

Metabolic Activities in Extracts of Mesophyll and Bundle Sheath Cells of *Panicum miliaceum* (L.) in Relation to the C₄ Dicarboxylic Acid Pathway of Photosynthesis¹

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ABSTRACT

The activities of certain enzymes related to the carbon assimilation pathway in whole leaves, mesophyll cell extracts, and bundle sheath extracts of the C₄ plant *Panicum miliaceum* have been measured and compared on a chlorophyll basis. Enzymes of the C₄ dicarboxylic acid pathway—phosphoenolpyruvate carboxylase and NADP-malic dehydrogenase—were localized in mesophyll cells. Carbonic anhydrase was also localized in mesophyll cell extracts. Ribose 5-phosphate isomerase, ribulose 5-phosphate kinase, and ribulose diphosphate carboxylase—enzymes of the reductive pentose phosphate pathway—were predominantly localized in bundle sheath extracts. High activities of aspartate and alanine transaminases and glyceraldehyde-3-P dehydrogenase were found about equally distributed between the photosynthetic cell types. *P. miliaceum* had low malic enzyme activity in both mesophyll and bundle sheath extracts.

Isolated bundle sheath cells were capable of converting aspartate to oxalacetate at rates approaching the aspartate transaminase activity of bundle sheath extracts. The bundle sheath cells had a light induced CO₂ fixation of 23 μmoles of CO₂/mg chl·hr in the absence of exogenous substrates.

The photorespiratory enzymes, hydroxypyruvate reductase and glycolic oxidase, were about 3 fold higher in bundle sheath extracts than in mesophyll extracts when compared on a chlorophyll basis.

bundle sheath cells of C₄ plants (1, 13, 17). Recent investigations have shown that considerable variation exists in the biochemical and anatomical features between plants having the C₄ pathway of photosynthesis (1, 6, 13, 18, 27). High malic enzyme activity has been demonstrated in a number of C₄ plants such as corn, sugarcane, sorghum, crabgrass, and *Panicum antidotale* (4, 13, 17). Where biochemical features and fine structure of the chloroplasts have been examined in these plants, there seems to be a correlation of high malic enzyme activity with malate as a predominant initial product of photosynthesis and a reduction in the number of grana in the bundle sheath chloroplasts (13). In these C₄ plants, malic enzyme is thought to be a key enzyme in catalyzing a carboxyl transfer from malate to the pentose pathway in bundle sheath cells.

However, substantial malic enzyme activity has not been demonstrated in all C₄ plants (1, 10, 13, 18); so the mechanism of carboxyl transfer from C₄ acids into the pentose pathway must continue to be considered. Recently, several C₄ plants having low malic enzyme activity were found to have high PEP carboxykinase which may catalyze the transfer of carbon from oxalacetate into the pentose pathway in bundle sheath cells (18). Examples of C₄ plants having high PEP carboxykinase are *Panicum maximum*, *Panicum texanum*, and *Sporobolus poiretti*. There is some evidence that these plants have aspartate as a predominant initial product of photosynthesis (13) and well developed grana in bundle sheath chloroplasts (7).

There are yet other C₄ plants such as *Panicum miliaceum*, *Amaranthus retroflexus*, and *Cynodon dactylon* which have been reported to have very low or undetectable levels of both malic enzyme (10, 13) and PEP carboxykinase (18) in leaf extracts. C₄ plants may have metabolic differences in the mechanism of carboxyl transfer from the C₄ dicarboxylic acids into the reductive pentose phosphate pathway. In addition, there may be differences in the distribution of enzymes of the C₄ pathway and the pentose pathway between mesophyll and bundle sheath cells, as well as in the biochemical basis for the lack of apparent photorespiration among C₄ species.

In this paper some of the metabolic characteristics of isolated bundle sheath cells and of extracts from whole leaves, mesophyll cells, and bundle sheath cells of *Panicum miliaceum* are presented.

MATERIALS AND METHODS

Plant Material. *Panicum miliaceum*, proso millet (variety red), was obtained from Olds Seed Company, Madison, Wisc. The millet was grown in a greenhouse in a 1:1:1 mixture of loam, peat, and perlite. Sunlight was supplemented with 100-w incandescent lamps to provide a day length of 14 hr. Day

The initial end products of photosynthesis in plants having the C₄ pathway² of photosynthesis are oxalacetate, malate, and aspartate, which are thought to be synthesized in mesophyll cells (21, 25). It has been suggested that these C₄ acids may serve as carboxyl donors to the pentose pathway in the bundle sheath cells; or more specifically to RuDP carboxylase in the

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² Abbreviations: C₄ pathway: C₄-dicarboxylic acid pathway; C₄ plants: plants having the C₄ pathway and reductive pentose phosphate pathway; pentose pathway: reductive pentose phosphate pathway; PEP: phosphoenolpyruvate; RuDP: ribulose-1,5-diphosphate; R-5-P: ribose-5-phosphate.

temperatures were maintained near 27 C and night temperatures near 22 C. Leaves were sampled when the plants were 3 to 4 weeks old. Plants older than 4 weeks gave relatively poor preparations of bundle sheath cells.

Enzyme Extraction and Assays. Grinding medium for extraction of enzymes included 0.6 M sorbitol, 5 mM MgCl₂, 50 mM Tricine buffer at pH 8.0, and 1 mM dithiothreitol. Four grams of leaves were cut into segments approximately 2 mm in width with a razor and placed in a mortar (Porcelain, Coors 522, No. 1). Ten milliliters of grinding medium were added, and the leaf segments were gently homogenized with a pestle. After several minutes the supernatant turned a light green color while the leaf segments maintained their original rectangular shape. The supernatant was filtered through a 20- μ nylon net (Nitex Nylon Monofilament Bolting Cloth [16]), and the filtrate was taken as the mesophyll extract. Seven milliliters of medium were added to the remaining leaf segments, and these were homogenized gently for about 5 min with a pestle until the leaf segments disintegrated and individual bundle sheath strands were released. Bundle sheath strands were collected according to previously described techniques (16) and resuspended in 1 or 2 ml of grinding medium. The bundle sheath strands were broken in a tight-fitting Ten-Broeck homogenizer, and the homogenate was passed through a 30- μ nylon net in a small filter unit. The filtrate was collected as the enzyme extract from bundle sheath cells. All steps for the enzyme preparations were carried out at 4 C.

To extract the enzymes from the whole leaf 1 g of tissue was cut into small segments and added to a mortar with 3 ml of grinding medium. After vigorous grinding, an additional 3 ml of grinding medium were added and the extract was further homogenized in a Ten-Broeck homogenizer. The homogenate was routinely examined to assure that the bundle sheath cells were broken. After filtration through a 20- μ nylon net the supernatant was taken as the whole leaf crude enzyme preparation.

Before assaying NADP-dependent malic dehydrogenase, an aliquot of the leaf extracts was preincubated in 25 mM dithiothreitol and 50 mM MgCl₂ for 15 min at room temperature. This preincubation was an absolute requirement for enzyme activation. Following this preincubation, the enzyme was assayed spectrophotometrically by measuring the oxidation of NADPH at 340 nm in 50 mM Tricine buffer, pH 8.0. Substrate levels were 0.2 mM NADPH and 3 mM oxalacetate. NAD-dependent malic dehydrogenase was assayed in a similar manner using NADH as substrate without preincubation of enzyme.

Other enzymes with reference to their method of assay were NADP-specific malic enzyme, in the direction of decarboxylation (32); ribose-5-P isomerase (2); ribulose-5-P kinase (28); carbonic anhydrase (9); glyceraldehyde-3-P dehydrogenase (29); cytochrome *c* oxidase (35); and hydroxypyruvate reductase (41). Catalase was assayed spectrophotometrically by following the disappearance of hydrogen peroxide at 240 nm (30). Glycolate oxidase was measured by following the formation of the glyoxylate phenylhydrazone at 324 nm (33). A control reaction was run without glycolate.

Aspartate transaminase (aspartate- α -ketoglutarate transaminase) and alanine transaminase (alanine- α -ketoglutarate transaminase) were assayed spectrophotometrically by following the decrease in absorbance at 340 nm in coupled reactions. The aspartate transaminase reaction mixture included 50 mM Tricine, pH 8.0; 2.5 mM α -ketoglutarate; 2.5 mM aspartate; 0.03 mM pyridoxal phosphate; 0.1 mM NADH; 2 mM EDTA; and enzyme extract in volume of 3 ml. The reaction was coupled to the endogenous malic dehydrogenase which was in excess. A similar assay was used for alanine transaminase except alanine re-

placed aspartate and 5 units of lactic dehydrogenase were added.

RuDP carboxylase was assayed by following the incorporation of radioactive bicarbonate in a reaction mixture of 0.15 ml containing the same concentration of components used in the grinding medium plus enzyme extract, 44 mM NaH¹⁴CO₃, and 1 mM RuDP. The reaction was initiated by adding RuDP, and the reaction rate was linear over the 8-min incubation period. Samples of 40 μ l were taken after 2, 4, and 8 min of incubation and added to 50 μ l of 20% trichloroacetic acid in scintillation vials for scintillation counting. In a similar assay for PEP carboxylase the reaction medium contained in addition to the compounds of the grinding medium: 5 mM, phenylhydrazine HCl, 1 mM PEP, 3 mM NaH¹⁴CO₃, and enzyme extract. The two carboxylases were assayed at 30 C. Other enzymes unless specified were assayed at room temperature.

CO₂ Fixation by Isolated Bundle Sheath Cells. The grinding medium for isolating the intact bundle sheath cells contained 0.6 M sorbitol, 2 mM EDTA, 5 mM MgCl₂, 1 mM NaNO₃, 1 mM MnCl₂, 0.5 mM K₂HPO₄, and 50 mM Tricine at pH 8.0. Bundle sheath strands were isolated by techniques similar to those for the enzyme preparation. The yield of bundle sheath cells from 4 g of leaves on a chlorophyll basis was about 0.2 mg. The cells were resuspended in 1 ml of grinding medium. Routine microscopic observations of bundle sheath cell preparations showed them to be free of mesophyll cells.

For experiments, aliquots were taken from the preparation with capillary tubes (1.6–1.8 mm outside diameter \times 100 mm) adapted to a 100- μ l syringe. Cells were isolated at room temperature and then kept on ice until used. The reaction medium for the CO₂ fixation studies included the same concentration of components as used in the grinding medium plus 3 mM NaH¹⁴CO₃, and bundle sheath cells.

Conversion of Aspartate to Oxalacetate by Isolated Bundle Sheath Cells. The grinding medium for isolating the cells contained 0.6 M sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM NaNO₃, 50 mM tris-HCl at pH 8.0, and 0.5 mM K₂HPO₄. Tricine buffer in the reaction medium was found to inhibit the colorimetric method for detecting the oxalacetate formation. The reaction medium included the same levels of components as used in the grinding medium in addition to bundle sheath cells, and various levels of aspartate and α -ketoglutarate in a total volume of 0.25 ml. After 5 and 10 min of incubation at 35 C, 0.05-ml samples were taken in duplicate and added to a tube containing 0.05 ml of 0.4 N HCl to stop the reaction. After 2 min 0.05 ml of 0.4 N KOH; 0.05 ml of 1 M tris-HCl, pH 8.0; and 0.4 ml of 0.5% of 6-benzamido-4-methoxy-*m*-toluidine diazonium chloride (Fast Violet B Salt) in 1% soluble polyvinylpyrrolidone were added. Two minutes after adding the diazonium salt, 2.4 ml of water were added, and readings were taken immediately at 530 nm. Control reactions without bundle sheath cells were run, and the absorbance at 530 nm was subtracted from the sample readings. The amount of oxalacetate formed was determined by preparing a standard curve (3) with various levels of oxalacetate in the reaction mixture without bundle sheath cells.

Chlorophyll Determination. Chlorophyll in the various extracts or in isolated bundle sheath cells was determined by the method of Wintermans and DeMots (39). The bundle sheath cells were incubated for 20 min with 96% ethanol prior to centrifugation to assure complete extraction of chlorophyll. The chlorophyll *a/b* ratio in whole leaf extracts (*WL*), mesophyll cell extracts (*MC*), and bundle sheath extracts (*BS*) was 3.45, 3.75, and 3.10, respectively. According to these ratios 57% of the chlorophyll in the whole leaf would be in mesophyll cells and 43% in bundle sheath cells. Chlorophyll percentages were derived using the formula where the %

chlorophyll in the mesophyll cells is equal to:

$$\frac{100(MC + 1)(WL - BS)}{(WL + 1)(MC - BC)}$$

RESULTS AND DISCUSSION

Enzyme Levels and Distribution Between Two Cell Types. In analyzing the levels of enzymes in whole leaves and the distribution of enzymes between the mesophyll and bundle sheath cell extracts of *P. miliaceum*, we found NADP-dependent malic dehydrogenase, PEP carboxylase, and carbonic anhydrase localized in the mesophyll extracts (Table I). The levels of PEP carboxylase and NADP-dependent malic dehydrogenase are within the rates of photosynthesis of 175 to 210 μ moles of CO₂ fixed per mg of chl per hr expected for C₄ plants (23). It is not known if carbonic anhydrase is involved in photosynthesis in C₄ plants. If the active "CO₂" for the PEP carboxylase reaction is HCO₃⁻, as suggested by Cooper (12), the carbonic anhydrase could be important in catalyzing the conversion of atmospheric CO₂ to HCO₃⁻ as it enters the mesophyll cells. Graham *et al.* (19) recently reported that carbonic anhydrase may be localized in the mesophyll cells of corn.

RuDP carboxylase and ribulose-5-P kinase, enzymes of the reductive pentose phosphate pathway, as well as R-5-P isomerase, were predominantly localized in the bundle sheath extracts (Table I). This suggests that the reductive pentose pathway is primarily associated with bundle sheath cells. Investigations on the localization of RuDP carboxylase in several other C₄ plants generally agree that the enzyme is predominant in the bundle sheath cells (4, 5, 17, 36), although one report suggests that the RuDP carboxylase is in mesophyll cells of sugarcane and corn (8).

Malic enzyme has been reported to be low in leaf extracts of *P. miliaceum* (18). We found low activity of malic enzyme in leaf extracts, mesophyll extracts, and bundle sheath extracts. We analyzed leaves of this species for other possible C₄ dicarboxylic acid decarboxylating enzymes which included assaying for PEP carboxykinase (18); aspartate decarboxylase by the method of Tate and Meister (38); pyruvate carboxylase by the method of Scrutton, Keech, and Utter (34); oxal-

acetate decarboxylase by the method of Stern (37) and Kosicki (26); and PEP carboxytransphosphorylase by an exchange reaction using NaH¹⁴C₃ as described by Wood, Davis, and Willard (40), without finding measurable activity.

Activities of aspartate transaminase and alanine transaminase were extremely high in leaf extracts of *P. miliaceum*, and the two enzymes were equally distributed between the two cell types. Downton (13) classified *P. miliaceum* as an "aspartate former" since 74% of the initial ¹⁴C-labeled products of photosynthesis was aspartate while 22% was malate. Aspartate may be a primary end product of CO₂ fixation in mesophyll cells through PEP carboxylase and aspartate transaminase. However, since PEP carboxylase is relatively low in bundle sheath cells, the aspartate transaminase could be playing a different role in the bundle sheath cells than in the mesophyll cells.

Karpilov (24) has suggested that aspartate might be transferred from mesophyll cells to bundle sheath cells in C₄ plants and after carboxyl transfer to the pentose pathway alanine may be transported back to the mesophyll cells as a precursor for the formation of PEP. As indicated by Johnson, Hatch, and Slack (23), it is conceivable that the aspartate and alanine transaminases in the two cell types of certain C₄ plants may be involved in the carbon assimilation pathway.

The NADH- and NADPH-dependent glyceraldehyde-3-P dehydrogenase was found in both cell types, which indicates that both cells have the capacity for carbon reduction in photosynthesis (Table I). Cytochrome *c* oxidase and NAD-dependent malic dehydrogenase were found in both cell extracts, which further demonstrate that some enzymes are prevalent in bundle sheath and mesophyll cells.

Among the photorespiratory enzymes analyzed, glycolate oxidase and hydroxypyruvate reductase were about 3-fold higher in bundle sheath extracts in *P. miliaceum* than in mesophyll extracts when compared on a chlorophyll basis. However, catalase showed little preferential localization between the two cells. Edwards and Black (17) previously found all three of these enzymes preferentially localized in bundle sheath cells of crabgrass. The activity of hydroxypyruvate reductase and glycolate oxidase was several-fold higher in the bundle sheath cells of *P. miliaceum* than in the bundle sheath cells of crabgrass. *P. miliaceum* has well developed grana in bundle sheath chloroplasts (7) while crabgrass has agranal bundle sheath chloroplasts (6). Downton (14) has reported higher levels of glycolate oxidase in leaves of several *Atriplex* species having well developed grana in bundle sheath chloroplasts than in corn or sorghum, which have agranal bundle sheath chloroplasts. C₄ plants having agranal bundle sheath chloroplasts may have a low capacity for glycolate synthesis (14, 33), which would correlate with a low level of enzymes of glycolate respiration in these plants. However, there are little conclusive data in the literature on the capacity of C₄ plants for glycolate synthesis.

CO₂ Fixation by Isolated Bundle Sheath Cells. The results of Table II show that isolated bundle sheath cells of *P. miliaceum* have a light-dependent fixation of CO₂ in the absence of an exogenous substrate of 22.7 μ moles of CO₂ fixed per mg of chl per hr. This suggests that the bundle sheath cells have some capacity for fixing CO₂ independent of the mesophyll cells. The rate is considerably higher than the light-induced rate of 1.2 μ moles of CO₂ fixed per mg of chl per hr by isolated bundle sheath cells of crabgrass (17) and corn (11). The addition of RuDP or R-5-P to crabgrass bundle sheath cells induced CO₂ fixation, which suggests that the isolated cells were unable to regenerate sufficient levels of pentose phosphate precursors for the RuDP carboxylation (17). Corn and crabgrass, which have malate as a primary initial product of CO₂ fixation, have reduced grana in the bundle sheath chloroplasts along with high

Table I. Enzyme Distribution between Mesophyll and Bundle Sheath Extracts of *Panicum miliaceum*

| Enzyme | Whole Leaf | Mesophyll Cells | Bundle Sheath Cells |
|--|------------|-----------------|---------------------|
| | | | |
| A. Enzymes with preferential localization | | | |
| PEP carboxylase | 299 | 565 | 26 |
| NADP-malic dehydrogenase | 77 | 201 | 15 |
| Carbonic anhydrase ¹ | 197 | 283 | 0 |
| RuDP carboxylase | 73 | 29 | 207 |
| Ru5P kinase | 804 | 158 | 1140 |
| R-5-P isomerase | 2355 | 499 | 3933 |
| Malic enzyme | 21 | 14 | 49 |
| B. Enzymes localized in both cells | | | |
| Glyceraldehyde-3-P dehydrogenase | | | |
| NADH | 765 | 793 | 777 |
| NADPH | 962 | 769 | 731 |
| Aspartate transaminase | 1293 | 1433 | 1493 |
| Alanine transaminase | 1433 | 1960 | 1717 |
| NAD-malic dehydrogenase | 7493 | 3887 | 6883 |
| Cytochrome <i>c</i> oxidase | 36 | 66 | 52 |
| C. Photorespiratory enzymes | | | |
| Hydroxypyruvate reductase | 361 | 207 | 610 |
| Glycolic oxidase | 16 | 9 | 30 |
| Catalase | 24300 | 24450 | 29683 |

¹ Enzyme assayed in an ice bath and activity expressed as enzyme units/mg chl according to Reference 9.

activities of malic enzyme and high chl *a/b* ratios in the bundle sheath cells (4, 6, 17, 22). These plants may be dependent on malic enzyme for providing part of the reducing power for the pentose pathway from the reduction of NADP during the decarboxylation of malate (20, 31). The grana-containing chloroplasts of the bundle sheath cells of *P. miliaceum* may be self-sufficient in providing reducing power for CO₂ fixation.

Additions of R-5-P increased the rate of CO₂ fixation by isolated bundle sheath cells of *P. miliaceum*, and the enhancement by R-5-P as expected was largely light-dependent (Table II). If a carboxyl transfer to the pentose pathway was occurring in the bundle sheath cells of *P. miliaceum* by a decarboxylation of malate, aspartate, or oxalacetate, addition of these substrates to the bundle sheath cells might reduce the rate of the light-dependent fixation of added NaH¹⁴CO₃ by causing a dilution of the radioactive bicarbonate pool inside the cell and by decreasing the concentration of pentose phosphate precursors for the RuDP carboxylation. The addition of 5 mM aspartate and 5 mM α -ketoglutarate or 5 mM aspartate or 10 mM malate had little effect on the rates of CO₂ fixation (Table II). An attempt to measure the decarboxylation of aspartate and malate by isolated bundle sheath cells was made by adding ¹⁴C-4-labeled substrates. No activity was lost from aspartate during a 60-min incubation period while 4 mM malate was decarboxylated at a rate of 8 μ moles/mg chl·hr at 35 C in the presence of 5 mM NADP.

Conversion of Aspartate to Oxalacetate by Bundle Sheath Cells. Figure 1 shows that isolated bundle sheath cells of *P. miliaceum* are capable of converting aspartate to oxalacetate. The conversion of aspartate to oxalacetate was dependent on the addition of α -ketoglutarate, which implies that the conversion is dependent on the aspartate transaminase reaction in the cell (Fig. 1A). No oxalacetate formation was detected when pyruvate was substituted for α -ketoglutarate as the amino group acceptor. Concentrations of α -ketoglutarate up to 50 mM were required to saturate the rate of oxalacetate formation while 10 mM aspartate provided a near maximal rate of oxalacetate formation with the isolated cells. Aspartate may be more permeable to the isolated cells than α -ketoglutarate. Much lower levels of aspartate and α -ketoglutarate saturated the rate of the aspartate transaminase reaction from extracts of bundle sheath cells with an apparent *K_m* of 0.5 mM for

Table II. Effect of Various Substrates on CO₂ Fixation by Isolated Bundle Sheath Cells of *Panicum miliaceum*

Concentrations of substrates were 3 mM NaH¹⁴CO₃, 5 mM aspartate, 5 mM α -ketoglutarate, 2 mM RuDP, 10 mM R-5-P, and 10 mM L-malate. Reactions with malate included 5 mM NADP. Light provided by an incandescent lamp was approximately 2500 ft-c. The temperature was 35 C.

| Condition | Rate of CO ₂ Fixation |
|--|----------------------------------|
| | μ moles/mg chl/hr |
| Dark | 0.1 |
| Light | 22.7 |
| Light, aspartate | 24.4 |
| Light, aspartate, α -ketoglutarate | 18.9 |
| Light, malate | 18.3 |
| Dark, R-5-P | 0.4 |
| Light, R-5-P | 40.0 |
| Light, R-5-P, aspartate | 38.1 |
| Light, R-5-P, aspartate, α -ketoglutarate | 38.7 |
| Light, R-5-P, malate | 25.2 |
| Dark, RuDP | 6.9 |
| Light, RuDP | 25.6 |

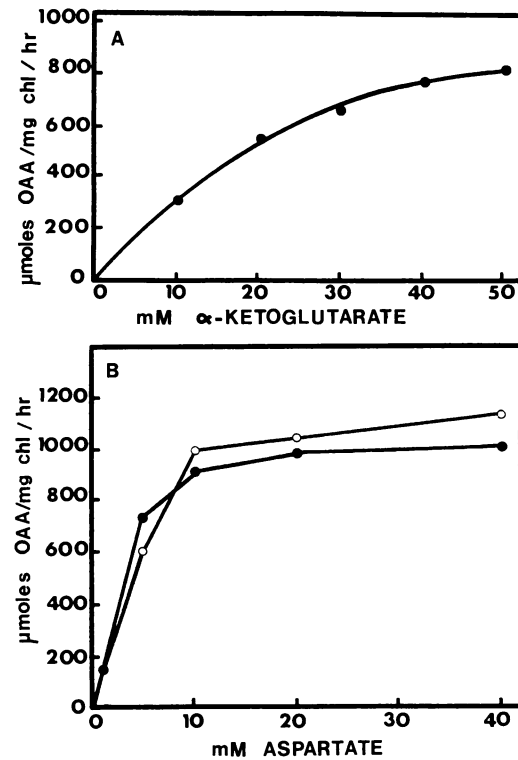


FIG. 1. Formation of oxalacetate from aspartate by isolated bundle sheath cells of *P. miliaceum*. ●: Dark; ○: light. Temperature was 35 C. A: Reaction contained 50 mM aspartate and varying amounts of α -ketoglutarate; B: reaction contained 50 mM α -ketoglutarate with varying amounts of aspartate.

aspartate and 0.11 mM for α -ketoglutarate when assayed at 35 C.

P. miliaceum may form aspartate as a primary initial product of photosynthesis through PEP carboxylase and mesophyll cell aspartate transaminase and subsequently transport the aspartate to the bundle sheath cells. Isolated bundle sheath cells have a high capacity for converting aspartate to oxalacetate which could be significant in photosynthesis if oxalacetate is a primary carboxyl donor to the pentose pathway in *P. miliaceum*. However, under the experimental conditions used, the oxalacetate formed was not rapidly converted to other compounds in the light (Fig. 1B), which might be expected if oxalacetate were a carboxyl donor in photosynthesis in bundle sheath cells.

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