Plant Physiol. (1984) 75, 993–996
0032-0889/84/75/0993/04/$01.00/0

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

III. REDUCTION OF PHOTORESPIRATION BY A LIMITED C_4 PATHWAY OF PHOTOSYNTHESIS IN *FLAVERIA RAMOSISSIMA*

Received for publication February 8, 1984 and in revised form April 9, 1984

MARY E. RUMPHO, MAURICE S. B. KU*, SHU-HUA CHENG, AND GERALD E. EDWARDS
Botany Department (M. E. R., M. S. B. K., S-H. C., G. E. E.) and Institute of Biological Chemistry (G. E. E.), Washington State University, Pullman, Washington 99164-4230

ABSTRACT

The initial products of photosynthesis by the C_3 species *Flaveria cronquistii*, the C_4 species *F. trinervia*, and the C_3–C_4 intermediate species *F. ramosissima* were determined using a pulse-chase technique with $^{14}CO_2$–$^{12}CO_2$. The intermediate species *F. ramosissima* incorporated at least 42% of the total soluble ^{14}C fixed into malate and aspartate after 10 seconds of photosynthesis in $^{14}CO_2$, as compared with 90% for the C_4 species *F. trinervia* and 5% for the C_3 species *F. cronquistii*. In both *F. ramosissima* and *F. trinervia*, turnover of labeled malate and aspartate occurred during a chase period in $^{12}CO_2$, although the rate of turnover was slower in the intermediate species. Relative to *F. cronquistii*, *F. ramosissima* showed a reduced incorporation of radioactivity into serine and glycine during the pulse period. These results indicate that a functional C_4 pathway of photosynthesis is operating in *F. ramosissima* which can account for its reduced level of photorespiration, and that this species is a true biochemical intermediate between C_3 and C_4 plants.

Naturally occurring species with photosynthetic characteristics intermediate between C_3 and C_4 plants have been identified in the genera *Mollugo* (19), *Panicum* (4, 14), *Moricandia* (2, 9), *Flaveria* (1, 10, 12) and, most recently, *Neurachne* (Hattersley, personal communication). The intermediate nature of these species includes a Kranz-like leaf anatomy, a lower Γ[2] (indicative of reduced photorespiration), and a reduced sensitivity of net photosynthesis to O_2. In recent years, elucidation of the biochemical basis for this intermediacy has been the focus of several investigations. The important question is whether the C_4 pathway of photosynthesis or some other mechanism is functioning in these species. In the intermediate species *Panicum milioides*, it is concluded from earlier studies (*e.g.* Ref. 17) that the operation of limited C_4 photosynthesis is responsible for its reduced level of photorespiration. However, recent studies (6, 8) clearly showed that *P. milioides* fixes CO_2 solely by the C_3 pathway. Similarly, C_4 photosynthesis is not involved in the intermediate species *Moricandia arvensis* (8, 9, 21). These results are consistent with the low activities of C_4 cycle enzymes in these species (6, 11, 15). The mechanism(s) for reducing photorespiration in these species thus remains unknown. In C_3–C_4 intermediate *Flaveria* species (particularly *F. ramosissima*), however, substantial activities of certain C_4 cycle enzymes were detected (12), suggesting that they may be capable of assimilating atmospheric CO_2 through the C_4 pathway. In this study, we demonstrated that in *F. ramosissima* a considerable portion of the exogenous CO_2 is fixed through the C_4 pathway, which may account for its intermediate photosynthetic characteristics.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Plants of *F. cronquistii* Powell, *F. ramosissima* Klatt, and *F. trinervia* Mohr were obtained by germination from seeds and grown in a growth chamber under conditions similar to those previously described (12). Plants were watered with dilute nutrient solution three times a week. The experiments reported here were performed on young and fully expanded leaves from 2- to 4-month-old plants.

Gas Exchange Measurements. The Γ was determined using an Anarad IR gas analyzer, AR-500R, in a differential mode. A newly expanded leaf (or leaves in some cases) was sealed into a Plexiglas chamber (volume, 300 cm^3) and illuminated with a PPFD of 1250 ± 50 $\mu mol/m^2 \cdot s$. Leaf temperature was maintained at 30 ± 0.2°C. A small fan inside the chamber provided air circulation. The CO_2 concentration in the chamber was reduced to less than 10 $\mu l/l$ and the time required for reaching the Γ to less than 20 min. A gas sample of 3 ml was withdrawn from the leaf chamber through a rubber septum at 5-min intervals and the concentration of CO_2 was determined immediately. The technique for determining CO_2 concentration in the gas sample is similar to that described by Atkins and Pate (3).

Whole leaf photosynthesis was measured with an open IRGA system as described in a previous paper (12). Leaf temperature was 30 ± 0.5°C; the PPFD, 1650 ± 50 $\mu mol/m^2 \cdot s$; and the CO_2 concentration, 310 ± 5 $\mu l/l$.

$^{14}CO_2$ Feeding and Identification of ^{14}C-Products. For the pulse-chase experiments, 1 to 2 leaf blades (approximately 0.20 g fresh weight) were cut from the plants 6 h into the light period. The leaf blades were immediately placed into a glass vial (final volume 35 ml), with the base of the leaf petiole submerged in distilled H_2O, and were preilluminated for 10 to 15 min with a PPFD of 1000 $\mu mol/m^2 \cdot s$ from three 150-w Westinghouse PAR lamps. The lights were filtered through a 5-cm thick water column to avoid excess heat. The vial was constantly flushed with a stream of humidified air (containing approximately 340 $\mu l/l$ CO_2 as determined by IRGA) during the preillumination. Prior to $^{14}CO_2$ feeding, the vial was briefly flushed with CO_2-free

[1] Supported in part by a grant from Washington State University Graduate School and by National Science Foundation Grant PCM 82-04625.

[2] Abbreviations: Γ, photosynthetic CO_2 compensation point; PPFD, photosynthetic photon flux density; PGA, 3-phosphoglyceric acid.

air and quickly sealed with a serum stopper. Three ml of air were then withdrawn from the vial through a serum stopper using a 5-ml medical syringe. Three ml of approximately 4000 μl/l $^{14}CO_2$, generated by injecting $NaH^{14}CO_3$ into H_3PO_4 in a separate vial, were then quickly added to the vial, giving an initial $^{14}CO_2$ concentration in the vial of 340 μl/l. For the chase in $^{12}CO_2$, the leaf blades were quickly pulled out of the $^{14}CO_2$-feeding vial and transferred to another vial with conditions similar to those described for preillumination. At intervals, the leaf blades were quickly killed by plunging into boiling 80% (v/v) ethanol. The leaves were boiled for an additional 5 to 10 min, ground thoroughly with a mortar and pestle with the aid of a small amount of acid-washed sand, and extracted again, once with 80% ethanol and twice with water. All extracts were pooled and concentrated to less than 0.5 ml. The radioactivity in the insoluble fractions of all preparations accounted for less than 7% of the total ^{14}C fixed. Separation and identification of the labeled photosynthetic products were accomplished using two-dimensional thin-layer electrophoresis and chromatography methods similar to those described previously (20). Recovery of radioactivity from the plates ranged from 90 to 110%.

RESULTS AND DISCUSSION

Previously published data on leaf anatomy, Γ, and O_2 inhibition of net photosynthesis (1, 12) have demonstrated that *F. trinervia* and *F. ramosissima* represent C_4 and C_3–C_4 intermediate *Flaveria* species, respectively. In addition to the earlier results (1, 16), further evidence obtained in this study establishes *F. cronquistii* as a C_3 species. *F. cronquistii* exhibited high Γ values (58 ± 3 μl/l), while net photosynthesis was inhibited by atmospheric O_2 by 32.8 ± 2.8%. These results indicate that *F. cronquistii* possesses a high level of photorespiration typical of C_3 plants, and in contrast with the C_4 species *F. trinervia* where net photosynthesis is insensitive to atmospheric O_2 (2.0 ± 1.4% inhibition) and apparent photorespiration is lacking (Γ < 1 μl/l). Furthermore, *F. ramosissima* exhibits intermediate photosynthetic characteristics to both of the above species, with reduced sensitivity of net photosynthesis to 21% O_2 (20.9 ± 2.0% inhibition) and a low Γ (6 ± 1 μl/l).

To determine the initial products of photosynthesis by the three *Flaveria* species, pulse-chase experiments with $^{14}CO_2$-$^{12}CO_2$ were performed on detached leaves. In the first experiment, the primary photosynthetic products labeled during the pulse in $^{14}CO_2$ were followed for up to 30 s in both the C_4 species, *F. trinervia*, and the C_3-C_4 intermediate species, *F. ramosissima* (Fig. 1). In *F. trinervia*, after 3 s of photosynthesis in $^{14}CO_2$, malate and aspartate accounted for 94% of the total soluble labeled products, while the label in PGA and sugar phosphates totaled less than 5%. Very little label was found in serine or glycine. The per cent label in malate and aspartate decreased,

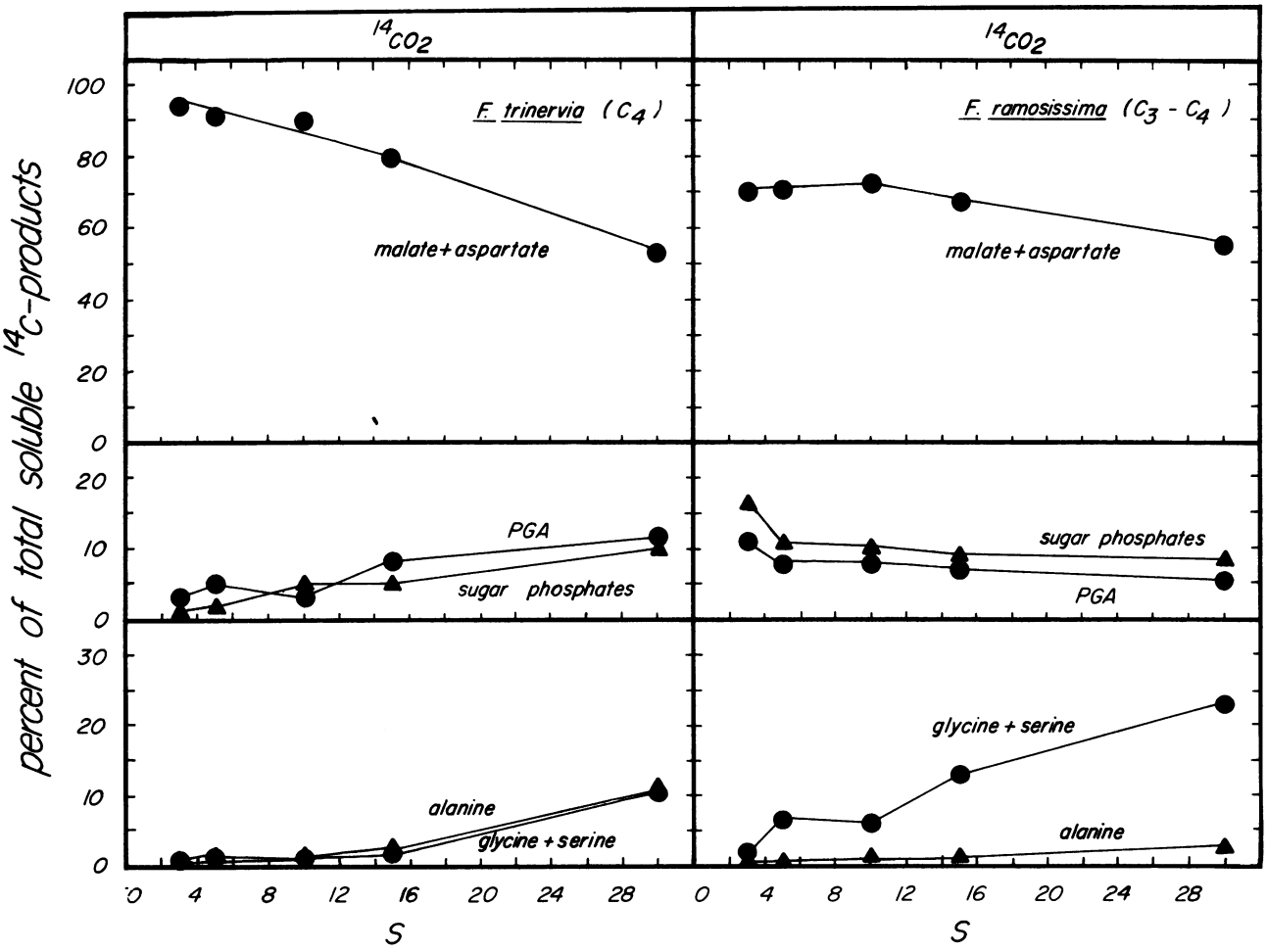

FIG. 1. Changes in the distribution of radioactivity among soluble ^{14}C-labeled products after pulsing for various time periods with $^{14}CO_2$ in *F. trinervia* and *F. ramosissima*. The pulse experiments were performed at 25 to 27°C. The initial $^{14}CO_2$ concentration for the pulse was 340 μl/l. The maximum depletion of CO_2 in the vial (*e.g.* after 30 s of photosynthesis) was estimated to be less than 50%, based on rates of photosynthesis and the sizes of leaves used.

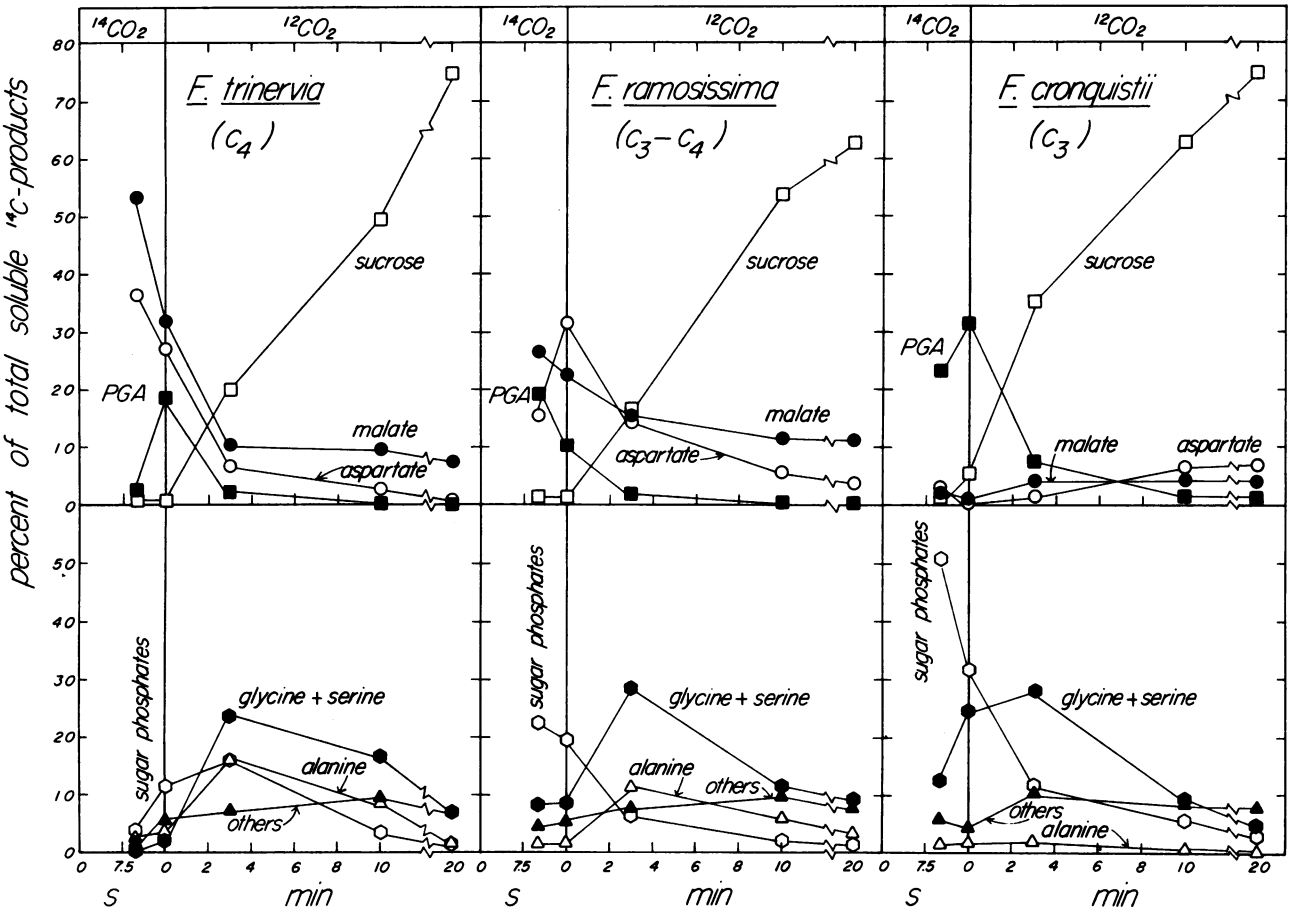

FIG. 2. Changes in the distribution of radioactivity among soluble ^{14}C-labeled products after pulsing with $^{14}CO_2$ and chasing with $^{12}CO_2$ in *F. trinervia*, *F. ramosissima*, and *F. cronquistii*. The pulse-chase experiments were performed at 25 to 27°C. The initial $^{14}CO_2$ concentration for the pulse was 340 μl/l. The maximum depletion of CO_2 in the vial (*e.g.* after 15 s of pulse-photosynthesis) was estimated to be less than 25%, based on rates of photosynthesis and the sizes of leaves used.

whereas the per cent label in PGA, sugar phosphates, and serine plus glycine increased with a longer pulse duration. These results are consistent with those previously recorded for typical C_4 plants (5, 7, 18). The extrapolation of per cent label in malate and aspartate to 100% at zero time of pulse indicates that atmospheric CO_2 is fixed directly through the C_4 pathway in *F. trinervia*. This is as would be expected in C_4 species if all the atmospheric CO_2 is fixed through the C_4 pathway in the mesophyll cells. In the C_3–C_4 intermediate species *F. ramosissima*, malate and aspartate also accounted for a large portion of the primary photosynthetic products, although less than found in *F. trinervia*. After 3 s of $^{14}CO_2$ fixation, 70% of the total soluble ^{14}C-products were found in the two C_4 acids and about 28% of the label was in PGA and sugar phosphates. The per cent label in malate and aspartate remained the same with a pulse period up to 10 s and then decreased slowly thereafter. As compared with *F. trinervia*, the per cent label in glycine and serine increased relatively faster with an increase in pulse duration, from 2% after a 3-s pulse to 23% after a 30-s pulse, indicating the operation of photorespiration. These results suggest that, in *F. ramosissima*, atmospheric CO_2 is being assimilated directly through the C_4 and C_3 pathways simultaneously, with the major portion of CO_2 fixation via the C_4 pathway. If phosphoenolpyruvate carboxylase and ribulose 1,5-bisphosphate carboxylase are differentially localized as in C_4 plants, simultaneous fixation of atmospheric CO_2 by the C_4 and C_3 pathways in *F. ramosissima* could be due to the availability of external CO_2 to both mesophyll and bundle sheath cells. Alternatively, it could be due to the presence of both carboxylases in the mesophyll cells. Information on the intercellular localization of key photosynthetic enzymes of the C_4 and C_3 pathways in *F. ramosissima* is needed to resolve this question.

In a further experiment, the primary photosynthetic products labeled during a pulse in $^{14}CO_2$, and the changes of the labeled products during a subsequent chase in $^{12}CO_2$, were determined for *F. cronquistii*, *F. ramosissima*, and *F. trinervia* (Fig. 2). After 10 s of photosynthesis in $^{14}CO_2$, malate and aspartate accounted for approximately 90% of the total label in soluble products in the C_4 species, *F. trinervia*, while PGA and sugar phosphates accounted for about 75% of the total soluble labeled products in the C_3 species *F. cronquistii*. Only 5% of the radioactivity was found in malate and aspartate in the C_3 species. The labeling pattern of primary photosynthetic products of the C_3–C_4 intermediate species, *F. ramosissima*, is intermediate between that of the C_4 and C_3 *Flaveria* counterparts. The intermediate species incorporated 42% of the total soluble ^{14}C-label into malate and aspartate and 42% into PGA and sugar phosphates. The results shown in both Figures 1 and 2 indicate a greater percentage of labeling (*e.g.* after 10 s) in C_4 acids in *F. trinervia* than in *F. ramosissima*. However, despite the variation in percentage of label appearing in malate and aspartate in *F. ramosissima* between the two experiments, a significantly high amount (at least 42%) of label was always recovered in the C_4 acids in the intermediate species. Some variation might be expected between experiments on separate plants when atmospheric CO_2 is fixed

simultaneously through two carboxylases. The experiments in both Figures 1 and 2 demonstrate that the intermediate *Flaveria* species assimilates exogenous CO_2 through both the C_4 and C_3 pathways of photosynthesis simultaneously.

The fate of labeled C_4-acids was further evaluated during a chase period of up to 20 min in $^{12}CO_2$. During the chase, labeled malate and aspartate decreased in both *F. trinervia* and *F. ramosissima*. However, the apparent turnover rate for the labeled C_4-acids was slower in *F. ramosissima* than in *F. trinervia*. For example, after a 3-min chase, labeled malate and aspartate decreased by 67% and 75%, respectively, in the C_4 *Flaveria* species. The corresponding values in the C_3–C_4 intermediate species *F. ramosissima* were 32% and 54%, respectively. The turnover of C_4 acids indicates that, in these species, they are further metabolized, presumably via the major C_4-acid decarboxylation enzyme detected, NADP-malic enzyme (12). The lower percentage labeling and slower turnover of C_4 acids in *F. ramosissima* may be due to lower activities of the C_4 cycle enzymes in this species (12). The slower turnover in *F. ramosissima* could also be accounted for if it has a larger active pool of C_4 acids than *F. trinervia*, or a longer distance of transport from the site of C_4-acid formation to the site of decarboxylation. Clearly, C_4 photosynthesis is functional and operating in this C_3-C_4 intermediate, although the capacity for C_4 photosynthesis is about one-half that of the C_4 species.

There was considerable label in glycine and serine in *F. cronquistii* (25%) but little in *F. trinervia* (2%) at the end of the 15-s pulse (Fig. 2). This would be expected for a C_3 and C_4 plant, respectively, if glycine and serine were being labeled via the photorespiratory cycle. Relative to *F. cronquistii*, the C_3–C_4 intermediate species *F. ramosissima* showed a reduced incorporation of label into these two intermediates at the end of the pulse (9%). This is consistent with the evidence, based on labeling of C_4 acids, that C_4 photosynthesis is functioning simultaneously with C_3 photosynthesis in this species, which would reduce the pool sizes of photorespiratory intermediates. Photorespiration could also be inhibited by elevated internal CO_2 levels (due to release of CO_2 from malate decarboxylation), since CO_2 and O_2 are competitive inhibitors with respect to each other for photorespiration *versus* photosynthesis (13).

The results show that the photosynthetic intermediate, *F. ramosissima*, is a true biochemical intermediate between a C_3 and C_4 plant with regard to CO_2 assimilation through the C_3 and C_4 pathways and with regard to photorespiratory activity. It is evident that a limited expression of C_4 photosynthesis in a species may prove beneficial in reducing photorespiration. In other C_3–C_4 intermediates such as *P. milioides* and *M. arvensis*, CO_2 is fixed solely by the C_3 pathway (6, 8, 11, 21), and the biochemical basis for intermediate photosynthetic characteristics is not yet known. It appears that the C_3–C_4 *Flaveria* species are biochemically more advanced than *P. milioides* and *M. arvensis* in expression of C_4 characteristics.

Acknowledgments—The authors are grateful to R. A. Kennedy for the use of his facilities and help on two-dimensional thin-layer electrophoresis and chromatography.

LITERATURE CITED

1. APEL P, I MAASS 1981 Photosynthesis in species of *Flaveria*. CO_2 compensation concentration, O_2 influence on photosynthetic gas exchange and $\delta^{13}C$ values in species of *Flaveria* (Asteraceae). Biochem Physiol Pflanz 197: 396–399
2. APEL P, H OHLE 1979 CO_2-compensation point and leaf anatomy in species of the genus *Moricandia* DC (Cruciferae). Biochem Physiol Pflanz 174: 68–75
3. ATKINS CA, JS PATE 1977 An IRGA technique to measure CO_2 content of small volumes of gas from the internal atmosphere of plant organs. Photosynthetica 11: 214–216
4. BROWN RH, WV BROWN 1975 Photosynthetic characteristics of *Panicum milioides*, a species with reduced photorespiration. Crop Sci 15: 681–685
5. CHEN TM, RH BROWN, CC BLACK 1971 Photosynthetic $^{14}CO_2$ fixation products and activities of enzymes related to photosynthesis in bermudagrass and other plants. Plant Physiol 47: 199–203
6. EDWARDS GE, MSB KU, MD HATCH 1982 Photosynthesis in *Panicum milioides*, a species with reduced photorespiration. Plant Cell Physiol 23: 1185–1195
7. HATCH MD 1971 C_4-pathway photosynthesis: evidence for an intermediate pool of carbon dioxide and the identity of the donor of C_4 dicarboxylic acid. Biochem J 125: 425–432
8. HOLADAY AS, R CHOLLET 1983 Photosynthetic/photorespiratory carbon metabolism in the C_3–C_4 intermediate species, *Moricandia arvensis* and *Panicum milioides*. Plant Physiol 73: 740–745
9. HOLADAY AS, AT HARRISON, R CHOLLET 1982 Photosynthetic/photorespiratory CO_2 exchange characteristics of the C_3–C_4 intermediate species, *Moricandia arvensis*. Plant Sci Lett 27: 181–189
10. HOLADAY AS, KW LEE, R CHOLLET 1984 C_3–C_4 intermediate species in the genus *Flaveria*: leaf anatomy, ultrastructure, and the effect of O_2 on the CO_2 compensation concentration. Planta 150: 25–32
11. HOLADAY AS, Y-J SHIEH, KW LEE, R CHOLLET 1981 Anatomical, ultrastructural and enzymic studies of leaves of *Moricandia arvensis*, a C_3–C_4 intermediate species. Biochim Biophys Acta 637: 334–341
12. KU MSB, RK MONSON, RO LITTLEJOHN, H NAKAMOTO, DB FISHER, GE EDWARDS 1983 Photosynthetic characteristics of C_3-C_4 intermediate *Flaveria* species I. Leaf anatomy, photosynthetic responses to O_2 and CO_2, and activities of key enzymes in the C_3 and C_4 pathways. Plant Physiol 71: 944–948
13. LORIMER GH 1981 The carboxylation and oxygenation of ribulose 1,5-bisphosphate: the primary events in photosynthesis and photorespiration. Annu Rev Plant Physiol 32: 349–383
14. MORGAN JA, RH BROWN 1979 Photosynthesis in grass species differing in carbon dioxide fixation pathways II. A search for species with intermediate gas exchange and anatomical characteristics. Plant Physiol 64: 257–262
15. MORGAN JA, RH BROWN, BJ REGER 1980 Photosynthesis in grass species differing in carbon dioxide fixation pathways III. Oxygen response and enzyme activities of species in the Laxa group of *Panicum*. Plant Physiol 65: 156–159
16. NAKAMOTO H, MSB KU, GE EDWARDS 1983 Photosynthetic characteristics of C_3–C_4 intermediate *Flaveria* species II. Kinetic properties of phosphoenolpyruvate carboxylase from C_3, C_4 and C_3–C_4 intermediate species. Plant Cell Physiol 24: 1387–1393
17. RATHNAM CKM, R CHOLLET 1979 Photosynthetic carbon metabolism in *Panicum milioides*, a C_3–C_4 intermediate species: evidence for a limited C_4 dicarboxylic acid pathway of photosynthesis. Biochim Biophys Acta 548: 500–519
18. REPO, E, MD HATCH 1976 Photosynthesis in *Gomphrena celosioides* and its classification amongst C_4-pathway plants. Aust J Plant Physiol 3: 863–876
19. SAYRE RT, RA KENNEDY 1977 Ecotypic differences in the C_3 and C_4 photosynthetic activity in *Mollugo verticillata*, a C_3–C_4 intermediate. Planta 134: 257–262
20. SCHÜRMANN P 1969 Separation of phosphate esters and algal extracts by thin-layer electrophoresis and chromatography. J Chromatogr 39: 507–509
21. WINTER K, H USUDA, M TSUZUKI, MR SCHMITT, GE EDWARDS, RJ THOMAS, RF EVERT 1982 Influence of nitrate and ammonium on photosynthetic characteristics and leaf anatomy of *Moricandia arvensis*. Plant Physiol 70: 616–625