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Photosynthetic Characteristics of C_3-C_4 Intermediate *Flaveria* Species[1]

I. LEAF ANATOMY, PHOTOSYNTHETIC RESPONSES TO O_2 AND CO_2, AND ACTIVITIES OF KEY ENZYMES IN THE C_3 AND C_4 PATHWAYS

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ABSTRACT

Four species of the genus *Flaveria*, namely *F. anomala*, *F. linearis*, *F. pubescens*, and *F. ramosissima*, were identified as intermediate C_3-C_4 plants based on leaf anatomy, photosynthetic CO_2 compensation point, O_2 inhibition of photosynthesis, and activities of C_4 enzymes. *F. anomala* and *F. ramosissima* exhibit a distinct Kranz-like leaf anatomy, similar to that of the C_4 species *F. trinervia*, while the other C_3-C_4 intermediate *Flaveria* species possess a less differentiated Kranz-like leaf anatomy. Photosynthetic CO_2 compensation points of these intermediates at 30°C were very low relative to those of C_3 plants, ranging from 7 to 14 microliters per liter. In contrast to C_3 plants, net photosynthesis by the intermediates was not sensitive to O_2 concentrations below 5% and decreased relatively slowly with increasing O_2 concentration. Under similar conditions, the percentage inhibition of photosynthesis by 21% O_2 varied from 20% to 25% in the intermediates compared with 28% in *Lycopersicon esculentum*, a typical C_3 species. The inhibition of carboxylation efficiency by 21% O_2 varied from 17% for *F. ramosissima* to 46% for *F. anomala* and were intermediate between the C_4 (2% for *F. trinervia*) and C_3 (53% for *L. esculentum*) values. The intermediate *Flaveria* species, especially *F. ramosissima*, have substantial activities of the C_4 enzymes, phosphoenolpyruvate carboxylase, pyruvate, orthophosphate dikinase, NADP-malic enzyme, and NADP-malate dehydrogenase, indicating potential for C_4 photosynthesis. It appears that these *Flaveria* species may be true biochemical C_3-C_4 intermediates.

All available evidence suggests that C_4 plants have evolved from ancestors possessing the C_3 pathway of photosynthesis and this has occurred independently many times in taxonomically diverse groups (3, 21). At present, the precise evolutionary transition, at the anatomical, physiological, and biochemical levels, from a C_3 to a C_4 plant is not clear. It is generally believed that studies of C_3-C_4 intermediate species might provide insight into the evolution of C_4 photosynthesis. In addition, since most of the world's important crops are C_3 plants, there has been considerable interest in improving their productivity by screening for mutants with reduced rates of photorespiration or by incorporating C_4 characteristics into C_3 plants (3, 19, 20). Thus, the search for naturally occurring C_3-C_4 intermediates and the study of their anatomical, physiological, and biochemical characteristics are of importance to both theoretical and applied disciplines of plant biology.

Since 1975, naturally occurring species intermediate between C_3 and C_4 plants have been found in the genera *Panicum* (6), *Mollugo* (22), and *Moricandia* (2). The intermediate nature of these species is based on Kranz-like leaf anatomy, low photosynthetic CO_2 compensation point, and a reduced level of photorespiration. Most recently, two species of *Flaveria* (*F. anomala* and *F. pubescens*) have also been identified as C_3-C_4 intermediates based on low CO_2 compensation point at 21% O_2 (1). In the present study, we examined the leaf anatomy, photosynthetic response to CO_2, sensitivity of net photosynthesis to O_2, and activity of key enzymes in C_3 and C_4 photosynthesis of several species of *Flaveria*, a genus apparently having C_3, C_4 and C_3-C_4 intermediate species (1, 21).

MATERIALS AND METHODS

Plant Material and Growth Conditions. Plants of *Flaveria anomala* Robinson, *F. linearis* Lag., *F. pubescens* Rydb., *F. ramosissima* Klatt, *F. trinervia* Mohr, and *Lycopersicon esculentum* Mill (C_3) were obtained by germinating the seeds on top of fine soil in peat pots which were placed in trays and watered by absorption or on moist filter paper in Petri dishes. After seedlings reached 1 to 3 cm in height, they were transplanted into larger pots filled with a mixture of peat and sand, and maintained in a growth chamber under a daily regime of 14 h of light at 27°C and 8 h of darkness at 22°C. Light was provided by a combination of fluorescent and incandescent lamps, giving a photosynthetic photon flux density of 80 nE/cm^2·s at plant height. Plants were watered with dilute nutrient solution three times a week. Young and newly expanded leaves from 2- to 4-month-old plants were used for experiments.

Leaf Anatomy. Samples (approximately 4 mm^2) of tissue were cut from young, fully expanded leaves and vacuum infiltrated with cold fixative (2% depolymerized paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate, pH 7.0). After 2 h, the tissue was washed with buffer, dehydrated in a graded ethanol series, and embedded in 'L.R. White' embedding medium according to the supplier's instructions (Polysciences, Inc.). Sections were cut at 2.5-μm thickness and stained with the periodic acid-Schiff reaction for insoluble carbohydrate (12).

Gas Exchange Measurements. CO_2 and water vapor exchange of intact individual leaves were measured with an open IR gas analysis system as described in a previous paper (18). Leaf temperatures were maintained at 30 ± 0.5°C using a peltier-cooled heat exchanger. A photosynthetic photon flux density of 180 nE/cm^2·s within the leaf chamber was provided by a combination of

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a 1-kw multivapor lamp and three 300-w medium flood lamps (Sylvania) after filtration through 20 cm of water. In the CO_2 response experiments, various gas mixtures were provided by mixing gases from cylinders containing 21% O_2 in N_2 or 2% O_2 in N_2 with pure CO_2 using a pair of Wösthoff mixing pumps. In the O_2 response experiments, the gas mixtures were generated by mixing gases from cylinders containing pure O_2, pure N_2, and 1% CO_2 in N_2 using a series of three Wösthoff mixing pumps. The rates of photosynthesis were measured after reaching steady state (usually within 30 min).

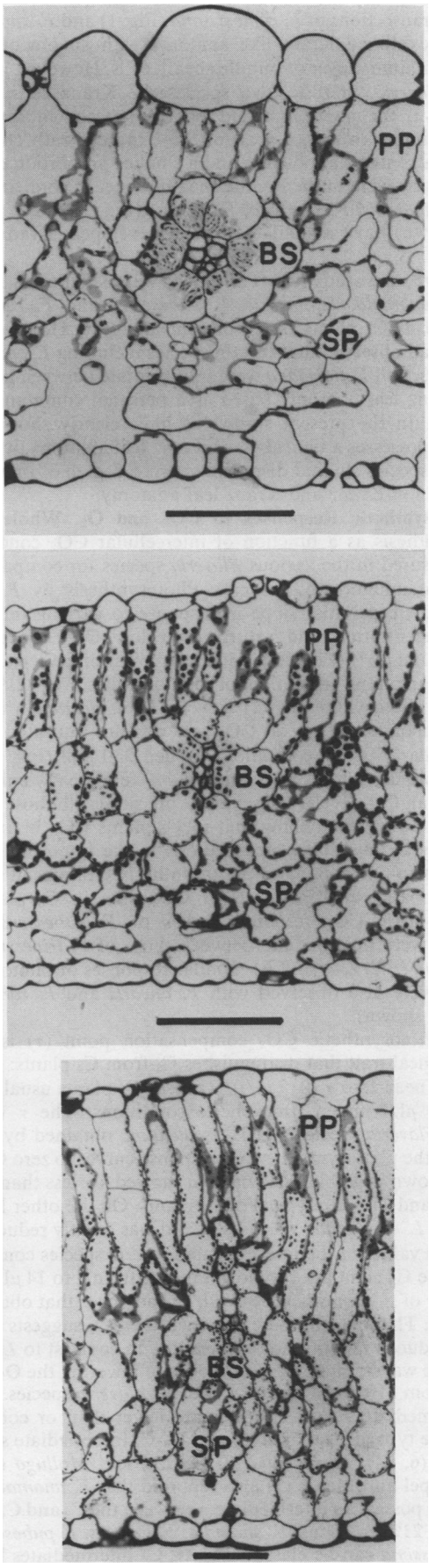
The photosynthetic CO_2 compensation points were determined by extrapolating the initial slope of the CO_2 response curve through the abscissa. Carboxylation efficiency was determined from the initial slope of the CO_2 response curve.

Enzyme Extraction. For the assay of PEP[3] carboxylase, pyruvate,Pi dikinase, and NADP-malic enzyme, leaf extracts were obtained at 4°C immediately after harvesting the tissue in 4 volumes of grinding medium containing 10 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM EDTA, 2.5 mM pyruvate, 100 mM DTE, and 2.5% (w/v) insoluble PVP. The crude extract was passed through one layer of Miracloth and the filtrate was rapidly desalted by passage through a small Sephadex G-25 column (0.8 cm in diameter and 5 cm in length). The column was pre-equilibrated with a buffer solution containing 50 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM pyruvate, 0.2% BSA, and 10 mM DTE. About 0.4 ml of the crude filtrate was applied to the column and desalted by centrifugation of the column at 1400*g* for 3 min at room temperature (10). The eluate obtained by this technique was not diluted and the protein yield was over 90% of the original sample. An aliquot was taken for Chl determination prior to applying the filtrate to the Sephadex column. For the assay of RuBP carboxylase, PEP carboxykinase, NAD-malic enzyme, and NADP-malate dehydrogenase, a buffer solution containing 50 mM Hepes-KOH, pH 7.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM DTE, and 2.5% (w/v) insoluble PVP was used for enzyme extraction. The same buffer solution without PVP was used for equilibration of the Sephadex column.

Enzyme Assays and Chl Determination. The following enzymes were assayed spectrophotometrically at 340 nm in a total volume of 1 ml at 25 to 27°C as previously described: PEP carboxylase (24), pyruvate,Pi dikinase (23), NADP-malic enzyme (13), NADP-malate dehydrogenase (13), and NAD-malic enzyme (9). RuBP carboxylase and PEP carboxykinase were assayed radiometrically in a total volume of 0.15 ml at 30°C using $NaH^{14}CO_3$ according to (17) and (7), respectively. Chl concentration and Chl *a/b* ratios were determined according to Wintermans and De Mots (26) after extraction in 96% (v/v) ethanol.

RESULTS AND DISCUSSION

Leaf Anatomy. The leaf anatomy of *F. trinervia*, as shown in Figure 1A, is characteristic of a C_4 or Kranz leaf. A layer of well developed bundle sheath cells surrounds the vascular tissue. The bundle sheath cells contain numerous chloroplasts in a centripetal position. Surrounding the bundle sheath cells are palisade parenchyma beneath the adaxial epidermis and spongy parenchyma above the abaxial epidermis, typical of a C_4 dicotyledonous leaf. The bundle sheath chloroplasts were considerably larger, appearing more elongated than those of the palisade and spongy parenchyma cells.



[3] Abbreviations: DTE, dithioerythritol; PEP, phosphoenolpyruvate; τ, photosynthetic CO_2 compensation point; CE, carboxylation efficiency; RuBP, ribulose 1,5-bisphosphate.

FIG. 1. Leaf transections of *F. trinervia* (upper), *F. ramosissima* (middle), and *F. linearis* (lower). BS, bundle sheath cell; PP, palisade parenchyma cell; SP, spongy parenchyma cell. Bar = 100 μm.

Leaf transections of *F. ramosissima* (Fig. 1) and *F. anomala* (not shown) exhibited Kranz-like anatomy with an obvious chloroplast-containing layer of bundle sheath cells. However, in contrast to *F. trinervia*, in these two species the Kranz cells were less distinctive; the mesophyll and bundle sheath chloroplasts appeared similar in size, not all of the bundle sheath chloroplasts were in a centripetal position and a smaller proportion of the leaf chloroplasts were in the bundle sheath tissue. In comparison to *F. ramosissima* and *F. anomala*, *F. linearis* (Fig. 1) and *F. pubescens* (not shown) have a similar, but less developed, Kranz-like leaf anatomy.

In a previous study (21), *F. trinervia* was classified as a C_4 plant and *F. anomala*, *F. linearis*, *F. pubescens*, and *F. ramosissima* classified as C_3 plants based on leaf anatomy. However, W. V. Brown had observed that certain species including *F. oppositifolia*, *F. linearis*, and *F. floridana* were intermediate between non-Kranz and Kranz leaf anatomy (cited as a personal communication in Ref. 21). In the present study, we have clearly shown that *F. trinervia* possesses a typical C_4 or Kranz leaf anatomy, while other *Flaveria* species studied display a varying degree of intermediacy between non-Kranz and Kranz leaf anatomy.

Photosynthetic Responses to CO_2 and O_2. Whole leaf net photosynthesis as a function of intercellular CO_2 concentration was measured in the various *Flaveria* species for comparing their carbon assimilation efficiencies. Photosynthesis by *F. trinervia* showed a steep initial slope in response to varying intercellular CO_2 concentration and saturated at low CO_2 concentrations (around 150 μl/l) (Fig. 2A), which is typical of a C_4 plant. There was no apparent inhibition of photosynthesis by 21% O_2. In contrast, photosynthesis at 21% O_2 by *L. esculentum*, a typical C_3 plant, responded slowly to CO_2 and was not saturated until the intercellular CO_2 concentration reached 330 μl/l (Fig. 2B). The rates of photosynthesis in *L. esculentum* were greatly enhanced at 2% O_2 at all CO_2 levels. These results are similar to those of earlier studies (4, 14) which show that in C_4 plants CO_2 is fixed more efficiently at low CO_2 concentrations in the intercellular spaces (a steeper initial slope) and photosynthesis saturates at relatively lower (subatmospheric) levels of CO_2 than in C_3 plants. The photosynthetic CO_2 response curves of *F. pubescens* and *F. anomala* were intermediate between those of *F. trinervia* and *L. esculentum* (Fig. 2, A and B). Similar responses of photosynthesis to CO_2 were also observed with *F. linearis* and *F. ramosissima* (data not shown).

The photosynthetic CO_2 compensation point (τ) is another physiological trait that distinguishes C_3 from C_4 plants: C_4 plants exhibit a near-zero τ (0–5 μl/l), whereas C_3 plants usually have τ about 50 μl/l under atmospheric conditions. The τ values of various *Flaveria* species and *L. esculentum*, obtained by extrapolation of the photosynthetic CO_2 response curves to zero CO_2 (Fig. 2), are shown in Table I. *F. trinervia* showed a τ less than 1 μl/l at both 2% and 21% O_2, typical of C_4 plants. On the other hand, the value for *L. esculentum* was 54 μl/l and was greatly reduced at 2% O_2. The τ values of the intermediate *Flaveria* species compared to that of the C_3 plant are very low, ranging from 7 to 14 μl/l at 21% O_2. The τ of *F. ramosissima* of 7 μl/l approaches that obtained for C_4 plants. The low τ in these species at 21% O_2 suggests that they have a reduced rate of photorespiration. In contrast to *L. esculentum*, there was no significant influence of lowering the O_2 concentration from 21% to 2% on the τ of these *Flaveria* species. The τ of the intermediate *Flaveria* species are lower than or comparable with those typically reported for the C_3-C_4 intermediate species in *Panicum* (6, 14), *Moricandia* (2, 11, 25), and *Mollugo* (22). Recently, Apel and Maass (1) also reported that *F. anomala* and *F. pubescens* possess an intermediate τ between the C_3 and C_4 *Flaveria* species at 21% O_2. Thus, *F. anomala*, *F. linearis*, *F. pubescens*, and *F. ramosissima* can be classified as C_3-C_4 intermediates based on leaf anatomy and photosynthetic CO_2 compensation point.

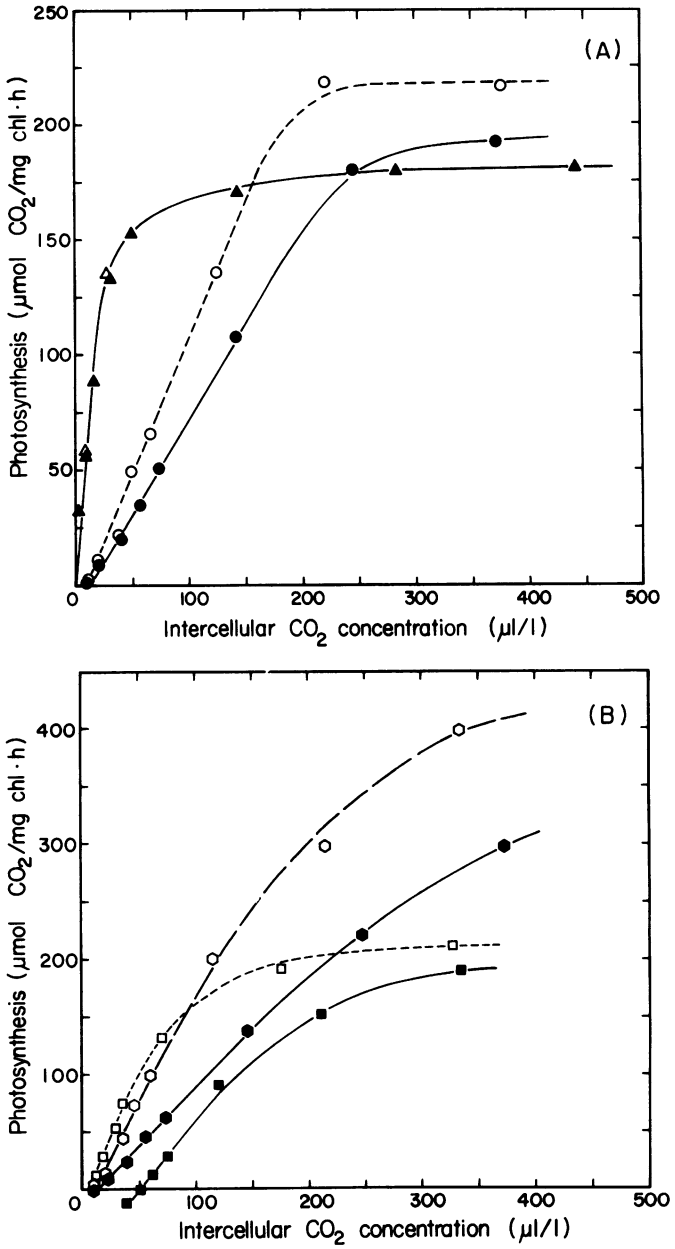


FIG. 2. The response of net photosynthesis to varying intercellular CO_2 concentration for various *Flaveria* species and *L. esculentum* (C_3) at 2% (open symbols) and 21% O_2 (solid symbols). A, *F. trinervia* (△, ▲; note, at each CO_2 level, rates of photosynthesis at the two O_2 levels coincided, giving essentially no effect of O_2), *F. pubescens* (○, ●); B, *F. anomala* (⬡, ⬢), *L. esculentum* (□, ■). The assay conditions were 180 nE/cm²·s photosynthetic photon flux density, 340 μl/l CO_2, and 30 ± 0.5°C leaf temperature. Note different scales for ordinates of A and B. Data presented represent one replication although two measurements were made for each species.

The intermediate species are also less sensitive to O_2 inhibition of photosynthesis, relative to C_3 species. CE, derived from the initial slope of the photosynthetic CO_2 response curve, measures the efficiency of different species in utilizing low levels of CO_2. The data of CE for various *Flaveria* species and *L. esculentum*, measured at 21% and 2% O_2, are presented in Table I. The C_4 species *F. trinervia* had the highest CE and exhibited essentially no inhibition of CE by 21% O_2, presumably due to the CO_2 concentration mechanism of the C_4 pathway of photosynthesis. *L. esculentum* had slightly higher CE than the intermediate *Flaveria*

Table I. *Photosynthetic CO_2 Compensation Point and Carboxylation Efficiency at 2% and 21% O_2 of Various Flaveria Species and Lycopersicon esculentum*

Species	CO_2 Compensation Point[a]		Carboxylation Efficiency[b]		
	21% O_2	2% O_2	21% O_2	2% O_2	Inhibition by 21% O_2
	μl/l				
F. anomala	12	11	1.02	1.89	46
F. linearis	8	6	0.94	1.63	42
F. pubescens	14	11	0.83	1.21	31
F. ramosissima	7	5	1.07	1.29	17
F. trinervia	<1	<1	4.07	4.15	2
L. esculentum	54	9	1.29	2.74	53

[a] Determined by extrapolating the initial slope of the photosynthetic CO_2 response curve through the abscissa.

[b] Determined from the initial slope of the photosynthetic CO_2 response curve and expressed as photosynthesis rate (μmol/mg Chl·h) per unit of intercellular CO_2 concentration (μl/l).

species at both 21% and 2% O_2. This may be due to a higher level of RuBP carboxylase in *L. esculentum* than in the intermediate *Flaveria* species. The CE of *L. esculentum* was inhibited more than 50% by 21% O_2, indicating a significant competitive O_2 inhibition of photosynthesis. The inhibitions of CE by atmospheric O_2 for the intermediate *Flaveria* species varied from 17% for *F. ramosissima* to 46% for *F. anomala*, giving values intermediate to the C_3 and C_4 species. The observation that exposure of the intermediates to 21% O_2 resulted in a greater effect on CE than on the CO_2 compensation point is consistent with previous studies on C_3-C_4 intermediate *Panicum* species (5). The results suggest that the mechanisms decreasing the amount of photorespiratory CO_2 loss from the leaves of these C_3-C_4 intermediates is relatively more efficient than the mechanisms decreasing the amount of competitive O_2 inhibition of photosynthesis, relative to C_3 species.

The sensitivity of net photosynthesis to O_2 in the *Flaveria* species was also assessed in a separate experiment by measuring the photosynthetic response to varying O_2 levels (Fig. 3). Photosynthesis by *F. trinervia* increased slightly with increasing O_2 up to 12%, was similar at 2% and 21% O_2, and then decreased substantially at 28% (Fig. 3A). Substantial inhibition of C_4 photosynthesis by O_2 above atmospheric levels has been reported in maize and *Amaranthus graecizans* (see Ref. 15). The basis for the O_2 inhibition of photosynthesis in C_4 plants remains unclear. With *L. esculentum*, there was a linear decrease in photosynthesis rate as O_2 was increased from 2% to 28% (Fig. 3B). However, photosynthesis by *F. anomala*, *F. linearis*, *F. pubescens*, and *F. ramosissima* showed little or no inhibition by 5% O_2. This response is similar to that reported for *Panicum milioides*, another C_3-C_4 intermediate (6). At atmospheric or subatmospheric O_2 concentrations, the degree of O_2 inhibition in these intermediate *Flaveria* species was always lower than that in the C_3 species *L. esculentum*. The percentage inhibition varied from 20% to 25% in the intermediates compared to 4% in *F. trinervia* and 28% in *L. esculentum*. These results indicate that the intermediate *Flaveria* species have reduced rates of photorespiration, consistent with the earlier results of photosynthetic CO_2 compensation points and the effect of O_2 on carboxylation efficiency (Table I).

Chlorophyll *a/b* Ratios and Enzyme Activity. The various *Flaveria* species which we have designated as C_3-C_4 intermediates based on the other criteria in this study have Chl *a/b* ratios lower than that of the C_4 species *F. trinervia* (Table II). *F. trinervia*, an NADP-malic enzyme-type C_4 plant as revealed by the enzyme

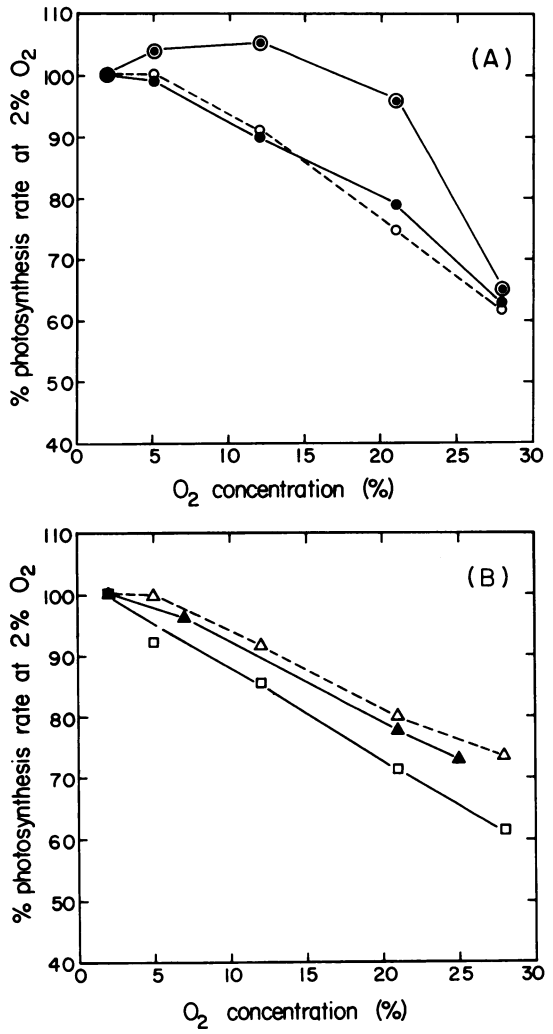


FIG. 3. The response of net photosynthesis to O_2 concentration for various *Flaveria* species and *L. esculentum*. The data were expressed as % photosynthesis rate at 2% O_2 for each species. The rates of photosynthesis (μmol/mg Chl·h) at 2% O_2 were: A, *F. trinervia* (⊙), 195; *F. pubescens* (●), 214; *F. anomala* (○), 352; B, *F. linearis* (△), 248; *F. ramosissima* (▲), 264; *L. esculentum* (□), 269. The assay conditions were 180 nE/cm^2·s photosynthetic photon flux density, 340 μl/l CO_2, and 30 ± 0.5°C leaf temperature. Each point represents the mean of two replicates.

study (Table II), has a Chl *a/b* ratio of 3.87. NADP-malic enzyme type C_4 plants have high Chl *a/b* ratios in bundle sheath chloroplasts which results in higher Chl *a/b* ratios for the whole leaf (approximately 4) compared to C_3 species (approximately 3) (16). Among the intermediate *Flaveria* species, *F. ramosissima* has a Chl *a/b* ratio of 3.40, more like that of the C_4 species *F. trinervia*.

The potential for C_4 photosynthesis in the intermediate *Flaveria* species was evaluated by examining the *in vitro* activity of several key enzymes of the C_4 pathway. *Panicum milioides*, another C_3-C_4 intermediate, was included for comparison. As shown in Table II, the C_4 species *F. trinervia* has high activities of PEP carboxylase, pyruvate,Pi dikinase (ATP- and Pi-dependent activity), NADP-malate dehydrogenase, and NADP-malic enzyme, but low activities of NAD-malic enzyme and PEP carboxykinase. Thus, *F. trinervia* is identified as an NADP-malic enzyme-type C_4 plant. The C_3-C_4 intermediate *Flaveria* species, particularly *F. ramosissima*, *F. pubescens*, and *F. anomala*, also have substantial activities of PEP carboxylase, pyruvate,Pi dikinase, NADP-malate dehydrogenase, and NADP-malic enzyme, although the levels were

Table II. *Activity of Several Key Enzymes of C_4 Photosynthesis in Leaf Extracts of Various Flaveria Species and Panicum milioides*

See "Materials and Methods" for the assay of enzymes. RuBPC, ribulose 1,5-bisphosphate carboxylase; PEPC, PEP carboxylase; PPDK, pyruvate,Pi dikinase; NADP-ME, NADP-malic enzyme; NAD-ME, NAD-malic enzyme; PEP-CK, PEP carboxykinase; NADP-MDH, NADP-malate dehydrogenase.

Species	Chl *a/b*	RuBPC	PEPC	PPDK	NADP-ME	NAD-ME	PEP-CK	NADP-MDH
				μmol/mg Chl·h				
F. anomala	2.82	564	123	23	25	22	0.6	133
F. linearis	2.92	561	123	3.7	66	50	0.6	23
F. pubescens	2.78	497	207	27	90	18	1.7	54
F. ramosissima	3.40	412	162	37	153	34	0.4	135
F. trinervia	3.87	309	908	290	1091	47	4.3	758
P. milioides	3.01		76	5.5	14	4.1	N.D.[a]	

[a] N.D., not detectable.

about one-tenth of those of the C_4 species. In *P. milioides*, the C_4 enzymes, particularly pyruvate,Pi dikinase and the C_4 acid decarboxylases, were very low in activity, which is consistent with the recent report that this species fixes CO_2 solely by the C_3 pathway (8). These results suggest that the C_3-C_4 intermediate *Flaveria* species, in contrast to *Moricandia arvensis* (11, 25) and *P. milioides*, may be capable of fixing some CO_2 through the C_4 pathway.

The C_4 pathway in the genus *Flaveria* occurs mostly in the advanced annual species and is proposed to have arisen from C_3 species relatively recently under arid, tropical conditions (21). The genus appears to contain a number of species which exhibit C_3-C_4 intermediate characteristics (1, 21). In the present study, *F. anomala*, *F. linearis*, *F. pubescens*, and *F. ramosissima* are identified as intermediate species between C_3 and C_4 plants based on leaf anatomy, photosynthetic CO_2 compensation point, sensitivity of photosynthesis to O_2, and activities of C_4 enzymes. Whereas the mechanism of reduced photorespiration in *M. arvensis* and *P. milioides* remains unknown, it appears that a limited degree of C_4 photosynthesis may be responsible for the lower CO_2 compensation points and reduced rates of photorespiration in the C_3-C_4 intermediate *Flaveria* species. Thus, some of the *Flaveria* species may be in the process of evolution from C_3 to C_4 photosynthesis at both the anatomical and biochemical levels.

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