CRASSULACEAN ACID METABOLISM IN THREE SPECIES OF THE C₄ GENUS PORTULACA

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The putative existence of Crassulacean acid metabolism was investigated in three succulent species of the C₄ genus Portulaca: P. grandiflora (a widely cultivated species), P. oleracea (a cosmopolitan weed), and P. mundula (found on rock outcrops throughout the southwestern United States). Patterns of diurnal conductance and CO₂ exchange, as well as diurnal tissue malic acid fluctuations, were measured in plants under well-watered and drought-stressed conditions. Net CO₂ uptake in both treatments occurred only during the day, although small amounts of nighttime CO₂ uptake occurred in a few individuals. Stomatal conductances in well-watered and drought-stressed P. mundula and drought-stressed P. oleracea were higher during the night than during the day, though these higher conductances were generally not accompanied by net CO₂ assimilation. No significant diurnal malic acid fluctuations occurred under well-watered conditions in any species; however, under drought stress, significant diurnal malic acid fluctuations occurred in the three species. The primary source of carbon for nocturnal malic acid production is presumed to be respiratory CO₂, as occurs in C₃ plants that undergo CAM-cycling. This study confirms past reports of CAM acid fluctuations in P. grandiflora and P. oleracea and marks the first report of CAM activity in P. mundula.

Introduction

The C₄ photosynthetic pathway is distinguished from the C₃ pathway by the involvement of phosphoenolpyruvate (PEP) carboxylase in the initial fixation of atmospheric CO₂, resulting in the production of four-carbon organic acids (Black 1973; Edwards and Walker 1983). In addition, leaves of C₄ plants display the distinctive Kranz anatomy, wherein mesophyll and bundle sheath cells are different in appearance and function. This combination of anatomical and biochemical features allows a reduction of photorespiration and, consequently, maintenance of net CO₂ fixation even under stressful conditions. Plants that utilize Crassulacean acid metabolism (CAM) also employ PEP carboxylase for the initial fixation of atmospheric CO₂, but this process occurs at night, when water loss is minimal (Kluge and Ting 1978; Winter 1985). During the day, the stomata of CAM plants are closed, resulting in very high diurnal water-use efficiencies. A fundamental difference between C₄ and CAM is that initial CO₂ fixation via PEP carboxylase is separated from the subsequent reduction of CO₂ via Rubisco spatially in C₄ plants while in CAM plants the separation is temporal.

Although the photosynthetic pathway of most plants can be categorized as C₃, C₄, or CAM, numerous species combine aspects of two pathways (Ehleringer and Monson 1993). For example, some "C₃-C₄ intermediates" have anatomical and biochemical features intermediate between those of C₃ and C₄ species. Combinations at the organ level occur as well, including species with CAM stems and C₃ leaves (Osmund et al. 1982). Furthermore, a few species exhibit seasonal switching between the C₄ and CAM pathways (Lüttge 1993). Finally, an increasing number of species has been found to exhibit "CAM-cycling" (Ting 1985; Martin 1995). These plants exhibit C₄ gas exchange patterns but show CAM-like diurnal acid fluctuations. As stomata are closed at night, respiration is presumably the source of CO₂ during nocturnal malic acid formation. Whether CAM-cycling directly benefits a plant is unclear. Several hypotheses have been posed, including a possible increase in water conservation (Martin et al. 1988; Harris and Martin 1991) and a potential reduction of photo-inhibition (Osmund et al. 1980; Osmond 1982). Accumulating evidence, however, suggests that CAM-cycling may simply be a precursor to "CAM-idling" and not directly benefit the plant (Rayder and Ting 1981; Sipes and Ting 1985; Martin 1995). Many plants that exhibit CAM or CAM-cycling when well-watered undergo CAM-idling under severe drought stress, during which CAM acid fluctuations occur despite 24-h stomatal closure. Presumably, by maintaining metabolic activity through the fixation of respiratory CO₂, these plants may resume uptake of atmospheric CO₂ more rapidly upon rehydration.

Because C₄ and CAM plants exhibit nearly identical biochemistry, the existence of C₄-CAM intermediates is not unexpected. There are only a few, scattered reports of such plants, however, all in the genus Portulaca. Under artificial conditions, Koch and Kennedy (1980) found that a short photoperiod (8 h) induced diurnal acid fluctuations indicative of CAM in the C₄ species Portulaca oleracea. Drought-stressed plants exhibited CAM acid fluctuations as well, albeit at reduced levels. On the other hand, subsequent fieldwork by the same authors indicated that drought-stressed individuals under long photoperiods (13 h) showed the greatest degree of CAM activity (Koch and Kennedy 1982). Ku et al. (1981) measured greater diurnal acid fluctuations in well-watered plants of Portulaca grandiflora than in drought-stressed individuals. Finally, in a recent survey of CAM in the family Portulaceae, Guralnick and Jackson (1993) reported diurnal acid fluctuations in several genera including the C₄ genus
Portulaca. Several studies of Portulaca species, however, either found no evidence of CAM activity or reported peculiarities in the C₄ pathway. In a survey of succulent species in North Carolina, Martin et al. (1982) found no diurnal acid fluctuations in either P. oleracea or Portulaca pilosa (note that Matthews and Levins [1985a, 1985b] treat P. pilosa as Portulaca mundula). Also, results of pulse-chase experiments with K-stressed plants of P. oleracea by Karadge (1986) indicated C₃ photosynthesis. Finally, Fathi and Schnarrenberger (1990) found that the NADP-malic enzyme of P. grandiflora displayed an immunoch­
terical cross-reaction more similar to that of C₄ species.

The approximate nighttime vapor pressure deficit (vpd) was 0.3–1.0 kPa, while the daytime vpd was ca. 1.8–2.0 kPa. Drought-stressed individuals of P. grandiflora and P. mundula, a small succulent native to the central and southwestern United States that grows in shallow, gravelly soil over limestone outcrops (Matthews and Levins 1985a, 1985b); and P. grandiflora, an ornamental cultivar, which grows best in sandy soil, originally from northern South America (Bailey and Bailey 1976). Based on anatomical (Welkie and Caldwell 1970; A. A. Kraybill, personal observation) and physiological characteristics (Tregunna and Downton 1967; Martin et al. 1982; δ¹³C of P. mundula = −13.8‰, unpublished data), these three species of Portulaca are clearly C₄ plants. A further objective was to determine whether the presence or degree of CAM is correlated with the potential aridity of the typical habitat of each species.

**Material and methods**

**PLANT COLLECTION AND CULTIVATION**

Plants of Portulaca oleracea L. were collected from a recently cultivated ornamental plot on the campus of the University of Kansas, Lawrence, Kansas. The plants were growing in full sun and well-drained topsoil. Mature plants of Portulaca grandiflora Hook were purchased locally. Seedlings of Portulaca mundula I. M. Johnst. were collected from thin pasture soil over limestone outcrops ca. 6.5 km southwest of Neodesha, Wilson County, Kansas.

Plants were potted in a 1:2 (v/v) mixture of sand and standard greenhouse soil (7:2:2:1 [v/v] mixture of clay loam, peat moss, Perlite, and vermiculite) and were grown in a greenhouse over limestone outcrops (Matthews and Levins 1985a, 1985b); and P. grandiflora, an ornamental cultivar, which grows best in sandy soil, originally from northern South America (Bailey and Bailey 1976). Based on anatomical (Welkie and Caldwell 1970; A. A. Kraybill, personal observation) and physiological characteristics (Tregunna and Downton 1967; Martin et al. 1982; δ¹³C of P. mundula = −13.8‰, unpublished data), these three species of Portulaca are clearly C₄ plants. A further objective was to determine whether the presence or degree of CAM is correlated with the potential aridity of the typical habitat of each species.

**GAS EXCHANGE**

CO₂ and water vapor exchange were measured with an open, differential infrared gas analysis system (Harris and Martin 1991; Gravatt and Martin 1992). Intact shoots were sealed in gas exchange cuvettes for 2 d; soil and roots remained outside the cuvettes. Environmental conditions inside the cuvettes were: PPFD at plant height of 1000 µmol m⁻² s⁻¹, 30/20°C day/night temperatures, and 2.4/0.5 kPa day/night leaf to air vpd. During well-watered treatments, the plants received water throughout the measurement period. Due to leaf morphology, an accurate measurement of leaf surface area was impossible to obtain from these plants. Leaf dry weight, however, was obtained and used as an indirect measure of area. Thus, all data are expressed on a dry weight basis. On the second day of gas exchange measurements, shoot tissue was removed from the cuvettes immediately prior to the final dark period. The plant tissue was frozen at −65°C upon sampling until subsequent malate analysis. The remaining shoot tissue was removed and frozen at the end of the dark period.

**MALATE ANALYSIS**

Leaf and stem tissue was sliced upon thawing, and cell sap (up to 10% of total tissue liquid) was extracted by centrifugation (Smith and Lütte 1985). Malate concentrations of the sap were determined using the enzymatic/spectrophotometric method of Gutmann and Wahlefeld (1974) and a standard curve based upon known malate concentrations. As the pH of the buffer used in this assay is quite high, most malic acid is converted to malate upon analysis. Thus, tissue malic acid concentration will be discussed, as it is more technically correct, though tissue malate concentrations were actually measured.

**STATISTICS**

The nonparametric Mann-Whitney U-test was used to compare two means. Low sample sizes made the normality of the data distribution difficult to determine, thus preventing the use of parametric statistics.

**Results**

**WELL-WATERED PLANTS**

Uptake of CO₂ was observed only during the day in all three species (figs. 1–3); no net uptake occurred at night. Daytime internal CO₂ concentrations were lowest and CO₂ uptake rates highest in Portulaca oleracea, followed by Portulaca grandiflora and Portulaca mundula. Stomatal conductance patterns of P. grandiflora matched assimilation; values were higher during the day and lower at night. In contrast, conductances for P. mundula were higher at night than during the day but were not accompanied by nighttime CO₂ assimilation. This phenomenon also was observed in P. oleracea during the second day/night period.

The amount of CO₂ assimilated throughout the day
by *P. oleracea* was over twice that of *P. grandiflora* and more than four times that of *P. mundula* (table 1). Total nighttime release of CO$_2$ was approximately equal in *P. grandiflora* and *P. oleracea*, while less CO$_2$ was released by *P. mundula*. Total integrated water use efficiency (WUE) was more positive in *P. oleracea* than in *P. mundula* and *P. grandiflora* (table 2).

Morning and evening tissue malic acid concentrations were not significantly different in leaves of the species under well-watered conditions (fig. 4) and in stems of *P. grandiflora* and *P. mundula* (fig. 5). Due to sampling difficulties, malic acid fluctuations were not measured in stems of well-watered *P. oleracea*.

**Drought-stressed plants**

Significant daytime CO$_2$ uptake was not detected in *P. grandiflora* under drought stress (fig. 1a). Likewise, drought-stressed individuals of *P. oleracea* exhibited only small amounts of net daytime CO$_2$ assimilation; no nighttime uptake was observed (fig. 2a). Relatively high rates of CO$_2$ assimilation, though generally lower than control rates, occurred during the day in all individuals of *P. mundula*, while net nocturnal CO$_2$ uptake was observed only in one plant (fig. 3a). The three species of *Portulaca* exhibited reduced nocturnal rates of respiration relative to well-watered plants (table 1).

Nocturnal stomatal conductances for *P. oleracea* were greater than daytime values, with higher conductances measured during the second than the first night.
were significantly different in all species under drought-stressed conditions (fig. 4). Diurnal malic acid fluctuations were greater in leaves of *P. mundula* and *P. grandiflora* than in *P. oleracea*. Low sample sizes made statistical interpretation of stem malic acid fluctuations difficult. Diurnal changes did occur, however, and the magnitude of the acid fluctuations for individuals of *P. oleracea* was generally higher than that of the other two species (fig. 5).

**Discussion**

While there were no significant differences in shoot water content between well-watered and drought-stressed plants in the current study (data not shown), other indications of drought stress were clearly evident. Plant midday water potentials were ca. −0.3 MPa under well-watered conditions, but were reduced by 0.3–0.6 MPa under drought stress conditions (data not shown), and daytime assimilation rates were reduced in all three species. Finally, under drought stress, significant diurnal malic acid fluctuations, which did not occur in well-watered plants, were observed in all species.

Carbon dioxide exchange patterns for the three species under well-watered conditions were similar to other *C₄* species, though rates were lower; CO₂ was taken up during the day and released at night. Under drought stress, gas exchange patterns were similar to those of well-watered individuals, albeit at reduced levels. Net nocturnal CO₂ assimilation did occur in a few individuals, however. Comparable CO₂ exchange patterns in *Portulaca oleracea* under drought stress were reported by Koch and Kennedy (1980, 1982).

Diurnal stomatal conductance patterns in well-watered individuals of *Portulaca mundula* were unlike those found in the other species; conductances were lower during the day than during the night. Higher nighttime conductances were observed despite high calculated leaf internal CO₂ concentrations and were generally not accompanied by CO₂ uptake. An explanation for these unusual findings is not available.

Significant diurnal fluctuations of malic acid occurred in the leaves of the three species of *Portulaca* under drought-stressed conditions. A similar trend was observed in the stem tissue, though low sample sizes made the statistical significance of the measurements questionable. The data is of interest, however, given the scarcity of stem CAM data in leaf succulents. While malic acid fluctuations in *P. oleracea* and *Portulaca grandiflora* had been documented in past studies, this observation marked the first report of CAM activity in the *C₄* species *P. mundula*. In addition, the magnitude of the stem and leaf malic acid fluctuations in *P. oleracea* and *P. grandiflora* differed markedly from past findings. Koch and Kennedy (1980, 1982) found greater malic acid fluctuations in the leaves than in the stems of drought-stressed *P. oleracea*. In our study, diurnal fluctuations of malic acid in the leaves of drought-stressed *P. oleracea* were comparable to those observed by Koch...
and Kennedy (1980), but stem acid fluctuations were much higher than previously reported (comparisons made on fresh weight basis, data not shown). In addition, fluctuations of malic acid in both stems and leaves of _P. grandiflora_ were much higher than previously reported by Ku et al. (1981; also compared on fresh weight basis). Finally, both Ku et al. (1981) and Koch and Kennedy (1980) reported that drought stress reduced the magnitude of diurnal malic acid fluctuations. In our study, however, significant malic acid fluctuations did not occur in well-watered plants while drought stress induced acid fluctuations indicative of CAM. While Martin et al. (1982) found no diurnal acid fluctuations in _P. oleracea_ or _Portulaca pilosa_ in the field in North Carolina, the results of our study would indicate that the plants were possibly well-hydrated at the time of measurement.

Though the anatomical and physiological characteristics of the three species of _Portulaca_ in this study indicate C₄ photosynthesis (see Introduction), our results indicate that these plants can also undergo CAM, or at least diurnal acid fluctuations. When nighttime CO₂ uptake occurs, the total amount of CO₂ fixed is approximately 5%–22% of the total malic acid produced that night. Presumably, the primary source of carbon for nighttime malic acid production is the recycling of respired CO₂. This presumption is further supported by a reduction in the amount of CO₂ respired by drought-stressed plants at night, relative to well-watered plants of the same species. Respiration rates in drought-stressed individuals of _P. oleracea_, _P. grandiflora_, and _P. mundula_ were ca. 40%, 30%, and 2% of the rates of well-watered plants, respectively. In addition, the changes in malic acid concentration in the three species under drought stress closely correlate with the reductions in respiration.

The recycling of respiratory CO₂ per se should not directly benefit the plants because energy must be expended to reduce the CO₂, regardless of its source (Martin 1995). On the other hand, this process might indirectly benefit the plants. High levels of malic acid in well-watered plants may constitute a pool of malic acid that could be utilized as an alternative source of carbon during drought stress. Nighttime fixation of respiratory CO₂ might then replenish malic acid stores without the water loss associated with a high daytime leaf to air vpd. In addition, the recycling of respiratory CO₂ would allow for maintenance respiration during severe drought. During the day, the release of additional CO₂ from stored malic acid might also prevent stress-induced damage to the photosynthetic apparatus.

If "C₄-CAM-cycling" is indeed an adaptation to drought stress, the greatest degree of acid fluctuations might be expected in _P. mundula_, which tends to grow in the most arid habitats. _Portulaca mundula_ did indeed exhibit malic acid fluctuations (stem and leaf) that are higher than those of _P. oleracea_ and _P. grandiflora_, which are approximately equal. Also, under drought stress, the highest WUE is observed in _P. mundula_. Finally, a drought period 1 wk longer than that of the two other species is required to effect an

### Table 1

<table>
<thead>
<tr>
<th>Species and treatment</th>
<th>Daytime CO₂ exchange (mmol kg⁻¹)</th>
<th>Nighttime CO₂ exchange (mmol kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uptake</td>
<td>Loss</td>
</tr>
<tr>
<td><em>P. grandiflora</em>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>7330 (2910)</td>
<td>0</td>
</tr>
<tr>
<td>No water for 4 d</td>
<td>6 (12)</td>
<td>161 (162)</td>
</tr>
<tr>
<td><em>P. oleracea</em>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>17,635 (4033)</td>
<td>0</td>
</tr>
<tr>
<td>No water for 4 d</td>
<td>37 (31)</td>
<td>-145 (76)</td>
</tr>
<tr>
<td><em>P. mundula</em>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>3631 (526)</td>
<td>0</td>
</tr>
<tr>
<td>No water for 11 d</td>
<td>1816 (558)</td>
<td>0</td>
</tr>
</tbody>
</table>

Note. Values are means (standard deviations in parentheses) of four plants and were derived from gas exchange analyses; representative diurnal gas exchange patterns are provided in figures 1–3. Data are expressed on a dry mass basis.

⁴ Positive values indicate net CO₂ uptake; negative values indicate net CO₂ loss.

### Table 2

<table>
<thead>
<tr>
<th>Species and treatment</th>
<th>WUE (mmol CO₂/mol H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. grandiflora</em>:</td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>4.94 (0.46)</td>
</tr>
<tr>
<td>No water for 4 d</td>
<td>na²</td>
</tr>
<tr>
<td><em>P. oleracea</em>:</td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>7.01 (0.63)</td>
</tr>
<tr>
<td>No water for 4 d</td>
<td>na²</td>
</tr>
<tr>
<td><em>P. mundula</em>:</td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>5.76 (1.19)</td>
</tr>
<tr>
<td>No water for 11 d</td>
<td>5.76 (0.53)</td>
</tr>
</tbody>
</table>

Note. Date are means (standard deviations in parentheses) of four plants.

² na = not available; negative net CO₂ exchange prevented calculation of WUE.
appreciable decrease in the shoot water potential of *P. mundula*, and yet CO$_2$ exchange rates are much less affected than those of the other species. Attempts to relate the potential degree of aridity of the habitat of the three species with the magnitude of CAM observed in the laboratory, however, are possibly confounded by greenhouse growth conditions. For example, the degree of CAM may have been greater in all plants when grown under full sunlight and continuous drought-stressed conditions.

In conclusion, this study confirms that some level of CAM can occur in these succulent species of the genus *Portulaca*. Given the biochemical similarities between CAM and C$_4$ plants, this combination of photosynthetic pathways is not completely unexpected.

The biochemical regulation of C$_4$ and CAM in the same tissue, however, is not fully understood (Ku et al. 1981). Also, as this combination of photosynthetic pathways appears to be rare among C$_4$ species the question of its ecological significance, if any, remains to be answered. These biochemical and adaptive questions require further investigation before this unusual combination of C$_4$ and CAM can be fully understood.

**Acknowledgments**

This study was funded in part by the College of Liberal Arts and Sciences of the University of Kansas. We would like to thank Mr. Maynard Struthers of Neodesha, Kansas, for providing access to his land for specimen collection.
Literature cited


