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Crassulacean Acid Metabolism in the Succulent C_4 Dicot, *Portulaca oleracea* L. Under Natural Environmental Conditions[1]

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ABSTRACT

Crassulacean acid metabolism (CAM) was examined under natural environmental conditions in the succulent C_4 dicot *Portulaca oleracea* L. Two groups of plants were monitored; one was watered daily (well watered), while the other received water once every 3 to 4 weeks to produce a Ψ of −8 bars (drought stressed). Gas exchange, transpiration rate, and titratable acidity were measured for 24-hour periods during the growing season. CAM activity was greatest in drought-stressed plants during late August which had 13 hour days and day/night temperatures of 35/15°C. Under these conditions net CO_2 uptake occurred slowly throughout the night. Diurnal fluctuations of titratable acidity took place in both leaves and stems with amplitudes of 17 and 47 microequivalents per gram fresh weight, respectively. Transpiration data indicated greater opening of stomata during the night than the day. CAM was less pronounced in drought-stressed *P. oleracea* plants in July and September; neither dark CO_2 uptake nor positive carbon balance occurred during the July measurements. In contrast, well-watered plants appeared to rely on C_4 photosynthesis throughout the season, although some acid fluctuations occurred in stems of these plants during September.

To determine the fate of the CO_2 assimilated at night in drought-stressed *Portulaca* plants, exposure to $^{14}CO_2$ during the night followed by 9 hours of ambient air in the light. Malate was the predominant compound labeled during the night, with some citrate and aspartate. No $^{14}CO_2$ release was detected during the following day and by midafternoon the majority of the label was found in the insoluble fraction (predominantly starch). These results substantiate our earlier work with growth-chamber-grown plants and show that limited CAM activity can occur in the succulent C_4 dicot *Portulaca oleracea* L. under certain natural environmental conditions.

Portulaca oleracea is the only C_4 species currently known to express CAM characteristics (10) although shifts from C_3 to CAM occur in a number of succulents (13, 26, 27, 28). In earlier studies, *P. oleracea* plants were shown to utilize the C_4 pathway of carbon fixation (9) and that products of photosynthesis were effected by NaCl (7, 8). We have also found this species to exhibit diurnal acid fluctuations typical of CAM when subjected to drought stress under 8-h photoperiods and day/night temperatures of 30/15°C (10). At night, detached leaves and stems both accumulated acid independently. In addition, malic acid was the most heavily labeled compound following $^{14}CO_2$ fixation in the dark. Gas exchange measurements showed limited CO_2 assimilation in the light and slow uptake in the dark.

During the normal CAM photosynthesis, night CO_2 uptake should account for a major portion of the carbon assimilated and be utilized for photosynthesis in the light. In our earlier experiments (10), relatively large portions of CO_2 were released at the start of each light period as commonly occurs in CAM plants grown in growth chambers (1, 4, 6). Because of this it was difficult to determine the importance of the nighttime CO_2 uptake to the overall carbon economy of drought-stressed *Portulaca* plants. We were also interested in whether endogenous CO_2 was recycled in *P. oleracea*, as observed in *Portulacaria* (5), a different succulent genus in the same family.

There are several differences between controlled environment conditions and those in the field that might affect the expression of CAM. First, outdoors, more gradual changes in temperature and light intensity could result in different patterns of CO_2 exchange. Second, *Portulaca* plants grown outdoors are typically much larger than those grown in growth chambers and the thicker stem tissues could influence the CAM activity of the whole plant. Irradiance is also much greater under natural conditions, which could affect the photosynthesis or carbohydrate reserves. Another consideration is the interaction between plant age and daily changes in photoperiod and temperature which occur under field conditions. Because of these differences, we were interested in determining if CAM activity occurs in *P. oleracea* under natural environmental conditions and, if so, its significance.

MATERIALS AND METHODS

Three-week-old seedlings of *P. oleracea* L. were transplanted to clay pots and separated into four groups. The first two groups were grown in growth chambers as described previously (10), with 9- or 16-h photoperiods. The remaining plants were placed outdoors in early June; one-half was watered daily and the pots surrounded with sphagnum to minimize soil drying (well-watered plants), while the other group of plants (drought stressed) were shielded from rain by moveable glass frames and watered at 3- to 4-week intervals. Under these conditions, a Ψ of −8 bars was maintained for extended periods of time (10).

Daily maximum and minimum temperatures were recorded during the 4-month growing period (Fig. 1) to show both the growth conditions of field-grown plants and when periods favorable for CAM typically occur in the natural environment. Frequent measurements were made during the course of the 24-h experiments.

Gas exchange and transpiration measurements were conducted in the laboratory with an IR gas analyzer and humidity sensor as described previously (11). Leaf resistances were calculated from temperature and transpiration data (20). Light and temperature changes which occurred outdoors throughout each experimental period were simulated in the laboratory by frequent adjustment of light intensity and temperature (11). Samples were killed in

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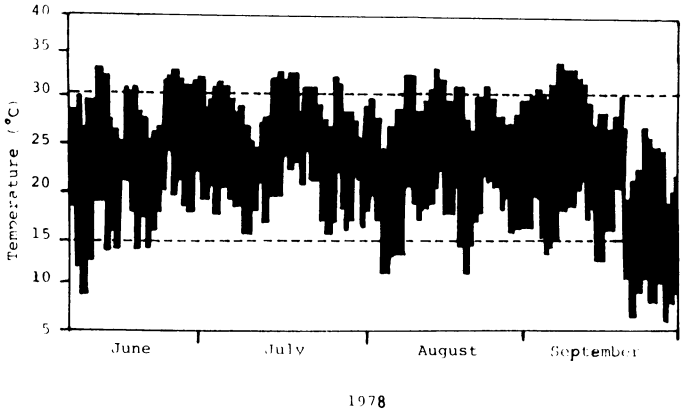

FIG. 1. Temperatures during the June to September 1978 growing period for *P. oleraca* grown outdoors in Iowa. (- - -), day/night temperatures used in growth chamber studies.

liquid N_2 and acids extracted by boiling crushed tissues in water. Titrations were conducted using 10 mM NaOH at 50°C as reported (10).

$^{14}CO_2$ assimilation experiments were done in late September using a single large intact *P. oleracea* plant in which net dark uptake of CO_2 had been recorded by gas analysis the preceding night. The above-ground portion of the plant was enclosed in a glass container and the soil surface sealed with wax. One h after sundown (1830), the chamber was covered with a black cloth and 245 μCi of $^{14}CO_2$ injected into the container, bringing the final $^{14}CO_2$ concentration to 300 μl/l (620 μl/l total CO_2). An additional 123 μCi $^{14}CO_2$ was added shortly after midnight. Before dawn, the plant was removed from the chamber and one of four large branches removed and killed in liquid N_2. The remaining plant was sealed inside a glass chamber in the laboratory where release of $^{14}CO_2$ and metabolism of labeled carbon compounds could be monitored. Light and temperature conditions were adjusted to approximate those occurring outside. Ambient air was circulated through the chamber and $^{14}CO_2$ evolved was trapped in 1.0 M monoethanolamine. The remaining branches were removed from the plant at 3-h intervals (800, 1100, and 1430) and killed in liquid N_2. Labeled compounds were extracted as described earlier (10) and thin-layer electrophoresis and chromatography used to separate and identify compounds. In addition, ion exchange chromatography was used to verify the identity of some compounds (2).

RESULTS AND DISCUSSION

Depending on the season and the water status, a wide range of CAM and C_4 activity took place in *P. oleracea* under natural environmental conditions. Drought-stressed *Portulaca* plants showed CAM activity to varying extents from July through September. The greatest CAM activity occurred in August while stress appeared to be greatest during July based on gas exchange data (Fig. 2A). No net uptake of CO_2 was recorded during the 24-h period studied in July, and CO_2 efflux varied between 0.7 and 2.4 mg dm^{-2} h^{-1} at night and 1.2 to 2.4 mg dm^{-2} h^{-1} during the day. Plants were no drier during this month, but carbon losses may have been greater for several reasons. First, the photoperiod in July was approximately 15 h long, while in a previous study, we found 16-h days to limit CAM expression in *P. oleracea* (10). In addition, July temperatures were unfavorable for maximum CAM activity because of the warmer nights occurring at this time (3, 4, 16). Finally, these young *Portulaca* plants may not have reached a sufficient age for CAM activity (6). During this period, some degree of CAM was still evident based on reduced CO_2 loss during the night and again the following day. Acid fluctuations also occurred in both stem and leaf tissues during this time, but only with amplitudes of 30 and 20 μeq g^{-1} fresh weight, respectively. Plants recovered after watering and were examined 4 weeks later after a second drought period.

CAM was greatest in drought-stressed *P. oleracea* during August as seen by acid fluctuations and net CO_2 uptake in the dark (Fig. 2B). Diurnal variations in acid levels occurred in both stems and leaves, and fluctuations accounted for 47 and 17 μeq g^{-1} fresh weight, respectively. The former is within the 30 to 300 μeq g^{-1} fresh weight range observed for CAM plants (24), and is similar to fluctuations recorded under drought conditions in another member of the Portulaca family, *Portulacaria afra* (65 μeq g^{-1} fresh weight) (26), in *Echeveria* (51 μeq g^{-1} fresh weight) (14), and several *Opuntia* species (11, 22). In the present study, maximum acidity was reached in stems by early morning, while leaves continued to acidify until early afternoon. The significance of CAM in these plants can also be seen in their day/night gas exchange; most CO_2 was fixed in the dark and a positive carbon balance resulted. Rates of CO_2 assimilation at night ranged from 0.4 to 0.7 mg dm^{-2} h^{-1}, with temperatures of 14 to 20°C. Comparable rates of dark CO_2 uptake (1.0–3.0 my dm^{-2} h^{-1}) have been induced after salt or drought stress in another CAM species, *Portulacaria afra* (26). In *P. oleracea*, a small amount of CO_2 was taken up in the morning, but was followed by a 4-h period of slow release to the light. Transpiration data also indicated a substantial reduction in water loss and stomatal movements typical of CAM. Resistances decreased to minimum values of 19 and 54 s cm^{-1} during August and September, respectively, but net CO_2 uptake has been recorded at resistances as high as 30 s cm^{-2} in stressed *Portulacaria* (26) and at 80 s cm^{-2} in *Brophyllum* (6). From the data in the present study, it appears that drought stressed *P. oleracea* depend on CAM for increased water and carbon economy.

Although carbon balance was negative in drought-stressed plants during September (Fig. 2C), CO_2 uptake at night helped reduce carbon loss. Both leaves and stems acidified throughout the night and during the first few h of daylight, reaching maximum levels at midmorning. The magnitude of these changes was 42 μeq g^{-1} fresh weight in stems and 28 in leaves, similar to the levels occurring in some CAM plants under field conditions (24). Daytime stomatal closure may also have aided the refixation of endogenous CO_2 like the recycling of carbon during stomatal closure in drought-stressed cacti (23).

Throughout most of the growing season, CAM was wholly or partially lacking in well-watered plants. During July and August (Figs. 2D and 2E), acid fluctuations were minimal and the difference between morning and late-day levels of titratable acidity remained less than 28 μeq g^{-1} fresh weight in stems and less than 9 μeq g^{-1} fresh weight in leaves. These values are both less than those observed in CAM plants (15, 24). In well-watered plants in the present study, both diurnal fluctuations and absolute values consistently remained lower in leaves than in stems. Gas exchange measurements during this time also showed no indication of CAM since CO_2 uptake occurred in the light and was released in the dark. On the other hand, leaf resistance measurements indicated stomata were at least partially open throughout the day/night cycle, as occurs in well-watered cacti (25).

Even in September, when acid fluctuations became CAM-like in stems of well-watered plants, whole-plant gas exchange measurements indicated that C_4 photosynthesis predominated (Fig. 2F). The amplitude of diurnal acid change in stems of these plants doubled, increasing to 48 μeq g^{-1} fresh weight. Stomatal movements and gas exchange patterns, however, were not typical of CAM. A small increase in the amount of CO_2 fixed or reassimilated by these well-watered plants at night could account for the increased stem acidity but effect nocturnal CO_2 exchange only minimally. The majority of CO_2 uptake would still be expected to occur in the light via the more active C_4 pathway. The increased

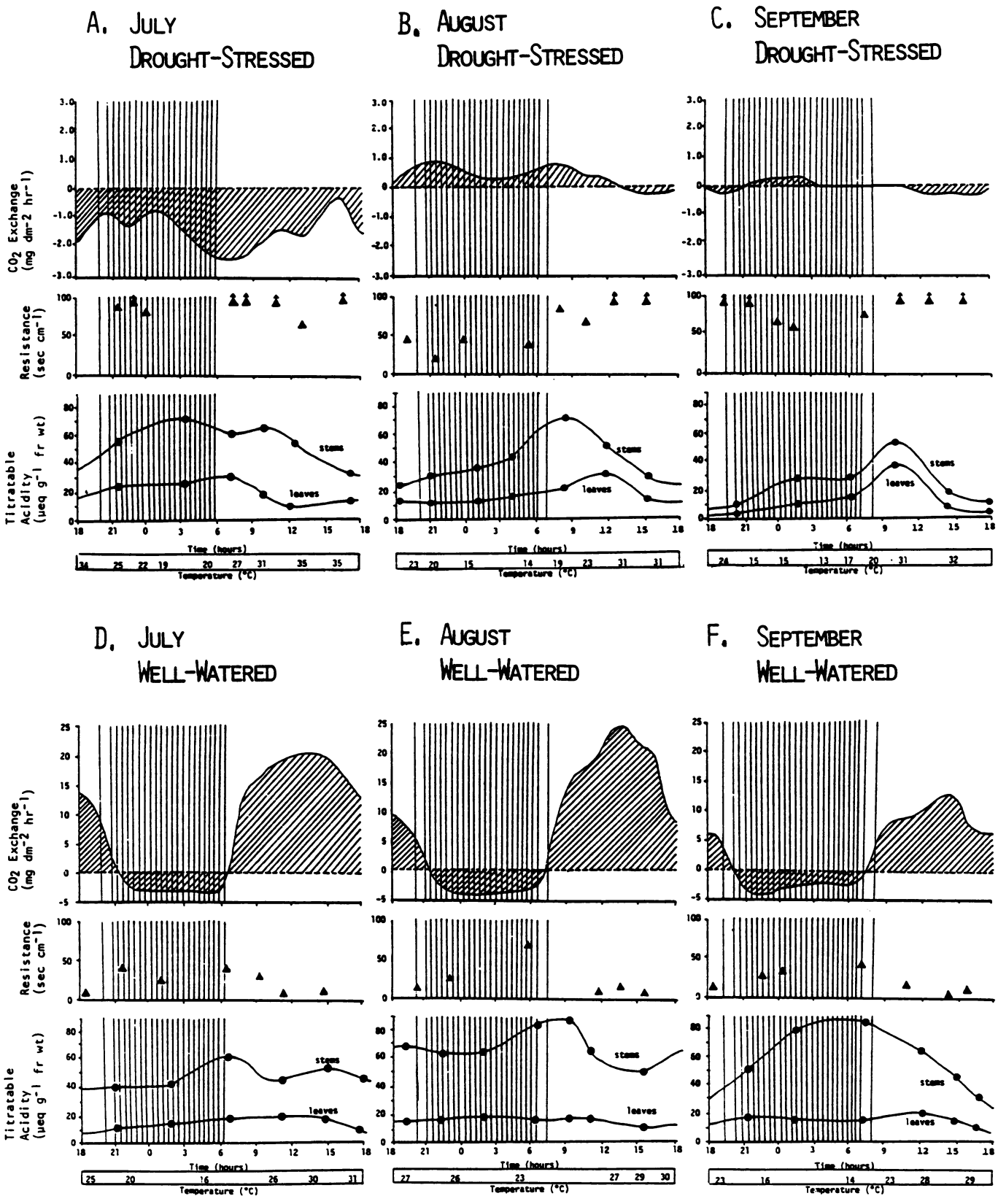

FIG. 2. CO_2 exchange, leaf resistance, and acid fluctuations of *P. oleracea* plants during 24-h studies in (A), September, drought-stressed; (B), August, drought-stressed; (C), September, drought-stressed; (D), July, well-watered; (E), August, well-watered; and (F), September, well-watered. Vertically shaded areas (▥) indicate night. Net CO_2 exchange is shown by diagonally shaded areas (▨); uptake when above the dashed center line and release when below. Temperatures recorded during the test period are shown in a bar across the base of each figure.

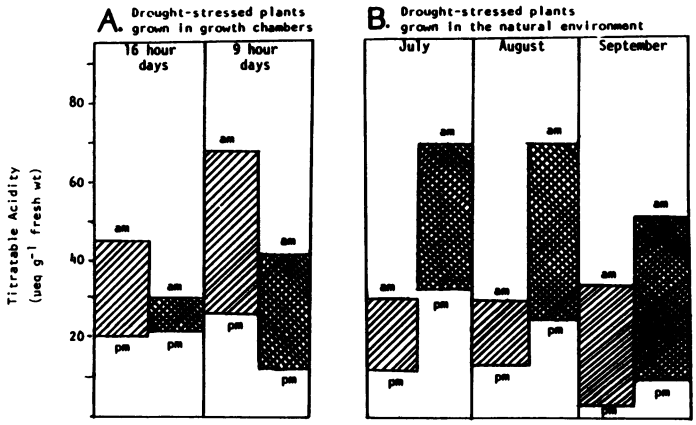

FIG. 3. Changes in titrable acidity of drought-stressed *P. oleracea* plants grown (A) in growth chambers and (B) in natural environments. Tops of bars represent maximum morning acid levels, the bottoms of bars indicate minimum late-day levels leaves (▨) and stems (▩).

Table I. *Distribution of ^{14}C in an Intact, Field-Grown Portulaca Plant*

An 11-h $^{14}CO_2$ fixation in the dark was followed by 9 h in ambient air in the light. Total ^{14}C in 530 sample was 24,402 dpm. Subsequent samples varied in size and contained 146,549, 233,330, and 428,477 dpm, respectively. The first sample (530) was taken immediately before the start of the light period. Net Co_2 uptake had been recorded the previous night in September (Fig. 2C).

Time of Day	530	800	1100	1430
	% ^{14}C of total			
Tissue				
Leaves	66	85	54	53
Stems	34	15	46	47

Table II. *Distribution of ^{14}C in an Intact, Field-Grown Portulaca Plant*

An 11-h $^{14}CO_2$ fixation in the dark was followed by 9 h in ambient air in the light. Total ^{14}C in samples are as in Table I. The first sample (530) was taken immediately before the start of the light period. Net CO_2 uptake had been recorded the previous night in September (Fig. 2C).

Time of Day	Leaves				Stems			
	530	800	1100	1430	530	800	1100	1430
	% of ^{14}C in tissue							
Assimilate								
Organic acids	100	22	0	16	80	32	57	9
Amino Acids	0	48	20	4	20	25	0	3
Sucrose and neutral assimilates	0	17	24	23	0	29	0	5
Insoluble Fraction	0	13	56	59	0	14	43	86

acid fluctuations during September are also consistent with earlier results, using short photoperiods in growth chambers (10) and with other reports of short-day effects on CAM (18). These changes in acid levels also occurred without prior drought stress, but the involvement of plant age (6) and/or temperature cannot be discounted (4, 13, 16).

Acid fluctuations of drought-stressed *Portulaca* plants grown outdoors were compared to those grown under controlled environmental conditions (Fig. 3). In field-grown plants, acid fluctuations were greater in stems than in leaves, while the reverse occurred in growth-chamber grown plants. The contrast between the two groups may have resulted from different light intensities. Plants grown under full sunlight had nearly seven times more total surface area and considerably larger stems for starch or acid storage. Thus, outdoor plants may also have greater amounts of carbohydrate available for PEP formation and subsequent acidification. Deacidification during the day has also been found to be closely associated with light intensity in some CAM plants (17) possibly explaining the decreased acidity found in the leaf tissues of *P. oleracea* grown outdoors.

The distribution of ^{14}C after an 11-h exposure to $^{14}CO_2$ is shown in Table I. At the end of the dark period, leaves and stems contained 66 and 34% of the total label, respectively, indicating that both tissues were able to accumulate ^{14}C assimilates at night. However, it is not clear whether this involved the movement of ^{14}C from one tissue to another. In addition, distribution of total acid levels measured the preceding night was the reverse; stem tissues had greater total titratable acid than leaves at the end of nighttime CO_2 assimilation (Fig. 2E). These results indicate the likely presence of unlabeled organic acid in stems and the importance of endogenous carbon sources to stem acidification. Limited gaseous diffusion in thick stems may occur which could favor refixation of endogenous CO_2. It is possible, however, that some ^{14}C was released by stems and refixed by leaves. Still, transfer from one tissue to another appears to have been minimal and nearly 50% of the ^{14}C-assimilate had not yet been translocated out of leaves at mid-afternoon.

The products of dark $^{14}CO_2$ fixation and their subsequent metabolism in the light are shown in Table II. At night, only organic acids (malic and citric) were labeled in leaves, while in stems, aspartate also contained 20% of the ^{14}C taken up. The label in leaves also moved more rapidly out of these compounds during the following day than occurred in stems. This is consistent with the higher photosynthetic rate in leaves, a factor considered important in regulating deacidification of *Bryophyllum* (12). Percentages of [^{14}C]sucrose, neutral compounds, and sugar phosphates increased steadily during the day in leaves while in stems substantial levels appeared only in the morning. The majority of the ^{14}C label in leaves and stems eventually accumulated in the insoluble fraction (predominantly starch) as is typical in CAM. The formation of a viscous carbohydrate at this time was similar to the glucan synthesis found in *Kalanchoë* (21). Starch formation via gluconeogenesis, as shown in stomata is also possible (19).

In summary, under natural environmental conditions, CAM contributes to the carbon balance and water retention in the C_4 dicot *P. oleracea* as we reported earlier for growth chamber-grown plants. Stomatal closure in the light appears to reduce water loss from the plants with insoluble compounds being synthesized from the CO_2 assimilated during the night. The process is apparently responsible for maintaining a positive carbon balance in *Portulaca* under severe drought conditions, especially as the season progresses. Thus, drought-stress, photoperiod, developmental state, and diurnal temperature changes all appear to be important in CAM expression in *P. oleracea*.

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