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Photosynthetic Enzyme Activities and Localization in *Mollugo verticillata* Populations Differing in the Levels of C_3 and C_4 Cycle Operation[1]

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ABSTRACT

Ecotypic differences in the photosynthetic carbon metabolism of *Mollugo verticillata* were studied. Variations in C_3 and C_4 cycle activity are apparently due to differences in the activities of enzymes associated with each pathway. Compared to C_4 plants, the activities of C_4 pathway enzymes were generally lower in *M. verticillata*, with the exception of the decarboxylase enzyme, NAD malic enzyme. The combined total carboxylase enzyme activity of *M. verticillata* was greater than that of C_3 plants, possibly accounting for the high photosynthetic rates of this species. Unlike either C_3 or C_4 plants, ribulose bisphosphate carboxylase was present in both mesophyll and bundle sheath cell chloroplasts in *M. verticillata*. The localization of this enzyme in both cells in this plant, in conjunction with an efficient C_4 acid decarboxylation mechanism most likely localized in bundle sheath cell mitochondria, may account for intermediate photorespiration levels previously observed in this species.

Plants which assimilate CO_2 by the C_4 pathway of photosynthesis are characterized by their initial incorporation of CO_2 into four-carbon acids, their high photosynthetic rates, and lack of detectable photorespiration (14, 25). In contrast, C_3 plants incorporate CO_2 directly into the Calvin or C_3 cycle; at high light intensities they generally have lower photosynthetic rates than C_4 plants and higher levels of photorespiration (35). While most plants can be classified as C_3 or C_4 based on their photosynthetic pathway, few plants have features which are intermediate between these two groups of plants (5, 21). These C_3-C_4 intermediates may represent evolutionary links between C_3 and C_4 plants and, interestingly, are all members of genera which contain both C_3 and C_4 species (31).

Mollugo verticillata was first reported (21) to be a C_3-C_4 intermediate based on its equal labeling of PGA[4] and C_4 acids during short term exposure to $^{14}CO_2$, its intermediate levels of photorespiration, and high photosynthetic rates. We have recently shown (21, 31) that *M. verticillata* populations may also exhibit ecotypic variation in their photosynthetic characteristics. Populations from warm, dry environments had greater C_4 activity than did those from cool, moist habitats, indicating, as often suggested (25), an adaptive advantage of C_4 photosynthesis over C_3 photosynthesis in such environments. The most C_4-like population of the four studied was from Kansas. This population had the highest photosynthetic rate and greatest labeling of four-carbon acids after short term exposures to $^{14}CO_2$ (31). The Kansas population also had an intermediate CO_2 compensation point of 25 μl/l and a 12% enhancement of photosynthesis under low O_2. Three other populations from Iowa, Mexico, and Massachusetts had lower photosynthetic rates and lower amounts of ^{14}C-labeled C_4 acids (31). These three populations also had identical CO_2 compensation points of 40 μl/l and their rates of photosynthesis were enhanced equally (21%) under low O_2.

In this report we present additional physiological characteristics of the photosynthetic and ecotypic variation in *M. verticillata*. Photosynthetic enzyme activities, enzyme localization, and leaf anatomies of four populations were studied and compared to those of C_3 and C_4 plants. The results indicate that the intermediate photosynthetic carbon metabolism and ecotypic differentiation among *M. verticillata* populations are due to differences in the levels of certain C_3 and C_4 cycle photosynthetic enzyme activity.

MATERIALS AND METHODS

All plants were grown and sampled as previously described (31). Chl and leaf protein levels were determined spectrophotometrically by the methods of Arnon and Lowry, respectively (1, 27). For interveinal measurements, leaf tissue was fixed in Nawashins fixative, aspirated, dehydrated with n-butyl alcohol, and embedded with Paraplast plus. Embedded material was sectioned to a thickness of 10 μm on an A0280 rotary microtome and stained with safranin and fast green.

Enzyme Assays. One g leaf tissue was ground at 4 C in a mortar and pestle containing a small amount of sand and 4 ml of grinding media consisting of 0.1 M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1.0 mM EDTA, 20 mM mercaptoethanol and 2% (w/v) PVP-10. Leaf extracts were then filtered through four layers of Miracloth and centrifuged at 10,000*g* for 10 min at 4 C. The supernatant was used for all enzyme assays, the assays were run at 25 C, and the rates expressed on a protein and fresh weight basis.

RuBP carboxylase/oxygenase (EC 4.1.1.39) activities were assayed under similar conditions except as follows. Crude enzyme extracts were incubated for 10 min in a solution of 0.1 M Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 5.0 mM mercaptoethanol, and 50 mM

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[4] Abbreviations: PGA: 3-phosphoglyceric acid; PEP: phosphoenolpyruvate; RuBP: ribulose 1,5-bisphosphate; PBS: phosphate-buffered saline, FITC: fluorescein isothiocynanate; OAA: oxaloacetate.

$NaH^{14}CO_3$. The reactions were started by the addition of 2.5 mM RuBP for the carboxylase and 0.5 mM RuBP for the oxygenase. The carboxylase assay was terminated by the addition of glacial acetic acid. For the carboxylase assay, an aliquot was removed, dried, and quantified by liquid scintillation counting. Oxygenase activity was determined by the depletion of O_2 in a water jacketed cuvette using a Clarke-type electrode. The initial O_2 concentration was 0.237 μM O_2 ml^{-1} at 25 C (30). Rates were linear for 5 min and control assays were complete except for the addition of RuBP.

PEP carboxylase (EC 4.1.1.31) was assayed by the incorporation of $H^{14}CO_3^-$ into acid-stable products. The reaction mixture contained 150 mM Tricine (pH 7.3), 50 mM $MgCl_2$, 40 mM mercaptoethanol, 10 mM $NaH^{14}CO_3$, 5.0 mM PEP, and 5.0 mM glutamate. Reactions were initiated by the addition of crude enzyme extract, terminated with acetic acid and counted as described above.

NAD malic enzyme (EC 1.1.1.38) and NADP malic enzyme (EC 1.1.1.40) activities were monitored spectrophotometrically by the reduction of pyridine nucleotide at 340 nm (12, 18). Assays were carried out in 3-ml cuvettes containing 2.5 mM Tris-HCl (pH 8.3), 0.5 mM EDTA, 2.5 mM malate, crude enzyme extract, and either 0.25 mM NADP or 2.0 mM NAD with 0.75 μM CoA. NAD malic enzyme reactions were initiated by the addition of 5.0 mM $MnCl_2$, and NADP malic enzyme assays were started by 5.0 mM $MgCl_2$. Absorbance changes averaged 0.06/min and an extinction coefficient of 6.23×10^6 mM^{-1} cm^{-1} was used.

PEP carboxykinase (EC 4.1.1.49) was assayed according to the method of Hatch (10). Reaction cuvettes contained 50 mM Hepes (pH 7.2), 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 3 units of pyruvate kinase (Sigma), 0.25 mM ATP, 0.5 mM OAA, and enzyme. The decrease in OAA was monitored spectrophotometrically at 280 nm using an extinction coefficient of 1,200 μM^{-1} cm^{-1}.

Oxidation of NADH at 340 nm was used to monitor malate dehydrogenase (EC 1.1.1.37) activity (28). The reaction cuvette contained 100 mM Tricine (pH 7.5), 0.2 mM EDTA, 0.1 mM NADH, and crude enzyme extract. OAA was added to start the reaction after determining a basal rate of NADH oxidation.

A coupled enzyme system was used to assay aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) (11, 13). The aspartate aminotransferase preparation contained 25 mM Tricine (pH 8.0), 1.5 mM pyridoxal phosphate, 2.0 mM EDTA, 0.1 mM NADH, 1.5 units malate dehydrogenase (Sigma), and crude enzyme extract. Reactions were started after a 10-min preincubation by the addition of 1.25 mM α-ketoglutarate. Alanine aminotransferase reaction mixtures contained 25 mM Tricine (pH 7.25), 2.5 mM DTT, 4.0 mM alanine, 0.03 mM pyridoxal phosphate, 2.0 mM EDTA, 0.1 mM NADH, 1.5 units of lactate dehydrogenase (Sigma), and crude enzyme extract. After a 10-min preincubation, 2.5 mM α-ketoglutarate was added to start the reaction. NADH oxidation was followed at 340 nm.

Pyruvate-Pi-dikinase (EC 2.7.9.1) activity was assayed spectrophotometrically using the coupled assay procedure of Sugiyama (33). Reaction cuvettes contained 0.1 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 1.25 mM pyruvate, 5.0 mM DTT, 0.16 mM NADH, 2.5 mM K_2KPO_4, 50 mM $NaHCO_3$, 2 units of corn PEP carboxylase, 3 units of malate dehydrogenase (Sigma), and enzyme. Reactions were started after a 3-min preincubation with 1.25 mM ATP and NADH oxidation was monitored at 340 nm.

A phenylhydrazone assay was used to measure glycolate oxidase (EC 1.1.3.1) activity. A 3-ml cuvette contained 67 mM phosphate buffer (pH 8.3), 3.3 mM cysteine HCl, 3.3 mM phenylhydrazine HCl, and 6.7 mM Na-glycolate. Reactions were started by adding 0.2 ml of crude enzyme extract and glycolate-phenylhydrazone formation was monitored at 324 nm.

Immunological Methods. Purified antigen was prepared from commercially obtained RuBP carboxylase (Sigma) according to the method of Sprey (32). Ten mg of protein in 0.1 M Tris HCl (pH 7.8) containing 4 mM $MgCl_2$ was fractionally precipitated with $(NH_4)_2SO_4$. Protein precipitating between 35 and 55% saturation was collected, dialyzed, and chromatographed on a Sephadex G-100 column. The protein peak corresponding to RuBP carboxylase, measured by enzyme activity, was reprecipitated with 55% $(NH_4)_2SO_4$ and stored at −4 C. Before use in animal immunization, protein was pelleted by centrifugation (10,000g for 10 min) and dissolved in 0.1 M Tris-HCl (pH 7.8), 4 mM $MgCl_2$ and 0.9% NaCl. Enzyme homogeneity was confirmed using starch gel electrophoresis (32).

Antisera to RuBP carboxylase were produced in New Zealand white rabbits by footpad injection of 5 to 6 mg of protein emulsified in complete Freund's adjuvant, followed by an intravenous boost with 1 mg protein 30 days later. Animals were partially bled from the marginal ear vein 7 days after the boost and the sera harvested by conventional methods. Animals were given a second boost of 3.5 mg 90 days after the primary injection and were exsanguinated by cardiac puncture on day 97. The antisera were tested for reactivity to RuBP carboxylase by double immunodiffusion in 1% agarose in 0.1 M Veronal buffer (pH 8.6) with 3% PEG 6000 (9).

Fresh leaf material for indirect immunofluorescent antibody labeling was prepared according to the method of Hattersley *et al.* (17). Sections (1 × 5 mm) were vacuum-infiltrated and fixed in 70% ethanol for 2 h. Leaves were sectioned (10- to 20-μm thick) with a mechanical microtome and washed in PBS (0.1 phosphate, 0.2 M NaCl, pH 7.5). The sections were then incubated in 0.9 ml of rabbit antisera or normal rabbit sera, 1:15 dilution with PBS, for 1 h. After rinsing three times in PBS (20 min), they were transferred to a vial containing 0.9 ml of FITC-labeled sheep antirabbit antisera (Miles Laboratories), 1:15 dilution PBS, for 1 h in the dark. The leaf sections were rinsed as before and mounted in 50% glycerol. *Zea mays* and *M. verticillata* sections were incubated in immunized sera and examined on a Leitz Orthaphan fluorescence microscope with a wide-band, blue, high intensity filter (E. Leitz, Inc., Rockleigh, N. J.). Photography was done with an Orthomat W. automatic camera (E. Leitz, Inc.) using Kodak high speed film (Ektachrome, ASA 200).

RESULTS

Interveinal distance has been used (16) as one criterion to distinguish between C_3 and C_4 grass species. The shorter interveinal distances of C_4 grasses have been suggested to facilitate the transfer of photosynthate from the chlorenchyma to the vasculature, accounting for the higher vascular loading rates reported in C_4 plants (16). Interveinal distance, leaf Chl, and protein levels of the four *M. verticillata* populations are shown in Table I. All four *M. verticillata* populations had interveinal distances of about 150 μm, a value intermediate between the average interveinal distances observed in C_3 and C_4 grasses. Chl *a/b* ratios were also between the values reported for C_3 and C_4 plants (5) and paralleled the relative degree of C_4 cycle metabolism in the four populations tested. Total Chl, ranging from 1.45 to 2.23 mg Chl g^{-1} fresh weight, appears to be unrelated to the degree of C_4 pathway activity recorded among the populations. No significant difference was observed among the populations in leaf protein levels with average values being equal to those reported (4) for other species.

On the basis of carboxylating enzymes, the Kansas population had the highest photosynthetic capacity of the four populations studied (Table II). This population has a RuBP carboxylase activity of 9.6 μmol mg^{-1} protein h^{-1}, a PEP carboxylase activity of 1.85 μmol mg^{-1} protein h^{-1}, and a RuBP carboxylase to PEP carboxylase ratio of 5.2 (Tables II and VI). The three other populations had lower amounts of these enzymes and were ranked in the order of Iowa, Mexico, and Massachusetts.

For PEP carboxylase, the relative enzyme activity of the four populations (Table II) also reflected levels of C_4 acid labeling during short term $^{14}CO_2$ exposures (31). This is in contrast to the relationship between early C_3 cycle product labeling and RuBP

Table I. *Chl, Soluble Leaf Protein, and Interveinal Distances for M. verticillata Populations*

Plant Species	Chl[1]	Chl *a/b* Ratio	Soluble Protein	Leaf Interveinal Distances
	mg/g fresh weight		*mg/g fresh weight*	*μm*
M. verticillata				
Kansas	2.18 ± 0.34	3.45 ± 0.18	24.97 ± 2.55	154.8 ± 19.4
Iowa	2.23 ± 0.34	3.25 ± 0.32	29.60 ± 6.46	145.9 ± 10.6
Mexico	1.69 ± 0.25	3.06 ± 0.04	27.65 ± 4.62	151.3 ± 15.5
Mass.	1.45 ± 0.18	2.79 ± 0.20	27.10 ± 3.93	143.0 ± 15.7

[1] ± SE; N = 6 to 8.

Table II. *Carboxylase Activity in Leaf Extracts*

Plant Species	RuBP Carboxylase[1]		PEP Carboxylase	
	μmol/g fresh weight·h	*μmol/mg protein·h*	*μmol/g fresh weight·h*	*μmol/mg protein·h*
M. verticillata				
Kansas	159 ± 19.5	9.6 ± 0.52	30.6 ± 1.42	1.85 ± 0.39
Iowa	146 ± 31.1	9.38 ± 0.37	19.4 ± 1.52	1.25 ± 0.42
Mexico	134 ± 20.4	8.43 ± 0.22	19.2 ± 3.87	1.21 ± 0.44
Mass.	126 ± 6.3	6.06 ± 0.26	20.4 ± 2.91	0.97 ± 0.48
Nicotiana sp.	140 ± 10.8	7.14 ± 0.91	17.1 ± 2.01	0.87 ± 0.10
P. oleracea	50 ± 3.0	4.37 ± 0.31	275.0 ± 18.9	25.8 ± 1.85

[1] ± SE; N = 6 to 8.

carboxylase activity, again reflecting the importance of C_4 pathway photosynthesis to carbon assimilation in this species. Although the Kansas population had the highest RuBP carboxylase activities, it had the lowest labeling of C_3 cycle products after 3-s $^{14}CO_2$ incorporations. Compared to *Nicotiana*, a C_3 plant, and *Portulaca oleracea*, a C_4 plant, *M. verticillata* (Kansas) had twice the PEP carboxylase activity of the C_3 species, but one-tenth that of the C_4 species (Table II).

In C_4 plants, three different C_4 acid-decarboxylating mechanisms are presently known, each being catalyzed by a different bundle sheath cell enzyme (12). PEP carboxykinase type C_4 plants decarboxylate OAA in the bundle sheath cytoplasm to release CO_2, whereas NADP malic enzyme and NAD malic enzyme C_4 plants decarboxylate malate in the chloroplasts and mitochondria, respectively. As shown in Table III, NAD malic enzyme activity of *M. verticillata* was approximately equal to that of *P. oleracea*, an NAD malic enzyme type C_4 plant. Also like NAD malic enzyme C_4 plants, the levels of the other decarboxylating enzyme, NADP malic enzyme and PEP carboxykinase, were negligible (12, 28). The actual levels of NAD malic enzyme in Kansas, Iowa, and Mexico populations were approximately 6.6 μmol mg^{-1} protein h^{-1}. The nature of the decarboxylating system in *M. verticillata* is also suggested by previous cytological investigations (24). Laetsch (24) observed numerous mitochondria adjacent to the inner bundle sheath cell wall of this species, as often seen in NAD malic enzyme type C_4 plants (21) such as *Mollugo cerviana* (24). These facts, plus the rapid C_4 acid turnover rates previously demonstrated (31) indicate a functional NAD malic enzyme system probably localized in the bundle sheath cells of *M. verticillata*.

Relative to C_3 plants, aspartate and alanine aminotransferase activities of C_4 plants are characteristically much greater (11). These enzymes catalyze the amination and deamination of C_4 cycle intermediates in mesophyll and bundle sheath cells. The activities of these enzymes in *M. verticillata* populations were less than half those of the C_4 plant, *P. oleracea*, but from 10 to 60% greater than *Nicotiana*, a C_3 species (Table IV). The Kansas population again had the highest aspartate aminotransferase enzyme activity of 5.1 μmol mg^{-1} protein h^{-1}, followed by the Iowa population (4.9 μmol mg^{-1} protein h^{-1}) and the Mexico and Massachusetts populations (3.5 μmol mg^{-1} protein h^{-1} each). Alanine aminotransferase activity was less than that of aspartate aminotransferase ranging from 4.5 μmol mg^{-1} protein h^{-1} for the Kansas population, to 3.1 μmol mg^{-1} protein h^{-1} for the Mexico population, values which are lower than those of both C_3 and C_4 species (Table IV). Although we obtained a pyruvate-Pi-dikinase activity of 106 μmol g^{-1} fresh weight h^{-1} in *P. oleracea*, no similar enzyme activity was detected in *M. verticillata*. The lack of detectable pyruvate-Pi-dikinase activity indicates an incomplete C_4 cycle in this C_3-C_4 intermediate species (15, 33).

In previous work (32) three of the *M. verticillata* populations (Iowa, Mexico, and Massachusetts) had identical CO_2 compensation points of 40 μl/l and their photosynthetic rates were enhanced to a similar degree under 2% O_2. The Kansas population, in similar analyses, had significantly lower photorespiratory activity. In the present report, RuBP oxygenase and glycolate oxidase enzymes were assayed to determine their relationship to the photorespiratory differences observed earlier among the four populations. In general, the RuBP oxygenase activity paralleled the RuBP carboxylase activity for each species or population (Tables II, V, and VI). Thus, the Kansas population had the highest RuBP oxygenase activity, while the lowest oxygenase activity was found in the Massachusetts population (Table V). All populations had RuBP carboxylase/oxygenase ratios averaging about 10 (Table VI).

Like RuBP oxygenase activity, there was no direct relationship between glycolate oxidase activity and the previously reported levels of photorespiration in the four populations. All four populations had similar glycolate oxidase activities, averaging 3.1 μmol

Table III. *Malic Enzyme and Malate Dehydrogenase Activity in Leaf Extracts*

Plant Species	NAD Malic Enzyme[1]		Malate Dehydrogenase	
	μmol/g fresh weight·h	*μmol/mg protein·h*	*μmol/g fresh weight·h*	*μmol/mg protein·h*
M. verticillata				
Kansas	111 ± 11.65	6.7 ± 0.44	1504 ± 178	86.7 ± 8.8
Iowa	103 ± 3.89	6.6 ± 0.44	1367 ± 191	76.1 ± 15.6
Mexico	101 ± 9.50	6.4 ± 0.05	1327 ± 97	65.8 ± 18.5
Mass.	98.2 ± 9.69	4.7 ± 0.18	1304 ± 97	66.0 ± 14.7
Nicotiana sp.	74.3 ± 4.99	3.8 ± 0.13	1516 ± 37	79.0 ± 3.48
P. oleracea	62.3 ± 2.73	6.8 ± 0.59	900 ± 26	89.3 ± 4.88

[1] NADP malic enzyme <1.5 μmol/g fresh weight·h; PEPCK <5.0 μmol/g fresh weight·h. ±SE; N = 6 to 11.

Table IV. *Aminotransferase Activity in Leaf Extracts*

Plant Species	Aspartate Aminotransferase[1]		Alanine Aminotransferase	
	μmol/g fresh weight·h	*μmol/mg protein·h*	*μmol/g fresh weight·h*	*μmol/mg protein·h*
M. verticillata				
Kansas	135 ± 13.5	5.1 ± 0.76	118 ± 17.9	4.5 ± 0.96
Iowa	141 ± 11.5	4.9 ± 0.73	96 ± 15.5	3.4 ± 0.77
Mexico	89 ± 13.5	3.5 ± 0.87	85 ± 7.84	3.1 ± 0.54
Mass.	90 ± 5.8	3.5 ± 0.24	92 ± 12.9	3.6 ± 0.83
Nicotiana sp.	65 ± 11.0	3.2 ± 0.24	117 ± 7.15	5.6 ± 1.14
P. oleracea	182 ± 5.8	12.3 ± 0.22	117 ± 9.82	10.4 ± 0.63

[1] ±SE; N = 6 to 11.

Table V. *RuBP Oxygenase and Glycolate Oxidase Activity in Leaf Extracts*

Plant Species	RuBP Oxygenase[1]		Glycolate Oxidase	
	μmol/g fresh weight·h	*μmol/mg protein·h*	*μmol/g fresh weight·h*	*μmol/mg protein·h*
M. verticillata				
Kansas	25.7 ± 2.11	0.99 ± 0.05	79.7 ± 9.22	3.29 ± 0.38
Iowa	23.2 ± 1.26	0.84 ± 0.05	89.7 ± 3.40	3.30 ± 0.23
Mexico	23.4 ± 1.64	0.76 ± 0.03	92.9 ± 6.36	3.10 ± 0.23
Mass.	19.8 ± 3.07	0.64 ± 0.03	82.3 ± 6.84	2.78 ± 0.34
Nicotiana sp.	21.6 ± 1.99	0.97 ± 0.04	79.4 ± 6.15	3.34 ± 0.28
P. oleracea	8.85 ± 1.59	0.42 ± 0.06	6.96 ± 1.15	0.49 ± 0.06

[1] ±SE; N = 6 to 11.

mg^{-1} protein h^{-1}, values which were approximately equal to those in the C_3 species, but much greater than the C_4 species (Table V).

The ratios of photosynthetic to photorespiratory enzymes and RuBP carboxylase/oxygenase enzyme kinetics have been used to model the photorespiratory activity of C_3 and C_4 plants (2, 20, 22, 26, 29). As shown in Table VI, neither the RuBP carboxylase/oxygenase or RuBP carboxylase/PEP carboxylase ratios were correlated with the CO_2 compensation point, another indicator of photorespiration. In contrast, the RuBP oxygenase/PEP carboxylase ratio was correlated with compensation point ($r = 0.95$, $P < 0.01$), as previously shown with *Panicum miliodes*, and best accounted for the photorespiratory differences among *M. verticillata* populations and between C_3 and C_4 species (22).

Although there is now general agreement on the location of RuBP carboxylase exclusively in the bundle sheath cells of C_4 plants (7, 17, 28), the localization of this enzyme in *M. verticillata*, with its anatomical features of both C_3 and C_4 plants, is unknown and its location is critical to any explanation of its intermediate C_3-C_4 photosynthesis. In the present study, RuBP carboxylase was localized microscopically using fluorescent antisera (17).

The purity of RuBP carboxylase protein used in immunization was confirmed by the observation of only a single, slow moving band in starch gel electrophoresis. Antisera were shown to be reactive against RuBP carboxylase by double immunodiffusion; no reactivity was shown by normal rabbit sera. Immunological specificity was also verified by the localization of RuBP carboxylase antisera fluorescence exclusively in the bundle sheath chloroplasts of *Zea mays* (Fig. 1). In *M. verticillata*, leaf sections treated with antisera to RuBP carboxylase exhibited FITC fluorescence in both mesophyll and bundle sheath chloroplasts (Figs 2 and 3). The presence of RuBP carboxylase in all chlorenchymatous tissue in *M. verticillata* is therefore similar to C_3 plants.

Table VI. *Enzyme Activity Ratios for Species with Different Levels of Apparent Photorespiration*

Plant Species	RuBPC/RuBPO	RuBPC/PEPC	RuBPO/PEPC	Compensation Point
				µl/l
M. verticillata				
Kansas	9.7	5.2	0.54	24
Iowa	11.2	7.55	0.67	40
Mexico	11.1	6.96	0.63	40
Mass.	9.5	6.18	0.66	40
Nicotiana sp.	7.4	8.18	1.11	60
P. oleracea	10.4	0.17	0.02	1–2

DISCUSSION

The results presented in this paper generally demonstrate that the ecotypic differences in photosynthetic gas exchange and $^{14}CO_2$-labeling patterns which we previously reported (31) are associated with corresponding variations in the C_3 and C_4 cycle enzyme activity among the four *M. verticillata* populations. This is best illustrated by the relationship between four-carbon acid synthesis under short term $^{14}CO_2$ exposures (31) and the PEP carboxylase activity of each population (Table II). Unlike the relationship between higher PEP carboxylase activity and greater levels of C_4 acid synthesis, populations which had higher RuBP carboxylase activity had lower levels of C_3 cycle product labeling upon first

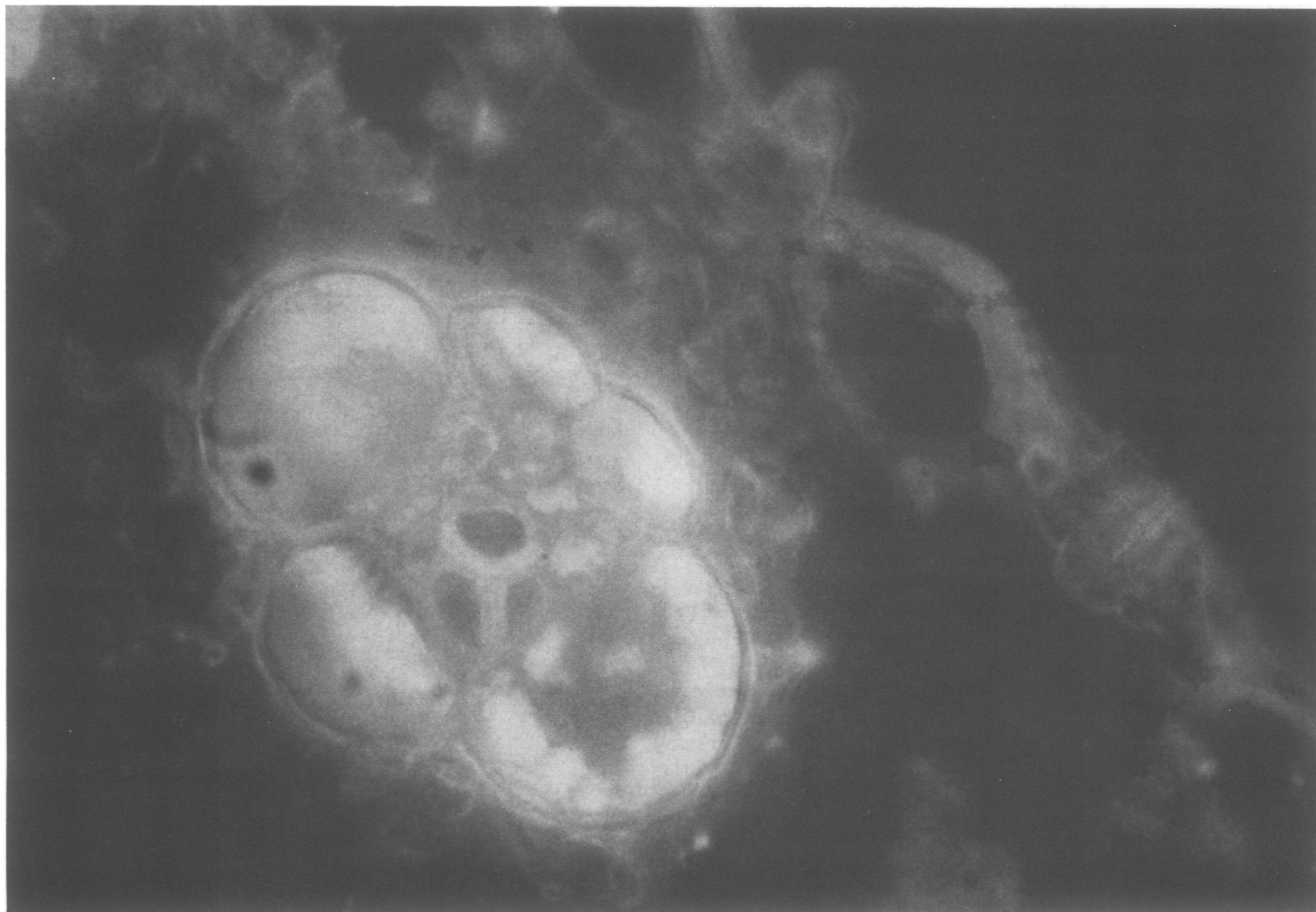

FIG. 1. Immunofluorescent labeling of RuBP carboxylase in *Z. mays* leaf cross-sections showing specific fluorescence localized only in bundle sheath cell chloroplasts of this C_4 plant.

FIG. 2. Immunofluorescent labeling of RuBP carboxylase in *M. verticillata* leaf sections showing specific fluorescence in all chlorenchymatous cells.

exposure to $^{14}CO_2$. Since the specific activities of PEP carboxylase in *M. verticillata* were much less than those found in C_4 plants and no more than one-fifth of their RuBP carboxylase activity, the disproportionately high level of early C_4 acid labeling may be best explained by the higher carboxylation efficiency or lower K_m for CO_2 of PEP carboxylase (34). This higher carboxylation efficiency, relative to RuBP carboxylase, may be dependent upon the cellular localization of PEP carboxylase, its greater affinity for CO_2 and/or its lack of O_2 inhibition (29, 30, 34).

Although the activities of aspartate and alanine aminotransferase and malate dehydrogenase in the four populations were generally lower than those of *P. oleracea*, a C_4 plant, they were greater than the PEP carboxylase specific activity for each population and are sufficient to account for the C_4 cycle activity in this intermediate species. NAD malic enzyme activity was equal to that of *P. oleracea*, an NAD malic enzyme type C_4 species. The high NAD malic enzyme activity in *M. verticillata* explains at least in part the previously observed rapid turnover of C_4 acids, as seen in ^{14}C kinetic studies (31).

One of the more intriguing aspects of carbon metabolism in this C_3-C_4 intermediate is its apparent lack of pyruvate-Pi-dikinase activity. In C_4 plants, the continued operation of the C_4 pathway is dependent upon this enzyme (15), but this may not be true in *M. verticillata.* The metabolism of pyruvate, its cellular localization, and the role of PEP in C_4 acid metabolism in *M. verticillata*, remain to be answered.

We found that the intermediate levels of photorespiration in *M. verticillata* and those of C_3 and C_4 plants were best correlated with RuBP oxygenase/PEP carboxylase ratios. The lower levels of photorespiration in C_4 plants are dependent upon it being the initial CO_2 fixation by PEP carboxylase (7, 29). Equally important is the subsequent decarboxylation of C_4 acid products, which elevate bundle sheath cell CO_2 concentrations and competitively reduce RuBP oxygenase activity (7, 25, 28). Other investigators (22) have also shown that RuBP oxygenase/PEP carboxylase ratios were correlated with the photorespiratory activity in C_3 and

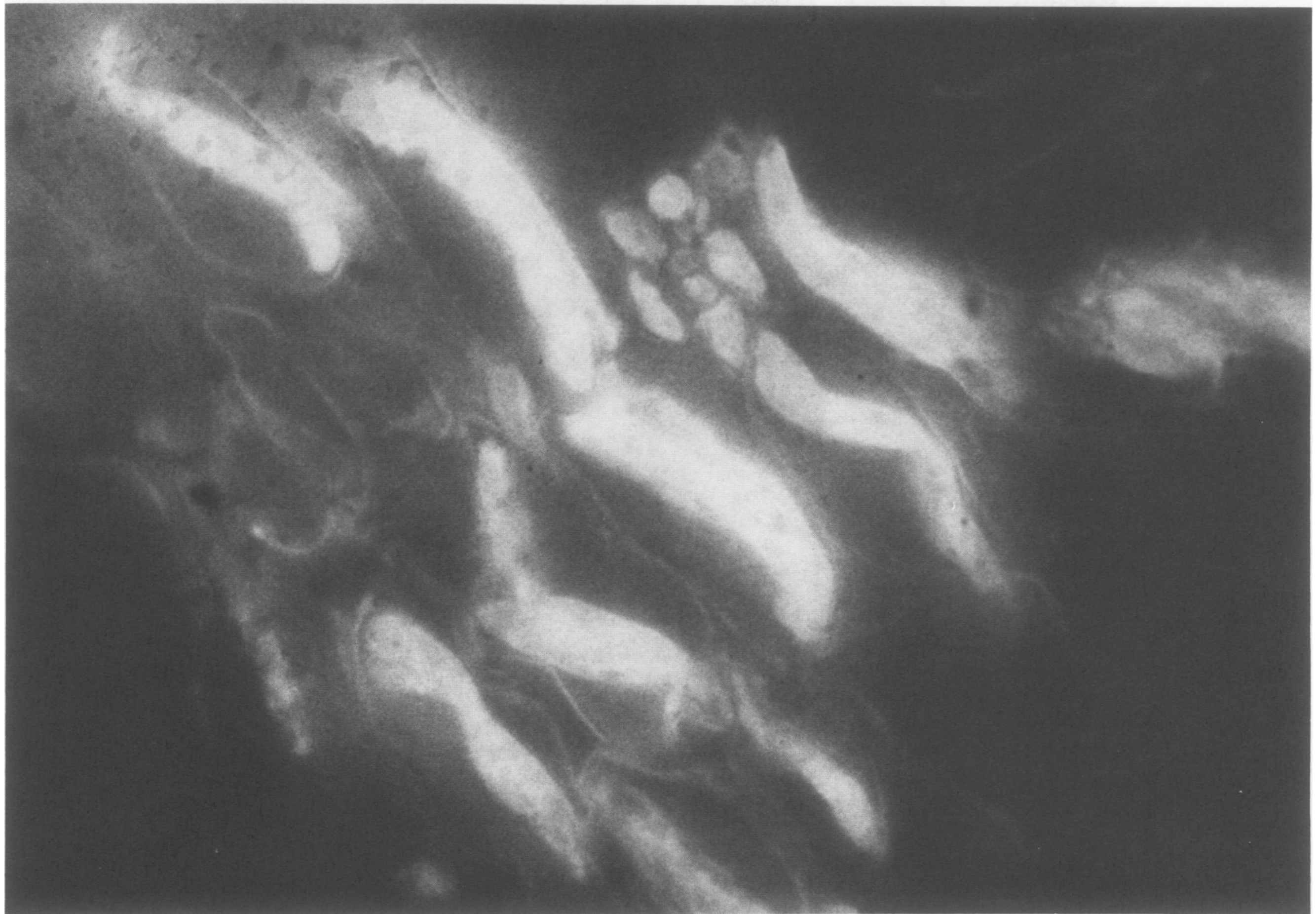

FIG. 3. Immunofluorescent labeling of RuBP carboxylase in *M. verticillata* leaf sections showing specific fluorescence in the chloroplasts of bundle sheath cells surrounding the vasculature.

C_4 species, and in *Panicum miliodes*, another C_3-C_4 intermediate. Later studies have questioned this relationship based on the low levels of C_4 acid metabolism in *P. miliodes* (8, 19, 20, 23). In contrast, C_4 acid metabolism made a significant contribution to photosynthesis (31) in *M. verticillata*.

Cytologically, the bundle sheath cells of *M. verticillata* appear similar to those of C_4 plants (21, 24). As shown by immunofluorescence, these cells have many large chloroplasts which contain the Calvin cycle enzyme RuBP carboxylase, in addition to numerous mitochondria (24). CO_2 released from the decarboxylation of C_4 acids may then be refixed within these cells and account for *M. verticillata's* intermediate photorespiration rates.

In this study, it was our purpose to characterize further the ecotypic differentiation of photosynthetic characteristics among *M. verticillata* populations and to compare them to C_3 and C_4 plants. The differential activity of C_3 and C_4 cycle enzymes among the four *M. verticillata* populations best explains the ecotypic variation in photosynthetic physiology. Additionally, the apparent lack of pyruvate-Pi-dikinase activity could indicate a noncyclic synthesis of PEP for C_4 photosynthesis in *M. verticillata*.

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