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ANATOMICAL, ULTRASTRUCTURAL AND ENZYMIC STUDIES OF LEAVES OF *MORICANDIA ARVENSIS*, A C_3-C_4 INTERMEDIATE SPECIES *

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The possibility that reduced photorespiration in the crucifer *Moricandia arvensis* is due to a limited C_4-photosynthesis system similar to that in the C_3-C_4 intermediate *Panicum milioides* (Rathnam, C.K.M. and Chollet, R. (1979) Biochim. Biophys. Acta 548, 500–519) was investigated. The anatomical, physiological and biochemical features of this crucifer which are similar to those of *P. milioides* are: (a) the presence of prominent leaf vascular bundle sheaths which contain numerous, centripetally arranged chloroplasts and mitochondria; (b) a CO_2 compensation concentration of 16 ± 3 μl/l at 21% O_2 and 25°C; (c) a C_3-type phospho*enol*pyruvate carboxylase exhibiting a maximal velocity which is 2–3-times that of the enzyme from C_3 plants; and (d) aspartate and alanine aminotransferase activities which are 2–3-fold higher than in a representative C_3 species. However, *M. arvensis* differs from the C_3-C_4 intermediate *Panicum* species in that the activities of the three known C_4 acid decarboxylating enzymes present in leaves of C_4 plants (NAD- and NADP-malic enzymes and phospho*enol*pyruvate carboxykinase) and pyruvate, orthophosphate dikinase (a key C_4-related enzyme) are low or undetectable. From these comparative results we conclude that the mechanism by which photorespiration is reduced in *M. arvensis* is qualitatively different from the limited C_4-like CO_2-concentrating system operating in *P. milioides*.

Introduction

Three *Panicum* species (*P. milioides*, *P. decipiens*, *P. schenckii*) and possibly one *Mollugo* species (*M. verticillata*) have been identified as being intermediate between C_3 and C_4 plants with respect to leaf anatomy and photorespiratory activity [1–3]. The bundle sheath cells of these intermediate plants contain numerous chloroplasts. Furthermore, in the C_3-C_4 *Panicum* species, the bundle sheath chloroplasts and prominent mitochondria are arranged centripetally as in NAD-malic enzyme-type C_4 plants [2–4]. The photosynthetic carbon metabolism of these naturally occurring intermediate plants has only been extensively characterized for *P. milioides*. Based on detailed comparative studies in our laboratory we recently proposed [4] that CO_2 fixation in this plant occurs via two pathways, a limited, but functional C_4 pathway and the conventional Calvin cycle. The Calvin cycle is the dominant pathway of CO_2 assimilation and occurs in both mesophyll and bundle sheath cells. The limited C_4 cycle, which resembles that in NAD-malic enzyme-type C_4 plants [2], is of sufficient activity to concentrate CO_2 in the bundle sheath and reduce the amount of O_2 fixation catalyzed by bundle sheath ribulosebisphosphate carboxylase/oxygenase, thereby reducing photorespiration.

Crookston [5] and Krenzer et al. [6] reported that the crucifer *Moricandia arvensis*, grown from seeds

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Abbreviations: Γ, CO_2 compensation concentration; Chl, chlorophyll; C_4 acids, malate and aspartate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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obtained from the Royal Botanic Gardens at Kew (U.K.), exhibited rates of photorespiration and values of Γ which were intermediate between those of representative C_3 and C_4 plants. However, these workers described the leaf anatomy as being typical of a dicotyledonous C_3 plant. More recently, studies in our own laboratory (Holaday, A.S. and Chollet, R., unpublished data) and those of Apel [7,8], using plants from the Botanical Garden at the University of Leipzig, revealed that several anatomical, physiological and CO_2-exchange characteristics of *M. arvensis* and a related species, *M. spinosa*, were similar to those of other C_3-C_4 intermediates (especially *P. milioides* [3,4,9,10]), including C_3-type $\delta^{13}C$ values. The results of these studies indicate that photosynthesis in *M. arvensis* and *M. spinosa* is less sensitive to O_2 than in typical C_3 plants, and that photorespiration is reduced relative to photosynthesis.

Biochemical studies in the laboratory of Apel [11] suggested that the reduction of photorespiration in *M. arvensis* was not due to altered kinetic properties of ribulosebisphosphate carboxylase/oxygenase. However, the phospho*enol*pyruvate carboxylase activity in whole leaf extracts prepared from this species was twice that in *M. foetida*, a related species with C_3-type CO_2-exchange characteristics [7,11]. Similarly, previous biochemical studies of *P. milioides* had revealed that in vitro phospho*enol*pyruvate carboxylase activity was greater than that of a related C_3 *Panicum* species [12].

Therefore, consistent evidence has been presented which indicates that *M. arvensis* is intermediate between C_3 and C_4 plants with respect to photorespiration. The results of preliminary anatomical and biochemical studies suggest that a limited C_4-like system similar to that in *P. milioides* might be responsible for reducing photorespiration in *M. arvensis*. This report presents the results of leaf ultrastructural studies and comparative enzymic analyses of whole leaf extracts using *M. arvensis*, *M. foetida* and representative C_3 and C_4 species to examine the possibility that a limited C_4 cycle exists in the crucifer *M. arvensis*.

Materials and Methods

Anatomical and enzymic analyses were conducted using *M. arvensis* (L.) DC. and *M. foetida* Bourg plants grown from seeds which were generously supplied by Dr. P. Apel. Plants of *M. arvensis* grown from seeds obtained from the Royal Botanic Gardens at Kew were compared with the Leipzig plants and these two cultivars were found to be identical in all features examined. Thus, only the information on the Leipzig plants is presented. Representative C_3 (*Lolium perenne* L.) and C_4 [*Zea mays* L. (NADP-malic enzyme-type C_4), *P. miliaceum* L. and *Portulaca oleracea* L. (NAD-malic enzyme-type C_4), *P. maximum* Jacq. (phospho*enol*pyruvate carboxykinase-type C_4)] plants were included in the enzymic analyses for comparison with the *Moricandia* species. All of the plants were grown in controlled environment rooms under a 16 h photoperiod (250 $\mu E \cdot m^{-2} \cdot s^{-1}$) except for *P. oleracea*, which was collected locally. The *Moricandia* and *Lolium* species were grown at 21°C/16°C (day/night) while the C_4 species were grown at 31°C/27°C. The plants were fertilized twice weekly with a modified Hoagland's solution. Young, fully expanded leaves of the *Moricandia* species were used in all studies.

For transmission electron microscopy, narrow tissue strips were dissected from the leaves midway between the leaf margin and midrib, cut into small pieces, and fixed, dehydrated and embedded in Epon 812 as previously described [13]. The specimens were sectioned with a diamond knife on an LKB Ultrotome III, stained for 5 min in 2% (w/v) aqueous uranyl acetate followed by 5 min in basic lead citrate [13], and viewed in a Philips 201 electron microscope operating at 60 kV. For light microscopy of the Epon-embedded material, 1- to 2-μm sections were cut with glass knives and stained with a solution of 0.5% toluidine blue in 1% H_3BO_3.

For the enzyme assays, whole leaf extracts were prepared by grinding 0.5–1 g fresh weight of preilluminated leaf material at 4°C in a Ten Broeck homogenizer. The grinding solutions for the analysis of phospho*enol*pyruvate carboxylase, NAD- and NADP-malic enzyme and aspartate and alanine aminotransferase activities contained, at pH 7.5, 50 mM Hepes-NaOH, 1 mM $MgCl_2$ (10 mM for phospho*enol*pyruvate carboxylase), 1 mM $MnCl_2$, 10 mM dithiothreitol, 0.1 mM Na_2EDTA and 2% (w/v) insoluble polyvinylpyrrolidone. After grinding, sufficient Triton X-100 was added to the extracts for NAD-malic enzyme analysis to yield a final concentration of

0.05% (v/v). For analysis of aspartate and alanine aminotransferase activities, pyridoxal phosphate was added to give a final concentration of 80 μM. The extraction solutions were the same as previously described for analysis of ribulosebisphosphate carboxylase [14], phospho*enol*pyruvate carboxykinase [15] and NADP-malate dehydrogenase [16]. For pyruvate, orthophosphate dikinase, the leaf tissue was homogenized in 50 mM Hepes-KOH (pH 7.6), 10 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM KH_2PO_4 and 2.5 mM sodium pyruvate. The leaf material was illuminated (10 klx) during homogenization for the latter two, light-activated enzymes. Prior to centrifugation (12 000 × *g*) of the crude extracts, aliquots were removed for determination of chlorophyll content [13]. The supernatant fluids were gel filtered through Sephadex G-25/PD-10 columns (Pharmacia Fine Chemicals) which had been equilibrated with the appropriate grinding solution. Enzyme assays were performed at 30°C using the gel-filtered extracts. Essentially identical results were obtained when centrifuged, crude leaf extracts were used.

Analysis and kinetic studies of phospho*enol*pyruvate carboxylase activity (0.075–2.4 mM phospho*enol*pyruvate, ±2 mM glucose 6-phosphate) were performed spectrophotometrically at 340 nm [17]. Activities were verified using an $H^{14}CO_3^-$-based assay procedure [18]. The activity of ribulosebisphosphate carboxylase was determined as described elsewhere [14].

Gel-filtered extracts to be analyzed for NAD-malic enzyme, NADP-malate dehydrogenase and pyruvate, orthophosphate dikinase activities were first incubated under N_2 for 1 h at 25°C. The spectrophotometric assay of Chapman and Hatch [19] was used for NAD-malic enzyme analysis. The inclusion of 75 μM CoA in the reaction medium (pH 7.8) gave the maximum activities for this Mn^{2+}-dependent enzyme. The spectrophotometric assay for NADP-malate dehydrogenase activity [16] contained (in 1 ml) 50 mM Tris-HCl (pH 8.8), 1 mM Na_2EDTA, 15 mM dithiothreitol, 0.25 mM NADPH, 2 mM oxaloacetate and 0.1 ml extract. The addition of substrate initiated the reaction. Pyruvate, orthophosphate dikinase activity was determined by measuring ATP-, pyruvate- and P_i-dependent $H^{14}CO_3^-$ fixation in the presence of 3 units each of wheat phospho*enol*pyruvate carboxylase (Boehringer Mannheim) and bovine heart NADH-malate dehydrogenase (Sigma) [20]. The reaction was initiated with ATP or 0.1 ml extract and terminated after 2 min with 0.1 ml of 4 M HCl.

The activity of NADP-malic enzyme was determined in a 1 ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.8), 0.2 mM Na_2EDTA, 5 mM dithiothreitol, 5 mM L-malate, 1 mM NADP, 10 mM $MgCl_2$ or $MnCl_2$, and 0.1 ml gel-filtered extract. The reaction was initiated by adding Mg^{2+} or Mn^{2+}. The inclusion of 75 μM CoA in the reaction mixture did not enhance the rate of NADP reduction, thus establishing that the NADP-dependent oxidation of malate at pH 7.8 was due to catalysis by NADP-malic enzyme. The assay used to determine phospho*enol*pyruvate carboxykinase activity was the $H^{14}CO_3^-$/oxaloacetate exchange reaction described by Dittrich et al. [15]. The reactions were terminated after 3 min by adding 0.1 ml of 4 M HCl saturated with phenylhydrazine. The assay procedures for aspartate and alanine aminotransferases were similar to those described by Hatch and Mau [21] except that 10 mM aspartate and 5 mM 2-oxoglutarate were used.

In the $H^{14}CO_3^-$-based assays outlined above, unfixed $^{14}CO_2$ was removed after acidification by drying the samples in a stream of air. The amount of acid-stable ^{14}C fixed was determined by liquid scintillation spectroscopy.

Results and Discussion

The presence of a chloroplast-containing bundle sheath is essential to the functioning of C_4 photosynthesis [2] and the limited C_4 cycle in *P. milioides* [4]. Light microscopic analysis of *M. arvensis* leaf cross-sections confirmed earlier findings [7] which suggested that the vascular tissue is surrounded by a sheath of large, chloroplast-containing cells (Fig. 1). More notably, these cells contain considerably more chloroplasts than do the mesophyll cells, and the majority of organelles in the bundle sheath cells are arranged centripetally. In contrast, those in the mesophyll cells are dispersed throughout the peripheral cytosol. The typical C_4 Kranz anatomy consisting of mesophyll cells encircling the bundle sheath is not present, however. Instead, the mesophyll of *M. arvensis* is differentiated into an upper palisade and a lower spongy layer of chlorenchyma (Fig. 1), as found in typical dicotyledonous C_3 plants and the

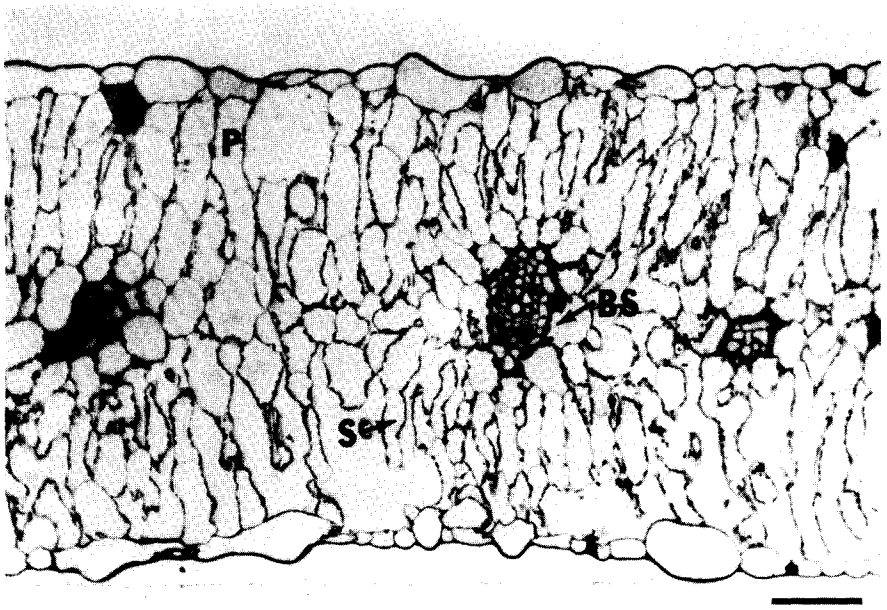

Fig. 1. Leaf cross-section of *Moricandia arvensis*. Note that the vascular tissue is surrounded by bundle sheath cells (BS) which contain numerous, centripetally arranged chloroplasts and that the mesophyll is differentiated into both palisade (P) and spongy (S) chlorenchyma layers. The horizontal bar represents 50 μm.

putative C_3-C_4 intermediate *Mollugo verticillata* [22].

Ultrastructural analysis of the bundle sheath region in *M. arvensis* (Fig. 2) highlighted the centripetal arrangement of most of the granal, starch-containing chloroplasts in this layer. Associated with these chloroplasts are numerous, prominent mitochondria which are clustered along the cell wall adjacent to the vascular tissue. The preponderance of mitochondria occurs only in the bundle sheath cells and not in the spongy and palisade mesophyll. The presence of numerous, closely associated chloroplasts and mitochondria arranged centripetally in the bundle sheath cells of *M. arvensis* is strikingly similar to the situation in NAD-malic enzyme-type C_4 plants and *P. milioides* [3,4,23]. In marked contrast, the bundle sheath cells in *M. Foetida* resemble the mesophyll cells in that they contain much fewer chloroplasts and mitochondria and these organelles are dispersed throughout the peripheral cytosol (Fig. 2).

The anatomical and ultrastructural similarities between *M. arvensis* and *P. milioides*, in conjunction with their similar CO_2-exchange characteristics (Refs. 1, 5–7, 9, 10; and Holaday, A.S. and Chollet, R., unpublished data), suggest that a limited C_4 cycle might also exist in *M. arvensis* to reduce photorespiration. To investigate this possibility, whole leaf extracts from *M. arvensis* were analyzed to determine the activities of various enzymes associated with C_4 and C_3 photosynthesis. An important feature of the biochemistry of the C_3-C_4 intermediate *P. milioides* is that the in vitro phospho*enol*pyruvate carboxylase activity is several times greater than that in related C_3 species [12,18]. Similarly, the activity of this enzyme in whole leaf extracts from *M. arvensis* is 2–3-times that in *M. foetida* and *L. perenne* (Tables I and II). In contrast, there is little difference in ribulosebisphosphate carboxylase activity between the three plants (Table I). Although the in vitro activity of phospho*enol*pyruvate carboxylase from *M. arvensis* is much less (8–25%) than that in representative C_4 plants (Table I), its similarity to the activity in extracts from *P. milioides* [12] suggests that the enzyme in *M. arvensis* might have sufficient in vivo activity to participate in a limited C_4-like system.

Leaf phospho*enol*pyruvate carboxylase in C_3 plants differs from that in C_4 species with respect to

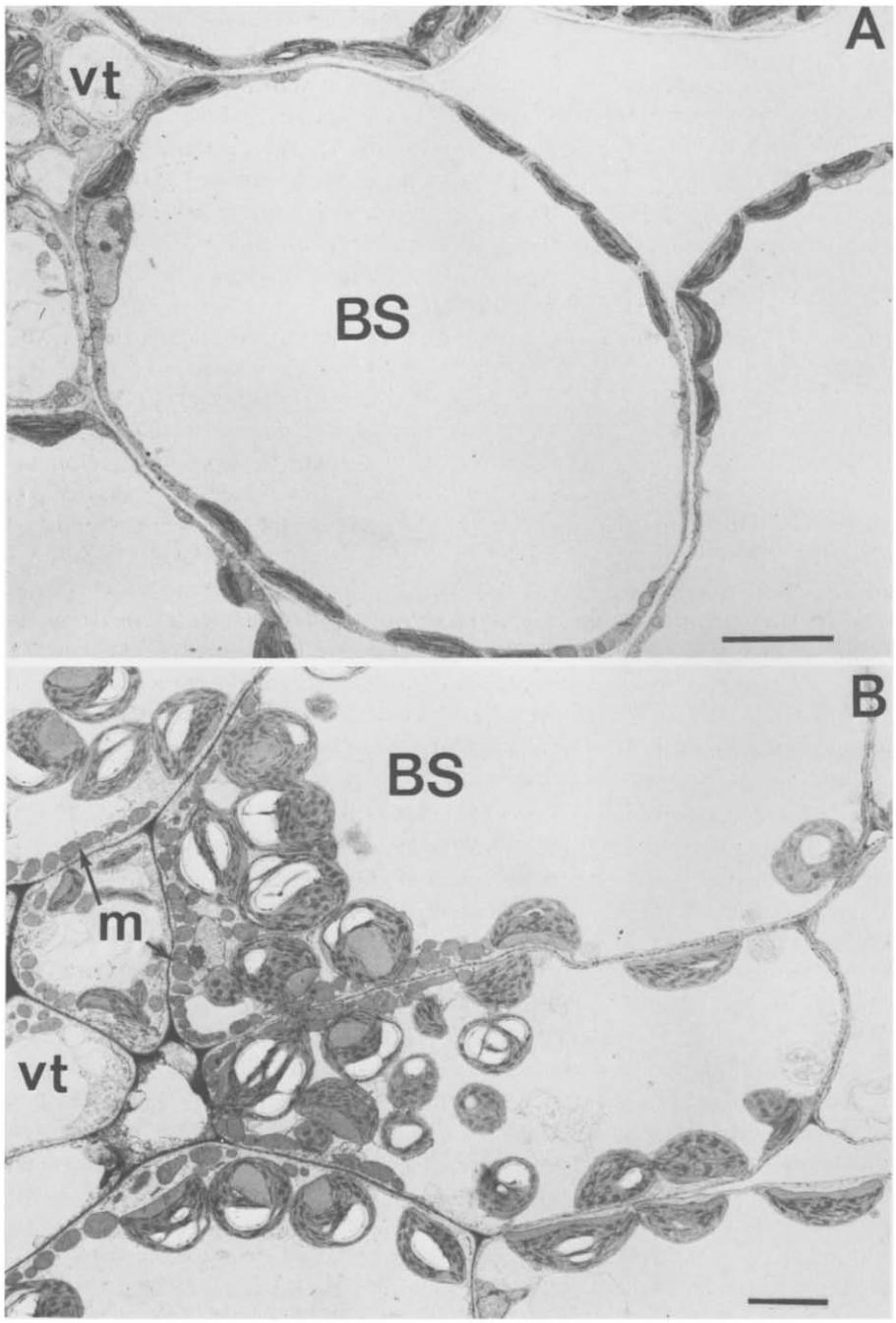

Fig. 2. *Moricandia* leaf ultrastructure. (A) *M. foetida* cross-section showing the presence of only a few mitochondria and granal chloroplasts dispersed throughout the peripheral cytosol of the bundle sheath (BS). (B) *M. arvensis* cross-section highlighting the centripetal arrangement of numerous granal, starch-containing chloroplasts and prominent mitochondria (m) in the bundle sheath (BS) layer. The horizontal bars represent 5 μm. vt, vascular tissue.

TABLE I

CARBOXYLASE ACTIVITIES IN LEAF EXTRACTS OF *M. ARVENSIS*, *M. FOETIDA* AND REPRESENTATIVE C_3 AND C_4 SPECIES

The centrifuged leaf extracts were gel filtered through Sephadex G-25 before analysis at 30°C. PEP, phospho*enol*pyruvate; RuBP, ribulosebisphosphate. Activities are expressed in μmol/mg Chl per h.

Species	PEP carboxylase	RuBP carboxylase
M. arvensis	101	614
M. foetida	54	549
L. perenne	36	513
Z. mays	401	158
P. oleracea [a]	1 202	

[a] Field-grown material.

kinetic properties and the effects of certain activators such as glucose 6-phosphate [24,25]. The enzyme in *P. milioides* was recently reported to have a K' (phospho*enol*pyruvate) value (similar to a K_m value) and other properties which are typical of the C_3 form of the enzyme [18]. In the absence of glucose 6-phosphate, the enzyme in *M. arvensis* extracts exhibits an apparent affinity for phospho*enol*pyruvate which is similar to that in *L. perenne* (Table II). In contrast, the K_m value for the *Z. mays* carboxylase is more than 10-fold higher than the value for the C_3 form of the enzyme (Table II and Ref. 24). The presence of glucose 6-phosphate decreases the K_m (phospho*enol*pyruvate) values only slightly and does not affect the V values for phospho*enol*pyruvate carboxylase from *M. arvensis* and *L. perenne* (Table II). However, as previously reported for the C_4 form of the enzyme [25], the addition of glucose 6-phosphate decreases the K_m (phospho*enol*pyruvate) value markedly and results in a small increase in the V value for the *Z. mays* enzyme (Table II). Thus, like the carboxylase in *P. milioides* [18], the enzyme from *M. arvensis* has kinetic and regulatory properties most similar to C_3 phospho*enol*pyruvate carboxylase.

TABLE II

THE EFFECT OF GLUCOSE 6-PHOSPHATE ON THE V AND K_m VALUES OF PHOSPHO*ENOL*PYRUVATE CARBOXYLASE EXTRACTED FROM LEAVES OF *M. ARVENSIS* AND REPRESENTATIVE C_3 AND C_4 SPECIES

Activities were measured at pH 8.3, 30°C, 5 mM $MgCl_2$ and 0.075–2.4 mM phospho*enol*pyruvate (PEP), ±2 mM glucose 6-phosphate (G6P). In all cases the phospho*enol*pyruvate saturation curves showed no trace of sigmoidicity, and double-reciprocal plots of velocity versus phospho*enol*pyruvate concentration yielded straight lines.

Species	K_m (PEP) (μM)		V (μmol/mg Chl per h)	
	−G6P	+G6P	−G6P	+G6P
M. arvensis	59	40	93	99
L. perenne	71	40	44	43
Z. mays	858	130	547	716

The in vitro phospho*enol*pyruvate carboxylase activity observed for *M. arvensis* would not be sufficient, per se, to account for lower photorespiratory rates. To function in this capacity, the enzyme must operate as part of a complete system, possibly like the limited C_4 cycle in *P. milioides*. If a similar system were operative in *M. arvensis*, then the activities of enzymes involved in C_4 acid decarboxylation and phospho*enol*pyruvate regeneration would also have to be sufficient to account for the reduction of photorespiration. In leaf extracts from *P. milioides*, sufficient activity of NAD-malic enzyme was detected to utilize C_4 acids at rates roughly comparable to those of C_4 acid synthesis catalyzed by phospho*enol*pyruvate carboxylase [12]. The CoA-stimulated activity of NAD-malic enzyme in *M. arvensis*, although 5-fold greater than that in *L. perenne*, is considerably less than the activity in leaf extracts of *P. milioides* [12] and *P. miliaceum*, an NAD-malic enzyme-type C_4 plant (Table III). In addition, even though the NADP-malic enzyme activity in *M. arvensis* extracts is considerably higher than that in *P. milioides* [12] and *L. perenne* (Table III), it is still less than 50% of the in vitro C_4 acid decarboxylase activity in *P. milioides* [12] and only 8% of the NADP-malic enzyme activity in *Z. mays* (Table III). Furthermore, *M. foetida* extracts have NAD- and NADP-malic enzyme activities which are essentially identical to those in *M. arvensis* (Table III). These data, together with the low phospho*enol*pyruvate carboxykinase activity in *M. arvensis* (Table III), indicate that, on a whole leaf basis, the potential for C_4 acid decarboxylation in this intermediate species is much less than that in *P. milioides*.

TABLE III

ACTIVITY OF VARIOUS ENZYMES ASSOCIATED WITH C_4 PHOTOSYNTHESIS IN LEAF EXTRACTS OF *M. ARVENSIS*, *M. FOETIDA* AND REPRESENTATIVE C_3 AND C_4 SPECIES

The centrifuged leaf extracts were gel filtered through Sephadex G-25 before analysis at 30°C. PEP, phospho*enol*pyruvate. n.d., no detectable ATP-, P_i-, and pyruvate-dependent activity.

Enzyme	Activity (μmol/mg Chl per h)					
	M. arvensis	*M. foetida*	*L. perenne*	*Z. mays*	*P. miliaceum*	*P. maximum*
NAD-malic enzyme (Mn^{2+})	22	23	4	19	215	
NADP-malic enzyme (Mg^{2+})	37	40	17	473		
PEP carboxykinase	5	2	0			561
NADP-malate dehydrogenase	216	192	183	946		
Aspartate aminotransferase	595	617	178	193	1501	
Alanine aminotransferase	560	1031	257	63	2099	
Pyruvate, orthophosphate dikinase	n.d.	n.d.	n.d.		249	

The activities of aspartate and alanine aminotransferases in *P. milioides* leaf homogenates, although not as great as in extracts prepared from *P. miliaceum*, are considerably greater than in a related C_3 species [12]. Relative to *L. perenne*, *M. arvensis* contains similarly high activities of these two enzymes (Table III). However, even higher activities are found in *M. foetida* extracts. Thus, the presence of very active aspartate and alanine aminotransferases appears to be a feature common to *Moricandia* species, irrespective of their relative activities of phospho*enol*pyruvate carboxylase (Table I) and rates of photorespiration.

A critical step in the operation of a C_4-like pathway is the synthesis of phospho*enol*pyruvate, the initial carboxylation substrate. In C_4 species, pyruvate, orthophosphate dikinase catalyzes the formation of phospho*enol*pyruvate from pyruvate, ATP and P_i. The activity of this enzyme in C_3 leaf tissue is not detectable, whereas in *P. milioides* the in vitro activity is similar to the elevated activity of phospho*enol*pyruvate carboxylase [12]. When extracts from both *Moricandia* species and *L. perenne* were analyzed for the presence of pyruvate, orthophosphate dikinase, no ATP-, P_i-, and pyruvate-dependent activity was found (Table III). The use of gel-filtered extracts and the results from reciprocal mixing experiments with crude leaf homogenates of *P. miliaceum* minimized the possibility that the lack of detectable activity of this key C_4 enzyme was an artifact due to the presence of low molecular weight inhibitors or specific proteases. Thus, these data indicate that in this important aspect of phospho*enol*pyruvate carboxylation, namely substrate formation, *M. arvensis* is unlike *P. milioides* or representative C_4 plants and suggest that reduction of photorespiration in this crucifer does not involve a limited C_4-like cycle identical to that operating in the intermediate *Panicum* species.

Concluding Remarks

Consistent evidence has been presented independently by several groups which indicates that *M. arvensis* is intermediate between C_3 and C_4 plants with respect to photorespiration [5–7]. Indeed, recent experiments in our laboratory indicate that Γ for this crucifer is 16 ± 3 μl CO_2/l at 21% O_2 and 25°C, compared to 45 ± 2 μl/l for soybean (Holaday, A.S. and Chollet, R., unpublished data). The similarities between *M. arvensis* and *P. milioides* with respect to certain CO_2-exchange characteristics, anatomical and ultrastructural features (Figs. 1 and 2), and phospho*enol*pyruvate carboxylase activity and properties (Tables I, II) suggest that these two species might possess a similar mechanism for reducing photorespiration. However, other C_4-related enzyme activity measurements (Table III) have revealed critical differences in the biochemistry of these two naturally

occurring intermediate plants. The low or undetectable activities of the C_4 acid decarboxylases and pyruvate, orthophosphate dikinase in *M. arvensis* suggest that a limited C_4-like CO_2-concentrating mechanism similar to that in *P. milioides* [4] is not responsible for reducing photorespiration in this crucifer. Moreover, whereas the C_3-C_4 intermediate *Panicum* and *Mollugo* species may represent evolutionary links or stable hybrids between C_3 and C_4 plants in these genera, no known C_4 species occur in the *Moricandia* genus or the Cruciferae family [6]. This fact diminishes the likelihood that a true C_4-like system exists in *M. arvensis*, since such a pathway would have had to develop directly from mutations in a C_3 system.

However, the results from the present whole leaf extract study do not totally negate the possibility that some type of phospho*enol*pyruvate carboxylase-mediated system exists in *M. arvensis* to reduce photorespiration. Indeed, certain ultrastructural features of the bundle sheath layer are suggestive of the presence of a C_4-like mechanism. It is possible that NAD- or NADP-malic enzyme might be localized exclusively in the bundle sheath cells, and, when analzyed on this basis, the activity may be sufficient to decarboxylate the C_4 acids synthesized by phospho*enol*pyruvate carboxylase. Similarly, the observation that phospho*enol*pyruvate synthesis in *M. arvensis* does not occur via pyruvate, orthophosphate dikinase may be offset by converting 3-phosphoglycerate to phospho*enol*pyruvate via the 3-phosphoglycerate mutase/enolase glycolytic sequence and by the high affinity of the *M. arvensis* carboxylase for phospho*enol*pyruvate. However, the activities of these two glycolytic enzymes are essentially identical to those in *L. perenne* (Holaday, A.S. and Chollet, R., unpublished data). Detailed studies of $^{14}CO_2$-incorporation patterns for intact leaves and biochemical analyses of the isolated leaf cell types are currently in progress to provide definitive insight into these possibilities and to investigate further the mechanism by which photorespiration is reduced in *M. arvensis*.

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