Photosynthetic Characteristics of the C₃-C₄ Intermediate
Parthenium hysterophorus

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ABSTRACT

The weedy species Parthenium hysterophorus (Asteraceae) possesses a Kranz-like leaf anatomy. The bundle sheath cells are thick-walled and contain numerous granal chloroplasts, prominent mitochondria, and peroxisomes, all largely arranged in a centripetal position. Both mesophyll and bundle sheath chloroplasts accumulate starch. P. hysterophorus exhibits reduced photorespiration as indicated by a moderately low CO₂ compensation concentration (20–25 micromolars per liter at 30°C and 21% O₂) and by a reduced sensitivity of net photosynthesis to 21% O₂. In contrast, the related C₃ species P. incanum and P. argentatum (guayule) lack Kranz anatomy, have higher CO₂ compensation concentrations (about 55 micromolars per liter), and show a greater inhibition of photoresynthesis by 21% O₂. Furthermore, in P. hysterophorus the CO₂ compensation concentration is relatively less sensitive to changes in O₂ concentrations and shows a biphasic response to changing O₂, with a transition point at about 11% O₂. Based on these results, P. hysterophorus is classified as a C₃-C₄ intermediate. The activities of diagnostic enzymes of C₄ photosynthesis in P. hysterophorus were very low, comparable to those observed in the C₃ species P. incanum (e.g. phosphoenolpyruvate carboxylase activity of 10–29 micromoles per milligram of chlorophyll per hour). Exposures of leaves of each species to ¹⁴CO₂ (for 8 seconds) in the light resulted in 3-phosphoglycerate and sugar phosphates being the predominant initial ¹⁴C products (77–84%), with ≤4% of the ¹⁴C-label in malate plus aspartate. These results indicate that in the C₃-C₄ intermediate P. hysterophorus, the reduction in leaf photorespiration cannot be attributed to C₄ photosynthesis.

Through considerable interest and effort over the last decade, many naturally occurring plant species with photosynthetic characteristics intermediate to C₃ and C₄ plants have been identified (see 10, 14, 20 for review). Most C₃-C₄ species have in common at least a partially developed Kranz leaf anatomy and reduced levels of photorespiration relative to C₃ plants, as indicated by decreased values of ¹³C and a decreased sensitivity of net photosynthesis or carboxylation efficiency to O₂. Most of the intermediate species occur in genera having also both C₃ and C₄ species (e.g. Panicum and Neurachne [Poaceae], Alternanthera [Amaranthaceae], Flaveria [Asteraceae], and Mollugo [Aizoaceae]. However, Moricandia (Brassicaceae) and Parthenium (Asteraceae) have one or more intermediate species, but no known C₅ species. Such circumstantial evidence suggests that the C₃-C₄ species are evolutionary intermediates, and not hybrid species.

Different mechanisms for reducing photorespiration are likely utilized among the various C₃-C₄ intermediate species (10, 14). In intermediate Panicum and Moricandia species, Brown (4), Winter et al. (30), and others (5, 9, 16) have suggested that the relatively large abundance of chloroplasts, mitochondria, and peroxisomes in the bundle sheath cells may contribute to enhanced refixation of photorespired CO₂ by RuBiCO in bundle sheath chloroplasts. If this is an effective mechanism, then one would expect a relatively large proportion of the leaf’s photorespiratory glycolate and/or glycine to be metabolized in the bundle sheath cells (20), but this is yet to be thoroughly tested. Utilizing apparently different mechanisms, the C₃-C₄ Flaveria species have many biochemical characteristics intermediate between those of C₃ and C₄ species, and possess a limited, but in some cases a considerable capacity for C₄ photosynthesis (3, 21, 27). Rumpho et al. (27) proposed that this capacity in F. ramosissima is sufficient to reduce photorespiration, presumably by elevating the CO₂ concentration at the site of RuBiCO carboxylation in the bundle sheath cells. Direct evidence of a light-dependent, CO₂-concentrating ability of leaf discs from F. ramosissima and the apparently more advanced Flaveria intermediates has been obtained (23). However, the possible refixation of photorespired CO₂ by PEP carboxylase may also contribute to reduced apparent photorespiration, particularly among the less advanced Flaveria intermediates (10).

A previous study showed that Parthenium hysterophorus possesses a Kranz leaf anatomy, but the bundle sheath chloroplasts were reported not to accumulate starch (13, 24) and the leaf Δ¹³C-values were C₃-like (25). Curiously, the data of Hedges and Patil (13, 25) and others (26) indicate that leaves of P. hysterophorus contain considerable activities of certain C₄ cycle enzymes, including PEP carboxylase, yet are capable only of C₃-type photosynthesis. Γ has been reported by different authors to range from 27 to 35 µl/L (24, 26), thus indicating that this species has relatively low photorespiration. However, the mechanism by which photorespiration may be reduced is not clear. In the present study, we have examined the leaf anatomy and ultrastructure, Γ and its response to O₂, the C₄ photosynthetic enzyme activities, and the initial products of ¹⁴CO₂ fixation in leaves of P. hysterophorus and two related C₃ species, P. incanum and P. argentatum (guayule). The results demonstrate that P. hysterophorus is a C₃-C₄ species which has Kranz-like anatomy with reduced photorespiration, but which shows no biochemical capacity for C₄ photosynthesis.
MATERIALS AND METHODS

Reagents and Supplies. Cellulose MN 300 was from Brinkmann Instruments Co. x-ray film (X-OMAT AR-5) and secbutyl alcohol were from Eastman Kodak Co. NaH\(^{14}\)CO\(_3\) (55.9 Ci/mol) was from ICN Biomedicals, Inc. Scintillation cocktail was from Research Products International Corp. Other supplies and reagents were of the highest quality available.

Plant Material. Plants of Parthenium argentatum, P. hysterophorus, and P. incanum were grown in soil and water regularly with a commercial nutrient solution (Peter’s fertilizer plus micronutrients). They were generally maintained in a growth chamber (27°C/20°C day/night thermoperiod, a 14 h photoperiod, and an irradiance of 350 \(\mu\)mol/m\(^2\)·s photon flux density at plant height), but in some experiments were grown in a greenhouse during winter months under similar conditions (25-27°C/18-20°C day/night thermoperiod, 300 \(\mu\)mol/m\(^2\)·s photon flux density). Young, fully expanded leaves of 2-month-old vegetative plants were used for the experiments, except as indicated otherwise.

Leaf Anatomy and Ultrastructure. Small samples were removed at midday from unshaded leaves and were immersed in a fixative composed of 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 50 mM Pipes buffer (pH 7.2). The samples were postfixed with 1% OsO\(_4\) in 25 mM cacodylate buffer (pH 7.2), dehydrated with ethanol, and embedded in Spurr’s epoxy resin. Sections for light microscopy were 1 \(\mu\)m thick, and were stained with Stevengil’s blue (6). Thin sections for transmission electron microscopy were stained with uranyl acetate and lead citrate, and examined with a Hitachi 300 TEM.

\(\text{CO}_2\)-Exchange Measurements.\ The photosynthetic \(\Gamma\) of intact leaves was determined in a closed, plexiglass leaf chamber using an Anarad IR gas analyzer in a differential mode. The technique for determining \(\text{CO}_2\) concentration in the cuvette was essentially as described by Atkins and Pate (2). One should note that this procedure is considered to be more accurate than those used by others in previous reports (IR gas analysis with analyser in absolute mode [26]; pH change of a bicarbonate solution after equilibrating with ambient \(\text{CO}_2\) in a closed vessel [24]). The measurements were made at a photon flux density of 600 \(\mu\)mol/m\(^2\)·s, a leaf temperature of 30°C, and varying \(\text{O}_2\) levels.

\(\text{CO}_2\) exchange rates of intact leaves were measured as previously described (19), with a photon flux density of 1500 \(\mu\)mol/m\(^2\)·s, a leaf temperature of 30 ± 0.2°C, and 325 \(\mu\)l CO\(_2/\text{L}\).

Enzyme Extraction and Assays. Leaves were weighed, powdered in liquid \(\text{N}_2\), and ground with a mortar and pestle in 6 volumes of medium with the following components: 100 mM Hepes-KOH (pH 7.5), 10 mM MgCl\(_2\), 10 mM DTE, 1 mM EDTA, 2.5 mM Na pyruvate, 0.5% (w/v) BSA, 1% (w/v) casein, 0.05% Triton X-100, and 2% (w/v) insoluble PVP. Extracts were filtered through Miracloth, centrifuged at 14,000 g for 1 min, and the clarified supernatant was immediately used for enzyme assays.

Spectrophotometric assays were done at 30°C, in 1-mL reaction volumes. PEP carboxylase (EC 4.1.1.32) was measured by coupling to NAD-MDH according to the procedure of Uedan and Sugiyama (25). Pyruvate, Pi dikinase (EC 2.7.9.1) was assayed in the medium described by Edwards et al. (8). Exogenous PEP carboxylase required in this coupled assay was partially purified from maize as described by Uedan and Sugiyama (25), using the earliest peaks of activity off a DE-52 column, which were devoid of pyruvate, Pi dikinase activity. Aspartate (-2-oxoglutarate) aminotransferase (EC 2.6.1.1) and alanine (-2-oxoglutarate) aminotransferase (EC 2.6.1.2) were assayed as described by Wendt and Gutierrez (7). NADP-malic enzyme (EC 1.1.1.40) and NADP-MDH (EC 1.1.1.82) were assayed according to Kanai and Edwards (17). The full reductive activation of NADP-MDH included a 1-min flush with \(\text{N}_2\) of the enzyme plus 10 mM DTE mixture, prior to incubation at 25°C. NAD-malic enzyme (EC 1.1.1.39) was measured according to Hatch et al. (12). PEP carboxykinase (ATP, EC 4.1.1.49) was measured according to Hatch (11).

\(\text{CO}_2\) Leaf Exposures and Identification of \(\text{^14}\)C Products. Detached leaves were exposed to \(\text{CO}_2\) (425 \(\mu\)l/L) for 8 s and killed in boiling 80% (v/v) ethanol as described by Moore et al. (22). Replicate leaf exposures were pooled and the soluble \(\text{\textsuperscript{14}}\text{C}\) products were extracted according to Rumpho et al. (27). Extracts were partitioned with CHCl\(_3\) and concentrated to about 0.5 ml. \(\text{\textsuperscript{14}}\text{C}\)-Labeled products were separated and identified by two-dimensional thin-layer electrophoresis and chromatography on cellulose plates, followed by autoradiography (28). Labeled metabolites were removed and their radioactivity quantified by liquid scintillation spectroscopy. The recovery of radioactivity from the plates was essentially 100%. The \(\text{\textsuperscript{14}}\text{C}\) label in glycerate was counted with that in 3-PGA. The authenticity of metabolic \(\text{\textsuperscript{14}}\text{C}\) glycate was demonstrated by showing relative co-migration with \(\text{\textsuperscript{14}}\text{C}\) glycate standard during TLC/TLC, and by HPLC analysis of the isolated metabolite.

Chl Measurement. Aliquots of filtered leaf homogenates were extracted in 96% ethanol, and the Chl contents calculated according to Wintermans and De Mots (31).

RESULTS

Mature leaves of P. hysterophorus have moderately developed Kranz anatomy, as seen in leaf cross-section, while those of P. incanum do not (Fig. 1). Encircling the smaller leaf veins of P. hysterophorus are 5 to 8 large, thick-walled bundle sheath cells (casual observation) which contain numerous organelles (Fig. 1B). In contrast, the bundle sheath cells of P. incanum are smaller, thin-walled parenchyma cells with few organelles (Fig. 1A). While each species has both leaf palisade and spongy mesophyll cells, these in general appear somewhat smaller in size and are more densely packed in P. hysterophorus, which has about four mesophyll cells between minor veins (observation). Chloroplasts, mitochondria, and peroxisomes are very prominent and mostly centripetally localized in the bundle sheath cells of P. hysterophorus, but are all rather scarce and peripherally located in the bundle sheath cells of P. incanum (Figs. 1 and 2). The bundle sheath chloroplasts of P. hysterophorus contain numerous well-developed grana and conspicuous, large starch granules (Fig. 2C). Both cell types of P. hysterophorus may accumulate high levels of starch as shown in thin section (Fig. 2C), or as observed by positive staining of leaf cross-sections with a periodic acid-Schiff’s reaction for polysaccharides (V Franceschi, unpublished data). However, bundle sheath chloroplasts of P. incanum do not similarly accumulate significant amounts of starch (Fig. 2B).

At 21% \(\text{O}_2\) and 30°C, \(\Gamma\) values in leaves of P. hysterophorus (20-25 \(\mu\)l/L) are less than one-half those observed in P. argentatum (about 56 \(\mu\)l/L) and P. incanum (about 51 \(\mu\)l/L, Fig. 3). Compared with the two \(\text{C}_3\) species, \(\Gamma\) in P. hysterophorus is also less sensitive to changing \(\text{O}_2\) concentrations, increasing particularly slowly from 2 to 11% \(\text{O}_2\) (from 1 → 7 \(\mu\)l CO\(_2/\text{L}\)). This results in a distinctly biphasic response of \(\Gamma\) as \(\text{O}_2\) levels are increased up to 50%. This response was not observed in P. argentatum or P. incanum, both in which \(\Gamma\) increased linearly and rapidly with increasing \(\text{O}_2\).

Rates of whole leaf photosynthesis by P. incanum and P. hysterophorus are equivalent on a Chl basis under 2% \(\text{O}_2\), but are relatively less inhibited by 21% \(\text{O}_2\) in P. hysterophorus (26% versus 34% inhibition in P. incanum, Table 1). Since P. hysterophorus has about 25% more Chl (\(\mu\)g/cm\(^2\)) than does P. incanum (from Table 1), photosynthesis on a leaf area basis is relatively higher in P. hysterophorus under either 2 or 21% \(\text{O}_2\).

We next sought to determine whether the apparent reduced photosynthetic activity of P. hysterophorus indicated by the
reduced Γ and O₂ inhibition values was accompanied by any C₄ photosynthesis. PEP carboxylase activity is quite low (10–29 μmol/mg Chl-h), as are the activities of pyruvate,Pi dikinase and the three C₄ acid decarboxylases found in C₄ plants (Table II). The activities of most of the C₄ cycle enzymes in leaves of P. hysterophorus are generally comparable to those in leaves of the related C₃ species, P. incanum. Aspartate aminotransferase is the only enzyme that had a considerably enhanced activity in P. hysterophorus. Leaf Chl a/b ratios are similar and of typical C₃ values in both species.

The implication from the enzyme-activity data of a low capacity for C₄ photosynthesis in P. hysterophorus was further evaluated by analyzing the initial photosynthetic products after exposure of leaves to a short pulse of ¹⁴CO₂. 3-PGA and sugarphosphates are the primary products of ¹⁴CO₂ fixation after an 8 s exposure of leaves of either P. hysterophorus or P. incanum (77–84% of soluble ¹⁴C products), with minimal ¹⁴C label recovered in malate plus aspartate (3–4%, Table III). Notably, the amount of label in glycollate after the pulse of ¹⁴CO₂ was substantially less in P. hysterophorus than in P. incanum (6% versus 17%).

DISCUSSION

At 30°C and 21% O₂, Γ in P. hysterophorus is 20 to 25 μL/L (Fig. 3), largely in agreement with previously estimated values of about 28 to 35 μL/L (26) and 27 μL/L (24). In P. hysterophorus, the response of Γ to increasing O₂ showed a transition point around 11% O₂, with a more rapid, linear increase in Γ with higher levels of O₂. Such a biphasic response has been observed in most other C₃-C₄ intermediates (10). Among intermediates of Flaveria which have some capacity for C₄ photosynthesis, such transitions generally occur at O₂ concentrations ≥21% (15). C₃ species show no such transition, and Γ increases proportionally and more rapidly with increasing O₂ up to 50% (e.g. Fig. 3). Γ in C₄ species also shows no such transitions, but shows minimal change with increasing O₂ (10). Thus, when such transitions occur at low O₂ levels as in P. hysterophorus (also as in Moricandia arvensis [1]; and Panicum milioides [18]) it may indicate a limited capacity for reducing photorespiration (10). Observation of only a moderately decreased sensitivity of net photosynthesis to 21% O₂ in leaves of P. hysterophorus (Table I) supports this interpretation.

The finding of negligible C₄ photosynthesis in leaves of P. hysterophorus (Table III) confirms previous data (13, 24) and indicates that a different mechanism may be used to partially reduce photorespiration. Such a mechanism may be as suggested for Panicum milioides (5, 9), and Moricandia arvensis (16, 30), i.e. some photospired CO₂ may be reassimilated by bundle sheath chloroplasts. Such chloroplasts are abundant, contain numerous grana, are capable of forming starch, and are in close proximity to large mitochondria and peroxisomes (Fig. 2). In most respects, similar features are also observed in bundle sheath cells of P. milioides (5) and M. arvensis (30). A previous study which concluded that bundle sheath chloroplasts of P. hysterophorus are incapable of forming starch (due to a lack of staining by I⁻-KI [24]) is clearly inaccurate. Also, these authors (13, 25) and others (26) reported that PEP carboxylase activities in mature leaves of P. hysterophorus range from 125 to 315 μmol/mg Chl-h, far in excess of any PEP carboxylase that we could detect in either chamber- or greenhouse-grown plant material (10–29 μmol/mg Chl-h, Table II). Patil and Hodge (25) additionally reported that aspartate aminotransferase has activities of only about 20 μmol/mg Chl-h, in contrast to the much higher, albeit rather variable, activities that we found (80–320 μmol/mg Chl-h, Table III). The reasons for these discrepancies between the data are unknown. However, the present data demonstrate low and comparable levels of PEP carboxylase in both P. hysterophorus and P. incanum, and therefore indicate that PEP carboxylase does not have a significant role in the observed reduction of photorespiration in P. hysterophorus.

We were unable to confirm a previous report that immature leaves of P. hysterophorus differ from the mature leaves in that the former lack Kranz anatomy and have relatively lower activities of C₄ cycle enzymes (25). We have observed Kranz-like anatomy in partially expanded leaves (data not shown), and could detect no significant differences in the expressed activities of C₄ cycle enzymes in immature leaves relative to mature leaves (Table II).

In summary, the weedy species Parthenium hysterophorus is classified as a C₃-C₄ intermediate based on Kranz-like leaf anatomy and reduced photorespiratory activity. The lack of appreciable activities of PEP carboxylase, pyruvate,Pi dikinase, and most other C₄ enzymes, plus the formation of C₃ products following short-term exposures of leaves to ¹⁴CO₂, indicate that P. hysterophorus assimilates atmospheric CO₂ solely by the C₃ pathway. The mechanism for reducing photorespiration in this
Fig. 2. TEM micrographs of thin sections from leaves of chamber-grown plants. A, Micrograph of bundle sheath cells (B) of *P. incanum*, magnified ×8600. Some vascular tissue can be seen at upper right. B, Micrograph of cells from *P. incanum* containing mesophyll (M) chloroplasts with large starch grains (S), facing a bundle sheath chloroplast with rudimentary starch grains, magnified ×12,700. C, Micrograph of cross-section of a minor leaf vein with surrounding bundle sheath cells (B) and adjoining mesophyll cells (M) of *P. hysterophorus*, magnified ×4400. Arrows designate bundle sheath mitochondria; P = peroxisomes. Conspicuous starch grains occur in chloroplasts of both mesophyll and bundle sheath cells.
Fig. 3. Response of CO₂ compensation point at 30°C to changing O₂ with leaves from chamber-grown plants of P. incanum (●), P. hysterophorus (○—○), and P. argentatum (□—□).

Table I. Photosynthesis Rates at 2% and 21% O₂, and the O₂ Inhibition of Photosynthesis in Leaves of P. incanum and P. hysterophorus

<table>
<thead>
<tr>
<th>Species</th>
<th>Photosynthesis Rate</th>
<th>Inhibition by 21% O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/mg Chl·h⁻¹</td>
<td>mg dm⁻³·h⁻¹</td>
</tr>
<tr>
<td>P. incanum</td>
<td>259 ± 15</td>
<td>171 ± 14</td>
</tr>
<tr>
<td>P. hysterophorus</td>
<td>254 ± 17</td>
<td>188 ± 15</td>
</tr>
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</table>

Table II. Chl a/b Ratios and Activities of C₃ Cycle Enzymes in Partially or Fully Expanded Leaves of P. incanum and P. hysterophorus

<table>
<thead>
<tr>
<th>Chl or Enzyme</th>
<th>P. incanum, Relative Leaf Age</th>
<th>P. hysterophorus, Relative Leaf Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl a/b</td>
<td>Young</td>
<td>Mature</td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>18</td>
<td>10 (29)</td>
</tr>
<tr>
<td>Pyruvate-Pi dikinase</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>NADP-malate dehydrogenase</td>
<td>5.2</td>
<td>4.9</td>
</tr>
<tr>
<td>NADP-malic enzyme</td>
<td>0.9</td>
<td>0.7 (4.6)</td>
</tr>
<tr>
<td>NAD-malic enzyme</td>
<td>20</td>
<td>5.7 (8.3)</td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>4.5</td>
<td>11 (95)</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>27</td>
<td>14 (64)</td>
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</table>

Table III. Percent Distribution of Soluble ¹⁴C Products in Leaves of P. incanum and P. hysterophorus after an 8 s Exposure to ¹⁴CO₂

<table>
<thead>
<tr>
<th>¹⁴C Product(s)</th>
<th>P. incanum</th>
<th>P. hysterophorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PGA</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td>Sugar phosphates</td>
<td>43</td>
<td>54</td>
</tr>
<tr>
<td>Malate</td>
<td>1.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Succinate</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Glycine, serine</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Alanine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Glycolate</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Others</td>
<td>1</td>
<td>0.1</td>
</tr>
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</table>

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