BRIEF COMMUNICATION

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Effect of NaCl on the photosynthetic pathways of halophytic leaf succulent *Sedum uniflorum*

C.E. MARTIN^{1,2#}, J.-M. CHIANG¹, and T.-C. LIN^{2,*}

Department of Life Science, Tunghai University, Taichung 40704, Taiwan¹

Department of Life Science, National Taiwan Normal University, Taipei 11677, Taiwan²

Abstract

The effect of NaCl on the photosynthetic pathways of the only halophytic subtropical species *Sedum uniflorum* was examined in the field in northern Taiwan and in the laboratory. Plants growing in the field exhibited CAM-cycling regardless of salt content in their tissues. In contrast, NaCl treatment in the laboratory induced CAM photosynthesis, while control plants exhibited a C₃-CAM intermediacy. This high level of photosynthetic pathway variability matches that of other *Sedum* species.

Additional key words: CAM, CAM-cycling, C₃-CAM intermediate, net CO₂ exchange, $\delta^{13}\text{C}$.

One of the more fascinating discoveries in plant ecophysiology in the past few decades is the environmentally-induced photosynthetic pathway flexibility found in certain species of succulents, exemplified by the coastal halophytes *Mesembryanthemum crystallinum* (Winter and Von Willert 1972, Winter *et al.* 1978) and *M. nodiflorum* (Winter and Troughton 1978) found in Mediterranean environments. During cool and moist winters and springs, seed germination and rapid growth of the young plants is, in part, enhanced by the C₃ photosynthetic pathway, which is not particularly water-conservative, yet lacks physiological and biochemical restrictions imposed by the crassulacean acid metabolism (CAM) pathway (Kluge and Ting 1978, Osmond 1978, Winter *et al.* 1978, Herrera 2009). Upon exposure to high salt concentrations and/or drought stress, these plants then switch from C₃ photosynthesis to the water-use efficient CAM photosynthetic pathway. Such plants are often referred to as C₃-CAM intermediates (Winter and Holtum 2014) despite the fact that the two photosynthetic pathways (*sensu stricto*) remain distinct in the two

seasons. A group of plants which always exhibits truly intermediate photosynthesis, *i.e.*, both the C₃ and CAM photosynthetic pathways occurring simultaneously, comprises a large number of phylogenetically disparate taxa, which undergo CAM-cycling (Ting 1985, 1994, Martin *et al.* 1988, Martin 1996, Herrera 2009). These plants exhibit a C₃ pattern of stomatal activity and, hence, gas exchange (*i.e.*, daytime stomatal opening and CO₂ uptake, followed by nighttime stomatal closure with zero CO₂ exchange or perhaps low rates of CO₂ loss), coincident with CAM diel fluctuations in tissue acidity. Because their stomata are closed at night during the accumulation of malic acid, it is assumed that the source of CO₂ required for acid accumulation is respiration (Patel and Ting 1987, Martin 1996, Winter and Holtum 2014). During the daytime, the stomata open, despite the release of CO₂ from the decarboxylation of malic acid in the mesophyll cells of the photosynthetic tissue (Martin 1996).

The ecophysiological significance of CAM-cycling is currently unclear (Martin 1996, Herrera 2009, Winter

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Abbreviations: CAM - crassulacean acid metabolism; PPFD - photosynthetic photon flux density.

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Current address: Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, Kansas 66045, USA.

* Corresponding author; e-mail: tengchiulin@gmail.com

and Holtum 2014). Although several authors have claimed that elevated daytime c_i in the photosynthetic tissues of such plants should effect partial stomatal closure with consequent reductions in water loss, supportive data are scarce. In addition, it has been argued that nighttime reductions in CO_2 loss might also conserve water, but such arguments remain conjectural. Finally, it is also claimed that the primary adaptive nature of CAM-cycling may be in preparing a plant to undergo CAM-idling, in which diel acid fluctuations continue during extreme drought stress, when the stomata of these plants remain closed during both day and night (Ting 1985, 1994, Martin 1996, Herrera 2009, Winter and Holtum 2014). In this case, CAM-idling may serve to allow such plants to recover quickly from drought stress, once the plants receive water. Once again, experimental support for this scenario is weak or absent.

Sedum is a large genus in the family *Crassulaceae* (Clausen 1975, Stephenson 1994), and the photosynthetic pathways of its species include CAM, CAM-cycling, and yet other forms of C_3 -CAM intermediacy (Martin and Jackson 1986, Martin *et al.* 1988, Borland and Griffiths 1990, Pilon-Smits *et al.*, 1991a,b, Gravatt and Martin 1992). Most species of *Sedum* grow in thin soils overlaying rock outcrops or among rocks often at high elevations and a wide diversity of other, mostly temperate habitats (Clausen 1975, Stephenson 1994). One curious species, *Sedum uniflorum*, is a halophytic succulent endemic to subtropical Taiwan and southern islands of Japan (Huang 1993) growing in sandy soils in dune slacks of coastal regions of these islands. The plants can grow in soils with varying salinities (judged by varying distances from the ocean). Whether or not salt can induce CAM-cycling, as in *Mesembryanthemum*, has not been previously investigated in any coastal succulent. Given this, coupled with the photosynthetic pathway diversity in the genus *Sedum*, the goal of this study was to determine the effects of NaCl on the photosynthetic behavior of *S. uniflorum* in the field in Taiwan and in the laboratory using plants grown from seeds collected in the same area.

Seeds of *Sedum uniflorum* Hook & Arn. were collected at the Baishawan beach, New Taipei City (25.284 °N, 121.519 °E) in northern Taiwan. Plants were growing in sandy soil along a transect perpendicular to the ocean from approx. 100 - 150 m away from the ocean water. The soil was moist at root level for all plants during the field work. Environmental conditions during this time approximated: a full sunlight of $2\,100\,\mu\text{mol m}^{-2}\text{ s}^{-1}$, a temperature of 33 °C, and a relative humidity of 85 %. Plants were sampled at approx. 25 m intervals away from the ocean along the transect.

In July 2013 at each of the seven locations along the transect, plants were sampled for titratable acidity and electrolytic conductivity measurements as follows: one leafy shoot was cut, placed in a plastic bag, then on ice in the evening (18:00) of the first day and again in the morning (07:00) of the following day. On the latter day,

all bagged samples were transported to the laboratory in Taipei and immediately frozen at -10 °C. Within the next two weeks, samples were thawed, weighed, then ground with a mortar and pestle plus a small amount of water. The resultant slurry was titrated to pH 7.0 with 0.01 M NaOH using a digital pH meter, then dried at 65 °C to recover the dry mass of the ground tissue, once the mass stabilized.

Leaves from plants at each of the transect locations were ground in deionized water (a total volume of 20 cm^3), and the electrolytic conductivity of this solution was measured with an LF320 conductivity meter (WTW Xylem Analytics, Weilheim, Germany). For this study, the shoot sap conductivity was used as an indicator of tissue NaCl content.

Seeds were collected from numerous plants in the middle of the transect, then transported to a greenhouse at the University of Kansas, whereupon they were germinated and grown in wet soil (a standard greenhouse mix and sand, 1:1) in plastic pots under a natural photoperiod with a photosynthetic photon flux density (PPFD) of approx. $500\,\mu\text{mol m}^{-2}\text{ s}^{-1}$ at midday with clear sky, day/night air temperatures of 30/20 °C, and relative humidities of 75/90 %. Plants were always watered with tap water (without salt) after soil drying.

After one year of growth in the greenhouse, plants were mature and flowering when used for the laboratory measurements. Two weeks prior to measurements, plants were placed in a growth chamber with these conditions: a 12-h photoperiod with a PPFD of $300\,\mu\text{mol m}^{-2}\text{ s}^{-1}$, 30/20 °C day/night air temperatures, and 60/80 % day/night relative humidities. Plants were then watered daily with 100, 200, 300, 400, 500, and 600 mM NaCl solutions for two days each. The salt solutions were administered sequentially, from lowest to highest. Plants were then kept watered daily with deionized water during three days of continuous day/night gas-exchange measurements. Approximately five shoots per plant were sealed into each of two gas-exchange cuvettes, which were water-jacketed for temperature control and constructed of polycarbonate to reduce CO_2 and H_2O vapor exchange between the air and the cuvette walls. Despite the latter, such a gas exchange was substantial and necessitated subtraction of near-simultaneous CO_2 and H_2O vapor exchange rates of an identical empty cuvette from both plant cuvettes (no gas exchange was observed by the soil and pots alone). On the morning (08:00) of the third day, the gas-exchange cuvettes with plants were opened, and two to three shoots (depending on the length) were removed from each and frozen (-10 °C) for subsequent measurement of titratable acidity. The cuvettes were then resealed until the remaining tissue was removed at 20:00 h for determination of fresh and dry masses.

Shoots of three greenhouse-grown plants and seven plants from along the field transect were dried at 65 °C, ground into a fine powder with a mortar and pestle, then

transported to Keck Paleoenvironmental and Environmental Stable Isotope Laboratory at the University of Kansas for stable carbon isotope analysis. For each sample, the powder was randomly subsampled, and the resultant material was combusted for determination of the $^{13}\text{C}/^{12}\text{C}$ ratio of the resultant CO_2 using a MAT 253 IRMS mass spectrometer (ThermoFinnigan, Bremen, Germany). The $\delta^{13}\text{C}$ value of the sample was expressed relative to the calibrated standard Vienna Pee Dee Belemnite.

The means of morning and evening acidities as well as of $\delta^{13}\text{C}$ values were compared by the Student t -test (field samples) and the paired t -test (laboratory samples) if the data met the assumptions for using parametric statistics (Sokal and Rohlf 2012). In cases in which the latter was not true, the Mann-Whitney U -test was used to compare the means. A linear regression was used to examine the relationship between acidity and shoot sap conductivity and distance from water. In all tests, statistically significant differences were assumed when $P \leq 0.05$.

Leaf titratable acidity increased overnight in all plants of *S. uniflorum* in the field near coastal dunes in northern Taiwan regardless of the conductance of the expressed leaf liquid (Fig. 1). Thus, nocturnal increases in shoot acidity were not correlated ($P > 0.05$) with shoot sap conductivity used here as an indicator of NaCl content. When nighttime increases in acidity (Δ acidity) were averaged for groups of plants along transect away from the ocean (Fig. 1 Suppl.), all acidity increases were highly significant ($P \leq 0.01$), whereas the regression between nocturnal acidity increases and distance from the seawater was not significant ($P > 0.05$).

Net CO_2 uptake occurred at night (Fig. 2) in both

Fig. 1. The response of tissue sap acidity in leaves of *Sedum uniflorum* sampled *in situ* in the northern Taiwan coast to tissue conductivity (an indicator of salt content). The Δ acidity values are the differences between morning and evening acidities. Plants were sampled along a 150-meter transect perpendicular to the ocean water; each data point represents one plant. The regression coefficient = 0.04; $P > 0.05$.

NaCl-treated and control (no-salt-treated) greenhouse-grown plants, whereas leaf tissue titratable acidity significantly increased overnight only in NaCl-treated

plants. The average Δ acidity (morning acidity values - evening values) of the control plants was not significantly different from that of the NaCl-treated plants (Fig. 2 Suppl.). The mean morning acidity was not significantly higher than the mean evening acidity in the control plants; therefore, the “ Δ acidity” of the latter plants cannot be interpreted as a true nocturnal increase in leaf acidity. On the other hand, the high variability in the acidity of control plants coupled with the low sample size of plants sampled could also be interpreted as an indicator that the Δ acidity of these plants is actually representative of at least some CAM biochemistry. The $\delta^{13}\text{C}$ values of *S. uniflorum* plants grown a year in the greenhouse (without salt) were significantly less negative than values of plants collected from the field in northern Taiwan (Fig. 4 Suppl.).

Fig. 2. Daily course (night indicated by thick black horizontal bars) of net CO_2 exchange (A) and net H_2O vapor exchange (B) of shoots of *Sedum uniflorum* grown in a greenhouse and watered for a week with solutions of increasing salinity (100 to 600 mM NaCl). Results shown are for one plant, but similar results were obtained for three other plants. Mean integrated gas-exchange values for all four plants are shown in Fig. 2 Suppl. and Fig. 3 Suppl.

Plants of *S. uniflorum* growing naturally near sand dunes along the coast in northern Taiwan exhibited CAM-cycling (Ting 1985, Martin 1996, Herrera 2009); *i.e.*, tissue acidity increased to varying degrees overnight in all plants examined (note the range of Δ acidity values in Fig. 1), which is indicative of CAM biochemistry. However, the $\delta^{13}\text{C}$ values of these plants were very negative and thus, characteristic of C_3 plants (Griffiths 1992). The increased tissue sap electrolytic conductivity (*i.e.*, a higher NaCl concentration) had no effect on the photosynthetic pathway of these plants; however, there

was a small trend toward increasing Δ acidity with increasing tissue sap conductivity, which matched the trend of increasing Δ acidity in plants closer to the ocean, where tissue contained more salts. Thus, all plants in the field growing along transect with varying degrees of salinity were performing CAM-cycling, and the degree of CAM-cycling appeared unrelated to leaf salt content.

In contrast, salt-treated plants grown for one year from seeds produced by the plants in the vicinity of those used for the field work, exhibited CAM photosynthesis indicated by nocturnal increases in tissue acidity, nighttime CO_2 uptake, followed by daytime stomatal closure (or at least minimal gas exchange during the day), and high (less negative) $\delta^{13}\text{C}$ values, similar to those of many other CAM taxa (Kluge and Ting 1978, Griffiths 1992). Application of NaCl to the greenhouse-grown plants appeared to induce CAM as nocturnal increases in leaf tissue acidity were measured only in the salt-treated plants. The apparent Δ acidity in the control plants was not statistically significant, yet these plants did exhibit CAM gas exchange and CAM $\delta^{13}\text{C}$ values; thus, these plants may have been using CAM, but certainly not CAM-cycling, as was found in the plants examined in the field in Taiwan.

Thus, the halophytic *S. uniflorum* exhibited CAM-cycling in the field, whereas greenhouse-grown plants exhibited CAM when treated with NaCl, and a possible mixture of CAM and C_3 photosynthesis when grown

without salt. These findings of a highly variable photosynthetic metabolism of *S. uniflorum* are not unlike those in other species (Kluge 1977, Schuber and Kluge 1981, Martin and Jackson 1986, Martin *et al.* 1988, Borland and Griffiths 1990, Gravatt and Martin 1992). Although both Δ acidity and salt content varied greatly among individuals of *S. uniflorum* in the northern Taiwan coast, there is no evidence here that salt stimulated or enhanced CAM-cycling in these plants. In contrast, only NaCl-treated greenhouse-grown plants clearly exhibited CAM instead of CAM-cycling, and control plants exhibited a mixture of CAM and C_3 photosynthesis. Therefore, the results of salt effects on the C_3 -CAM flexible halophytic species of *Mesembryanthemum* can be extrapolated, to some degree, to this C_3 -CAM intermediate halophytic species of *Sedum*.

Overall, it is clear that photosynthetic metabolism in the genus *Sedum* is highly variable, both within and among species, and much of this variability can be attributed to the environmental conditions under which the plants grow. It is also clear, based on the results of the current study, that salt may have dramatic effects on photosynthesis in the sole halophytic species of *Sedum* known, and that the effects of salt are highly variable, and, in some degree, not unlike what has been found in other studies of halophytic CAM or facultative CAM plants.

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