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Characteristics of Crassulacean Acid Metabolism in the Succulent C_4 Dicot, *Portulaca oleracea* L.[1]

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ABSTRACT

Crassulacean acid metabolism (CAM) was investigated in leaves and stems of the succulent C_4 dicot *Portulaca oleracea* L. Diurnal acid fluctuations, CO_2 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 acidification most likely resulted from refixation of respiratory CO_2. In water-stressed plants maximum acid accumulations were reduced under both long and short photoperiods. At night, these plants showed short periods of net CO_2 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 CO_2 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 C_3 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 C_4 plant species. *Portulaca oleracea* or purslane was chosen for this study for several reasons. First, many aspects of its C_4 physiology are well established, such as four-carbon acid metabolism (9, 10), enzyme activities (8, 9), compensation point (9, 31), anatomy and cytology (7), photorespiration (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 C_4 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 C_4 and CAM activity since this family contains C_3, C_4, 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).[4] 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), Well Watered (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 N_2. 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 N_2. Tissue was then ground at −196 C in a mortar and pestle and acid was extracted by boiling for 1 h in glass-distilled H_2O. Additional glass-distilled H_2O 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 CO_2. 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 N_2, products extracted as previously described (10), and thin layer electrophoresis used to separate labeled compounds.

Rates of CO_2 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 these 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 *Kalanchoë diagremontiana* (1), with amplitudes of 102 and 88 μeq g^{-1} fresh weight in leaves and stems, respectively. LD-WW plants

Table I. *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.

Tissue	LD-WW	SD-WW	LD-STR	SD-STR
	g fresh weight			
Whole plant	12.75	2.72	8.08	1.74
Leaf	5.36	1.26	2.22	0.46
	(42%)	(47%)	(28%)	(26%)
Stem	6.89	1.06	5.52	1.08
	(54%)	(39%)	(68%)	(62%)
Fruit	0.50	0.37	0.34	0.20
	(4%)	(14%)	(4%)	(12%)

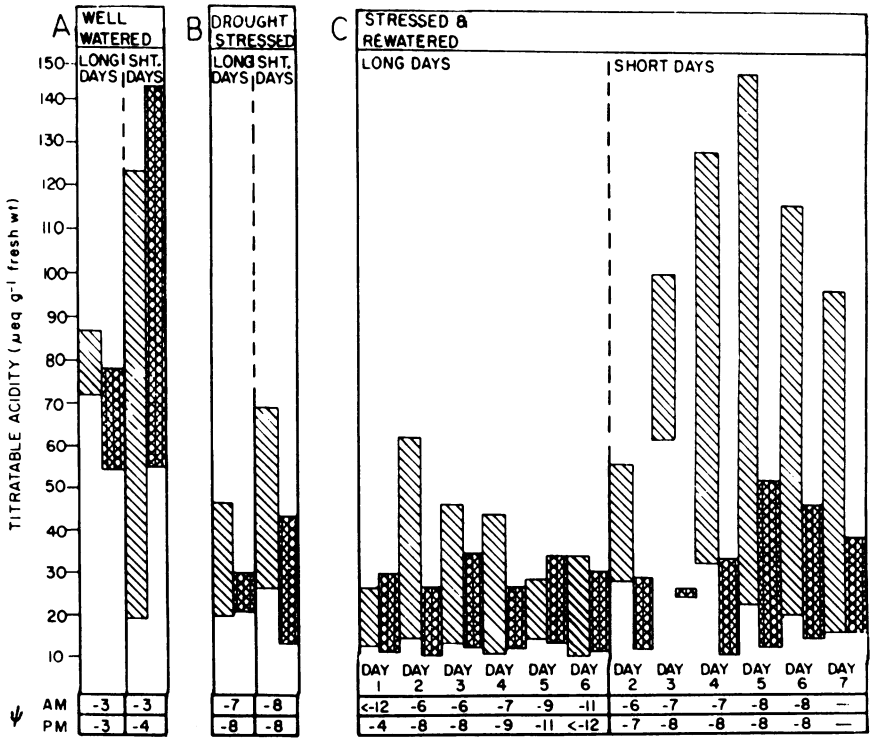

FIG. 1. Amplitude of acid fluctuations in *P. oleracea* under different growing conditions. Top of bar represents maximum early morning acid levels; bottom of bar indicates the minimum late day acid levels in leaves (▨) and stems (▩), respectively. Stressed and rewatered plants were given water on day 0.

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.

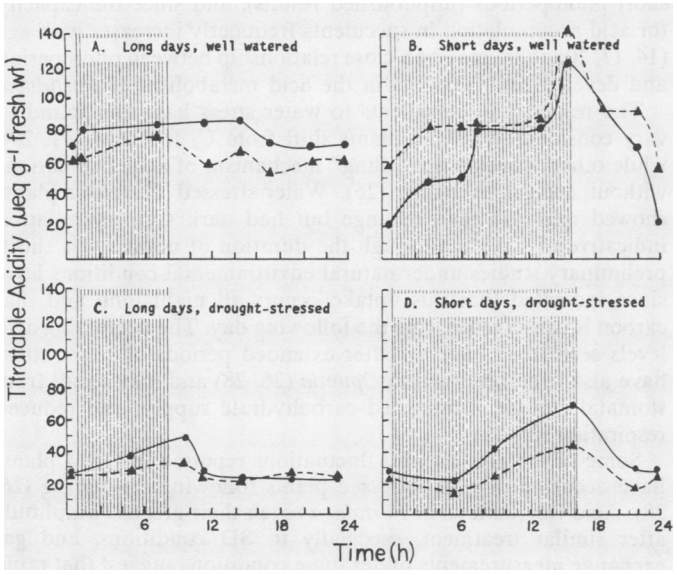

FIG. 2. Diurnal fluctuations of titratable acidity in *P. oleracea* under different growing conditions. Gas exchange and leaf resistance measurements during these experiments are shown in Figure 3. Values represent mean of four plants titrated individually; leaf acidity (●——●); stem acidity (▲- - -▲).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

Table II. *Changes in Titratable Acidity of Leaves and Stems of P. oleracea*

Leaves were removed from plants grown under SD-WW conditions and placed on moist filter paper in the bottom of covered beakers. Each figure represents the average of determinations from four separate plants.

Tissue	Dark Acidification	Light Deacidification
	μequivalents g^{-1} fresh weight	
Stems		
Intact	+92	−73
Defoliated	+37	−91
Leaves		
Intact	+101	−100
Defoliated	+113	−12

Table III. *Compounds Labeled in Intact SD-WW P. oleracea Plants after a 45-min Exposure to $^{14}CO_2$ in the Dark*

Tissue	Malate	Aspartate	Citrate	Glutamate
	% total ^{14}C incorporated			
Stems	47.7	31.6	17.5	3.2
Leaves	52.6	26.6	15.4	5.4

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.

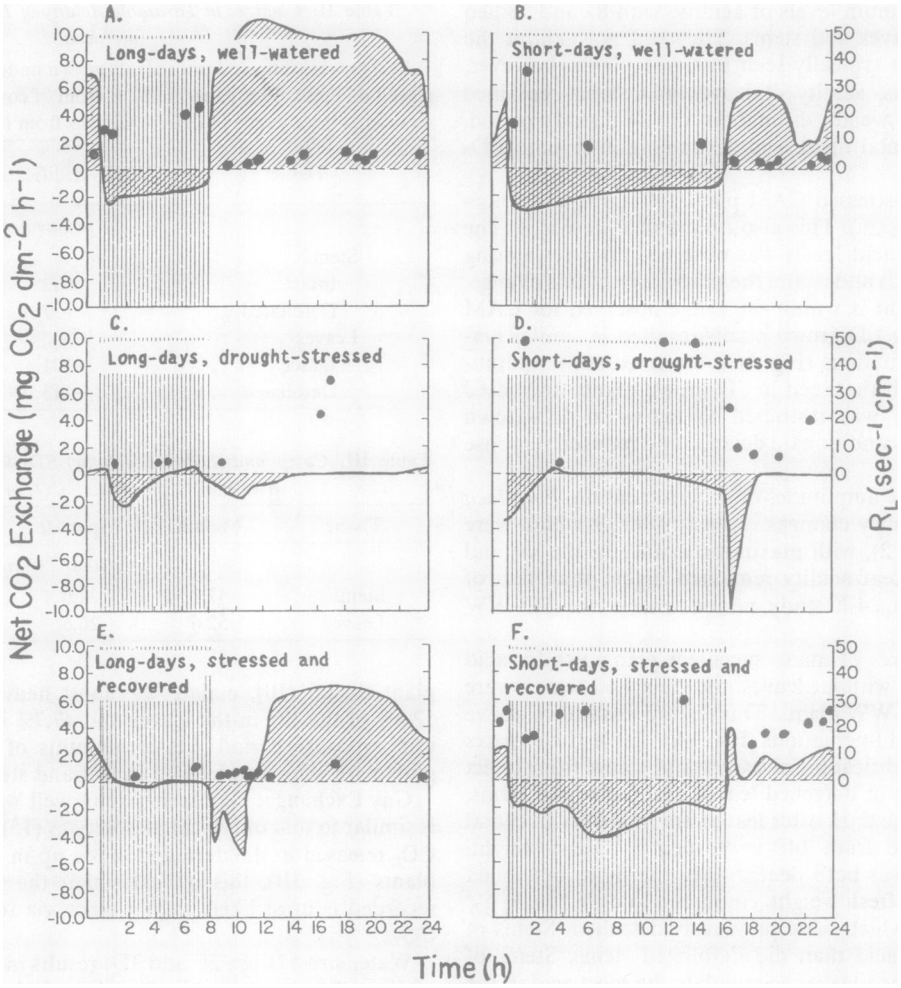

FIG. 3. Gas exchange and leaf resistance of *P. oleracea* under different growing conditions. Acid fluctuations measured during these experiments are shown in Figure 2. CO_2 uptake is shown above the dashed center line; CO_2 release when below (▨). Points show leaf resistance values. Vertically shaded areas (▥) indicate night.

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 C_4 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 CO_2 source for photosynthesis. Such deacidification was observed in SD-grown *P. oleracea* plants, but it seldom occurred under long photoperiod conditions when *Portulaca* exhibited C_4 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 blosfeldiana* Poellniz, 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 C_3 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 CO_2 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 grestest acid fluctuations reported in CAM plants have occurred in water-stressed plants following rewatering (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 rewatering.

CONCLUDING REMARKS

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

Table IV. *Summary of Relative Expression of CAM Characteristics of P. oleracea Grown under Different Photoperiods and Watering Regimes*

Characteristic	Acidity		CO_2 Exchange		Stomata
	Amplitude	Pattern	Net dark uptake	Daytime release	Low nocturnal leaf resistance
Well watered					
Long days	0[a]	0	0	0	0
Short days	+++	+	0	0	+
Drought-stressed					
Long days	0	+	+	+	+
Short days	+	+	+	+++	+
Stressed and rewatered					
Long days	+	+	0	++	+
Short days	++++	+	0	0	0

[a] 0 indicates no expression of a given CAM characteristic, + indicates relative degree of CAM activity. Acidity—amplitude: +(30–60 μeq g^{-1} fresh weight); ++(60–90 μeq g^{-1} fresh weight); +++(90–120 μeq g^{-1} fresh weight); ++++(120–150 μeq g^{-1} fresh weight). Acidity—pattern: +(nighttime acidification and daytime deacidification). CO_2 exchange: +(less than 3 mg dm^{-2} h^{-1}); ++(3–6 mg dm^{-2} h^{-1}); +++(6–9 mg dm^{-2} h^{-1}). Stomata: +(R_L less than 10 s cm^{-1} at some time during the night).

metabolism which were observed in *P. oleracea* under various conditions are: (*a*) acid fluctuations having an amplitude of 136 μeq g^{-1} fresh weight, comparable to those found in CAM plants (13); (*b*) diurnal pattern of acid fluctuations with greatest acidity at the end of the dark period and least near the end of the light period; (*c*) apparent photoperiod sensitivity with increases in CAM-like characteristics under SD treatments; (*d*) amplification in acid fluctuations on rewatering after stress; (*e*) net dark CO_2 assimilation under water stress into C_4 acid compounds; (*f*) periods of reduced CO_2 uptake in the light possibly due to an endogenous CO_2 supply; and (*g*) CO_2 release proportional to acidification of the previous night, similar to growth chamber responses in CAM plants (14). In addition, leaves and stems of SD-grown plants both have the capacity for acid synthesis in the dark. The apparent discrepancy observed in *Portulaca* between periods of maximum acid accumulations and those of net dark CO_2 uptake can occur in other succulents (26, 27) and are considered a result of respiratory variations.

To determine if true CAM occurs in *P. oleracea* or related species under specific conditions will require further study, but data presented here strongly suggest it. Whether or not this process is of functional significance to the carbon balance or water retention of *Portulaca* in the natural environment is the subject of a subsequent investigation. This would broaden the spectrum of CAM activity to include a succulent C_4 plant capable of considerable CAM-like activity under certain conditions. There are many biochemical and anatomical similarities between CAM and C_4 plants (12), so that CAM induction in a succulent C_4 plant would require only small adjustments and little or no major biochemical reorganization. Finally, the adaptive advantage would be considerable for a C_4 annual capable of CAM activity. Favorable water status earlier in the season would permit rapid photosynthetic production typical of C_4 plants (24) while later induction of CAM would enable life cycle completion under conditions of water stress.

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