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 C_4 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 CO_2 exchange, as well as diurnal tissue malic acid fluctuations, were measured in plants under well-watered and drought-stressed conditions. Net CO_2 uptake in both treatments occurred only during the day, although small amounts of nighttime CO_2 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 CO_2 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 CO_2, as occurs in C_3 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 C_4 photosynthetic pathway is distinguished from the C_3 pathway by the involvement of phosphoenolpyruvate (PEP) carboxylase in the initial fixation of atmospheric CO_2, resulting in the production of four-carbon organic acids (Black 1973; Edwards and Walker 1983). In addition, leaves of C_4 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 CO_2 fixation even under stressful conditions. Plants that utilize Crassulacean acid metabolism (CAM) also employ PEP carboxylase for the initial fixation of atmospheric CO_2, 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 C_4 and CAM is that initial CO_2 fixation via PEP carboxylase is separated from the subsequent reduction of CO_2 via Rubisco spatially in C_4 plants while in CAM plants the separation is temporal.

Although the photosynthetic pathway of most plants can be categorized as C_3, C_4, or CAM, numerous species combine aspects of two pathways (Ehleringer and Monson 1993). For example, some "C_3-C_4 intermediates" have anatomical and biochemical features intermediate between those of C_3 and C_4 species. Combinations at the organ level occur as well, including species with CAM stems and C_3 leaves (Osmond et al. 1982). Furthermore, a few species exhibit seasonal switching between the C_3 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 C_3 gas exchange patterns but show CAM-like diurnal acid fluctuations. As stomata are closed at night, respiration is presumably the source of CO_2 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 (Osmond et al. 1980; Osmond 1982). Accumulating evidence, however, suggests that CAM-cycling may simply be a precursor to "CAM-idling" and may 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 CO_2, these plants may resume uptake of atmospheric CO_2 more rapidly upon rehydration.

Because C_4 and CAM plants exhibit nearly identical biochemistry, the existence of C_4-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 C_4 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 Portulacaceae, Guralnick and Jackson (1993) reported diurnal acid fluctuations in several genera including the C_4 genus

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Portulaca. Several studies of *Portulaca* species, however, either found no evidence of CAM activity or reported peculiarities in the C_4 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 C_3 photosynthesis. Finally, Fathi and Schnarrenberger (1990) found that the NADP-malic enzyme of *P. grandiflora* displayed an immunochemical cross-reaction more similar to that of C_3 and CAM plants than to that of C_4 species.

Given the limited and conflicting nature of information regarding the existence of C_4-CAM intermediates, it was the purpose of the present study to examine the photosynthetic pathway(s), under well-watered and drought-stressed conditions, of three annual species of C_4 succulents in the genus *Portulaca*: *P. oleracea*, a cosmopolitan weed that grows best in freshly disturbed soil (Matthews et al. 1993); *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; $d^{13}C$ of *P. mundula* = $-13.8\%o$, unpublished data), these three species of *Portulaca* are clearly C_4 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 the greenhouse under the following conditions: average maximum PPFD of 1000 μmol m^{-2} s^{-1}, daytime temperature range of 27°–36°C, nighttime temperature range of 15°–26°C, daily watering, and weekly fertilization (solution of 18% of each of total N, P_2O_5, K_2O; including trace elements). The approximate nighttime vapor pressure deficit (vpd) was 0.3–1.0 kPa, while the daytime vpd was ca. 1.8–3.6 kPa. Drought-stressed individuals of *P. grandiflora* and *P. oleracea* received no water for 4 d prior to gas exchange analysis; *P. mundula* plants received no water for 11 d prior

to experimentation. A reduction in the midday plant water potential was used as a preliminary indicator of drought stress. The xylem water potential of the well-watered plants of the three species was approximately -0.3 MPa. Water was withheld until plants exhibited a xylem water potential of -0.6 to -0.8 MPa, as determined by Scholander pressure chamber measurements of shoot xylem tension.

GAS EXCHANGE

CO_2 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 Lüttge 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 CO_2 was observed only during the day in all three species (figs. 1–3); no net uptake occurred at night. Daytime internal CO_2 concentrations were lowest and CO_2 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 CO_2 assimilation. This phenomenon also was observed in *P. oleracea* during the second day/night period.
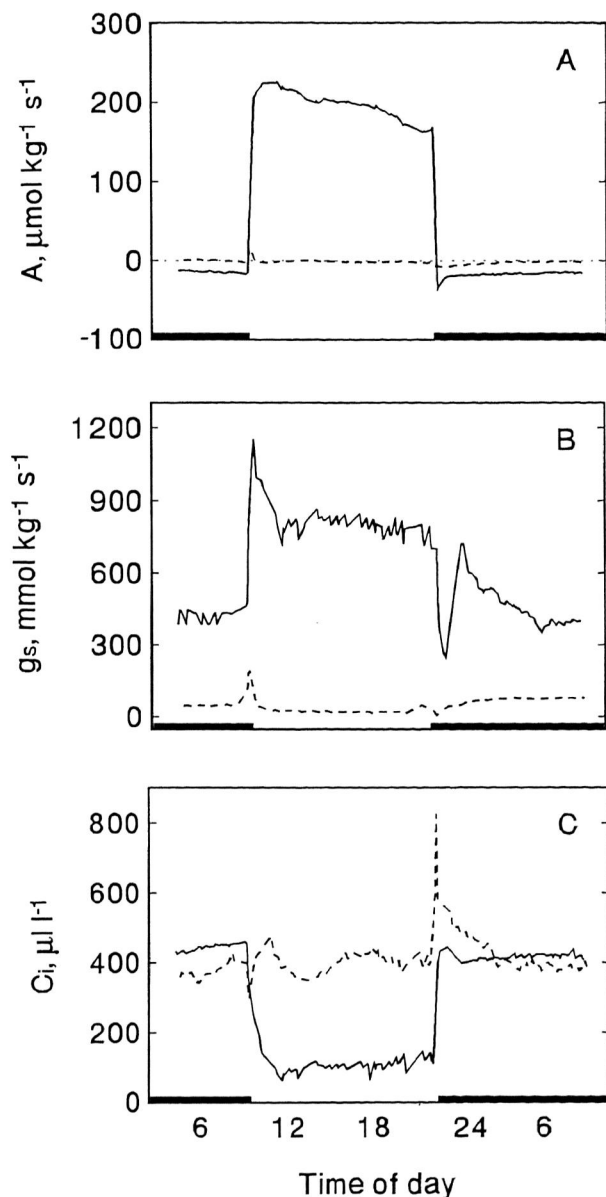
The amount of CO_2 assimilated throughout the day

Fig. 1 Diurnal patterns of net CO$_2$ assimilation (A, fig. 1A), stomatal conductance (g$_s$, fig. 1B), and leaf internal CO$_2$ concentration (C$_i$, fig. 1C) of shoots of *Portulaca grandiflora* under well-watered conditions (solid line) and drought-stressed conditions (dashed line). Results from the same plants are shown in each graph; mean day and night CO$_2$ exchange of four individuals are given in table 1. Data are expressed on a dry mass basis. Black bars indicate night.
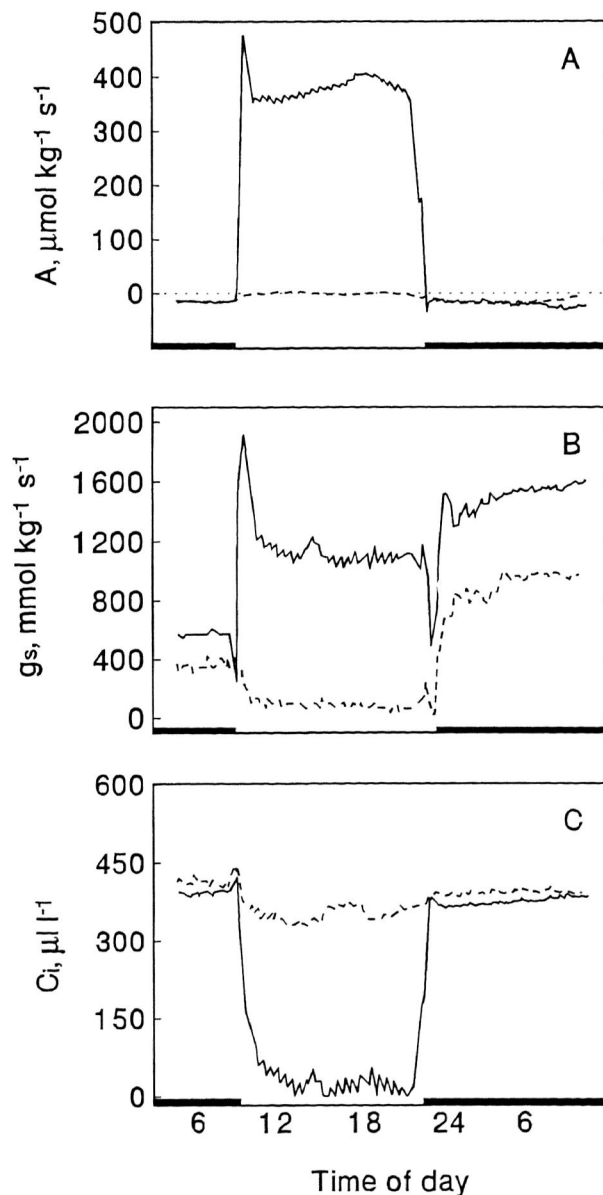
Fig. 2 Diurnal patterns of net CO$_2$ assimilation (A, fig. 2A), stomatal conductance (g$_s$, fig. 2B), and leaf internal CO$_2$ concentration (C$_i$, fig. 2C) of shoots of *Portulaca oleracea* under well-watered conditions (solid line) and drought-stressed conditions (dashed line). Results obtained as in fig. 1.

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
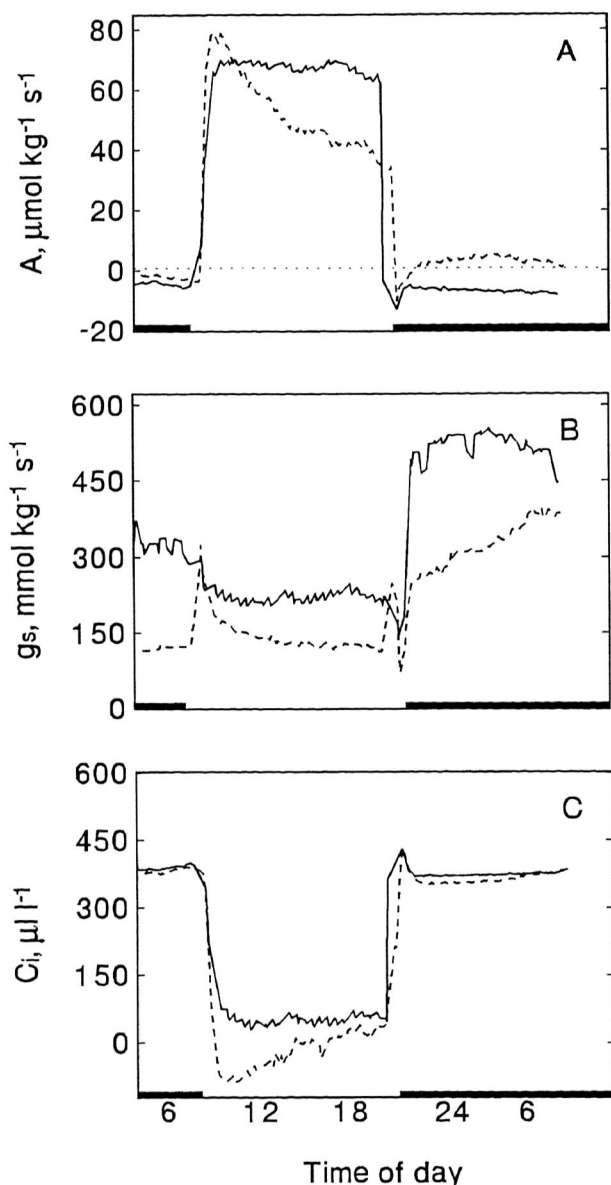
Fig. 3 Diurnal patterns of net CO_2 assimilation (A, fig. 3A), stomatal conductance (g_s, fig. 3B), and leaf internal CO_2 concentration (C_i, fig. 3C) of shoots of *Portulaca mundula* under well-watered conditions (solid line) and drought-stressed conditions (dashed line). Results obtained as in fig. 1. Note: negative C_i values resulted from instrument limitations in measuring extremely low conductances concomitant with high rates of CO_2 uptake.

(fig. 2b). In addition, conductances observed in *P. oleracea* during both light and dark periods were higher than in the other species. Conductances for shoots of *P. mundula* were similar during the first day and night but increased during the second night (fig. 3b). The stomatal conductance of *P. grandiflora* was lower than that of the other species and changed little throughout the measurement period (fig. 1b).

The total integrated WUE of *P. mundula* was similar under drought-stressed conditions and under well-watered conditions (table 2). *Portulaca grandiflora* and *P. oleracea,* however, exhibited no net CO_2 uptake under drought-stressed conditions, therefore WUE was not calculated.

Morning and evening leaf malic acid concentrations

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 C_4 species, though rates were lower; CO_2 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 CO_2 assimilation did occur in a few individuals, however. Comparable CO_2 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 CO_2 concentrations and were generally not accompanied by CO_2 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 C_4 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

INTEGRATED DAY AND NIGHT CO_2 EXCHANGE OF WELL-WATERED AND DROUGHT-STRESSED PORTULACA GRANDIFLORA,
PORTULACA OLERACEA, AND PORTULACA MUNDULA

Species and treatment	Daytime CO_2 exchange (mmol kg^{-1})			Nighttime CO_2 exchange (mmol kg^{-1})		
	Uptake	Loss	Net[a]	Uptake	Loss	Net[a]
P. grandiflora:						
Well-watered	7330 (2910)	0	7330 (2910)	0	1013 (425)	−1013 (425)
No water for 4 d	6 (12)	161 (162)	−155 (170)	10 (20)	310 (388)	−301 (398)
P. oleracea:						
Well-watered	17,635 (4033)	0	17,635 (4033)	0	864 (156)	−864 (156)
No water for 4 d	37 (31)	−145 (76)	−109 (47)	0	356 (192)	−356 (192)
P. mundula:						
Well-watered	3631 (526)	0	3631 (526)	0	320 (49)	−320 (49)
No water for 11 d	1816 (558)	0	1816 (558)	41 (69)	47 (23)	−6 (88)

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.

[a] Positive values indicate net CO_2 uptake; negative values indicate net CO_2 loss.

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 C_4 photosynthesis (see Introduction), our re-

TOTAL INTEGRATED MOLAR WATER USE EFFICIENCY (WUE) OF WELL-WATERED AND DROUGHT-STRESSED PORTULACA GRANDIFLORA, PORTULACA OLERACEA, AND PORTULACA MUNDULA OVER THE ENTIRE MEASUREMENT PERIOD

Species and treatment	WUE (mmol CO_2/mol H_2O)
P. grandiflora:	
Well-watered	4.94 (0.46)
No water for 4 d	na[a]
P. oleracea:	
Well-watered	7.01 (0.63)
No water for 4 d	na[a]
P. mundula:	
Well-watered	5.76 (1.19)
No water for 11 d	5.76 (0.53)

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

[a] na = not available: negative net CO_2 exchange prevented calculation of WUE.

sults indicate that these plants can also undergo CAM, or at least diurnal acid fluctuations. When nighttime CO_2 uptake occurs, the total amount of CO_2 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 CO_2. This presumption is further supported by a reduction in the amount of CO_2 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 CO_2 per se should not directly benefit the plants because energy must be expended to reduce the CO_2, 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 CO_2 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 CO_2 would allow for maintenance respiration during severe drought. During the day, the release of additional CO_2 from stored malic acid might also prevent stress-induced damage to the photosynthetic apparatus.

If "C_4-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
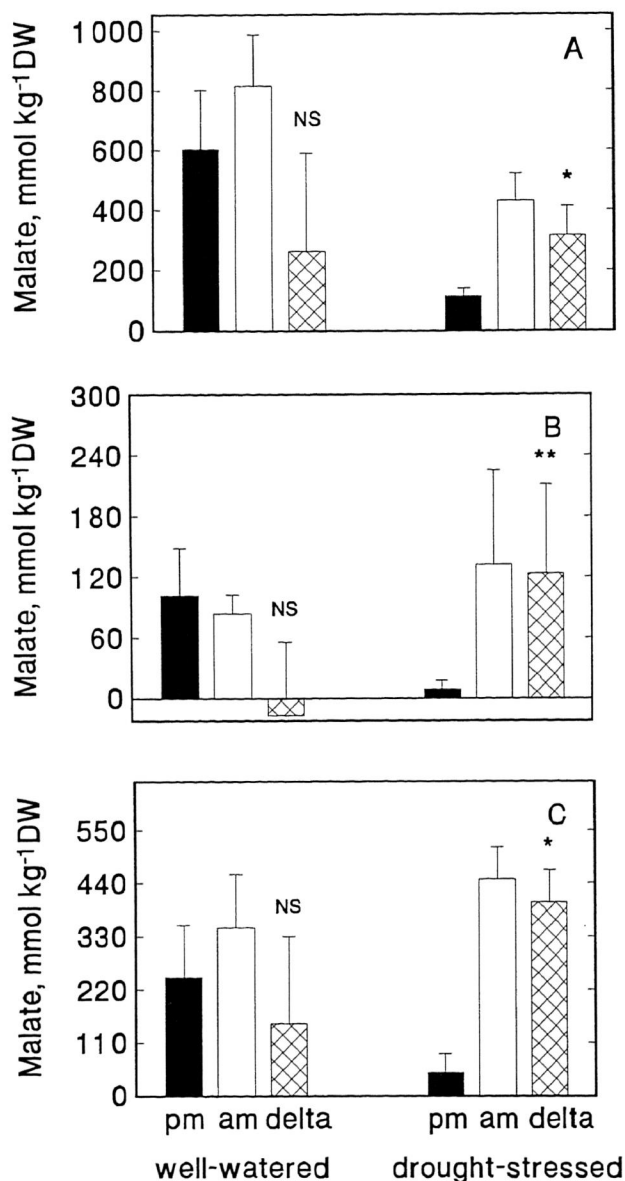
Fig. 4 Morning and evening leaf malic acid concentrations and diurnal malic acid fluctuations (delta) in well-watered and drought-stressed individuals of *Portulaca grandiflora* (fig. 4A), *Portulaca oleracea* (fig. 4B), and *Portulaca mundula* (fig. 4C). Data are expressed on a dry mass basis. Values are means of four plants; error bars represent standard deviations. *NS* = not significant; * = $P \leq$ 0.05; ** = $P \leq$ 0.01.

Fig. 5 Morning and evening stem malic acid concentrations and diurnal malic acid fluctuations (delta) in well-watered and drought-stressed individuals of *Portulaca grandiflora* (fig. 5A), *Portulaca oleracea* (fig. 5B), and *Portulaca mundula* (fig. 5C). Data are expressed on a dry mass basis. Sample sizes are noted above each bar; error bars represent standard deviations.

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.
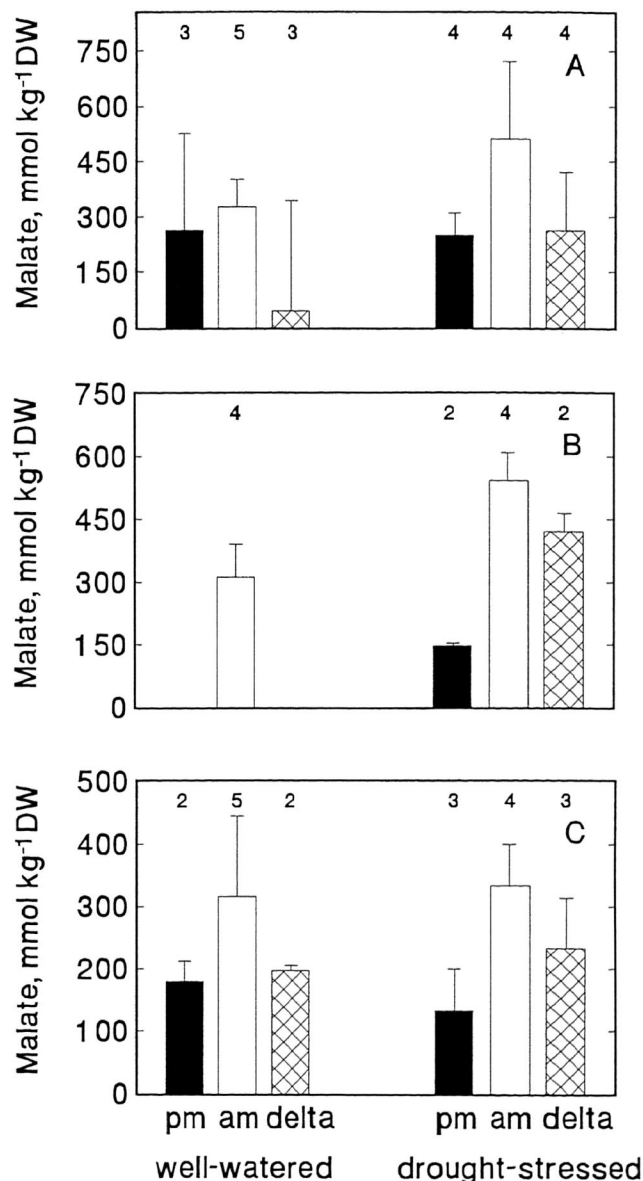
The biochemical regulation of C_4 and CAM in the same tissue, however, is not fully understood (Ku et al. 1981). Also, as this combination of photosynthetic pathways appears to be rare among C_4 species the question of its ecological significance, if any, remains to be answered. These biochemical and adaptive questions require further investigation before this unusual combination of C_4 and CAM can be fully understood.

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