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Age-specific changes of acidity, phosphoenolpyruvate carboxylase, ribulose-1,5-bisphosphate carboxylase/oxygenase, abscisic acid and leaf water potential in *Mesembryanthemum nodiflorum*

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Abstract

Age-induced changes in *1*) nocturnal and diurnal acidity fluctuations that coincide with the ongoing environmental conditions, *2*) the build up of abscisic acid (ABA) in plant roots and leaves during sunrise, midday, and sunset in all growing stages, *3*) the changes in phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) activities as key enzymes of the photosynthetic pathways of C_3 and CAM, *4*) leaf water potential (ψ_l), and *5*) K_m and V_{max} for PEPC to express its activity and affinity, were studied in *Mesembryanthemum nodiflorum* during transition from C_3 to CAM mode of CO_2 fixation. The acidity during sunset in mature stage was higher than in earlier stages and reflected the impact of environmental conditions on physiological and metabolic changes. Moreover, the higher acidity during sunrise and sunset was observed during the senescence than the mature stage; this might be due to CO_2 release and oxygen intake during senescence induced ethylene formation that lead to increased malic acid formation. The ABA concentration was high in *M. nodiflorum* leaves, but stomatal closure was insensitive to elevated ABA concentrations recorded. V_{max} of PEPC, K_m, and the affinity of PEPC during later stages indicated the ability of PEPC to fix CO_2 taking up at night in CAM cycle of *M. nodiflorum*. Less affinity during sunrise indicated inhibitory effect of malate on PEPC during the release of CO_2. The second peak of PEPC activity before sunset caused CO_2 fixation. The RuBPCO was inactive at night. Slight increase in ABA during sunset, and night drop in air temperature and increase in relative humidity reduced markedly transpiration rate without decreasing ψ_l.

Additional key words: Crassulacean Acid Metabolism, plant age, sunrise, sunset.

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Abbreviations: CAM - Crassulacean acid metabolism; PEPC - phosphoenolpyruvate carboxylase; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase.

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Introduction

The Crassulacean Acid Metabolism (CAM) plants are characterized by partial stomatal closure during the day, and by formation of oxaloacetic acid, malate (and in some plant species of aspartate); these changes are realized during the dark period *via* PEPC activity in the cytosol. These steps are followed by breakdown of the formed organic acids by decarboxylation and shift to C_3 cycle during daylight (Kluge and Ting 1979). The role of RuBPCO is to fix CO_2 lost from organic acids similarly as in the bundle sheath of C_4 plants. Obligatory CAM species maintain CAM photosynthesis under all environmental conditions. During severe drought, stomata of many obligatory CAM plants close during day and night, and plants keep up with very low metabolic activities (Szarek and Ting 1974, Osmond 1978, Svensson *et al.* 1995). Non-obligatory CAM plants are able to shift photosynthetic mode from C_3 into CAM and thus adapt to the environment (Guralnick *et al.* 1984). Seasonal shift from C_3 to CAM in *Mesembryanthemum crystallinum* was recorded as soil water potential decreased (Winter *et al.* 1978, Bloom and Troughton 1979).

M. nodiflorum L. is a non-obligatory CAM species. Its photosynthetic cycle shifts from C_3 in wet cool winter season to CAM in hot dry summer season. The detailed study of nocturnal and diurnal changes in activities of photosynthetic enzymes at different growing stages in CAM plants could reflect those during shift from C_3 to CAM. Abscisic acid (ABA) plays a key role in water regulation and conservation in the plants during drought (*e.g.* ElAmry 1990). Changes in ABA concentrations during the C_3-CAM shift need to be studied in field grown CAM plants.

This study investigates *M. nodiflorum* during a shift between C_3 and CAM cycles: *1*) nocturnal and diurnal organic acid fluctuation, *2*) the build up of ABA in plant roots and leaves during sunrise, midday, and sunset, *3*) changes in PEPC and RuBPCO as related to changes in environment, *4*) the chagnes in leaf ψ_l, and *5*) determination of V_{max} and K_m following Michaelis-Menten kinetics. These measurements are indicative of the current photosynthetic pathway (Franco *et al.* 1990, Leport *et al.* 1996) and coincide with the prevalent climatic and edaphic conditions. The measurements were done during wet and dry seasons in four age stages: seedling, juvenile, mature, and senescence.

Materials and methods

Plants: *Mesembryanthemum nodiflorum* L. (*Aizoaceae*) was studied at different ages. In seedling stage, the plants were very small and therefore 21 seedlings of uniform size were used for each analysis (seven seedlings, three replicates). In juvenile stage, three replicates of four plants; and in mature and senescence stages three replicates of two plants were used.

The site of study was at Baltim (31° 30' N, 31° 15' E), about 500 m from the Mediterranean coast marsh. The site was located in transient between sand dunes and salt marsh, where *M. nodiflorum* forms well defined zone of monospecific stand. In few stands it was co-associated with *M. crystallinum* L.

Acidity: Plant samples were randomly collected during sunrise and sunset. Fresh mass of the samples was immediately determined on field torsion balance, then they were ground fresh in a porcelain mortar, filtered, made up to a volume, and titrated with 0.01 NaOH to pH 7.0 end point using the phenolphthaline indicator.

Abscisic acid was analysed as described by ElAmry (1990). The internal standard was (±)-[G^{3}H] ABA (69 Bq mmol^{-1}, 0.07 ng of ABA; *Amersham*). Gas chromatography was equipped with *ECD-^{63}Ni* and 4 (internal diameter) × 180 mm glass column packed with mixture of *OV-17* (1 % *sp 2250*) and *OV-210* (2 % *sp 2401*). ABA recovery was 73.1 ± 4.11 %, such correction was applied for all reported values. Samples of shaded plants were compared to normal plants exposed to day light.

PEPC activity was measured following the method of Meyer *et al.* (1988). Each sample was ground with a cool all-glass *Beckman* homogenizer in 10 volumes of extraction medium containing 50 mg insoluble polyvinylpyrrolidone (PVP) in 50 mM glycylglycine buffer, pH 7.4, with 10 mM of 2-mercaptoethanol. Samples were centrifuged at 65 000 *g* for 40 min. Crude extracts were desalted on *Sephadex G-25* columns (*Pharmacia*), kept in refrigerator, and assayed in a mixture including 50 mM ACES, pH 7.0, 5 mM free Mg^{2-}, 5 mM $NaHCO_3$, 160 μM NADH, 12 IU MDH plus 5.5 IU LDH. Mg^{2+} was from Mg ACES (Rustin *et al.* 1988). Enzyme activity per mg protein was calculated from consumption of NADH (oxidation or decrease in the absorbance) during the reduction of oxaloacetate resulting from carboxylation of phosphoenolpyruvate (for routine assay 1 mM PEP was added). NADH was monitored using a *Zeiss* spectrophotometer (Jena, Germany) at 340 nm and 25 °C. Soluble protein of the extract was measured as a protein dye-complex formation using the *G-250* (Commassie blue) reagent. Bovine serum albumin (BSA) series of dilutions were used to get a standard curve. Dye-binding proteins were measured at 595 nm using a *Zeiss* spectrophotometer. V_{max} and K_m were calculated based on fitting rate of reactions at different PEP concentrations to the Michaelis-Menten equation.

RuBPCO was determined as described by Bhaskaran *et al.* (1983). Leaves were ground in an activation medium containing 20 mM Tris buffer (pH 8.2), 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DDT, and 10 mM $NaHCO_3$ with some washed sand and 0.3 g insoluble PVP per 1 g of leaf fresh mass. After centrifugation at 65 000 *g* for 20 min, supernatant solution was assayed using labelled $NaH^{14}CO_3$ (0.2 Bq mol^{-1}). Then, the reaction was initiated with 0.1 cm^3 of ribulose bisphosphate (RuBP) giving the final RuBP concentration of 0.5 mM, and terminated after 45 s with 0.1 cm^3 of 6 M HCl (Vu *et al.* 1984). The control used had no RuBP. The incorporation of acid stable labelled carbon was measured by liquid scintillation spectrometry as a mean of three determinations from three subsamples of combined plants with uniform size. The RuBPCO activity was measured on chlorophyll (Chl) basis. Chlorophyll was determined by the method of Arnon (1949). The RuBPCO extract was purified from contamination with other pentose phosphates, phosphoriboisomerase and phospho-ribulokinase activity, and ATP was removed (Seemann and Sharkey 1986).

Leaf ψ_l was measured using both the densitometric and the gravimetric methods described by Shardakov (1948).

Statistical analyses (McClave and Dietrich 1982) included running averages of at least three samples, *t*-test for comparison of two independent means, and analysis of variance (*F*-test) to compare and contrast closely related data.

Results

Titratable acidity: Noticeable variation in acidity was not recorded in seedling and juvenile stages as indication of CAM photosynthesis. Due to the wet cool winter period suitable for C_3 cycle, sunrise and sunset acidity of plants were low and not significantly different (Fig. 1). During the mature and senescence stages, acidity was high during sunrise and decreased by about 90 % during sunset.

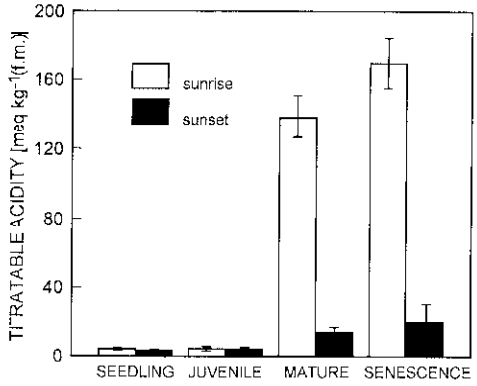

Fig. 1. Changes in titratable acidity during sunrise and sunset in the four age stages of *Mesembryanthemum nodiflorum*.

Abscisic acid: Concentrations of ABA in seedling and juvenile roots were about 100 μg kg^{-1}(d.m.) (Fig. 2). Roots of plants in mature and senescence stages did not show increase in ABA in comparison to the earlier stages. The ABA concentrations in roots were not significantly different at sunrise, midday and sunset.

Concentrations of ABA in leaves of seedling and juvenile stages were between 108 to 140 μg kg^{-1}(d.m.) during sunrise, midday, and sunset. In the mature leaves, ABA concentration was almost double of that in seedling and juvenile stages (Fig. 2). In the mature stage, shading of the leaves resulted in significantly decreased ABA concentration. There was a significant difference in ABA concentration between leaves and roots in all stages.

Activity of carboxylases: No significant difference in the PEPC activity occurred between the two early stages (Fig. 3); the average activities were only 27 % of the maximum PEPC activity in the mature stage. The mature and senescence stages

showed three peaks in PEPC activity. The first peak reached maximum 30 min after sunrise in the mature stage and after 60 min in the senescence stage. The second peak had smooth top run for about five afternoon hours in both mature and senescence stages, then the PEPC activity rapidly declined to a trough, the value of which was higher than that reached in the morning hours. The third peak was 75 and 60 % of the second peak in the mature and senescence stages respectively, and occurred during nocturnal CO_2 fixation.

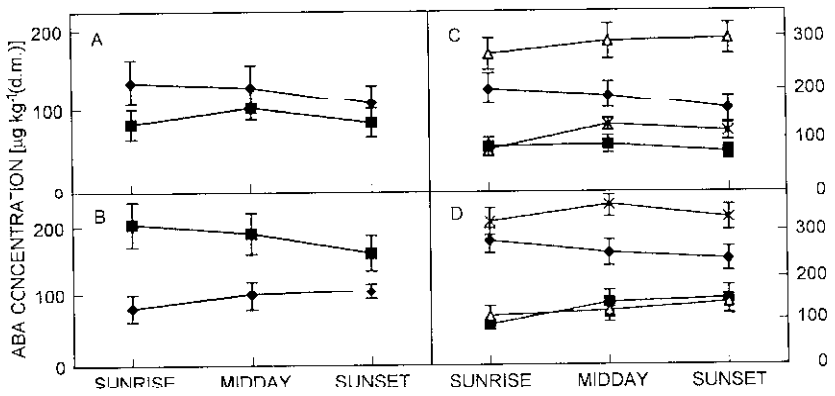

Fig. 2. Abscisic acid concentrations in roots (*squares*) and leaves (*circles*) during sunrise, midday, and sunset for sunny plants (*open symbols*) and shaded plants (*closed symbols*) in the seedling (*A*), juvenile (*B*), mature (*C*) and senescence (*D*) stages. *Bars* indicate S.E.

During day hours PEPC activity was inhibited by increasing malate accumulation and probably by degradation of glucan (Winter 1982, Leegood and Osmond 1990). Therefore, the PEPC kinetics were calculated only during sunrise and sunset. During

Table 1. Kinetic parameters of PEPCase (MgPEP as substrate). Values calculated from multiple regression analysis of 3 determinations of 3 subsamples (mean ± S.E.).

Stage	Daytime	V_{max} [μmol s^{-1}]	K_m [mM]	Affinity
Seedling	sunrise	167.17 ± 1.50	0.192 ± 0.007	5.02 to 5.41
	sunset	97.00 ± 1.83	0.224 ± 0.004	4.38 to 4.55
Juvenile	sunrise	101.50 ± 1.37	0.164 ± 0.002	6.02 to 6.18
	sunset	108.33 ± 1.82	0.294 ± 0.007	3.33 to 3.48
Mature	sunrise	301.17 ± 3.50	0.261 ± 0.003	3.79 to 3.88
	sunset	367.00 ± 3.33	0.153 ± 0.004	6.36 to 6.72
Senescence	sunrise	330.17 ± 3.50	0.247 ± 0.004	3.99 to 4.11
	sunset	418.17 ± 5.57	0.151 ± 0.009	6.25 to 7.05

sunrise, V_{max} was higher in the seedling stage than in the juvenile stage (Table 1), and increased by about 300 % in mature and senescence stages indicating mass increase

of PEPC relative to the substrate availability. The sunset V_{max} values in mature and senescence stages were much higher than in the earlier stages (Table 1). The K_m values and affinities of PEPC to substrate were consistent in all stages. The affinities of PEPC in seedling and juvenile stages were higher during sunrise than sunset and *vice versa* in the later stages.

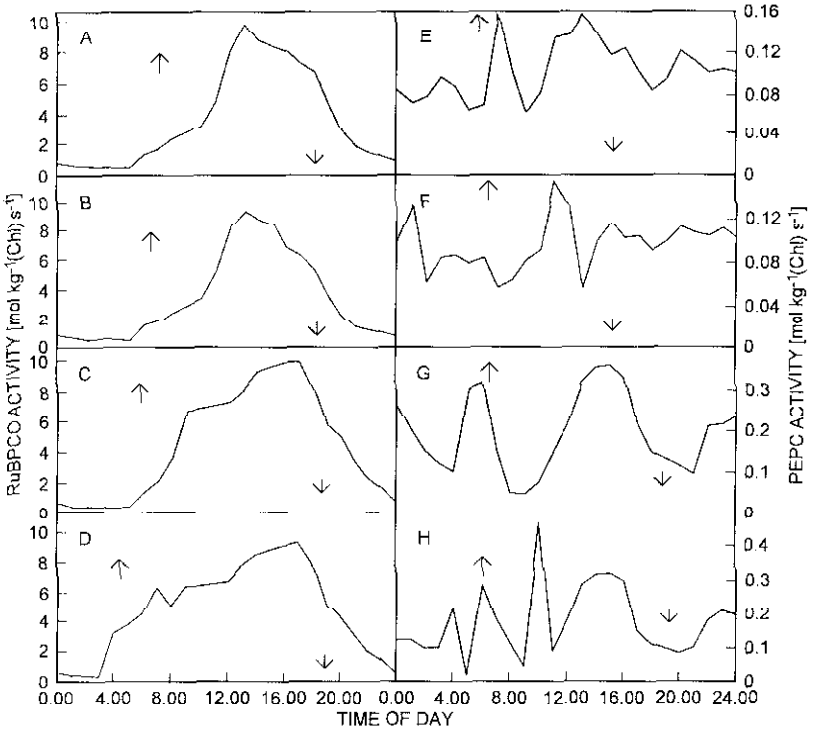

Fig. 3. Phosphoenolpyruvate (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) activities in leaves of *M. nodiflorum* plants in the seedling (*A,E*), juvenile (*B,F*), mature (*C,G*) and senescence (*D,H*) stages during day and night. *Upward arrow* indicates sunrise and *downward arrow* indicates sunset.

Gradual increase in RuBPCO activity occurred 70, 60, 20, and 80 min before sunrise in the four age stages, respectively (Fig. 3). In the seedling and juvenile stages, the RuBPCO activities reached maximum around the 13.00 and then started to decline slowly. At mature and senescence stages, the maximum RuBPCO activity was *ca.* 2 h before sunset; the minimum RuBPCO activity was 6 to 7 h after sunset.

Leaf ψ_l: At the seedling and juvenile stages, the plants had a high water availability (Table 2). In sunrise ψ_l in mature and senescence stages was about five times lower than that of the juvenile stage but the range was still out of inducible stress. Sunset ψ_l values were always lower than the sunrise ones.

Table 2. Leaf water potential, ψ_l, of *M. nodiflorum* during four growth stages at sunrise and at sunset (mean ± S.E., $n = 4$).

Stage	ψ_l [MPa] sunrise	sunset
Seedling	-0.02 ± 0.00	-0.09 ± 0.01
Juvenile	-0.04 ± 0.01	-0.12 ± 0.09
Mature	-0.21 ± 0.03	-0.35 ± 0.17
Senescence	-0.18 ± 0.04	-0.31 ± 0.12

Discussion

Titratable acidity: Organic acids, mainly malic acid serve as large temporary store of CO_2 in tonoplast and represented the nocturnal CO_2 uptake and subsequent fast depletion by fixation. In that respect, acidity and organic acid formation and storage in the tonoplast play an important role in CO_2-induced nocturnal stomatal opening mechanisms. Fluctuation in acidity during mature and senescence stages was accompanied by a shift in photosynthetic pathway from C_3 to CAM and reflected the impact of dry hot environment on physiology and metabolism of the studied species. In general, acidity, diurnal and nocturnal CO_2 uptake and fixation are not unique (Nobel *et al.* 1996, Leegood and Osmond 1990). Our new observation is that the acidity during sunset in mature stage is higher than in earlier stages. Moreover, the senescence stage has higher acidity during sunrise and sunset than the mature stage. Metabolic activities and growth rate in senescence are expected to be less than in the mature stage, which is not the case here. We hypothesize explanation as follows: senescence is accompanied by a large synthesis of ethylene from 1-amino cyclopropane 1-carboxylic acid (ACC). These result in an intake of oxygen and release of ammonium, formic acid and CO_2. Thus the added CO_2 and the depleted O_2 during ethylene production participate in furnishing biochemical line for PEPC activity for more fixation of CO_2 and formation of malic acid which may increase the acidity in senescence stage more than in the mature stage.

Abscisic acid: ABA concentrations in roots are not high enough to trigger changes in root hydraulic conductivity or play a role in stomatal closure (ElAmry 1990). We might conclude that ABA concentration in roots of different age stages was not affected by, nor played any apparent role, during the shift of *M. nodiflorum* from C_3 to CAM. High ABA content in leaves and roots is associated with stomatal closure (Ting 1987, Neales *et al.* 1989) and low photosynthetic rate. Thus, in mature and senescence stages one would expect lower ABA at midday (diurnal stomatal closure and low E values) than at sunset (nocturnal open stomata and fixation of CO_2), but ABA was not significantly different among sunrise, midday, or sunset samples (Fig. 2). Hence we conclude that: *1*) ABA concentration is species specific and it is high in *M. nodiflorum* leaves, and *2*) stomatal closure mechanism in *M. nodiflorum* is

relatively insensitive to these elevated ABA concentrations. Moreover, in shaded plants during mature and senescence stages the concentration of ABA decreased continuously from sunrise to midday and sunset. Further work on changes in ABA variations in response to climatic changes is required for CAM plants in their natural habitats.

Carboxylases: The first peak of PEPC activity during mature stage had sharper uphill-downhill cycle than during senescence. The second peak was similar in both stages. The V_{max}, K_m, and the affinity of PEPC during later stages indicated the ability of PEPC to fix CO_2 taking up at night. The relatively less affinity during sunrise indicated the inhibitory effect of malate concentration on PEPC during release of CO_2 which was fixed again by RuBPCO. But why RuBPCO and not PEPC fix CO_2 during the day, though both enzymes have almost equal affinity toward dissolved form of CO_2 (Kramer 1983, Salisbury and Ross 1984) and the cytosol location of PEPC should allow it to reach CO_2 before RuBPCO? Why the RuBPCO activity decreases slowly after sunset and diminishes totally during night while the PEPC peaks three times, two of them during day hours? Why the PEPC third peak at night was lower than the other two peaks of the day, though it had maximum CO_2 fixation by that time?

In CAM plants, both PEPC and RuBPCO are present in all chloroplast-containing cells. We may suggest that starch hydrolysis and decrease of glucan concentration preceding stomatal opening during sunset, result in high concentration of triose-P and hexose-P which in turn increase the activity of PEPC (Leegood and Osmond 1990). Then, fast movement of malic acid into the vacuoles prevents build up of the malate that inhibit PEPC locally. This inhibition occurs at high malate concentration which explains why RuBPCO and not PEPC refixes CO_2 during day time. Thus for malic acid to be formed, moved and accumulated in the vacuoles, PEPC must be active and RuBPCO must be inactive (mainly during phase I), and *vice versa* during de-acidification in phase III (Osmond *et al.* 1988). After pooling of malate in photosynthesis in phase IV, PEPC become active and participate with RuBPCO in CO_2 fixation. These steps may explain the presence of the recorded peaks of PEPC activity as questioned above. RuBPCO may be adjusted to be inactive at night because of the absence of light stimulation or presence of specific inhibitors. More studies required in that respect. The PEPC lower peak at night as compared with the other two peaks may reflect three issues to be tested: the formation of triose-P is gradual and continues to occur during CO_2 fixation; the synthesis and movement of malic acid may be fast enough to show much PEPC activity and probably have little inhibitory effect; the level of CO_2 uptake and PEP build up may cause adjustment of the tissues to permit a suitable level of PEPC activity which appears as lower night peak of activity.

Leaf ψ_l: Any loss of water can be potentially harmful for plants growing in arid zones. Thus, by closing of stomata during day time the CAM plants greatly reduce transpiration rate (E) and increase water use efficiency, though they essentially eliminate the influx of CO_2 during day light. In arid zones during sunset, the slight

increase in ABA in the leaves (during mature and senescence stages) could have decreased E without decrease in water potential. Also night drop in air temperature and increase in relative humidity reduce E markedly. These steps also result in high water use efficiency (Nobel 1984).

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