

RELATIONSHIP BETWEEN LEAF NITROGEN AND PHOTOSYNTHETIC RATE FOR THREE NAD-ME AND THREE NADP-ME C₄ GRASSES¹

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Theoretical considerations have suggested that there may be differences in photosynthetic nitrogen use efficiency (PNUE) among plants that use different biochemical variants of C₄ photosynthesis. To test this hypothesis we examined the leaf nitrogen content and photosynthetic rates of six grass species (three of C₄ subtype NAD-ME and three of C₄ subtype NADP-ME) grown over a wide range of nitrogen supply. While there were significant differences among the species in various traits, there were no consistent differences between the C₄ subtypes in either leaf nitrogen content at a given level of nitrogen supply or in the leaf nitrogen-photosynthesis relationship. We suggest that species-level variation in photosynthetic nitrogen use efficiency among C₄ species is large enough to mask any differences that may be due to C₄ subtype.

Key words: C₄ photosynthesis; grasses; leaf nitrogen; NAD-ME photosynthesis; NADP-ME photosynthesis; nitrogen use efficiency; photosynthetic nitrogen use efficiency; Poaceae.

Numerous studies have identified differences between C₃ and C₄ plants in nitrogen usage and photosynthetic nitrogen use efficiency (PNUE; photosynthesis per unit leaf nitrogen), which result from their different modes of carbon fixation (Brown, 1978; Bolton and Brown, 1980; Sage and Percy, 1987). The ability of C₄ plants to concentrate CO₂ at the sites of carboxylation allows them to attain higher photosynthetic rates for a given content of RUBISCO (ribulose biphosphate carboxylase) than C₃ plants, resulting in a higher photosynthetic nitrogen use efficiency (Ehleringer and Monson, 1993). The mechanism of C₄ photosynthesis, however, is not uniform among C₄ plants; there are three distinct biochemical pathways of C₄ photosynthesis, each performed by different C₄ species (Hatch, 1987). Previous research has suggested that the biochemical differences among these C₄ variants have implications for their nitrogen usage (Bowman, 1991), but very little work has been done comparing nitrogen use among C₄ species that use different C₄ pathways.

The three different pathways of C₄ photosynthesis differ in the mechanisms used to transport CO₂ to bundle sheath cells and to regenerate phosphoenolpyruvate (PEP). They are termed the NAD-ME, NADP-ME, and PCK pathways, after the principal bundle sheath decar-

boxylation enzyme used in each (NAD malic enzyme, NADP malic enzyme, and phosphoenolpyruvate carboxylase, respectively; Hatch, 1987). Of these, the NAD-ME and NADP-ME are the most common and have the widest taxonomic distribution, and we will confine our discussion to these two types.

Bowman (1991) points out that several of the differences between these pathways might have implications for their efficiency of nitrogen use. He notes that the NAD-ME pathway is more complex than the NADP-ME, involving a greater number of transformations of intermediates. This biochemical complexity in NAD-ME species imposes an extra nitrogen cost for the construction of the enzymes that mediate each reaction. In addition, the NAD-ME pathway requires a pool of nitrogen for the amination of OAA to aspartate, and of pyruvate to alanine. On the basis of these differences in carbon metabolism, Bowman (1991) hypothesized that NADP-ME species would have a higher rate of photosynthesis than NAD-ME species at a given leaf nitrogen concentration. If this is the case, NAD-ME species must either have lower photosynthetic rates than NADP-ME species, or they must have higher nitrogen concentrations in their photosynthetic organs than NADP-ME species.

There is some empirical evidence that NAD-ME and NADP-ME species differ in the nitrogen concentration of their photosynthetic tissues. Bowman (1991), comparing four NAD-ME and two NADP-ME members of the grass genus *Panicum*, found that at high levels of nitrogen availability the NAD-ME species had higher shoot nitrogen concentrations than the NADP-ME species. LeCain and Morgan (1998) found that leaf nitrogen concentration was higher in three NAD-ME grass species than in three NADP-ME grasses. These findings suggest that NAD-ME species may compensate for a lower PNUE with greater allocation of nitrogen to photosynthetic organs. However, no previous study has compared the relationship between leaf nitrogen concentration and photosynthesis among C₄ subtypes to see whether the biochemical differences among the pathways translate into the ex-

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pected differences in leaf-level physiological performance.

A lower photosynthetic return on nitrogen allocated to leaves for NAD-ME than NADP-ME photosynthesis might have consequences for the distribution of species that use the different C₄ subtypes across natural environmental gradients of nitrogen. Pons, Van der Werf, and Lambers (1994) found that C₃ monocot species with a low PNUE have low potential growth rates (the growth rate achievable under ideal growing conditions). Ecologically, low potential growth rates are associated with species found in nitrogen-poor habitats (Lambers and Poorter, 1992), and a low PNUE for NAD-ME species might similarly limit them to low-fertility habitats.

To explore possible differences in the photosynthetic response of C₄ subtypes to nitrogen, we compared the relationship between leaf nitrogen content and photosynthetic rate for three species of NAD-ME and three species of NADP-ME grass, grown over a wide range of nitrogen supply. In addition to the photosynthesis-leaf nitrogen relationship, we also compared the relationship between nitrogen availability (fertilizer N concentration) and leaf nitrogen concentration to test the robustness of previous research, which found higher nitrogen concentration in the photosynthetic organs of NAD-ME species.

MATERIALS AND METHODS

Experimental procedures—Seeds for the experiment were collected from populations in Suffolk County, New York, USA in the autumn of 1994. Collections were made from similarly situated sites within an area of ~2 km² to minimize differences among the populations in adaptations to climate. The six co-occurring species chosen were *Eleusine indica*, *Eragrostis ciliaris*, and *Panicum virgatum* with the NAD-ME subtype and *Schizachyrium scoparium*, *Digitaria ischaemum*, and *Setaria glauca* with the NADP-ME subtype (Hattersley, 1987; Hattersley and Watson, 1992).

Seeds were germinated in flats of sand and transferred to plastic pots containing 600 mL of washed sand (one plant per pot). Sand particle size was predominantly between 500 and 1400 μm. Plants were grown under natural light from 3 May to 7 August 1995 in the Life Sciences greenhouse at the State University of New York in Stony Brook, New York, USA (40°55'20" N, 73°07'50" W). Maximum photosynthetic photon flux density was ~1200 μmol photons·m⁻²·s⁻¹. Mean daily minimum and maximum temperatures were 20.8°C (SD = 3.3) and 33.0°C (SD = 4.2), respectively. Pots were watered to excess daily with a nutrient solution containing 2 mmol/L K₂PO₄, 1 mmol/L CaSO₄, 2 mmol/L MgSO₄, 40 μmol/L FeNa EDTA, 50 μmol/L KCl, 33 μmol/L H₃BO₃, 2 μmol/L MnSO₄, 2 μmol/L ZnSO₄, 0.5 μmol/L CuSO₄, and 0.5 μmol/L MoO₃. Nitrogen supply was varied as NH₄NO₃ at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 10, 20, and 40 mmol/L.

Gas exchange measurements were made with an LI-6400 gas exchange system (LI-COR, Lincoln, Nebraska, USA). Measurements were made on the newest fully expanded leaf on plants 7–14 wk after seedling emergence. One leaf was measured per plant. Between 16 and 18 plants were measured for each species. Light was supplied by the LI-6400's LED light source at a photosynthetic photon flux density of 1500 μmol photon·m⁻²·s⁻¹ (maximum irradiance at 670 nm). Air entering the leaf chamber was drawn from a buffered volume of ambient greenhouse air (occasionally the air stream was dried partially over desiccant to avoid condensation in the system). Intake CO₂ levels were ~370 ppm. After the leaf had acclimated to the chamber, as indicated by steady photosynthetic rates, measurements were made at 30°C, following which the temperature was raised to 37°C, the leaf allowed to re-acclimate, and photosynthesis and transpiration were measured again. Leaf

temperature was controlled by use of the LI-6400's Peltier thermo-electric heat exchangers and a hot-air gun. Measurements were made with leaf temperatures held within 0.5° of the target. Net photosynthetic rate (Ps) was calculated as per LI-COR (1995) by the LI-6400 software (Open 1.0). Immediately following the gas exchange measurements, leaf area was recorded with an LI-3000A area meter (LI-COR, Lincoln, Nebraska, USA) and the leaf was excised from the plant and dried at 65°C. Leaf nitrogen concentration was determined using a Carlo Erba (Milan, Italy) EA 1108 CHNS analyzer at the Analytical Laboratory Facility of the Marine Sciences Research Center, State University of New York at Stony Brook.

PNUE—There are a variety of ways to calculate PNUE, of which the simplest and most commonly used is the ratio of photosynthetic rate to leaf nitrogen concentration. An alternative approach, followed here, is to derive PNUE from the regression of photosynthesis on leaf nitrogen; PNUE is indicated by the height of the regression line at a given nitrogen concentration. This approach facilitates both comparison of PNUE at any given leaf nitrogen concentration and examination of how PNUE varies with leaf nitrogen (e.g., comparison of regression slopes). When discussing PNUE differences in our own results, it is in this sense, as indicating differences in the relative height of this regression; in discussing the results of others we assume the definition of PNUE used by the original authors of these studies.

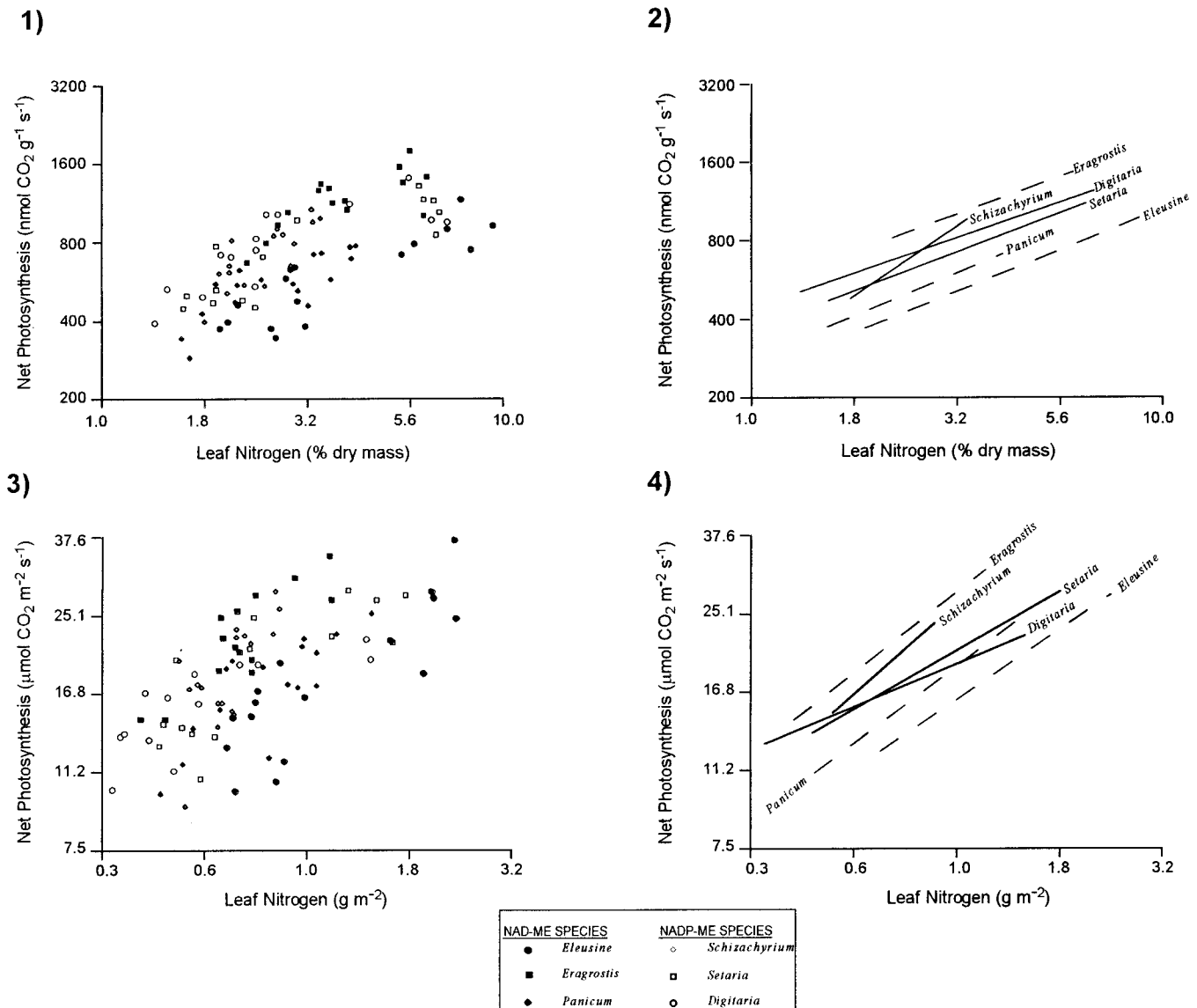
Statistical analyses—In this study, our interest was primarily in potential differences among species and C₄ subtypes in the relationships between pairs of measured variables. These included the relationships of leaf nitrogen concentration with photosynthetic rate and of fertilizer nitrogen with leaf nitrogen concentration. To test the significance of differences among the species and between the C₄ subtypes we examined these relationships with separate analysis of covariance (ANCOVA) models. For each model, the first variable of a pair listed above (e.g., leaf nitrogen concentration) was included as a covariate with the second (e.g., photosynthetic rate) as the dependent variable and C₄ subtype as a categorical factor. Each model also included species as a factor nested within C₄ subtype. As the covariates used in these analyses were measured with error, a Model II approach would have been strictly correct. However, in the absence of a well-developed Model II ANCOVA methodology (Sokal and Rohlf, 1995) we followed general custom (Goldberg and Scheiner, 1993) and used a Model I approach. Each regression was based on measurements for 16–18 plants.

Recent studies have suggested that the relationship between leaf nitrogen concentration and photosynthetic rate captures different aspects of the functional biology of leaves when these variables are expressed on a leaf area vs. a leaf mass basis (Reich and Walters, 1994; Reich, Walters, and Ellsworth, 1992). We therefore present data on both bases for comparison.

Leaf nitrogen concentration, photosynthetic rate, and nitrogen fertilizer concentration were all log transformed prior to significance testing to better meet the assumptions of the statistical tests used. These transformations led both to more normal distributions for these variables and to better linear fits between pairs of variables. Statistical analyses were performed using the linear model procedures (Fit Model routine) of the JMP 3.0.2 package for the Macintosh (SAS Institute, Cary, North Carolina, USA).

RESULTS AND DISCUSSION

The rate of photosynthesis obtained for a given leaf nitrogen concentration on a leaf-mass basis differed substantially among the species used in this study (Figs. 1, 2). With the exception of *Schizachyrium*, there is a clear ranking of the species in this regard across the entire observed range of leaf nitrogen concentrations (i.e., the regression lines do not cross), with *Eragrostis* > *Digi-*



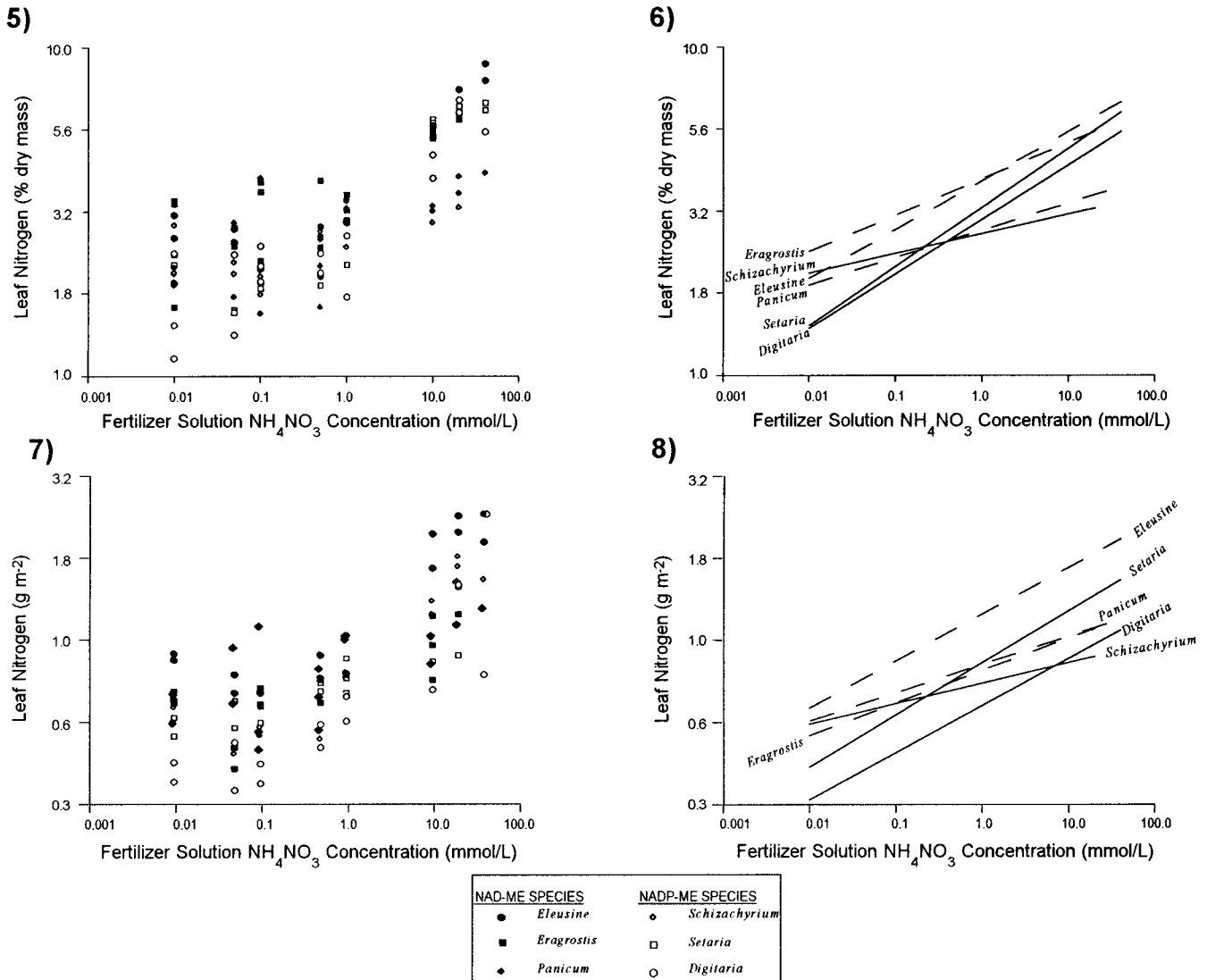
Figs. 1–4. Relationship between leaf nitrogen concentration and photosynthetic rate for three NAD-ME and three NADP-ME grass species. **1.** Individual leaves on a per-leaf mass basis. **2.** Linear regression lines for the data in Fig. 1 for each species. **3.** Individual leaves on a per-leaf area basis. **4.** Linear regression lines for the data in Fig. 3 for each species. NAD-ME species are indicated by filled symbols and dashed lines and NADP-ME species by open symbols and solid lines. Each regression line is shown only over the range observed. Regressions are shown separately from data for clarity (when depicted on the same graph with the data, the figure is too cluttered to be informative).

taria > *Setaria* > *Panicum* > *Eleusine* (Fig. 2). There is no clear pattern of difference between the NAD-ME and NADP-ME species. Two of the NAD-ME species, *Panicum* and *Eleusine* had the lowest PNUE of the six species, as hypothesized by Bowman (1991). However, the third NAD-ME species, *Eragrostis* had the highest rate of photosynthesis of any of the species at any given leaf nitrogen concentration.

These impressions are confirmed statistically. There were significant differences among the species in photosynthetic rates at a given leaf nitrogen concentration on a per-leaf mass basis (Figs. 1 and 2 for 30°C; data for 37°C not shown as relationships are essentially identical to those at 30°C; $df = 4,80$; $F = 26.8$; $P < 0.001$ at 30°C; $df = 4,69$; $F = 21.2$; $P < 0.001$ at 37°C). However,

the NAD-ME and NADP-ME species did not differ significantly in either the slope ($df = 1,4$; $F = 0.80$; $P > 0.25$ at 30°C; $df = 1,4$; $F = 0.52$; $P > 0.50$ at 37°C) or intercept ($df = 1,4$; $F = 0.50$; $P > 0.50$ at 30°C; $df = 1,4$; $F = 0.03$; $P > 0.75$ at 37°C) of the leaf nitrogen-photosynthesis relationship at either 30°C or 37°C (Fig. 2 for 30°C; data for 37°C omitted for brevity, as relationships are substantially the same as those at 30°C).

Very similar patterns were found when leaf nitrogen and photosynthetic rate were expressed on a leaf area rather than leaf mass basis. The rankings of the species in PNUE were similar to those found on a leaf mass basis, with *Eragrostis* again having the highest, and *Eleusine* the lowest PNUE of the species (Figs. 3, 4). There were statistically significant differences among



Figs. 5–8. Relationship between fertilizer solution nitrogen concentration and leaf nitrogen concentration for three NAD-ME and three NADP-ME grass species. **5.** Individual leaves on a per-leaf mass basis. **6.** Linear regression lines for the data in Fig. 5 for each species. **7.** Individual leaves on a per-leaf area basis. **8.** Linear regression lines for the data in Fig. 7 for each species. NAD-ME species are indicated by filled symbols and dashed lines and NADP-ME species by open symbols and solid lines. Each regression line is shown only over the range observed. Regressions are shown separately from data for clarity (when depicted on the same graph with the data, the figure is too cluttered to be informative).

the species in photosynthetic rates at a given leaf nitrogen concentration on a leaf-area basis (Figs. 3 and 4 for 30°C; data for 37°C not shown as relationships are essentially identical to those at 30°C; $df = 4,80$; $F = 9.8$; $P < 0.001$ at 30°C; $df = 4,69$; $F = 10.31$; $P < 0.001$ at 37°C). However, the NAD-ME and NADP-ME species did not differ significantly in either the slope ($df = 1,4$; $F = 0.14$; $P > 0.50$ at 30°C; $df = 1,4$; $F = 0.02$; $P > 0.75$ at 37°C) or intercept ($df = 1,4$; $F = 0.56$; $P > 0.25$ at 30°C; $df = 1,4$; $F = 0.002$; $P > 0.75$ at 37°C) of the leaf nitrogen-photosynthesis relationship at either 30°C or 37°C (Fig. 4 for 30°C; data for 37°C omitted for brevity, as relationships are substantially the same as those at 30°C).

Leaf nitrogen concentration shows a similar pattern. There are substantial differences among the species in

leaf nitrogen concentration at a given level of nitrogen availability, whether leaf nitrogen was expressed on a per-leaf mass (Figs. 5, 6), or a per-leaf area basis (Figs. 7, 8). There are differences in the rankings among the six species in leaf nitrogen concentration depending on which basis of measurement is used. On a per-mass basis, the highest leaf nitrogen concentrations over most of the range of fertilizer nitrogen used here were for two of the NAD-ME species, *Eleusine* and *Eragrostis*. However, the third NAD-ME species, *Panicum*, had leaf nitrogen concentrations comparable to the NADP-ME species. On a leaf area basis, *Eleusine* had the highest leaf nitrogen concentration of any of the species, and at any level of fertilizer nitrogen, three out of the four species with the highest leaf nitrogen were NAD-ME (Fig. 8). These results are consistent with previous observations of higher

leaf and shoot nitrogen concentrations in NAD-ME than in NADP-ME grasses (Bowman, 1991; LeCain and Morgan, 1998).

Overall, the slope of the regression of leaf nitrogen concentration on fertilizer nitrogen concentration differed significantly among species on either a leaf-mass basis (Fig. 6, $df = 4,86$; $F = 5.4$; $P < 0.001$), or a leaf-area basis (Fig. 8, $df = 4,81$; $F = 3.7$; $P < 0.01$), but there was no significant difference in this regard between the NAD-ME and NADP-ME species ($df = 1,4$; $F = 0.29$; $P > 0.50$ on a mass basis; $df = 1,4$; $F = 0.14$; $P > 0.50$ on an areal basis).

These results point to a pattern of substantial variation in PNUE and in leaf nitrogen concentration at the level of the species. Of the three NAD-ME species in this study, only *Eleusine* conformed to the predicted patterns of a low PNUE and a high leaf nitrogen concentration. *Eragrostis* had high leaf nitrogen concentration, but also had the highest PNUE of any species in the study. *Panicum*, on the other hand, combined low PNUE with a moderate leaf nitrogen concentration. A relatively low nitrogen concentration (for an NAD-ME species) appears to be a consistent attribute of *Panicum virgatum*. Both Bowman (1991) and LeCain and Morgan (1998) found that *P. virgatum* had the lowest shoot or leaf nitrogen of the NAD-ME species in their studies.

While we do not know the causes of the interspecific variation observed, some possibilities can be suggested by reference to other studies that have compared the use of nitrogen for photosynthetic machinery among a number of species (Evans, 1989; Usuda, Ku, and Edwards, 1984). One possible explanation for the differences among the species in photosynthetic rates at a given level of leaf nitrogen is that it is due to differences in the proportion of leaf nitrogen that is used for photosynthetic enzymes rather than for other leaf constituents (structural proteins, chlorophyll, nucleic acids, etc.). This has been proposed as a partial explanation for differences in photosynthetic nitrogen use efficiency among C_3 plants (Evans, 1989; Pons, Van der Werf, and Lambers, 1994), and such variation may be expected to occur among C_4 species as well. The species may also differ in their leaf concentrations of inorganic nitrogen.

Future experiments that distinguish among leaf nitrogen partitioned to these various compartments may be sensitive to differences among the C_4 subtypes that are missed with the whole-leaf nitrogen approach followed here. For example, there may be differences between the NAD-ME and NADP-ME subtypes in photosynthetic rates per-leaf organic nitrogen or per-leaf soluble protein even in the absence of differences in PNUE on a total nitrogen basis.

This study, having sampled only three species each of the NAD-ME and NADP-ME C_4 subtypes, also cannot rule out the possibility that there might exist small but consistent differences between NAD-ME and NADP-ME species in leaf total nitrogen concentration or PNUE, as suggested by Bowman (1991). Indeed, while we could detect no consistent differences between the C_4 subtypes, Bowman (1991) and LeCain and Morgan (1998) found NAD-ME species to have higher nitrogen concentrations than NADP-ME species in shoots and leaves, respectively, and we detected a trend in the same direction. How-

ever, there appears to be substantial overlap in PNUE between NAD-ME and NADP-ME species.

This result stands in stark contrast with the comparison of PNUE in C_3 vs. C_4 species. The difference in photosynthetic nitrogen use efficiency on a total leaf nitrogen basis between these photosynthetic types is large and has been consistently found, even in studies comparing unrelated C_3 and C_4 species (Bolton and Brown, 1980; Sage and Percy, 1987). The present study shows that any differences in photosynthetic nitrogen use efficiency between the C_4 subtypes must be small and any such differences between the C_4 subtypes occur against the background of substantial interspecific variation in PNUE.

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