Characteristics of Crassulacean Acid Metabolism in the Succulent C₄ Dicot, Portulaca oleracea L.¹

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ABSTRACT
Crassulacean acid metabolism (CAM) was investigated in leaves and stems of the succulent C₄ dicot Portulaca oleracea L. Diurnal acid fluctuations, CO₂ gas exchange, and leaf resistance were monitored under various photoperiod and watering regimes. No CAM activity was seen in well watered plants grown under 16-hour days. Under 8-hour days, however, well watered plants showed a -CAM-like pattern of acid fluctuation with amplitudes of 102 and 90 microequivalents per gram fresh weight for leaves and stems, respectively. Similar patterns were also observed in detached leaves and defoliated stems. Leaf resistance values indicated that stomata were open during part of the dark period, but night adicification most likely occurred from refixation of respiratory CO₂. In water-stressed plants, maximum acid accumulations were reduced under both long and short photoperiods. At night, these plants showed short periods of net CO₂ uptake and stomatal opening which continued all night long during preliminary studies under natural environmental conditions. Greatest acid fluctuations, in P. oleracea, with amplitudes of 128 microequivalents per gram fresh weight, were observed in water-stressed plants which had been rewatered, especially when grown under short days. No net CO₂ uptake took place, but stomata remained open throughout the night under these conditions. These results indicate that under certain conditions, such as water stress or short photoperiods, P. oleracea is capable of developing an acid metabolism with many similarities to CAM.

Some succulents have a unique capacity to shift their photosynthetic mode between the C₃ type and CAM, depending on environment and age (16, 17, 32). CAM has been shown to be affected by salt treatment (29, 34), water stress (1, 11, 15, 17, 26, 34) photoperiod (19), temperature fluctuations between hot days and cool nights (11, 14), stage of maturity (6, 17, 20), and flowering (3).

The purpose of the present study was to examine the possible occurrence of CAM or facultative CAM in a succulent C₄ plant species, Portulaca oleracea or purslane, which was chosen for this study for several reasons. First, many aspects of its C₄ physiology are well established, such as four-carbon acid metabolism (9, 10), enzyme activities (8, 9), compensation point (9, 31), anatomy and cytology (7), photosynthesis (8), photosynthetic rate (9), and response to salt and water stress (9). Also, previous work has shown that the water use efficiency of P. oleracea is particularly high, even for a C₄ plant (24) and is close to that of a CAM plant. Finally, the Portulacaceae is a likely group in which to find a species with both C₄ and CAM activity since this family contains C₃, C₄, and CAM plants (2, 5, 29).

MATERIALS AND METHODS

P. oleracea L. plants were grown in the greenhouse until 3 to 4 weeks old. Young, uniform plants with three to four leaf pairs were then individually potted and transferred to a growth chamber for a minimum of 7 weeks before experimental use (3). Incandescent and fluorescent bulbs provided an intensity of 380 µE m⁻² s⁻¹ at plant level. Temperatures of 30 °C day/15 C night were maintained with RH of 45%. Six different combinations of daylength and watering regimes were tested.

1. Long Day (16-h Light/8-h Dark), Well Watered (LD-WW). Plants were watered every 2 days. Tissue Ψ at the time of the experiment was -3 to -4 bars.

2. Short Day (8-h Light/16-h Dark), Water-stressed (SD-WW). Plants were watered every 3 days. Tissue Ψ at the time of the experiment was -3 to -4 bars.

3. Long Day (16-h Light/8-h Dark), Water-stressed (LD-STR). Plants were well watered for 3 weeks, then watered weekly for 5 weeks. Tissue Ψ at the time of the experiment was -7.5 to -8.5 bars.

4. Short Day (8-h Light/16-h Dark), Water-stressed (SD-STR). Plants were watered as in regime 3 with 9 days between waterings. Tissue Ψ at the time of the experiment was -7.5 to -8.5 bars.

5. Long Day (16-h Light/8-h Dark), Stressed and Rewatered. Plants were watered as in regime 3, but then water was withheld for 12 days before a single rewatering. Experiments were conducted for 6 days after this final watering and tissue Ψ became more negative as the plants dried.

6. Short Day (8-h Light/16-h Dark) Stressed and Rewatered. Plants were treated as in regime 5, but water was withheld 14 days before a single rewatering. Experiments were conducted for 6 days after this final watering and tissue Ψ became more negative as the plants dried.

Twenty-four-h measurements of changes in titratable acidity were made under each of the above regimes. Every 3-4 hours, plants were removed from the growth chamber and immediately submerged in liquid N₂. Leaves and stems of individual plants were then separated and weighed while frozen. To prevent thawing of frozen specimens, material was frequently reimmersed in liquid N₂. Tissue was then ground at -196 °C in a mortar and pestle and acid was extracted by boiling for 1 h in glass-distilled H₂O. Additional glass-distilled H₂O was added to bring the final volume to 100 times the original fresh weight and titrated to an end point.

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4 Abbreviations: LD-WW: long-day, well-watered; SD-WW: short-day, well-watered; LD-STR: long-day, stressed; SD-STR: short-day, stressed; PEP: phosphoenolpyruvate.
of pH 6.9. During this procedure, extracts were kept at 50 C and monitored with a thermally adjusted pH electrode to eliminate error due to dissolved CO₂. Since diffusion can occur between leaf and stem tissues of intact plants, CAM activity in stems alone would be expected to influence the acid fluctuations of the whole plant. For this reason, the leaves and stems were tested individually in one experiment. Leaves were removed from SD-WW plants and were placed on moist filter paper in the bottom of covered beakers. One group of both detached leaves and defoliated stems was placed in the growth chamber during the light period and another during the dark period. Samples were taken by the same method used for intact plants, and all titrations were run in quadruplicate.

Labeling experiments were conducted on SD-WW plants during the first and last hours of the dark period. Whole branches were then killed with liquid N₂, products extracted as previously described (10), and thin layer electrophoresis used to separate labeled compounds.

Rates of CO₂ exchange were determined using an open IR gas analysis system (21). Four intact plants from each of the six regimes tested were enclosed inside a Plexiglas chamber maintained at 30 C in the light and 15 C in the dark. Light intensity in which the plants were grown was duplicated using gauze filters and a 1,000-w Lucalux lamp. Water vapor concentrations in the chamber were recorded with a RH sensor and data, along with temperature readings, were used to calculate leaf resistance and transpiration rates (23).

Tissue water potential was determined using a Scholander pressure chamber (22).

RESULTS

Anatomy and Morphology. Although the leaves and stems of *P. oleracea* are fleshy, the cellular succulence, as discussed by Kluge (11), was determined by microscopy. Large vacuole to cytoplasm ratios were observed with the light microscope and in transmission and scanning electron micrographs.

The morphology of *P. oleracea* plants varied considerably under different growing conditions. The most notable differences, plant fresh weight, leaf size, and fruit production, are shown in Table I. When photoperiod was shifted from 16 h to 8 h, *Portulaca* plants accumulated approximately one-quarter the fresh weight, produced small leaves, synthesized more red pigment, and increased their relative fruit production. The effects of water stress were similar to those of short photoperiods in reducing leaf size and increasing red pigment content, most likely betalaines (33). Total fresh weight decreased an average of approximately 36% when water was withheld. Relative fruit production was little affected by water stress.

Acid Fluctuations. CAM plants generally show diurnal changes in titratable acidity with a range between maximum and minimum levels (amplitude) of 30-300 µeq g⁻¹ fresh weight (13). Large changes in titratable acidity of this magnitude were exhibited by *Portulaca* plants grown under SD-WW conditions (Fig. 1A). These changes were comparable to those of CAM species, such as *Kalanchoe diagremontiana* (1), with amplitudes of 102 and 88 µeq g⁻¹ fresh weight in leaves and stems, respectively. LD-WW plants

<table>
<thead>
<tr>
<th>Tissue</th>
<th>LD-WW</th>
<th>SD-WW</th>
<th>LD-STR</th>
<th>SD-STR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g fresh weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole plant</td>
<td>12.75</td>
<td>2.72</td>
<td>8.08</td>
<td>1.74</td>
</tr>
<tr>
<td>Leaf (42%)</td>
<td>1.26</td>
<td>2.22</td>
<td>1.08</td>
<td>0.46</td>
</tr>
<tr>
<td>Stem (54%)</td>
<td>1.06</td>
<td>5.52</td>
<td>5.86</td>
<td>0.58</td>
</tr>
<tr>
<td>Fruit (6%)</td>
<td>0.37</td>
<td>0.34</td>
<td>0.20</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 1. Leaf, Stem, Fruit, and Total Fresh Weights of *P. oleracea* Plants Grown under Different Photoperiods and Watering Regimes

Each figure represents the average of at least 20 individual plants. Numbers in parentheses are weights expressed as per cent of the whole plant.

![Fig. 1. Amplitude of acid fluctuations in *P. oleracea* under different growing conditions. Top of bar represents maximum early morning acid levels; bottom of bar indicates the minimum late day acid levels in leaves (m) and stems (s), respectively. Stressed and rewatered plants were given water on day 0.](image-url)
also showed high maximum levels of acidity, with 87 and 78 µeq g\(^{-1}\) fresh weight in leaves and stems. They did not exhibit the daytime deacidification typically seen in CAM plants, however, and in LD-WW plants, acidity of leaves and stems remained above 54 µeq g\(^{-1}\) fresh weight throughout the 24-h study period. Water stress reduced total night acid accumulation in both LD- and SD-grown plants.

Rewatering drought-stressed CAM plants often increases nocturnal acidification (25, 26). This also occurs in \(P.\) \(oleracea\). The greatest fluctuation in acid levels was observed after rewatering SD-STR plants (Fig. 1C) and again, the magnitude of this change, 126 µeq g\(^{-1}\) fresh weight is within the range observed for CAM plants (13). Rewatering LD-grown plants resulted in similar amplification of acid fluctuations (Fig. 1C), but maximum accumulation was less than half that seen in SD-grown plants. Increased nocturnal acidification was restricted to leaves in LD-grown plants, while under SD conditions, a delayed and reduced response was also seen in stems.

Along with CAM-like amplitudes of acid fluctuation, \(Portulaca\) plants also showed hourly changes in acidity levels which were like CAM plants (Fig. 2), with maximum acidity in the AM and least acid in the PM. Leaf acidity remained higher than that of stems throughout each 24-h study period, except for SD-WW plants.

Since stems and leaves of many succulent plants exhibit acid production (16), stems without leaves and detached leaves were tested separately in SD-WW plants. This regime was shown above to have the greatest acid fluctuations (Fig. 1A). Table II compares acidification and deacidification of leaves and stems from intact plants to that observed in detached leaves and defoliated stems. Stems also acidify in the dark after leaves are removed, but total acid levels are reduced from 148 to 93 µeq g\(^{-1}\) fresh weight. Excised and intact leaves both deacidify in the light, but intact leaves lose 100 µeq g\(^{-1}\) fresh weight, compared to only 12 µeq g\(^{-1}\) fresh weight in those which were previously detached. Stems of whole plants lost less acid than did defoliated stems. Stems of intact plants and detached leaves accumulate the most acid in the dark; whereas, in the light, stems and leaves of intact plants deacidify the most.

The compounds labeled during \(^{14}\)CO\(_2\) fixation in the dark are shown in Table III. As is typical for dark \(^{14}\)CO\(_2\) fixation in many plant tissues (10), malate was most heavily labeled, containing 52.6% of the \(^{14}\)C in the leaves and 47.7% in the stems after a 45-min exposure period. Lesser amounts of aspartate, citrate, and glutamate were also labeled in leaf and stem tissues.

Gas Exchange. Gas exchange in well watered \(Portulaca\) plants is similar to that of typical mesophytes (15) (Fig. 3, A and B), with CO\(_2\) released in the dark and taken up in the light. In SD-grown plants (Fig. 3B), this indicates that the nighttime acidification recorded is most likely taking place via refixation of respiratory CO\(_2\).

Water stress (Figs. 2C and 3D) results in considerable alteration of diurnal gas exchange in \(Portulaca\). In both SD- and LD-grown plants, there is a period of CO\(_2\) release at the beginning of the light period, as often seen in CAM plants under similar growth chamber conditions (1, 6, 13). The maximum rate of CO\(_2\) evolution was 10.0 mg CO\(_2\) dm\(^{-2}\) h\(^{-1}\) in SD-grown plants and 1.5 mg dm\(^{-2}\) h\(^{-1}\) in LD-grown plants. Net CO\(_2\) assimilation in water-stressed plants took place in the dark under both photoperiods. This CO\(_2\) uptake was observed for short periods, with rates of 0.6 and 0.9 mg CO\(_2\) dm\(^{-2}\) h\(^{-1}\), but took place continuously during later studies under natural environmental conditions (in preparation).

Depending on the photoperiod, gas exchange patterns differed after plants had been stressed and rewatered. Under long days, nocturnal release of respiratory CO\(_2\) was reduced to nearly zero in spite of relatively low leaf resistance. The light period began with a period of CO\(_2\) efflux, similar to that seen in water-stressed \(Portulaca\) plants, and atmospheric CO\(_2\) was assimilated during the remaining hours of light. SD-grown plants, on the other hand, released CO\(_2\) throughout the night and showed irregular gas exchange rates during both the dark and light periods. As often seen in CAM plants (1, 32), a "burst" in CO\(_2\) fixation occurred just after the start of illumination, followed by a period of reduced assimilation until nearly the end of the day.

Stomata. Leaf resistance values are low during the day in well watered \(P.\) \(oleracea\) (Fig. 3, A and B) and remain relatively low in the dark. Similar observations have been reported during periods of favorable water status in the CAM plants \(Opuntia\) (28) and \(Echinocereus\) (4). Under LD-STR conditions, leaf resistance increased substantially (Fig. 3C), but only during the day and last half of the night. The greatest stomatal opening appeared to be in the first part of the dark period. Rewatering stressed plants results in low leaf resistances under long photoperiods, and higher, variable resistances in SD-grown plants.

![Fig. 2](image-url) Diurnal fluctuations of titratable acidity in \(P.\) \(oleracea\) under different growing conditions. Gas exchange and leaf resistance measurements during these experiments are shown in Figure 3. Values represent mean of four plants titrated individually; leaf acid (●—●); stem acidity (▲—▲). Shaded areas designate night.

### Table II. Changes in Titratable Acidity of Leaves and Stems of \(P.\) \(oleracea\)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dark Acidification</th>
<th>Light Deacidification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µequivalents g(^{-1}) fresh weight</td>
<td></td>
</tr>
<tr>
<td>Stems</td>
<td>Intact</td>
<td>Defoliated</td>
</tr>
<tr>
<td></td>
<td>+92</td>
<td>-73</td>
</tr>
<tr>
<td></td>
<td>+37</td>
<td>-91</td>
</tr>
<tr>
<td>Leaves</td>
<td>Intact</td>
<td>Defoliated</td>
</tr>
<tr>
<td></td>
<td>+101</td>
<td>-100</td>
</tr>
<tr>
<td></td>
<td>+113</td>
<td>-12</td>
</tr>
</tbody>
</table>

### Table III. Compounds Labeled in Intact SD-WW \(P.\) \(oleracea\) Plants after a 45-min Exposure to \(^{14}\)CO\(_2\) in the Dark

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Malate</th>
<th>Aspartate</th>
<th>Citrate</th>
<th>Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% total (^{14})C incorporated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stems</td>
<td>47.7</td>
<td>31.6</td>
<td>17.5</td>
<td>3.2</td>
</tr>
<tr>
<td>Leaves</td>
<td>52.6</td>
<td>26.6</td>
<td>15.4</td>
<td>5.4</td>
</tr>
</tbody>
</table>
The relative CAM activity recorded under each of the six conditions is summarized in Table IV.

A striking characteristic of CAM plants is generally considered to be their nocturnal acidification (16). As seen in the present study, Portulaca showed high morning acidity under several conditions. As a C₄ plant, P. oleracea has the PEP carboxylase activity needed for acid synthesis in the dark (8, 10, 16), and as a succulent, it has the large vacuoles believed necessary for acid accumulation (11). Together, this succulence and PEP carboxylase activity could account for the high tissue acidity accumulated at night under SD conditions and maintained continually under LD conditions. Another important aspect of CAM, however, is the deacidification which occurs in the subsequent light period, supplying an internal CO₂ source for photosynthesis. Such deacidification was observed in SD-grown P. oleracea plants, but it seldom occurred under long photoperiod conditions when Portulaca exhibited C₄ activity (8-10).

Developmental factors may also be involved in the response to SD conditions, since the morphology of P. oleracea changed with photoperiod. Short days promoted formation of flowers and small leaves and resulted in greater allocation of fresh weight into fruit production (Table I), as reported by Zimmerman (35) between 12- and 16-h days. Similar effects of photoperiod on the flowering of other succulents have coincided with increased acid synthesis and parallel induction of the two processes has been proposed in Kalanchoë blossfeldiana Poellnitz, cv. Tom Thumb (13). In addition, P. oleracea seems to complete its life cycle more rapidly under short photoperiods (unpublished results), and since the capacity for acid accumulation in succulents frequently increases with age (14, 17, 20), there may be a close relationship between photoperiod and developmental factors in the acid metabolism of Portulaca.

The response of succulents to water stress has been found to vary considerably. Some plants shift from C₃ to CAM (17, 29), while others develop an "idling" mechanism of acid fluctuations without net gas exchange (26). Water-stressed Portulaca plants showed minimal gas exchange but had dark CO₂ assimilation indicative of CAM. Although the duration of uptake was short, preliminary studies under natural environmental conditions have since indicated that this uptake occurs all night long and that carbon is conserved during the following day. The decreasing acid levels seen in P. oleracea after extended periods of water stress have also been observed in Opuntia (26, 28) and may result from stomatal closure, diminished carbohydrate supply, and reduced respiration (26-28).

Some of the greatest acid fluctuations reported in CAM plants have occurred in water-stressed plants following rewetting (26, 28). Acid fluctuations in Portulaca reach their greatest amplitude after similar treatment, especially in SD conditions, and gas exchange measurements under these conditions suggest that rapid changes in metabolism may occur on rewetting.

**CONCLUDING REMARKS**

Recent studies have found considerable variation in the CAM activity of succulents (16). Some of these CAM-like aspects of
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