HMP13). Flow rates were monitored with an electronic mass flow meter (Matheson model 8142). Leaf temperatures were measured with fine-wire iron-constantan thermocouples. Light was provided by 400 w metal arc lamps (Sylvania M400-BU-HOR). Calibration of the CO_2 analyzer at various CO_2 concentrations was accomplished with a precision mixing pump (H. Wösthoff O. H. G. type M201a-F).

The photosynthetic responses of the youngest, fully expanded pair of leaflets on four 23-day-old plants were measured by

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simultaneously inserting pairs of leaflets from two separate plants into the gas-exchange chamber. Initial measurements were made under saturating light conditions at 21 C and ambient CO_2 concentrations of 320 to 350 $\mu l \cdot l^{-1}$. After steady state CO_2 exchange was attained at this level the ambient CO_2 concentration was decreased in steps to approximately 250, 150, and 50 $\mu l \cdot l^{-1}$. Apparent photosynthesis, leaf conductance to water vapor, and intercellular CO_2 concentrations were calculated from measurements of CO_2 flux, water vapor flux, and leaf temperature measurements according to Jarvis (10). Mesophyll conductance was calculated from the slope of the linear portion of the line representing the effect of changing intercellular CO_2 concentrations on the rate of CO_2 uptake as described by Jarvis (10).

Apparent photosynthesis of the whole shoot of six 24-day-old plants inoculated with a specific strain of *R. leguminosarum* was measured on individual plants in a Plexiglas chamber illuminated from the top and sides. Temperature and irradiance were maintained at levels similar to those in the original environmental chamber. The CO_2 concentration was 320 $\mu l \cdot l^{-1}$.

Growth Parameters. After 25 days of growth the plants were harvested. Leaf area was measured with an electronic leaf area meter (LI-COR LI-3000). The dry weight of plant parts was taken after 48 h at 75 C. Nitrogen content of the various plant parts was determined by Kjeldahl analysis (6). Symbiotic N_2 fixation was calculated by subtracting initial seed N content from Kjeldahl N values of harvested plants. Changes in foliar N associated with symbiotic N_2 fixation were measured by subtracting the foliar N content of plants inoculated with ineffective (non-nodulating) strains of *R. leguminosarum*.

RESULTS

Nitrogen Effects on Plant Development. Plants obtaining differing amounts of N from symbiotic N_2 fixation in association with differing rhizobial strains showed no significant differences in developmental stage after 25 days of growth. All plants were flowering at the seventh node at the time of harvest. Differences in total leaf area measured reflected primarily changes in leaf size rather than leaf number.

Nitrogen Allocation in Whole Plant. Plants deriving varying amounts of N from N_2 fixation allocated different relative proportions of total N content to leaves, stems, and roots (Fig. 1). The relative amount of N in the root decreased with increased N_2 fixation (Fig. 1C), whereas the relative amount of N in leaves increased and approached a maximum with greater N_2 fixation (Fig. 1A). The relative amount of N allocated to the stem initially decreased with intermediate levels of N_2 fixation but was restored to nearly 25% of total plant N with the highest levels of N_2 fixed (Fig. 1B).

Allocation of Foliar Nitrogen. Although the relative amount of N allocated to leaves approached a maximum at the high levels of N_2 fixation (Fig. 1A), there was a linear increase in the absolute amount of N allocated to leaves with increased amounts of N_2 fixation in the 25-day-old pea plants (Fig. 2). Absolute foliar N was calculated as Δ foliar N by subtracting foliar N content of plants inoculated with ineffective *Rhizobium* bacteria from foliar N content of effectively nodulated plants. Initial increases in absolute foliar N had large effects on per cent foliar N content until the foliar N content approached a maximum at approximately 5% of the leaf dry weight (Fig. 3). There was an increasing curvilinear relationship between absolute foliar N and leaf dry weight (Fig. 4). Thus N added to the foliar portion of growing pea seedlings through symbiotic N_2 fixation had complementary effects on per cent leaf N content and leaf dry weight.

Relationships between Changes in Foliar N and Leaf Photosynthesis. Apparent photosynthesis on a leaf-area basis was highly correlated with per cent leaf N content on a dry weight basis (R^2 = 0.971). Both parameters increased rapidly with initial increases

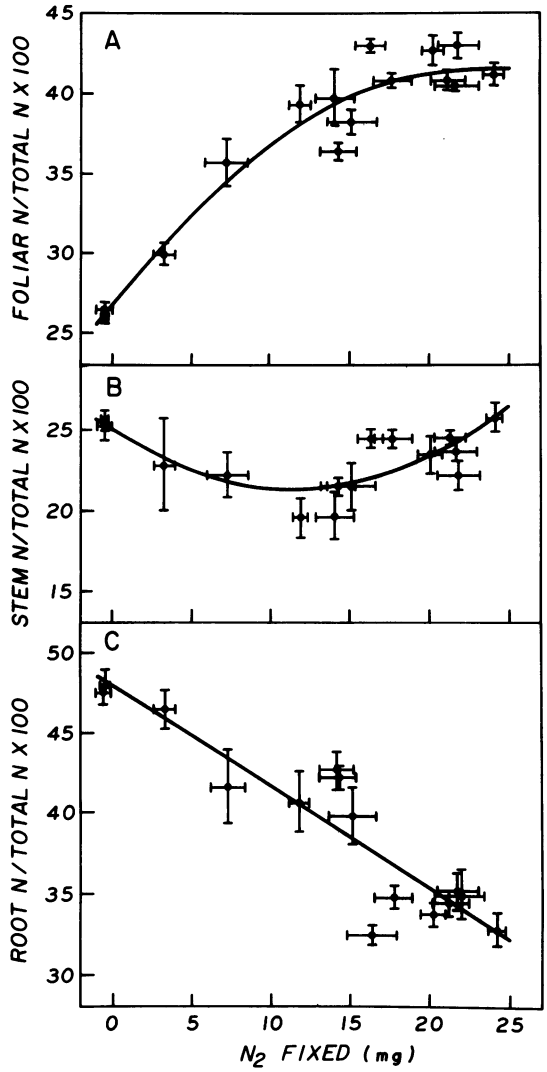

FIG. 1. Relationship between total symbiotic N_2 fixed and foliar, stem, and root N relative to total N in 25-day-old-pea seedlings inoculated with different strains of *Rhizobium*. Total N_2 fixed was calculated by subtracting initial seed N content from Kjeldahl N values of the harvested plants. Unless noted, mean ± SE values in all figures were computed from eight replicates. A: foliar N allocation. $Y = 26.8 + 1.28X - 0.028X^2$; $R^2 = 0.939$; B: stem N allocation. $Y = 25.1 - 0.646X + 0.028X^2$; $R^2 = 0.601$; C: root N allocation. $Y = 47.9 - 0.628X$; $R^2 = 0.845$.

in foliar N but approached a maximum at higher levels of foliar N (Figs. 3 and 5A). Increases in apparent photosynthesis were accompanied by greater mesophyll conductance to CO_2 and leaf conductance to water vapor (Fig. 5, B and C). There was a small decrease in internal CO_2 concentration with increased foliar N levels (Fig. 5D), even though leaf conductances increased at higher levels of foliar N (Fig. 5C).

Relationships between N_2 Fixation, Whole Plant Photosynthesis, and Growth. Whole plant apparent photosynthesis increased curvilinearly with increasing amounts of N_2 fixed (Fig. 6). That response was similar to the relationship between leaf area and N_2 fixation (Fig. 7). Thus, although apparent photosynthesis on a leaf-area basis approached a maximum in plants with higher N content (Fig. 5A), whole plant apparent photosynthesis continued to increase as leaf area increased (R^2 = 0.994).

Plant growth expressed as a change in dry weight relative to that of plants inoculated with ineffective (non-nodulating) strains of *R. leguminosarum* increased curvilinearly with greater N_2 fixa-

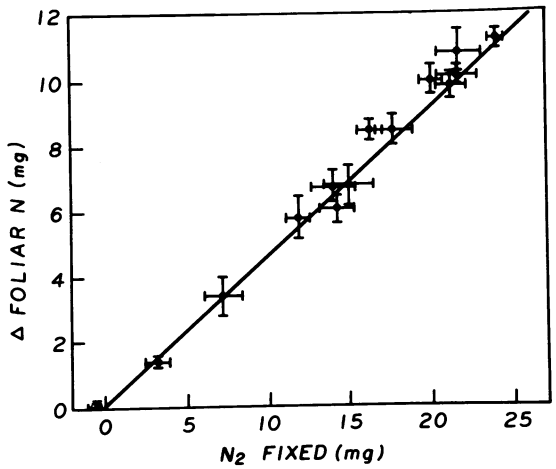

FIG. 2. Relationship between total symbiotic N_2 fixed and the absolute change in foliar N of plants inoculated with different strains of *Rhizobium*. The change in foliar N due to N_2 fixation was calculated by subtracting the foliar N of plants inoculated with ineffective (non-nodulating) *Rhizobium* strains in Figures 2, 3, 4, and 5. $Y = 0.078 + 0.472X$; $R^2 = 0.991$.

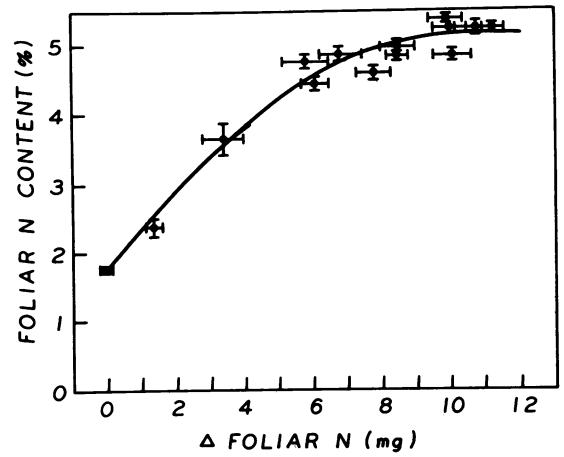

FIG. 3. Relationship between the absolute change in foliar N and relative foliar N content on a dry weight basis of plants inoculated with different strains of *Rhizobium*. $Y = 1.74 + 0.649X - 0.030X^2$; $R^2 = 0.983$.

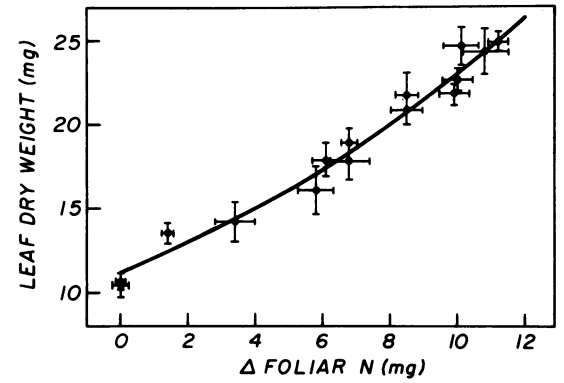

FIG. 4. Relationship between the absolute change in foliar N and leaf dry weight of plants inoculated with different strains of *Rhizobium*. $Y = 11.1 + 0.836X + 0.037X^2$; $R^2 = 0.977$.

tion (Fig. 8). The strongly curvilinear response shows that small changes in N content of the more N-sufficient plants can have large effects on plant dry weight.

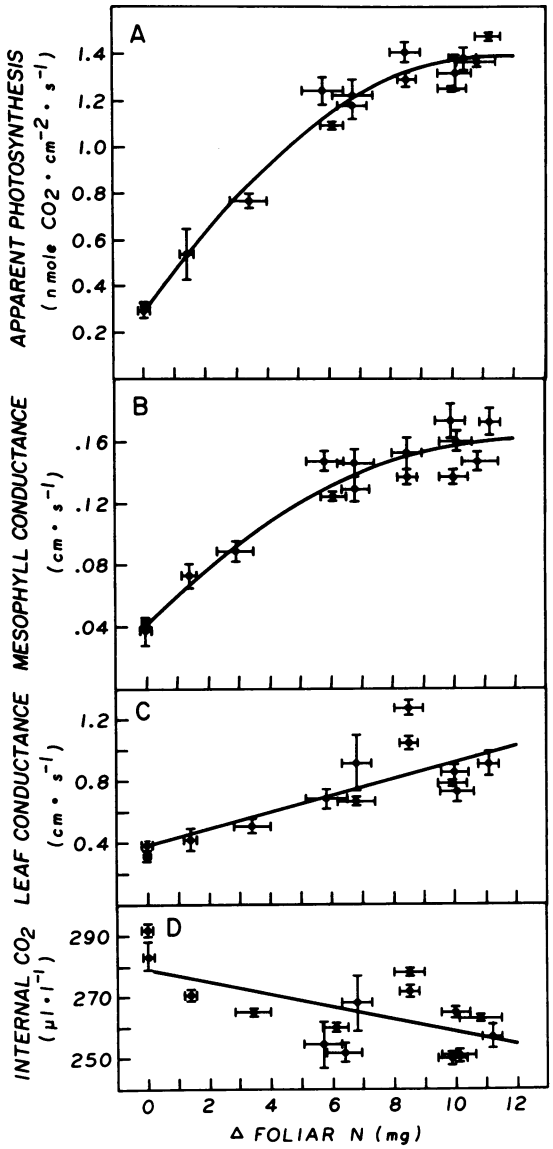

FIG. 5. Relationship between the absolute change in foliar N and apparent photosynthesis, mesophyll conductance, leaf conductance, or internal CO_2 concentration of young, fully expanded leaflets of plants inoculated with different strains of *Rhizobium*. Photosynthetic values were derived from measurements on four plants; other parameters were measured on eight replicate plants. A: apparent photosynthesis. $Y = 0.287 + 0.193X - 0.0084X^2$; $R^2 = 0.973$; B: mesophyll conductance. $Y = 0.0422 + 0.0196X - 0.0008X^2$; $R^2 = 0.930$; C: leaf conductance. $Y = 0.379 + 0.541X$; $R^2 = 0.640$; D: internal CO_2 concentration. $Y = 279.0 - 2.06X$; $R^2 = 0.414$.

DISCUSSION

Interpreting data from this study depends on an understanding of the N sources available to the pea seedlings. As the seedlings were grown with a nutrient solution lacking combined N, they were entirely dependent on seed N and symbiotically fixed N for growth. Bethlenfalvay and Phillips (3) reported that N_2 fixation did not begin until peas were more than 15 days old. Thus, under the present experimental conditions, plants probably were entirely dependent on seed N for the first 2 weeks of growth. After the initial period of dependence on seed N, N_2 fixation developed gradually until the day of harvest. The differences in pea seedlings inoculated with different strains of *Rhizobium* primarily reflect changes that occurred in the last 10 days of plant growth. The

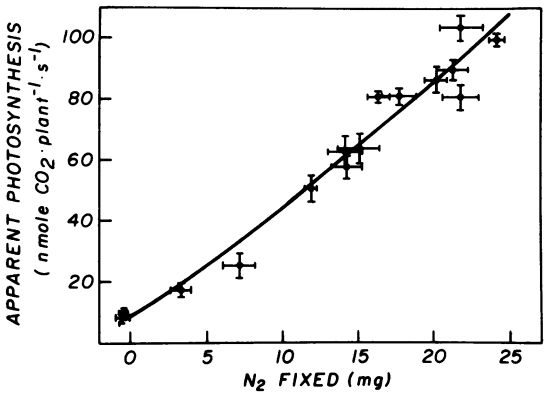

FIG. 6. Relationship between total symbiotic N_2 fixed and whole plant apparent photosynthesis in 25-day-old pea seedlings inoculated with different strains of *Rhizobium*. Photosynthetic values were derived from six replicates; N_2 fixation was measured on eight replicate plants. $Y = 8.40 + 3.38X + 0.0236X^2$; $R^2 = 0.961$.

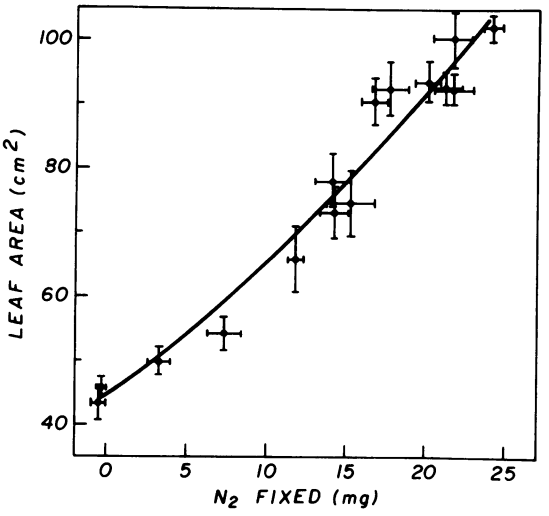

FIG. 7. Relationship between total symbiotic N_2 fixed and plant leaf area of 25-day-old pea seedlings inoculated with different strains of *Rhizobium*. $Y = 44.0 + 1.87X + 0.0264X^2$; $R^2 = 0.959$.

comparisons between plants inoculated with non-nodulating strains of *Rhizobium* and strains capable of different levels of N_2 fixation are an indication of how the additional N derived from N_2 fixation was allocated within the seedlings and the effects that the additional N had on plant function and growth. The effects of differing levels of added N on seedling function and growth indicate that N was a primary limiting factor during the period of seedling development. Because uniform plant material and environmental conditions were used, N availability through N_2 fixation, rather than CO_2 fixation, can be considered the independent variable.

The fact that all plants in this study exhibited a similar stage of morphological development is consistent with previous observations that variations in nitrogen nutrition do not affect flowering in intact, early pea varieties (16).

A deficiency in available mineral nutrients is associated with a greater root to shoot biomass ratio, and increasing mineral availability enhances shoot growth relative to root growth (5). The distribution of relative N content in organs of pea plants containing different levels of symbiotically fixed N (Fig. 1) is consistent with that concept, but physiological explanations of these observations may differ. Brouwer (5) proposed that since minerals are taken up by the root, under mineral-deficient conditions, the

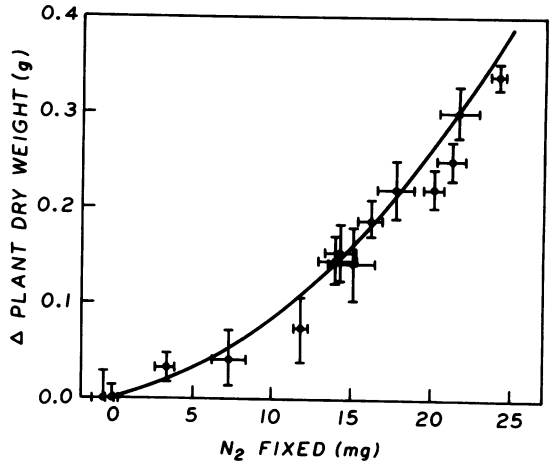

FIG. 8. Relationship between total symbiotic N_2 fixed and the absolute increase in plant dry weight of seedlings inoculated with different effective strains of *Rhizobium*. The absolute change in dry weight due to N_2 fixation was calculated by subtracting the mean dry weight of plants inoculated with ineffective (non-nodulating) strains of *Rhizobium*. $Y = 0.0037 + 0.0028X + 0.0005X^2$; $R^2 = 0.979$.

minerals will be used in the root before they can reach the shoot. In the present experiments, the only source of N available to the non-nodulated pea plants was in the seed. It would seem that such cotyledonary N should be equally available to both the shoot and the root, if the priorities for N were equal. Data in Figure 1C, however, show that roots have a high priority for the utilization of seed N in the N-deficient peas. The linear relationship between absolute changes in foliar N and total N_2 fixed over the growing period (Fig. 2) indicates that a constant percentage of the total N_2 fixed was allocated to the leaves. The apparent maximum in percentage of total N in the leaves (Fig. 1A) probably indicates that such plants were approaching an equilibrium with respect to relative N distribution in the whole plant.

The response of apparent photosynthesis to changes in foliar N (Fig. 5) is consistent with reports on the effect of N nutrition on photosynthesis in other crop species (17). The reason photosynthesis decreases under N-deficient conditions is not clear. Some workers have reported changes in mesophyll conductance and/or stomatal conductance in response to changes in plant nitrogen status (17). The data reported in this study agree with those previously reported for both mesophyll conductance and leaf conductance (Fig. 5, B and C). However, the internal CO_2 concentrations in leaves were similar or slightly higher in the more N-deficient plants although leaf conductance was lower (Fig. 5, C and D). If the decrease in leaf conductance associated with N stress (Fig. 5C) actually caused the decrease in apparent photosynthesis, one should find decreases in internal CO_2 concentration, rather than the relationship shown in Figure 5D. Raschke (20) and Wong *et al.* (22) proposed that stomatal aperture is related to the capacity of the mesophyll tissue to fix carbon. In their model, stomata act to regulate internal CO_2 concentrations when the capacity of the mesophyll tissue to fix CO_2 is altered by various means. The data presented in this paper are consistent with that model. It is apparent that leaf N status affected mesophyll conductance. If the model is correct, the differences in the leaf conductance can be interpreted as a response to the differences in the capacity of the mesophyll tissue to fix CO_2 at the different levels of foliar N.

The similarities between functions relating changes in foliar N content to per cent foliar N content, apparent photosynthesis, and mesophyll conductance (Figs. 3, 5, A and B) suggest that the differences in leaf photosynthetic activity are associated with changes in leaf N content. These data support previous work (17)

and are consistent with Nátr's general concept (17) that N affects photosynthesis by altering the levels and activity of ribulose bisphosphate carboxylase.

Although leaf photosynthesis approached a maximum in plants that attained high levels of N_2 fixation (Fig. 5A), whole plant apparent photosynthesis tended to increase with each increment in N_2 fixed (Fig. 6). Such results apparently were achieved by balancing N allocation to enhance both the quality (Fig. 3) and quantity (Fig. 4) of leaf tissue while continually increasing the total leaf area with each additional amount of N_2 fixed (Fig. 7). These data re-emphasize the importance of considering the intact plant canopy when studying the relationships between N_2 fixation or N nutrition and photosynthesis.

The relationship between the change in plant dry weight and N_2 fixation (Fig. 8) results from the integrative consequences of the N allocation between and within various plant parts. Thus, it is difficult to ascribe the curvilinear nature of the change in dry weight to any particular aspect of distribution of N described in this study. The plants that responded the least to increases in total N_2 fixation were the more N-deficient plants which had not achieved the maximum per cent foliar N content. Those plants may have been allocating a greater proportion of fixed N to increasing N content of existing tissue rather than to making new tissue. More detailed studies relating actual growth rates to N_2 fixation are needed to clarify such relationships.

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