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Partitioning of Nitrogen among Ribulose-1,5-bisphosphate Carboxylase/Oxygenase, Phosphoenolpyruvate Carboxylase, and Pyruvate Orthophosphate Dikinase as Related to Biomass Productivity in Maize Seedlings[1]

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ABSTRACT

Maize (*Zea mays* L. cv Golden Cross Bantam T51) seedlings were grown under full sunlight or 50% sunlight in a temperature-controlled glasshouse at the temperatures of near optimum (30/25°C) and suboptimum (17/13°C) with seven levels of nitrate-N (0.4 to 12 millimolars). The contents of phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPD), and ribulose-1,5-P_2 carboxylase/oxygenase (RuBisCO) were immunochemically determined for each treatment with rabbit antibodies raised against the respective maize leaf proteins (anti-PEPC and anti-PPD) or spinach leaf protein (anti-RuBisCO). The content of each enzymic protein increased with increasing N and raised under reduced temperature. The positive effect of light intensity on their contents was evident only at near optimal temperature. The relative increase in PEPC and PPD content with increasing N was significantly greater than that of RuBisCO irrespective of growth conditions. These enzymic proteins comprised about 8, 6, and 35% of total soluble protein, respectively, at near optimal growth condition. In contrast to significant increase in the proportion of soluble protein allocated to PEPC and PPD seen under certain conditions, the proportion allocated to RuBisCO decreased reciprocally with an increased biomass yield by N supply.

These results indicated that the levels of PEPC and PPD parallel to maize biomass more tightly than that of RuBisCO at least under near optimal growth condition.

One of the characteristic differences between C_3 and C_4 species is their N use efficiency, defined as biomass production per unit of N in the plants. Brown (3) has put forward the hypothesis and supporting data that C_4 species utilize N more efficiently than C_3 species; this hypothesis also has been supported by Schmitt and Edwards (13) with maize (C_4) and wheat and rice (C_3) plants. The main difference in the N use efficiency of these species appears to be based on partitioning of N among leaf proteins and the related carbon assimilation pathway (3). In C_3 species the proportion of protein allocated to RuBisCO,[2] a major leaf protein, appears to rise with increased leaf protein levels (2, 4, 12), the amount correlating strongly with the rate of photosynthesis which itself largely responds to biomass produced. In contrast, C_4 species usually have a lower content of this RuBisCO (1, 9). In a previous study we reported no significant correlation of RuBisCO content to maize biomass induced by varying N level when the plants are grown at 25°C (day temperature) (16). In addition, tissue soluble protein allocated to RuBisCO was found to decrease with increasing N level, even though the increased N resulted in increased biomass production. We did observe, though, highly positive correlations of the activities of both PEPC and PPD with maize biomass production. These observations led us to the view that the levels of the enzymes in the CO_2 trapping system of C_4 photosynthesis may be more critical than that of RuBisCO as determinants to the biomass productivity of maize plants.

To examine the effect of growth condition on partitioning of N among major carbon-assimilating enzymes and associated

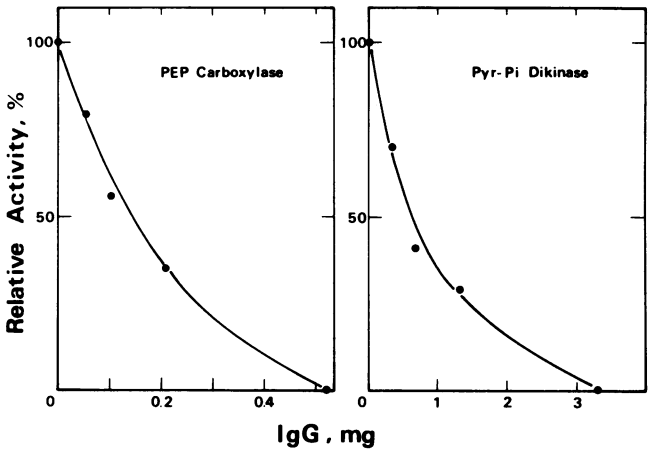

FIG. 1. Immunotitration of PEP carboxylase and pyruvate Pi dikinase activities in a crude extract of maize leaves. Constant amounts of crude extract (208 μg protein) of young leaves of 1-month-old plant were incubated for 3 h at 25°C with increasing amounts of either anti-PEPC, anti-PPD, or nonimmune IgGs in a total volume of 0.15 ml. After centrifugation of mixture at 14,000*g* for 5 min, resultant supernatants were assayed for PEPC and PPD. Relative activity was plotted taking the control with nonimmune IgG as 100.

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[2] Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; PEPC, phosphoenolpyruvate carboxylase; PPD, pyruvate orthophosphate dikinase; IgG, immunoglobulin G.

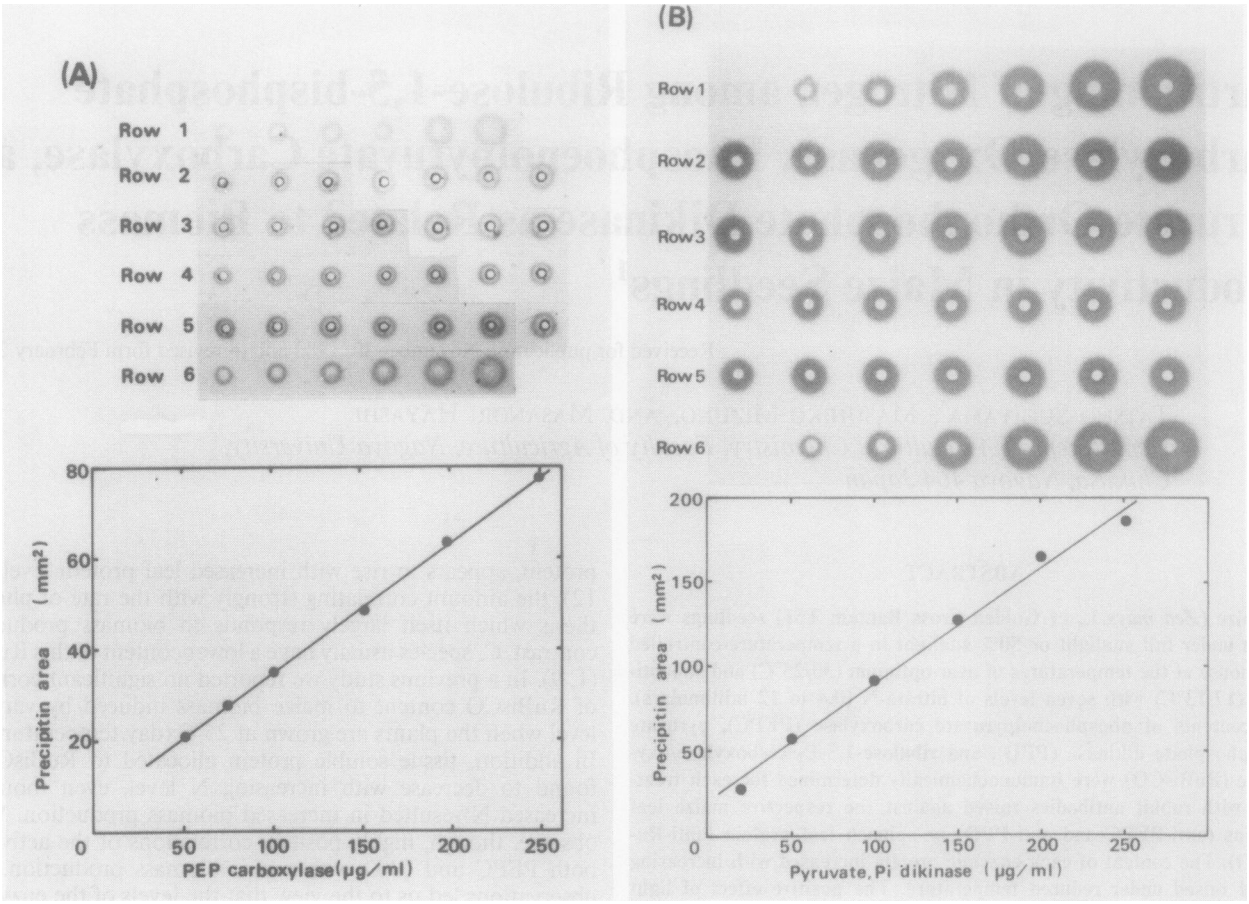

FIG. 2. Measurement of PEP carboxylase and pyruvate Pi dikinase contents by single radial immunodiffusion. A, PEP carboxylase. Standard wells (rows 1 and 6), from left to right, contained 5 μl of standard PEPC solutions containing 50, 75, 100, 150, 198, and 250 μg/ml. Gel plate (8 × 8.5 × 0.2 cm) contained 4.5 mg of anti-PEPC IgG. B, Pyruvate Pi dikinase. Standard wells (rows 1 and 6), from left to right, contained 5 μl of standard PPD solutions containing 20, 50, 100, 150, 200, and 251 μg/ml. Gel plate (8 × 9 × 0.2 cm) contained 0.57 ml of anti-PPD serum. Rows 5, 4, 3, and 2 in (A) and 2, 3, 4, and 5 in (B) are for the samples derived from biomass tissues obtained at 30/25°C, 100% sunlight, 30/25°C, 50% sunlight, 17/13°C, 100% sunlight, and 17/13°C, 50% sunlight, respectively. In each row wells (from left to right) contained 5 μl each of 4-fold diluted (PEPC) or nondiluted (PPD) extracts from plants grown with increasing nitrate supply from low (0.4 mM) to high (12 mM) levels.

biomass production, we grew maize plants at near optimal and suboptimal conditions and compared the biomass produced relative to N nutrition.

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays* L. cv Golden Cross Bantam T51) plants were grown in varying N concentrations (0.4, 0.8, 1.6, 3.2, 5.3, 8.0, and 12 mM as KNO_3) as described previously (16) in a temperature-controlled glasshouse between September and October of 1982 at Chikusa, Nagoya. The day/night temperature cycles were either 30/25°C or 17/13°C. At each temperature regime the plants were divided into two groups with one of them being shaded with nylon net to reduce the incident light by about 50%. Growth periods for the plants grown at 30/25°C, 100% sunlight, 30/25°C, 50% sunlight, 17/13°C, 100% sunlight, and 17/13°C, 50% sunlight were 14, 18, 25, and 28 d, respectively. For all the experiments, after a randomly selected group of plants had been harvested, shoots above the laminar joint of the second leaf were used as sources for protein and enzyme analyses and designated as the biomass after drying at 75°C for 48 h.

Enzyme Extraction and Analytical Methods. Enzymes were extracted from 2 g of fresh tissue according to the method described previously (16). Eluates after gel filtration with Sephadex G-25 columns were assayed immediately for PEPC (16, 17). For assaying other components the eluates were stored at −80°C until use. PPD was assayed as previously described (15, 16). All enzymes were assayed at 25°C. One unit of activity was defined as the amount of enzyme catalyzing the release of 1 μmol of product per min under the specified conditions. TCA (10%, w/v) insoluble protein was determined (11) using BSA as a standard. N in dry matter was estimated by Kjeldahl analysis.

Preparation of Antibodies against PEPC and PPD. PEPC and PPD were purified from the mature young leaves of 1-month-old maize according to the methods reported previously (15, 17). Using these methods, PEPC and PPD were judged to be 92 and 89% pure, respectively, by SDS-polyacrylamide disc gel electrophoresis. These preparations were used as standards for immunochemical study. Further purification of these proteins for use as antigens was achieved by preparative SDS polyacrylamide gel electrophoresis using a total monomer concentration of 10% (10). Proteins were visualized by placing gels, after completion of electrophoresis, into 4 M sodium acetate (7). Gels containing the subunits of the respective enzymes were sliced and protein eluted by electrophoresis (14). SDS was removed from the eluted proteins by passage through a column of Dowex 1 × 8 which had been equilibrated with 6 M urea in 50 mM Tris-acetate, pH 7.8, and 10 mM 2-mercaptoethanol according to the method of Weber and Kuter (18). Resultant protein solution was concentrated utilizing membrane filter (Amicon Immersible CX-10)

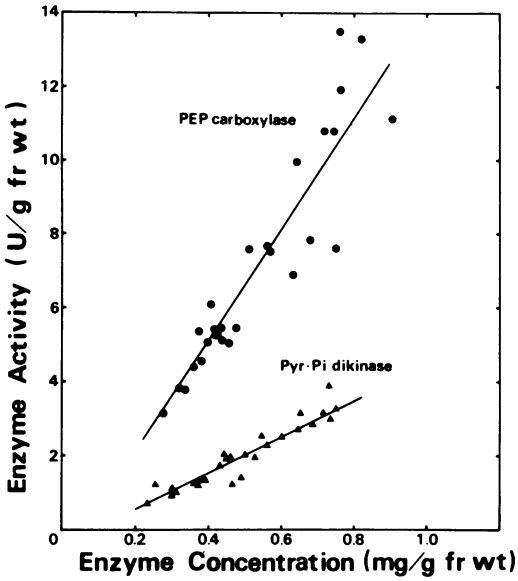

FIG. 3. Enzyme activities and concentrations of PEP carboxylase and pyruvate Pi dikinase proteins in extracts of various biomass tissues of maize.

and dialyzed for 1 h against 500 volumes of phosphate buffered saline to reduce the concentration of urea present. Antibodies to the purified SDS-polypeptides of PEPC and PPD were raised in New Zealand White rabbits after subcutaneous injection of the antigens with Freund complete adjuvant. IgG fractions in antisera were purified by precipitating with rivanol and $(NH_4)_2SO_4$ as described by Hurn and Chantler (8).

Single Radial Immunodiffusion. RuBisCO was determined as described previously (16) using rabbit IgG raised against spinach leaf RuBisCO, a kind gift of Dr. T. Akazawa. The final preparation of the enzyme which was purified from maize leaves according to the method described previously (19) after modification was judged as electrophoretically homogeneous and used as a standard for calibration. PEPC and PPD were determined similarly. Five μl of diluted purified maize leaf enzymes, or 2- to 4-fold dilutions of leaf extracts were placed in wells in gel plates and incubated for 50 to 72 h at 25°C to allow radial diffusion. After diffusion, gels were dried, protein stained by Coomassie Brilliant blue, and the area of precipitin rings measured after enlargement. For each experiment, values reported are means of at least duplicate.

RESULTS

Immunochemical Quantification of PEPC and PPD Proteins. Specific rabbit antibodies raised against the SDS-polypeptide fraction of each enzyme (as judged by Ouchterlony double immunodiffusion, data not shown) were used to quantitate PEPC and PPD proteins. Immunotitration of the enzymes with the corresponding antibody specifically inhibited enzyme activities in crude extracts (Fig. 1). Single radial immunodiffusion with the antibodies allowed the quantification of PEPC and PPD proteins by comparing the area of precipitin rings generated by the known amounts of pure antigens as shown in typical assay plates and derived calibration curves (Fig. 2). Some opacity was noted within the precipitin ring in PPD immunodiffusions. This result of the relatively less concentration of antibody compared to the concentration of antigens used. The values determined for extracts from several different plant leaves by this and conventional immunoprecipitin methods were comparable within experimental errors (data not shown).

The relationships between enzyme activities and the tissue concentration of these two proteins as determined by single radial immunodiffusion are depicted in Figure 3. Both PEPC and PPD showed highly significant correlations between the two parameters with average specific activities of 14 and 4 units per mg of immunochemically determined protein, respectively.

Effects of N, Temperature, and Light Intensity on RuBisCO, PEPC, and PPD Proteins and Biomass. Based on immunochemical measurements, we examined the effect of N nutrition on the contents of RuBisCO, PEPC, and PPD proteins in plants grown at near optimal and suboptimal conditions of temperature and irradiance. No significant N-deficiency symptoms were observed in biomass tissues used, although minimal chlorosis appeared at harvest in the oldest leaves of plants grown with the lowest two levels of N under high light and temperature conditions.

Figure 4 shows the effect of N level on the contents of total soluble protein and three enzymic proteins expressed on a fresh weight basis in the populations of biomass obtained under various environmental conditions. The content of soluble protein in tissues reached its maximum at nitrate levels higher than about 4 mM except the case of the growth condition of higher temperature/reduced sunlight where the content continued to increase with increasing nitrate up to 12 mM. Response of total

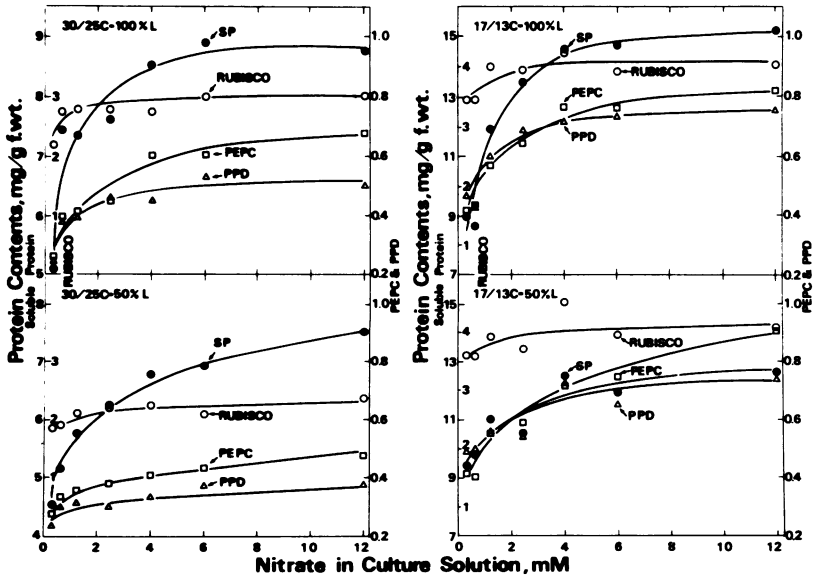

FIG. 4. The effects of nitrogen supply and growth temperature and light on the contents of total soluble protein, RuBP carboxylase/oxygenase, PEP carboxylase, and pyruvate Pi dikinase proteins in biomass tissues of maize. Each point is the average of duplicate analyses.

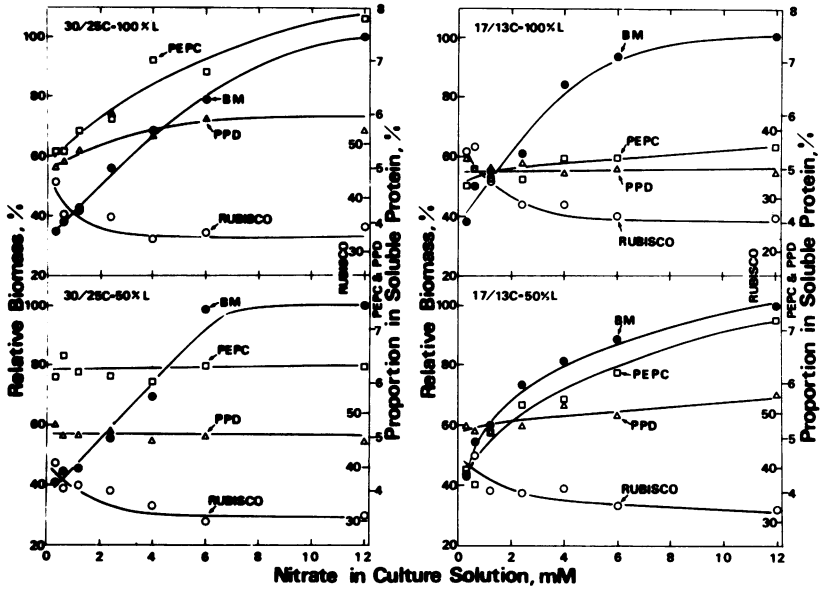

FIG. 5. The effects of nitrogen supply and growth temperature and light on biomass yield and the percentages of soluble protein allocated to RuBP carboxylase/oxygenase, PEP carboxylase, and pyruvate Pi dikinase in biomass tissues of maize. Biomass yield was plotted as percentages taking the maximum value at each growth condition as 100. The maximum values at the growth conditions of 30/25°C, 100% sunlight, 30/25°C, 50% sunlight, 17/13°C, 100% sunlight, and 17/13°C, 50% sunlight were 70.6, 96.3, 60.8, and 41.5 mg of dry matter/plant, respectively.

N content in tissues to N nutrition (data not shown) was similar to that of soluble protein. The content of each enzymic protein increased with increasing N with somewhat different responses seen among proteins. The relative increase in PEPC and PPD content with increasing N was greater than that for RuBisCO irrespective of growth conditions. The content of three enzymic proteins was higher in plants grown at lower temperature, as seen in the case of soluble protein. The effect of light intensity on the contents of these proteins and soluble protein was significant only in plants grown at higher temperature where the content of all the proteins raised with increased light intensity at all the N levels.

When the amount of three enzymic proteins was expressed on a soluble protein basis, the response of each protein to N nutrition varied depending on the growth condition (Fig. 5). Under near optimal temperature, the proportion of each protein in soluble protein was greater for the full sunlight treatment than for the reduced sunlight treatment except for the case of PEPC at low N levels. The proportion of PEPC at low N was less for the full sunlight treatment than for the reduced sunlight treatment, but at high N the proportions were reversed. Being consistent apparently with the greater proportion of these proteins, the N level required for saturating biomass shifted toward higher value. The PEPC and PPD proportions increased with increasing N at full sunlight with the maximum values of about 8 and 6%, respectively. At reduced sunlight the proportions were not affected significantly by N level despite the increasing biomass with the values of about 6.3 and 5% for PEPC and PPD, respectively.

Under suboptimal temperature, the situation exhibiting a greater proportion in three enzymic proteins was reversed with respect to light intensity: their proportions were greater for the reduced sunlight than for the full sunlight treatment. Similarly, a reversed situation was also observed in the level of N required for saturating biomass yield. The PEPC and PPD proportions increased with increasing N at reduced sunlight with the maximum values of about 7 and 6%, respectively, while at full sunlight the proportions were not much affected by N with nearly constant values of 5.0 to 5.5%.

In contrast to significant increase in PEPC and PPD proportions seen under certain growth conditions, the proportion of RuBisCO in soluble protein which was greatest among three enzymes decreased almost exponentially with increasing N at any growth conditions with its maximum value of 25 to 35% at high N. The data of Figures 4 and 5 collectively indicate that the level of PEPC and PPD in shoot tissues correlates to its biomass and soluble protein content more tightly than that of RuBisCO at least under the optimal growth condition.

DISCUSSION

A strong correlation between assayed enzyme activity and immunochemically determined enzyme mass was observed (Fig. 3). The specific activities calculated from this relationship largely agree with measured specific activities of the purified proteins (15, 17). A slightly lower specific activity in the case of PEPC is attributed to the presence of some interfering substance(s) present in crude extracts (17) previously seen during purification of the maize leaf enzyme. The lack of a zero intercept may be mainly due to statistical matter. In the case of PPD, however, the presence of a positive intercept on the enzyme concentration axis will be partly attributed to the presence of inactive form in the tissue extracts which would contribute to protein but not activity estimates. PPD activity is known to be under light/dark regulation (5) and, hence, subject to underestimation if not fully activated.

PEPC and PPD together accounted for about 14% of total soluble protein under the growth conditions of high N, light, and temperature (Fig. 5). The allocation of soluble protein to PEPC and PPD may vary somewhat with growth conditions, as suggested by activity measurements for both enzymes by Hatch *et al.* (6). In addition, unpublished observations from our laboratory suggest that PEPC and PPD account for 14 and 8% of total soluble protein in maize plants grown in midsummer under full sunlight. Thus, the proportion of total soluble protein allocated to these two enzymes plus RuBisCO in maize leaf appears to be similar to that allocated to RuBisCO alone in the leaves of C_3 species, as was hypothesized earlier (13).

The responses of enzyme amount and biomass yield to N fertilization in plants varied depending on its growth condition. From the viewpoint of partitioning of N into PEPC and PPD proteins, however, there was a similarity between plants grown under the conditions of two extremes (*i.e.* near optimal temperature/full sunlight and suboptimal temperature/reduced sunlight) and also between plants grown under the conditions where either temperature or light intensity is suboptimal (*i.e.* near optimal temperature/reduced sunlight and suboptimal temperature/full sunlight). Such similarities appear to be the result of counteraction of two environmental factors affecting the partitioning of N. At the latter set of growth conditions the proportion

of both enzymes in soluble protein was near constant despite the increasing N, whereas at the former growth conditions the proportion increased with increasing N although there was less parallelism between the two enzymes. The increase in percentage of RuBisCO (and concomitant decreased proportion of soluble protein allocated to PEPC and PPD) at a lower N supply seen at the latter set of growth conditions may indicate that RuBisCO formation has a higher priority than other leaf soluble proteins when N is limiting, thereby compensating the lower capacity of CO_2 trapping function due to the insufficient fulfillment of PEPC and PPD. Increasing N would lead to greater formation of PEPC and PPD and eventually result in the biomass increased. Among two enzymes, PEPC level may be more critical to biomass yield in maize than PPD as is seen from its greater increase in proportion to soluble protein with N supply compared to PPD under the optimal growth condition.

Regardless of growth condition, the decrease in soluble protein proportion allocated to RuBisCO (about 10–15%) is not fully accounted for by increase in the proportions allocated to PEPC and PPD. This decrease may be recovered in other proteins which presumably limit the biomass formation of plants. Nevertheless, the close relationships between maize biomass and the levels of PEPC and PPD raise the possibility that these enzymes might prove to be useful targets for increased productivity through increases in N use efficiency in maize. The parallels also underscore the significance of the CO_2 trapping mechanism to maize productivity.

Increased N nutrition usually causes some leaf anatomical changes. The association between effects of N nutrition on proportions of these proteins and reflection of the effects to leaf anatomy needs to be evaluated under various environmental conditions.

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