Photosynthetic Characteristics of C₃-C₄ Intermediate Flaveria Species

I. LEAF ANATOMY, PHOTOSYNTHETIC RESPONSES TO O₂ AND CO₂, AND ACTIVITIES OF KEY ENZYMES IN THE C₃ AND C₄ 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 C₃-C₄ plants based on leaf anatomy, photosynthetic CO₂ compensation point, O₂ inhibition of photosynthesis, and activities of C₄ enzymes. F. anomala and F. ramosissima exhibit a distinct Kranz-like leaf anatomy, similar to that of the C₄ species F. trinervia, while the other C₃-C₄ intermediate Flaveria species possess a less differentiated Kranz-like leaf anatomy. Photosynthetic CO₂ compensation points of these intermediates at 30°C were very low relative to those of C₄ plants, ranging from 7 to 14 micromoles per liter. In contrast to the C₄ plants, net photosynthesis by the intermediates was not sensitive to O₂ concentrations below 5% and decreased relatively slowly with increasing O₂ concentration. Under similar conditions, the percentage inhibition of photosynthesis by 21% O₂ varied from 20% to 25% in the intermediates compared with 28% in Lycopernicum esculentum, a typical C₃ species. The inhibition of carboxylation efficiency by 21% O₂ varied from 17% for F. ramosissima to 46% for F. anomala and was intermediate between the C₃ (2% for F. trinervia) and C₄ (53% for L. esculentum) values. The intermediate Flaveria species, especially F. ramosissima, have substantial activities of the C₄ enzymes, phosphoenolpyruvate carboxylase, pyruvate, orthophosphate dikinase, NADP-malic enzyme, and NADP-malate dehydrogenase, indicating potential for C₄ photosynthesis. It appears that these Flaveria species may be true biochemical C₃-C₄ intermediates.

All available evidence suggests that C₄ plants have evolved from ancestors possessing the C₃ 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 C₃ to a C₄ plant is not clear. It is generally believed that studies of C₃-C₄ intermediate species might provide insight into the evolution of C₄ photosynthesis. In addition, since most of the world's important crops are C₃ plants, there has been considerable interest in improving their productivity by screening for mutants with reduced rates of photorespiration or by incorporating C₄ characteristics into C₃ plants (3, 19, 20). Thus, the search for naturally occurring C₃-C₄ 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 C₃ and C₄ plants have been found in the genera Panicum (6), Mollugo (22), and Morecandia (2). The intermediate nature of these species is based on Kranz-like leaf anatomy, low photosynthetic CO₂ compensation point, and a reduced level of photorespiration. Most recently, two species of Flaveria (F. anomala and F. pubescens) have also been identified as C₃-C₄ intermediates based on low CO₂ compensation point at 21% O₂ (1). In the present study, we examined the leaf anatomy, photosynthetic response to O₂, sensitivity of net photosynthesis to O₂, and activity of key enzymes in C₃ and C₄ photosynthesis of several species of Flaveria, a genus apparently having C₃, C₄, and C₃-C₄ 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 (C₄) 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/cm²-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 mm³) 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. CO₂ 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/cm²-s within the leaf chamber was provided by a combination of

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PHOTOSYNTHESIS OF C_3-C_4 INTERMEDIATE FLAVERIA SPECIES

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 Wosthoff 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 Wosthoff 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^14CO_3 according to (17) and (7), respectively. Chl concentration and Chl a/b ratios were determined according to Wintemans 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_3 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; r, photosynthetic CO_2 compensation point; CE, carboxylation efficiency; RuBP, ribulose 1,5-bisphosphate.

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**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 transactions 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 C₄ plant and *F. anomala*, *F. linearis*, *F. pubescens*, and *F. ramosissima* were classified as C₃ 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 C₄ 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 CO₂ and O₂.** Whole leaf net photosynthesis as a function of intercellular CO₂ 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 CO₂ concentration and saturated at low CO₂ concentrations (around 150 μl/l) (Fig. 2A), which is typical of a C₄ plant. There was no apparent inhibition of photosynthesis by 21% O₂. In contrast, photosynthesis at 21% O₂ by *L. esculentum*, a typical C₃ plant, responded slowly to CO₂ and was not saturated until the intercellular CO₂ concentration reached 330 μl/l (Fig. 2B). The rates of photosynthesis in *L. esculentum* were greatly enhanced at 2% O₂ than at all CO₂ levels. These results are similar to those of earlier studies (4, 14) which show that in C₃ plants CO₂ is fixed more efficiently at low CO₂ concentrations in the intercellular spaces (a steeper initial slope) and photosynthesis saturates at relatively lower (subatmospheric) levels of CO₂ than in C₃ plants. The photosynthetic CO₂ 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 CO₂ were also observed with *F. linearis* and *F. ramosissima* (data not shown).

The photosynthetic CO₂ compensation point (τ) is another physiological trait that distinguishes C₃ from C₄ plants: C₃ plants exhibit a near-zero τ (0–5 μl/l), whereas C₄ 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 CO₂ response curves to zero CO₂ (Fig. 2), are shown in Table I. *F. trinervia* showed a τ less than 1 μl/l at both 2% and 21% O₂, typical of C₄ plants. On the other hand, the value for *L. esculentum* was 54 μl/l and was greatly reduced at 2% O₂. The τ values of the intermediate *Flaveria* species compared to that of the C₃ plant are very low, ranging from 7 to 14 μl/l at 21% O₂. The τ of *F. ramosissima* of 7 μl/l approaches that obtained for C₃ plants. The low τ in these species at 21% O₂ suggests that they have a reduced rate of photosorption. In contrast to *L. esculentum*, there was no significant influence of lowering the O₂ 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 C₄-C₃ intermediate species in Panicum (6, 14), Moricandia (2, 11, 25), and Mollugo (22). Recently, Apel and Maas (1) also reported that *F. anomala* and *F. pubescens* possess an intermediate τ between the C₄ and C₃ *Flaveria* species at 21% O₂. Thus, *F. anomala*, *F. linearis*, *F. pubescens*, and *F. ramosissima* can be classified as C₃-C₄ intermediates based on leaf anatomy and photosynthetic CO₂ compensation point.

![Fig. 2](https://example.com/fig2.png)

FIG. 2. The response of net photosynthesis to varying intercellular CO₂ concentration for various *Flaveria* species and *L. esculentum* (*Ca*) at 2% (open symbols) and 21% O₂ (solid symbols). A, *F. trinervia* (C, A; note, at each CO₂ level, rates of photosynthesis at the two O₂ levels coincided, giving essentially no effect of O₂), *F. pubescens* (O, A; B, *F. anomala* (O, O), *L. esculentum* (C, B). The assay conditions were 180 μE/cm².s photosynthetic photon flux density, 340 μl/l CO₂, 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 O₂ inhibition of photosynthesis, relative to C₃ species. CE, derived from the initial slope of the photosynthetic CO₂ response curve, measures the efficiency of different species in utilizing low levels of CO₂. The data of CE for various *Flaveria* species and *L. esculentum*, measured at 21% and 2% O₂ are presented in Table I. The C₃ species *F. trinervia* had the highest CE and exhibited essentially no inhibition of CE by 21% O₂, presumably due to the CO₂ concentration mechanism of the C₄ pathway of photosynthesis. *L. esculentum* had slightly higher CE than the intermediate *Flaveria* species. The intermediate species are also less sensitive to O₂ inhibition of photosynthesis, relative to C₃ species. CE, derived from the initial slope of the photosynthetic CO₂ response curve, measures the efficiency of different species in utilizing low levels of CO₂. The data of CE for various *Flaveria* species and *L. esculentum*, measured at 21% and 2% O₂ are presented in Table I. The C₃ species *F. trinervia* had the highest CE and exhibited essentially no inhibition of CE by 21% O₂, presumably due to the CO₂ concentration mechanism of the C₄ pathway of photosynthesis. *L. esculentum* had slightly higher CE than the intermediate *Flaveria* species.
species at both 21% and 2% O₂. 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% O₂, indicating a significant competitive O₂ inhibition of photosynthesis. The inhibitions of CE by atmospheric O₂ for the intermediate *Flaveria* species varied from 17% for *F. ramosissima* to 46% for *F. anomala*, giving values intermediate to the C₃ and C₄ species. The observation that exposure of the intermediates to 21% O₂ resulted in a greater effect on CE than on the CO₂ compensation point is consistent with previous studies on C₃-C₄ intermediate *Panicum* species (5). The results suggest that the mechanisms decreasing the amount of photorespiratory CO₂ loss from the leaves of these C₃-C₄ intermediates is relatively more efficient than the mechanisms decreasing the amount of competitive O₂ inhibition of photosynthesis, relative to C₃ species.

The sensitivity of net photosynthesis to O₂ in the *Flaveria* species was also assessed in a separate experiment by measuring the photosynthetic response to varying O₂ levels (Fig. 3). Photosynthesis by *F. trinervia* increased slightly with increasing O₂ up to 12%, was similar at 21% and 2% O₂, and then decreased substantially at 28% (Fig. 3A). Substantial inhibition of C₃ photosynthesis by O₂ above atmospheric levels has been reported in maize and *Amaranthus graecizans* (see Ref. 15). The basis for the O₂ inhibition of photosynthesis in C₃ plants remains unclear. With *L. esculentum*, there was a linear decrease in photosynthesis rate as O₂ 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% O₂. This response is similar to that reported for *Panicum milioides*, another C₃-C₄ intermediate (6). At atmospheric or subatmospheric O₂ concentrations, the degree of O₂ inhibition in these intermediate *Flaveria* species was always lower than that in the C₃ 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 CO₂ compensation points and the effect of O₂ on carboxylation efficiency (Table I).

**Chlorophyll a/b Ratios and Enzyme Activity.** The various *Flaveria* species which we have designated as C₃-C₄ intermediates based on the other criteria in this study have Chl a/b ratios lower than that of the C₄ species *F. trinervia* (Table II). *F. trinervia*, an NADP-malic enzyme-type C₄ plant as revealed by the enzyme study (Table II), has a Chl a/b ratio of 3.87. NADP-malic enzyme type C₄ plants have high Chl a/b ratios in bundle sheath chloroplasts which result in higher Chl a/b ratios for the whole leaf (approximately 4) compared to C₃ 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 C₄ species *F. trinervia*.

The potential for C₄ photosynthesis in the intermediate *Flaveria* species was evaluated by examining the *in vitro* activity of several key enzymes of the C₄ pathway. *Panicum milioides*, another C₃-C₄ intermediate, was included for comparison. As shown in Table II, the C₄ 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 C₄ plant. The C₃-C₄ 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 C₄ Photosynthesis in Leaf Extracts of Various Flaveria Species and Panicum milioides

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* N.D., not detectable.

about one-tenth of those of the C₃ species. In P. milioides, the C₄ enzymes, particularly pyruvate, Pi dikinase and the C₄ acid dehydratases, were very low in activity, which is consistent with the recent report that this species fixes CO₂ solely by the C₃ pathway (8). These results suggest that the C₃-C₄ intermediate Flaveria species, in contrast to Moricandia arvensis (11, 25) and P. milioides, may be capable of fixing some CO₂ through the C₄ pathway. The C₄ pathway in the genus Flaveria occurs mostly in the advanced annual species and is proposed to have arisen from C₃ species relatively recently under arid, tropical conditions (21). The genus appears to contain a number of species which exhibit C₃-C₄ intermediate characteristics (1, 21). In the present study, F. anomala, F. linearis, F. pubescens, and F. rosamositoma are identified as intermediate species between C₃ and C₄ plants based on leaf anatomy, photosynthetic CO₂ compensation point, sensitivity of photosynthesis to O₂, and activities of C₄ enzymes. Whereas the mechanism of reduced photorespiration in M. arvensis and P. milioides remains unknown, it appears that a limited degree of C₄ photosynthesis may be responsible for the lower CO₂ compensation points and reduced rates of photorespiration in the C₃-C₄ intermediate Flaveria species. Thus, some of the Flaveria species may be in the process of evolution from C₃ to C₄ photosynthesis at both the anatomical and biochemical levels.

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