

First Finding of Succulence and C<sub>4</sub>/CAM-Cycling Photosynthesis in a  
Grass: Ecophysiology of *Spinifex littoreus* in Coastal Regions of Taiwan

By

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## Abstract

*Spinifex littoreus* (Brum. f.) Merr. is a halophytic grass that is distributed on coastal sand dunes in Taiwan (and throughout Southeast Asia). This study is the first report of leaf succulence in a grass, with 64 % of the leaf cross-sectional area occupied by water-storage parenchyma (hydrenchyma). Leaf water content, saturated water content and mesophyll succulence indices were also similar to other reported succulent plants. In addition to the previous report of  $C_4$  photosynthesis, the current study found diel acid fluctuation and nocturnal stomatal closure, which indicates CAM photosynthesis (in a CAM-cycling form), thus indicating that *S. littoreus* is a grass with  $C_4$ /CAM-cycling photosynthesis. This finding is the first report of any grass with CAM photosynthesis of any kind. The presence of CAM acid fluctuation was found to be associated with exposed areas and at the edges of plant colonies. Also, the presence of CAM acid fluctuation in some populations was not consistent across years, and was not correlated with temperature or precipitation differences. Photosynthetic rates among populations varied, which might either be the result of different environmental conditions, or genotypic variability among populations throughout Taiwan. This study comprises the first report of a succulent grass, and the first report of  $C_4$ /CAM-cycling photosynthetic pathway in monocots. This plant may have an important prospect as a model organism for studying the regulation and evolutionary history of  $C_4$  and CAM photosynthesis, as well as the potential value for experimental breeding programs, improving drought and salt tolerance in cereal crops.

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## Chapter 1. General Introduction

### Photosynthetic pathways

Plants can be categorized into three major groups, C<sub>3</sub>, C<sub>4</sub>, and Crassulacean acid metabolism (CAM), based on their photosynthetic pathways. These different pathways allow plants to adapt to diverse environments (Larcher 2001; Taiz and Zeiger 2002). Most C<sub>4</sub> plants (except single-cell C<sub>4</sub> plants; Voznesenskaya *et al.* 2001) have Kranz anatomy composed of bundle sheath cells (BSC) with chloroplasts that surround their vascular tissues. CO<sub>2</sub> is first fixed by PEP carboxylase to the 4-carbon OAA in the mesophyll cells (MC), then OAA is transported to BSC, broken down and CO<sub>2</sub> is then fixed into photosynthetic carbon reduction (PCR) cycle by Rubisco (Larcher 2001; Taiz and Zeiger 2002). Because PEP carboxylase has a high affinity to CO<sub>2</sub> and no affinity to O<sub>2</sub> in bundle sheath cells, this spatial distribution allows CO<sub>2</sub> to accumulate in high concentrations thus reducing photorespiration. The C<sub>4</sub> pathway reportedly evolved in the middle Oligocene to early Miocene when ambient CO<sub>2</sub> concentration decreased dramatically (Tippie and Pagani 2007). In modern times, this spatial CO<sub>2</sub> concentrating mechanism (CCM) reduces photorespiration. Such CCM is considered to be an adaptation to high light and high temperature (Taiz and Zeiger 2002). Another photosynthetic pathway, CAM, also uses PEP carboxylase to fix CO<sub>2</sub>. CAM plants open stomata at night, fix CO<sub>2</sub> to malic acid, which is then stored in vacuoles. During the day, this malic acid is released and fixed into PCR cycle by Rubisco. Stomatal opening during the night reduces water loss, thus comprises an adaptation to drought environments (Larcher 2001; Taiz and Zeiger 2002). CAM pathway is often found in epiphytes and desert plants that frequently face drought stress (Larcher 2001).

## ***Spinifex littoreus***

*Spinifex littoreus* (Brum. f.) Merr. (syn. *S. squarrosus*; Poaceae, subfamily Panicoideae; tribe Paniceae) is a perennial halophytic grass that is distributed in coastal regions throughout southeastern Asia such as Japan, China, Taiwan, Indonesia, and India. This grass grows on sand dunes, which are often considered as a highly stressful environment (Bermúdez and Retuerto 2014). Both water and nitrogen are important limiting factors for vegetation in this ecosystem, as well as high temperature, high light, salt spray and soil salinity (Hesp 1991; Larcher 2001; Maun 2009; Bermúdez and Retuerto 2014). Rama Das and Raghavendra (1977) have reported *S. littoreus* as a NADP-ME type of C<sub>4</sub> grass by the presence of Kranz anatomy and biochemical activities. Carbon stable isotope values of *S. littoreus* were reported as -12.2 ‰ (Brown 1977), which is also consistent with C<sub>4</sub> photosynthesis.

## **Aim of the study**

*S. littoreus* has been studied since the 1970s; however, the thickness and succulence of the leaf has never been reported. Tissue succulence is often correlated with CAM, and recent data have supported a causal relationship between succulence and CAM (Ripley *et al.* 2013). Although both C<sub>4</sub> and CAM pathways use the same enzyme PEP carboxylase, their enzyme regulations are different. *Portulaca* is the only genus that has been reported to contain C<sub>4</sub> and CAM pathways in different tissues of a species (Guralnick *et al.* 2002; Kraybill and Martin 1996; Koch and Kennedy 1980; Sage 2002; Winter and Holtum 2014). Because of the causal relationship between succulence and CAM photosynthesis, *S. littoreus* might have the ability to undergo CAM photosynthesis. The aim of this study is to determine the degree of succulence in *S. littoreus*, its photosynthetic pathway(s), and how these results compare among different populations in Taiwan.

## **Chapter descriptions**

Chapter 2 addresses the definition of succulence, different methods in quantifying the degree of succulence, and how the results of *S. littoreus* are compared to other species. Chapter 3 discusses the photosynthetic pathway(s) of *S. littoreus* using gas exchange, acidity and carbon stable isotope values. Chapter 4 compares the amount of diel acid fluctuations, gas-exchange rates, and microhabitats among six populations from northern to southern coastal regions in Taiwan. Chapter 5 summarizes all the results and provides a future perspective of *S. littoreus*.

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## **Chapter 2. First Report of Leaf Succulence in a Grass: *Spinifex littoreus* (Poaceae) on Coastal Sand Dunes in Taiwan**

### **Introduction**

One common limitation for most terrestrial plants is the retention and/or acquisition of water, especially in arid lands or during periods without precipitation. Growing under long-term desiccation may impair physiological processes and ultimately result in death of the plant. A wide variety of adaptive features, both morph-anatomical and physiological (Turner and Kramer 1980; Levitt 1980; Larcher 2001), allow plant survival during drought stress, and one such morphological adaptation comprises the storage of large amounts of water inside the leaf. Such stored water may serve as a supplemental supply of water to the photosynthetic tissue, allowing plants to maintain their physiological activity even under drought conditions (Nowak and Martin 1997; Herrera *et al.* 2000; Chiang *et al.* 2013). Tissue water storage is often termed “succulence”, and there are different approaches to define the degree of succulence of a leaf, including leaf thickness (Teeri *et al.* 1981; Winter *et al.* 1983; Gibson and Nobel 1986), amount of water storage parenchyma (hydrenchyma; Chiang *et al.* 2013), fresh mass per leaf area (Borland *et al.* 1998), and the ratio of water mass (fresh mass minus dry mass) to tissue fresh mass (von Willert *et al.* 1992). In an attempt to relate succulence to photosynthetic pathways, Kluge and Ting (1978) defined succulence as the ratio of water mass to chlorophyll mass (g/mg), which they termed “mesophyll succulence” (or  $S_m$ ), and which they claimed correlates well with the CAM photosynthetic pathway. Furthermore, Ogburn and Edwards (2012) suggested that saturated water content (SWC), the difference between leaf mass after full hydration and dry mass divided by leaf dry mass, is a meaningful way to

quantify succulence because it relates to tissue capacitance, and it is also highly correlated with leaf thickness and water storage cell size, both of which are also indicators for succulence.

Succulent plants have been found in many families; however, no succulent plants have been reported in the Poaceae, one of the largest angiosperm families in the world and which includes many species growing in arid and saline regions. The current study comprises the first report of leaf succulence in the grass, *Spinifex littoreus*, a halophyte with thick, stiff leaves found on coastal sand dunes in Southeast Asia. The goal of this study was to examine the degree of succulence in leaves of *S. littoreus* using different indices of leaf succulence and to compare these results with other succulent plants.

## **Materials and Methods**

### **Plant collection**

Unshaded leaves of *Spinifex littoreus* (Brum. f.) Merr. were collected from coastal sand dunes in Taiwan at six localities: Baishawan beach (New Taipei City; 25.284°N, 121.519°E), Fulong Beach (New Taipei City; 25.020°N, 121.943°E), Haishan Fishing Port (Hsinchu; 24.765°N, 120.906°E), Chingshui Beach (Yilan; 24.685°N, 121.836°E), Rouzongjiao beach (Lukang; 24.075°N, 120.25°E) and Tainan Gold Coast (Tainan; 22.938°N, 120.175°E). *Spinifex littoreus* usually grows alone or with *Ipomoea pes-caprae*, *Bidens pilosa* L. var. *radiata*, and *Vitax rotundifolia*. Because *S. littoreus* mainly grows by spreading rhizomes, individuals were collected at least 5 m apart or at different sand dunes.

For laboratory work in Taiwan, one leaf from each of five individuals was collected at each population in July 2015 for leaf anatomy; and two leaves (one at 7-8 am; one at 4-5 pm) were collected from each plant (n = 22-30 plants in June or July 2015; n = 8-10 plants in July 2016 [it

was not possible to collect leaves in Haishan Fish Port 2016 due to bad weather]) at each location for determination of water content. One leaf was collected from one of six individuals at Tainan Gold Coast in July 2016 for the saturated water content experiment. All plant materials were immediately placed in zipper plastic bags on ice after excision and transported to the laboratory in Taipei. Leaves for anatomy were prepared immediately, and other leaves were stored in the freezer and analyzed no more than two weeks after collection.

For laboratory work at the University of Kansas, plants of *S. littoreus* were collected in February 2015 from Rouzongjiao beach (n = 5) and Baishawan beach (n = 3), transported to Kansas and were grown in a growth chamber until mesophyll succulence measurements in May 2016. Plants were watered three times a week and the day/night temperature (photo- and thermo-period of 12-hours) was ca. 30/20 °C. PPFD was ca. 100-150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and the day/night RH was 10-50%.

#### Proportion of water storage tissue

Thin sections (ca. 200-300  $\mu\text{m}$ ) were cut from the middle of each leaf. For each section, one photograph was taken with a stereo microscope (Olympus SZX10, Tokyo, Japan), and ImageJ (NIH, Baltimore MD, U.S.A.) was used for calculating the fraction of achlorophyllous water storage parenchyma area of the total cross-sectional area.

#### Water content

Whole leaves were used for determination of water content, which was calculated as:

$$\text{Leaf water content} = \frac{\text{Fresh mass} - \text{Dry mass}}{\text{Fresh mass}}$$

where DM = oven (65 °C) for at least 7 days until the difference of the dry mass was less than 0.001 g.

#### Saturated Water Content (SWC)

Leaves fresh mass was weighed, and saturated mass was weighed after soaking in distilled water for 48 hours and gently wiped with Kimwipes before weighing. Dry mass was then weighed until constant weight (at least 7 days) at 65 °C. SWC was calculated as:

$$\text{Saturated water content} = \frac{\text{Leaf mass at full hydration} - \text{Dry mass}}{\text{Dry mass}}$$

#### Mesophyll succulence (Sm)

One leaf per individual was excised, weighed and sliced into 1 mm-thick strips into a scintillation vial containing 10 mL of Dimethylformamide for 3 days, until all the tissues became transparent, and chlorophyll concentration was determined spectrophotometrically according to Moran (1982). Mesophyll succulence was calculated as:

$$\text{Sm (g/mg)} = \frac{\text{Fresh mass} - \text{Dry mass}}{\text{Chlorophyll mass}}$$

#### Statistical analysis

Mean proportions of hydrenchyma and mean water contents among populations were compared with a One-Way Analysis of Variance (One-Way ANOVA) or Kruskal-Wallis One-Way Analysis of Variance on Ranks if data were not normally distributed. All statistical tests were performed using SigmaStat (SystStat, Chicago, IL, USA), and differences among means were considered significant when  $P \leq 0.05$ .

## Results

Leaves of *S. littoreus* are 1 - 1.5 mm thick (middle of the leaf), with chlorenchyma cells and vascular bundles only on the abaxial side of the leaf (Figure 1). Hydrenchyma, defined as achlorophyllous tissue with large cells, occupied more than 60% of the cross-sectional leaf area in all populations examined (Table 1), and this proportion did not differ among populations ( $P > 0.05$ ). Leaf water contents of all populations exceeded 0.8 and were significantly different among populations. Water contents were the lowest in the two northernmost populations (Baishawan beach and Fulong Beach) in 2016. In addition, water content varied at different times of the day. Water contents in AM (at the end of the night) leaves were significantly higher than PM (at the end of the day) leaves in all populations in both years ( $P < 0.05$ , statistical data not shown) except Chingshui Beach and Tainan Gold Coast in 2015 (Table 2). Saturated water content (SWC) of *S. littoreus* in Tainan Gold Coast was  $6.92 \pm 0.26$  (mean  $\pm$  SE), while mesophyll succulence ( $S_m$ ) was  $1.03 \pm 0.08$  (mean  $\pm$  SE) at Rouzongjiao beach and  $0.84 \pm 0.04$  (mean  $\pm$  SE) at Baishawan beach.

## Discussion

*Spinifex littoreus* is distributed on coastal sand dunes throughout Southeast Asia, including Japan, China, Taiwan, Indonesia and India (Global Biodiversity Information Facility, GBIF; Rama Das and Raghavendra 1977). The leaves of this grass are clearly succulent based on general morphology, cross-sectional anatomy, and different succulent indices. Sixty four percent of the leaf area was occupied by hydrenchyma, which is similar to or higher than found in other succulent plants, such as *Pyrrosia lanceolata* (Chiang *et al.* 2013), *Peperomia magnoliaefolia* (Schmidt and

Kaiser 1987), *Tillandsia ionantha* (Nowak and Martin 1997), and *Peperomia carnevalii* (Herrera *et al.* 2000). In the latter studies, the hydrenchyma shrank under drought stress, and there is evidence that the water from the hydrenchyma moved into the chlorenchyma following a gradient in osmotic potential, thus maintaining photosynthetic function of the chlorenchyma and, hence, the leaf.

The water content of leaves of *S. littoreus* was more than 0.8, which is similar to or higher than values for other succulent plants (von Willert *et al.* 1990). Higher leaf water contents in the morning relative to the late afternoon might be the result of daytime stomata opening (and water loss) and nighttime stomata closure (Table 3 in Chapter 3). Water content in Tainan 2015 was much lower than that in 2016, which might be the result of different amounts of precipitation during the month of collection between the two years (23.0 mm in Jun. 2015 and 352.5 mm in Jul. 2016; Table 9 in Chapter 4). Although the leaf anatomy (*e.g.*, 64 % hydrenchyma) and water contents indicate that the leaves of *S. littoreus* are clearly succulent, values of saturated water content of the leaves were lower than many of other succulents (Ogburn and Edwards 2012).

Kluge and Ting (1978) measured mesophyll succulence ( $S_m$ ) for a number of non-CAM and CAM plants. Generally, the  $S_m$  of  $C_3$  plants are less than 1 while those of CAM plants ranged from 1 to 10 (Ting *et al.* 1983). It has also been reported, however, that the  $S_m$  of some CAM plants such as *Cissus gongylodes* and *Senecio petasitis* are much less than 1 (Virzo de Santo *et al.* 1983; Fioretto and Alfani 1988).  $S_m$  of *S. littoreus* in two populations were intermediate between  $C_3$  and CAM plants. It has been proposed that tissue succulence is highly correlated, or even causing CAM photosynthesis (Ripley *et al.* 2013). Chapter 3 also provides evidence of photosynthetic pathway intermediacy in *S. littoreus*.

von Willert *et al.* (1990) suggested that “utilizable water” can either be stored in mesophyll cells or in specialized hydrenchyma cells in the leaves; the latter being the case for *S. littoreus*. Hydrenchyma tissue in succulent leaves provides a source of water for the photosynthetic tissue, maintaining plant function, especially photosynthesis, under drought stress, as mentioned above. Leaves of *S. littoreus* had clearly defined hydrenchyma tissue, and the proportion of which did not vary among populations reflecting the consistency of this trait. In this regard, it is surprising that Rama Das and Raghavendra (1977) did not illustrate or mention the presence of hydrenchyma in their anatomical study of *S. littoreus* (= *S. squarrosus*) growing in India. Although succulence appears beneficial by providing leaves with an internal supply of water, it has also been considered maladaptive, as a result of increasing the distance of water and sugar transportation to the vascular tissues of the leaf, thus potentially reducing photosynthetic rates. This concern was refuted by Ogburn and Edwards (2013) who found a strong correlation between leaf succulence and 3-dimensional venation, which provides an efficient water transport system in leaves. Despite this correlation, 3-dimensional venation was not found in *S. littoreus*, which has vascular bundles only on the abaxial side of the leaf. This inconsistency might be a result of Kranz anatomy, the anatomy that has been reported (Rama Das and Raghavendra 1977), as constraint.

Because *S. littoreus* is a halophyte, leaf succulence may constitute an adaptation to soil salinity. Many halophytes have succulent leaves, and this type of succulence is considered to be an adaptation that dilutes the concentration of toxic  $\text{Na}^+$  and  $\text{Cl}^-$  ions (Levitt 1980, Repp 1958).

In conclusion, based on different measurements, the leaves of *S. littoreus* are clearly succulent, which is the first report of succulence in the grass family Poaceae. Leaf succulence in this coastal dune species may play a role in drought tolerance and/or protection from salt toxicity.

Furthermore, leaf succulence in *S. littoreus* may relate to the photosynthetic pathway(s) of this grass.



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Table 1. Mean ( $\pm$  SE, n=5) of the proportion of hydrenchyma for *S. littoreus* in six populations in Taiwan. BSW. Baishawan beach. FL. Fulong Beach. HS. Haishan Fishing Port. CS. Chingshui Beach. RZJ. Rouzongjiao beach. TN. Tainan Gold Coast. The populations are arranged in order from north (BSW) to south (TN) on the island. Proportions of hydrenchyma are not significantly different among populations ( $P > 0.05$ ).

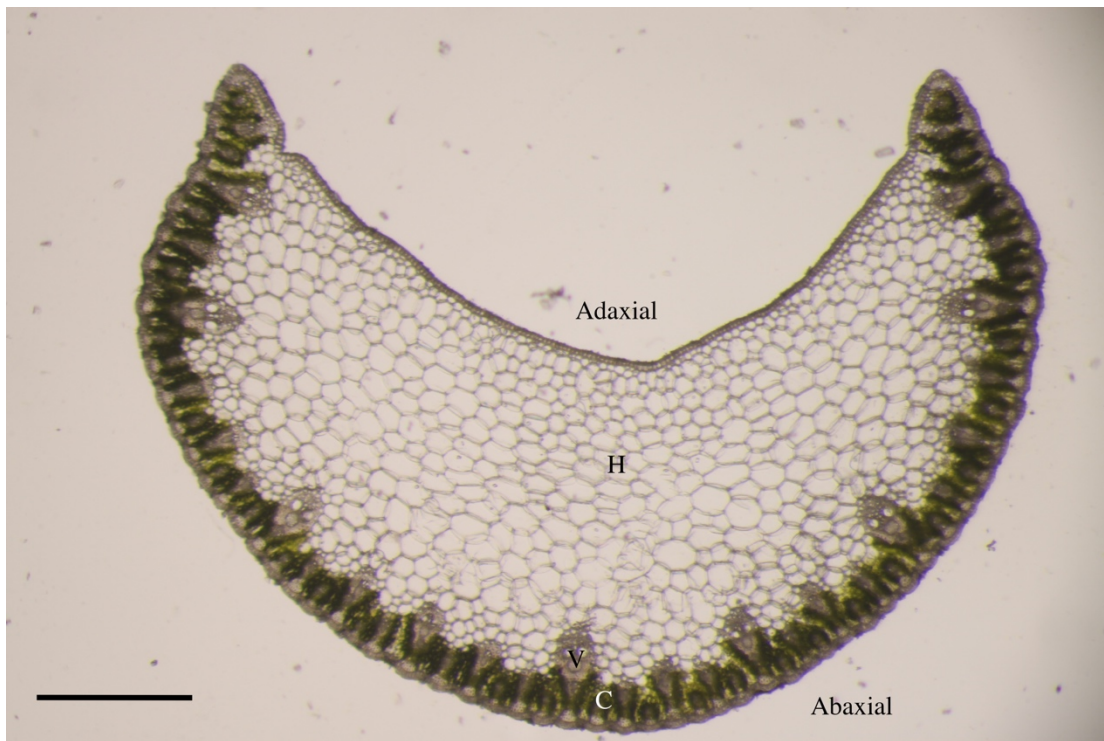
Population	Proportion of hydrenchyma (%)
BSW	65.5 $\pm$ 0.7
FL	66.0 $\pm$ 0.9
HS	63.5 $\pm$ 0.8
CS	62.3 $\pm$ 1.1
RZJ	64.8 $\pm$ 0.8
TN	63.0 $\pm$ 1.2
Mean	64.2 $\pm$ 0.4

Table 2. Mean  $\pm$  SE of water content of *S. littoreus* among populations in June or July 2015 and July 2016. See Table 1 for population abbreviations. Data did not pass normality test, so data were analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks (n=22-30 in 2015; n=8-10 in 2016). Means among populations are significantly different at  $P < 0.05$ , and letters denote the differences among populations.

Population	2015		2016	
	WC (AM)	WC (PM)	WC (AM)	WC (PM)
BSW	0.842 $\pm$ 0.003 <sup>ab</sup>	0.832 $\pm$ 0.005 <sup>b</sup>	0.822 $\pm$ 0.005 <sup>b</sup>	0.802 $\pm$ 0.006 <sup>bc</sup>
FL	0.833 $\pm$ 0.004 <sup>b</sup>	0.825 $\pm$ 0.004 <sup>b</sup>	0.807 $\pm$ 0.004 <sup>b</sup>	0.789 $\pm$ 0.005 <sup>c</sup>
HS	0.844 $\pm$ 0.004 <sup>ab</sup>	0.834 $\pm$ 0.003 <sup>ab</sup>	N.D.	N.D.
CS	0.836 $\pm$ 0.004 <sup>b</sup>	0.835 $\pm$ 0.003 <sup>ab</sup>	0.837 $\pm$ 0.002 <sup>ab</sup>	0.823 $\pm$ 0.003 <sup>ab</sup>
LK	0.861 $\pm$ 0.004 <sup>a</sup>	0.850 $\pm$ 0.004 <sup>a</sup>	0.852 $\pm$ 0.003 <sup>a</sup>	0.833 $\pm$ 0.004 <sup>a</sup>
TN	0.826 $\pm$ 0.004 <sup>b</sup>	0.805 $\pm$ 0.015 <sup>b</sup>	0.857 $\pm$ 0.007 <sup>a</sup>	0.839 $\pm$ 0.007 <sup>a</sup>
Mean	0.841 $\pm$ 0.0017	0.831 $\pm$ 0.00274	0.835 $\pm$ 0.00347	0.817 $\pm$ 0.004

N.D. Not determined

Figure 1. Leaf cross-section of *S. littoreus*. H. hydrenchyma V. vascular bundle C. chlorenchyma Bar = 500  $\mu\text{m}$ .



### **Chapter 3. Photosynthetic Pathway(s) of *Spinifex littoreus*, a Leaf Succulent Grass in Taiwan**

#### **Introduction**

Among the three major photosynthetic pathways, C<sub>4</sub> and CAM photosynthesis are considered to be specialized adaptations to high light, high temperature, and more arid environments (Taiz and Zeiger 2002). C<sub>4</sub> and CAM photosynthesis evolved multiple times in different lineages and use the same initial CO<sub>2</sub> fixation enzyme, PEP carboxylase. However, it is more common to find C<sub>3</sub>/CAM or C<sub>3</sub>/C<sub>4</sub> photosynthetic pathways in the same species. Although C<sub>4</sub> and CAM plants share similar enzymes and are distributed in similar environments with drought/high light stress (Edwards and Walker 1983; Taiz and Zeiger 2002), there is only one genus *Portulaca* that has been found to have C<sub>4</sub>/CAM photosynthetic pathways occurring together. Sage (2002) proposed several possibilities of C<sub>4</sub>/CAM incompatible at the cell level. Even though C<sub>4</sub> and CAM photosynthesis both use PEP carboxylase, the enzyme activated time is different. In addition, Kranz anatomy has been proposed to be required for C<sub>4</sub> photosynthesis, while tissue succulence is a common feature in CAM plants. However, the anatomical requirements for both pathways have been questioned. Single-cell C<sub>4</sub> and non-succulent CAM plants have been reported (Voznesenskaya *et al.* 2001, Martin *et al.* 2009), which were exceptions for these features. The low percentage of intercellular space due to highly packed cells and large vacuole in CAM plants is also not found in C<sub>4</sub> plants. Last but not least, the selective pressures for these two pathways might be different. It has been proposed that photorespiration caused by low ambient CO<sub>2</sub> may be the selective force of CO<sub>2</sub>-concentrating mechanism in C<sub>4</sub> plants, whereas nighttime CO<sub>2</sub> uptake evolved in CAM plants reduces water loss during the day, and also is beneficial for some aquatic



plants to take up CO<sub>2</sub> at night when the concentration is higher due to low temperature. There is only one genus, *Portulaca*, that has been found to perform both C<sub>4</sub> and CAM photosynthesis in some species (Guralnick *et al.* 2002; Kraybill and Martin 1996; Koch and Kennedy 1980; Sage 2002). A study of *Portulaca grandiflora* showed that C<sub>4</sub> photosynthesis occurred in Kranz cells periphery of the leaf, whereas CAM photosynthesis performed in water storage cells interior of the leaf (Guralnick *et al.* 2002).

Although C<sub>4</sub> and CAM evolved independently across lineages, and despite the fact that they grow in similar habitats and share similar biochemical pathways, it has never been reported to have CAM photosynthesis in Poaceae, one of the biggest families in angiosperms, mainly contributes to the leaf morphology: thin and not succulent. However, *Spinifex littoreus*, a grass that has been reported as C<sub>4</sub> photosynthesis (Rama Das and Raghavendra 1977), grows on coastal sand dunes in Southeast Asia, and the degrees of leaf succulence were measured and quantified by different indices in 2015 and 2016 (Chapter 2). Although tissue succulence does not always correlate with CAM photosynthesis (Martin *et al.* 2009), it has been proposed to be a prerequisite for evolving CAM photosynthesis (Ripley *et al.* 2013). It is possible that leaf-succulent *S. littoreus* has the ability to accumulate CAM acid. The aim of this study was to determine whether CAM photosynthesis occurs in *S. littoreus*.

## **Materials and Methods**

### **Sample collection**

Leaf tissues of *Spinifex littoreus* (Brum. f.) Merr. (syn. *S. squarrosus*; Poaceae, subfamily Panicoideae; tribe Paniceae) were collected from six populations in Taiwan along a latitudinal gradient: Baishawan beach (New Taipei City; 25.284°N, 121.519°E), Fulong Beach (New Taipei

City; 25.020°N, 121.943°E), Haishan Fishing Port (Hsinchu; 24.765°N, 120.906°E), Chingshui Beach (Yilan; 24.685°N, 121.836°E), Rouzongjiao beach (Lukang; 24.075°N, 120.25°E) and Tainan Gold Coast (Tainan; 22.938°N, 120.175°E). Because *S. littoreus* mainly grows by spreading rhizomes, individuals were determined at least 5 m apart or at different sand dunes. All plant materials (except *in situ* gas exchange experiment) were immediately placed in zipper plastic bags on ice after excision and transported to the laboratory in Taipei. Leaf samples were stored in the freezer (except samples for stomatal density were stored in 4 °C refrigerator) and analyzed no more than two weeks after collection.

For acidity titration in June and July 2015, samples were the same as for water content experiment in Chapter 2. In July 2016, two unshaded leaves from each of 8-10 individuals in five populations were used for gas-exchange experiments. After the gas-exchange measurements, three leaves at each time (7-8 am; 4-5 pm) were collected for acidity titration (samples were also the same as for water content experiment in Chapter 2). Because data from 2015 showed high variation within population, three leaves were used in 2016 in order to decrease the possible variation.

In January 2017, one unshaded leaf from each of ten individuals was collected for stomatal density (and osmotic potential in Chapter 4) measurement.

#### Leaf $\delta^{13}\text{C}$ Measurement

Leaf mid-section was excised from 5 individuals per population and were dried a week at 70°C, ground into a fine powder with a mortar and pestle, then transported to the Keck Paleoenvironmental and Environmental Stable Isotope Laboratory at 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  $\text{CO}_2$  using

a ThermoFinnigan (Bremen, Germany) MAT 253 IRMS Mass Spectrometer. The  $\delta^{13}\text{C}$  value of each sample is expressed relative to that of the calibrated standard Vienna Pee Dee Belemnite. The C isotope determination error was 0.10 ‰.

#### Acidity titration

Leaf samples were weighed, cut into 1-2 mm strips, ground with deionized water by mortar and pestle into a homogeneous slurry. Samples were titrated with 0.01N NaOH until pH = 7. After titration, ground tissue was dried at 65 °C for at least 7 days until the differences of dry mass was less than 0.001 g.

#### Gas exchange analysis

Net gas exchange of two middle of leaves of each individual *in situ* in the field was measured with a LI-COR (Lincoln, NE, USA) LI-6400 Portable Photosynthesis System and a 2 by 3 cuvette at 10 am to 2 pm (mid-day) and 10 pm to 2 am (mid-night) on sunny days in July 2016. Also, because leaves were succulent and concave, leaf cuvette was sealed by gas-tight putty (Chu Lun Stationery Co., LTD, Taiwan). Before measurements, cuvette was tested until the CO<sub>2</sub> did not leak. Cuvette block temperature was 40/30 °C day/night which was similar to ambient temperature, and CO<sub>2</sub> was 400 ppm. Vpd (vapor pressure deficit) was ca. 2.5-3.5 kPa at mid-day and 1.1-1.4 kPa at mid-night. Flow rate was 300  $\mu\text{mol s}^{-1}$ . PPFD during the daytime measurement was 2000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (similar to the ambient at noon). Each individual at each time was measured five times (except population in Chingshui Beach was measured three times) and average was used to represent the value of each individual. Each measurement was taken after pressed “match” to the reference and sample analyzers in LI-6400.

### Stomatal density

Nail polish was used on both sides of the mid-leaf and affixed to slides by transparent tape. The number of stomata average from six images per side of a sample was calculated using Olympus Optical Company Model BHC (Tokyo, Japan). Stomatal density on adaxial and abaxial side were added as total stomatal density.

### Statistical analysis

Mean values of PM and AM acidity was compared with a paired t-test in different populations and years. Mean values of CAM acid fluctuation, daytime and nighttime net gas exchange rate, stomatal conductance, intercellular CO<sub>2</sub> concentration, transpiration rates, water-use efficiency, and vpd among populations were compared by a One-Way Analysis of Variance. If data were not normally distributed, the Wilcoxon's Signed-Rank Test or Kruskal-Wallis Test were used. All statistical tests were performed using SigmaStat (SystStat, Chicago, IL, USA), and differences among means were considered significant when  $P \leq 0.05$ .

## **Results**

### Leaf $\delta^{13}\text{C}$ Measurement

Mean leaf  $\delta^{13}\text{C}$  value of *S. littoreus* was  $-14.247 \pm 0.158$  ‰, and values were not different among populations ( $P > 0.05$ ; Table 1).

### Diurnal acidity fluctuation

On a dry mass basis, acidity of AM leaves in 2015 and 2016 were significantly higher than PM leaves except Tainan in 2015 and Baishawan beach in 2016 (Table 2, Figure 1, Figure 2). In addition, of those populations that showed diurnal acid fluctuation, the amount of accumulated acid was not different among populations in both years ( $P > 0.05$  in 2016). Moreover, the acidity differences in 2016 were significantly higher than 2015 ( $P < 0.05$ ) except for Baishawan population ( $P > 0.05$ ; Table 2, Figure 3).

#### Gas exchange analysis

Gas exchange analysis of *S. littoreus* indicated daytime CO<sub>2</sub> uptake and daytime stomatal opening in five populations in July 2016 (Table 3, Figure 4). In addition, daytime net CO<sub>2</sub> exchange rates, nighttime respiration rates, daytime stomatal conductance, and daytime transpiration rates all differed significantly among populations with a trend of increasing from northern to southern Taiwan (Table 3). Nighttime stomatal conductance and transpiration were significantly higher in Chingshui population (Table 3).

#### Stomatal density

Stomata of *S. littoreus* were dumbbell-shape, mainly distributed on the abaxial side of a leaf (where chlorenchyma cells distributed), and were occasionally found at the edge of a leaf on the adaxial side. Stomatal density was not different among populations ( $P < 0.05$ ; Table 4).

### **Discussion**

Kranz anatomy and PEPC activity in *Spinifex littoreus* has been reported by Rama Das and Raghavendra (1977), and these results, combined with the leaf  $\delta^{13}\text{C}$  values (Table 1), showed C<sub>4</sub>

photosynthesis in *S. littoreus*. Photosynthetic rate of *S. littoreus* has also been reported for 63.2 mg CO<sub>2</sub> dm<sup>-2</sup> hr<sup>-1</sup> (39.9 μmol m<sup>-2</sup> s<sup>-1</sup>; Rama Das and Rachavendra 1977), which was similar to some of the populations in Taiwan (Table 3). This photosynthetic rate is in the average range of C<sub>4</sub> photosynthesis (30-60 μmol m<sup>-2</sup> s<sup>-1</sup>) that have been reported under optimal condition for each species (Larcher 2001), and is much higher than other coexisting species (Morrison and Reekie 1995, Santiago *et al.* 2000, Ramani *et al.* 2006). In addition, stomatal conductance of *S. littoreus* was also similar to other grasses (0.25-0.45 mol m<sup>-2</sup> s<sup>-1</sup>), although the stomatal density was higher than average range of other grasses (50-100 per mm<sup>2</sup>, Larcher 2001). Water use efficiency of *S. littoreus* is similar to other C<sub>4</sub> plants (ca. 4. Taiz and Zeiger 2002).

Diel acidity fluctuation in most of the populations of *S. littoreus* (Figure 1, Figure 2) with daytime net CO<sub>2</sub> uptake (Table 3) indicated that *S. littoreus* exhibits CAM-cycling photosynthesis at night. CAM-cycling plants recycle nocturnal respiratory CO<sub>2</sub>, is different from obligate CAM plants that open stomata and fix ambient CO<sub>2</sub> at night. This type of CAM photosynthesis is also found in *Portulaca*, a genus in dicot family Portulacaceae that has ever been reported for C<sub>4</sub>/CAM intermediacy. Other two genera, *Anacampseros* and *Grahamia*, in the same clade Anacampseroid has been investigated, but no C<sub>4</sub> photosynthesis ability was found (Guralnick 2008). Although it has been proposed that CAM-cycling in water storage tissue may play a role in water conservation and water supply to C<sub>4</sub> cells under drought stress (Guralnick *et al.* 2002), the adaptive significance of such small acidity accumulations is still unclear (Martin 1996; Winter *et al.* 2015).

In populations of Baishawan beach and Tainan Gold Coast, there was no consistency of acid fluctuation across years. Moreover, the nighttime acid accumulation in different populations was much higher in 2016 (Figure 3). These may be associated with environmental factors, as it has been reported in *Portulaca* that CAM-cycling photosynthesis occurs under environmental (usually

drought) stress (Guralnick *et al.* 2002; Kraybill and Martin 1996; Koch and Kennedy 1980; Sage 2002). In addition, daytime net CO<sub>2</sub> assimilation rates, transpiration rates, as well as stomatal conductance values increased from northern to southern Taiwan (except Tainan population) in 2016 (Table 3), might also be the result of environmental variation. The microhabitat comparisons of *S. littoreus* in different populations will be addressed in Chapter 4.

In conclusion, based on carbon stable isotope, acidity titration and gas-exchange experiments, *S. littoreus* exhibits C<sub>4</sub>/CAM-cycling photosynthetic pathway in different populations in Taiwan. This is the first report of C<sub>4</sub>/CAM-cycling apart from *Portulaca*, and also the first report of this photosynthetic intermediacy in the monocot family Poaceae. Inconsistency of nocturnal acid accumulation showed that biotic and/or abiotic microhabitat conditions might be associated with CAM-cycling ability.

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Table 1. Mean ( $\pm$  SE; n = 5) of leaf carbon stable isotope values of *S. littoreus* in six populations. See Table 1 for population abbreviations. Means are not significantly different among populations in Taiwan ( $P > 0.05$ ).

Population	Leaf $\delta^{13}\text{C}$ (‰)
BSW	-13.55 $\pm$ 0.13
FL	-14.27 $\pm$ 0.26
HS	-14.46 $\pm$ 0.36
RZJ	-14.19 $\pm$ 0.39
TN	-14.77 $\pm$ 0.42
Mean	-14.25 $\pm$ 0.16

Table 2. Mean ( $\pm$  SE;  $n = 25-40$  in 2015 and  $n = 8-10$  in 2016) values of leaf PM/AM acidity ( $\mu\text{mol H}^+ \text{g}^{-1} \text{DM}$ ), and diurnal acidity fluctuations (delta acidity, AM value minus PM value) in 2015 and 2016 on a dry mass basis. See Table 1 for population abbreviations. Significant level for means between AM and PM acidity:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ). Diurnal acidity fluctuations did not differ among populations that showed CAM acid increase in both years ( $P > 0.05$ ).

Population	Year 2015			Year 2016		
	PM	AM	AM-PM	PM	AM	AM-PM
BSW	60.19 $\pm$ 4.08	68.05 $\pm$ 4.47	7.86 $\pm$ 4.32 *	71.01 $\pm$ 7.88	93.87 $\pm$ 5.99	22.86 $\pm$ 11.32 (ns)
FL	79.34 $\pm$ 5.24	92.06 $\pm$ 5.45	12.72 $\pm$ 3.39 ***	64.89 $\pm$ 12.98	101.18 $\pm$ 12.87	36.29 $\pm$ 5.04 ***
HS	81.26 $\pm$ 4.92	96.07 $\pm$ 4.76	14.81 $\pm$ 3.80 ***	N.D.	N.D.	N.D.
CS	52.37 $\pm$ 3.61	65.87 $\pm$ 4.08	13.50 $\pm$ 4.41 ***	61.68 $\pm$ 7.53	93.45 $\pm$ 5.80	31.77 $\pm$ 3.39 ***
RZJ	72.20 $\pm$ 5.01	88.65 $\pm$ 5.25	16.45 $\pm$ 5.11 **	38.79 $\pm$ 2.67	75.80 $\pm$ 3.28	37.01 $\pm$ 3.59 ***
TN	53.54 $\pm$ 4.19	53.22 $\pm$ 3.26	-0.32 $\pm$ 3.19 (ns)	95.44 $\pm$ 12.28	125.76 $\pm$ 14.88	30.31 $\pm$ 6.31 ***

N.D. Not determined

Table 3. Mean ( $\pm$  SE;  $n = 8-10$ ) of mid-day and mid-night gas exchange rate (A. photosynthetic rate; R. respiration rate), stomatal conductance (gs), intercellular CO<sub>2</sub> concentration (Ci), transpiration rate (E), water-use efficiency (WUE) and vapor pressure deficit (vpd) of *Spinifex littoreus* in five populations in 2016. See Table 1 for population abbreviations. Means of different parameters among populations are significantly different at  $P < 0.05$ , and letters denote the differences among populations. Plants in these populations (except BSW) were also exhibited diel acid fluctuations (Fig. 2).

Population	mid-day A	mid-night R	mid-day g <sub>s</sub>	mid-night g <sub>s</sub>	mid-day C <sub>i</sub>	mid-day E	mid-night E	WUE	vpd
	$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$	$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$	$\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$	$\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$	$\mu\text{mol CO}_2 \text{ mol}^{-1}$	$\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$	$\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$	$A/E \times 1000$	kPa
BSW	$16.23 \pm 2.00^c$	$-0.83 \pm 0.16^a$	$0.100 \pm 0.011^b$	$0.012 \pm 0.003^b$	$109.70 \pm 10.30^{ab}$	$3.36 \pm 0.35^c$	$0.16 \pm 0.04^b$	$4.709 \pm 0.196^b$	$3.37 \pm 0.04^a$
FL	$35.83 \pm 2.95^b$	$-2.29 \pm 0.27^{bc}$	$0.283 \pm 0.023^{ab}$	$0.011 \pm 0.002^b$	$143.54 \pm 5.81^a$	$6.70 \pm 0.56^b$	$0.15 \pm 0.03^b$	$5.372 \pm 0.144^a$	$2.52 \pm 0.079^c$
CS	$48.95 \pm 2.95^{ab}$	$-1.69 \pm 0.32^{ab}$	$0.379 \pm 0.036^a$	$0.044 \pm 0.011^a$	$121.60 \pm 10.00^{ab}$	$9.58 \pm 0.76^{ab}$	$0.55 \pm 0.13^a$	$5.174 \pm 0.163^{ab}$	$2.81 \pm 0.048^{bc}$
RZJ	$56.17 \pm 3.28^a$	$-2.19 \pm 0.20^{bc}$	$0.398 \pm 0.036^a$	$0.005 \pm 0.002^b$	$89.72 \pm 6.22^b$	$10.09 \pm 0.61^a$	$0.06 \pm 0.02^b$	$5.583 \pm 0.131^a$	$2.9 \pm 0.073^b$
TN	$41.55 \pm 4.09^b$	$-2.62 \pm 0.13^c$	$0.371 \pm 0.052^a$	$0.014 \pm 0.004^b$	$139.60 \pm 12.10^a$	$9.43 \pm 1.00^{ab}$	$0.19 \pm 0.05^b$	$4.49 \pm 0.181^b$	$2.96 \pm 0.093^b$

Table 4. Mean ( $\pm$  SE; n = 10) of total stomatal density (no. mm<sup>-2</sup>) of *S. littoreus* in six populations in Taiwan. See Table 1 for population abbreviations. Total stomatal density values were not different among populations ( $P > 0.05$ ).

Population	Stomatal density
BSW	186.2 $\pm$ 16.3
FL	166.1 $\pm$ 10.8
HS	171.2 $\pm$ 7.0
CS	173.4 $\pm$ 3.9
RZJ	169.4 $\pm$ 10.4
TN	152.8 $\pm$ 7.7

Figure 1. Mean ( $\pm$  SE;  $n = 25-40$ ) of morning (AM) and late afternoon (PM) leaf acidities of *S. littoreus* in six populations in 2015. Data are expressed on a dry mass basis. See Table 1 for population abbreviations. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).

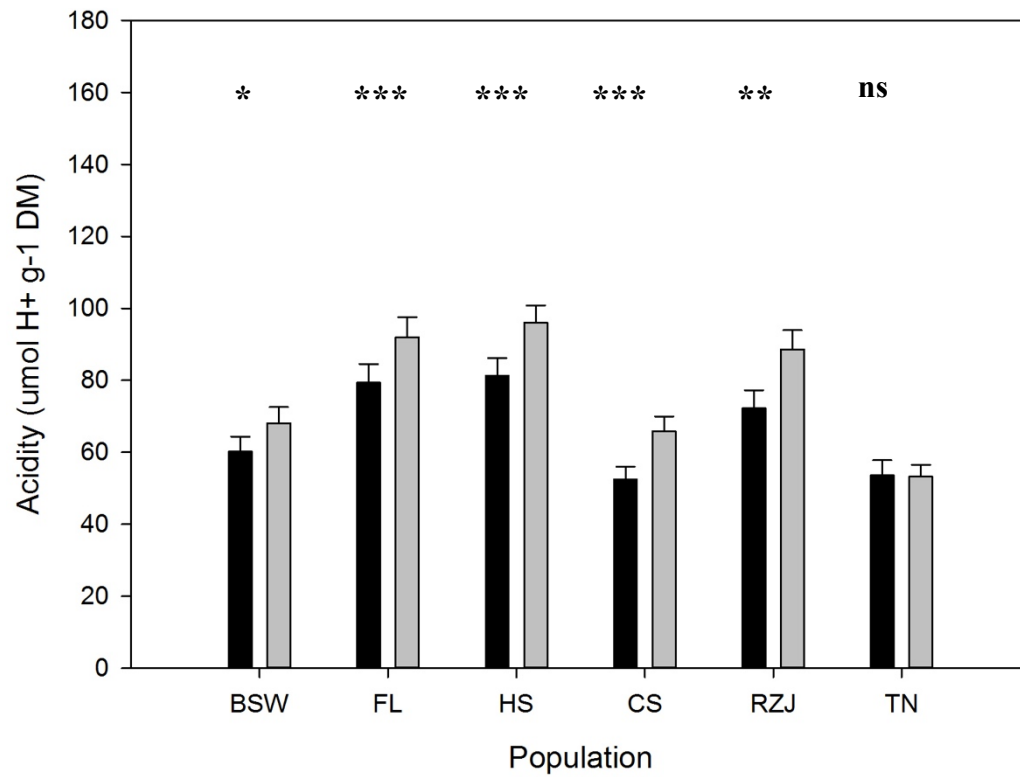


Figure 2. Mean ( $\pm$  SE;  $n = 8-10$ ) of morning (AM, grey bars) and late afternoon (PM, black bars) leaf acidities of *S. littoreus* in six populations in 2016. Data are expressed on a dry mass basis. See Table 1 for population abbreviations. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).

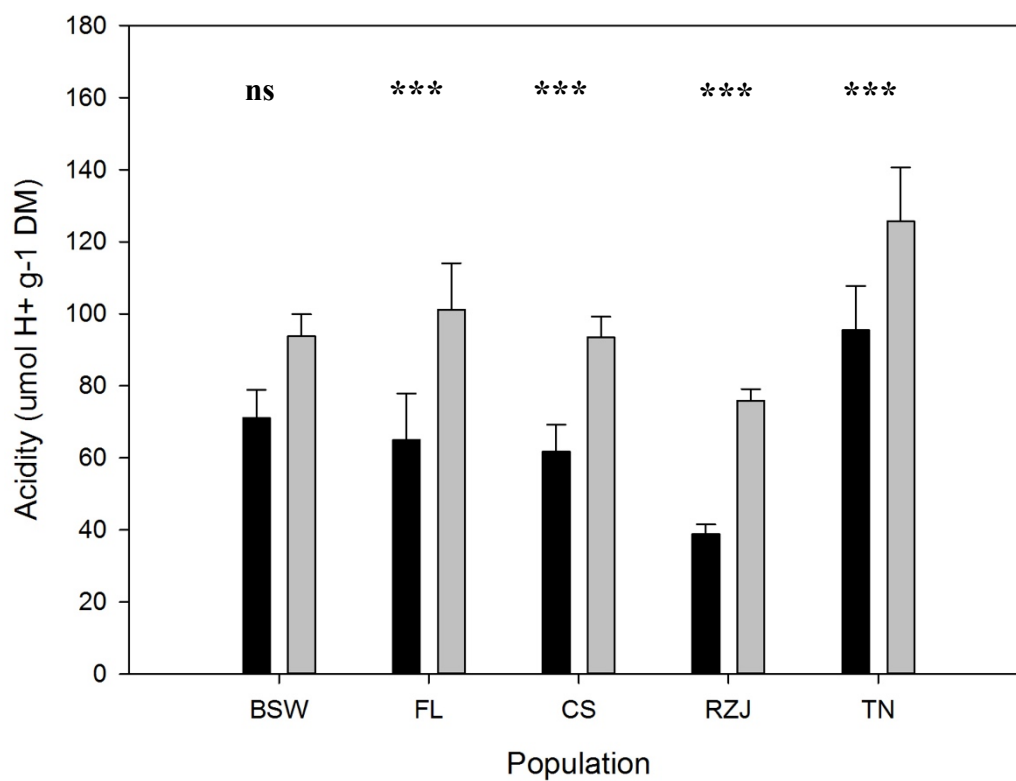
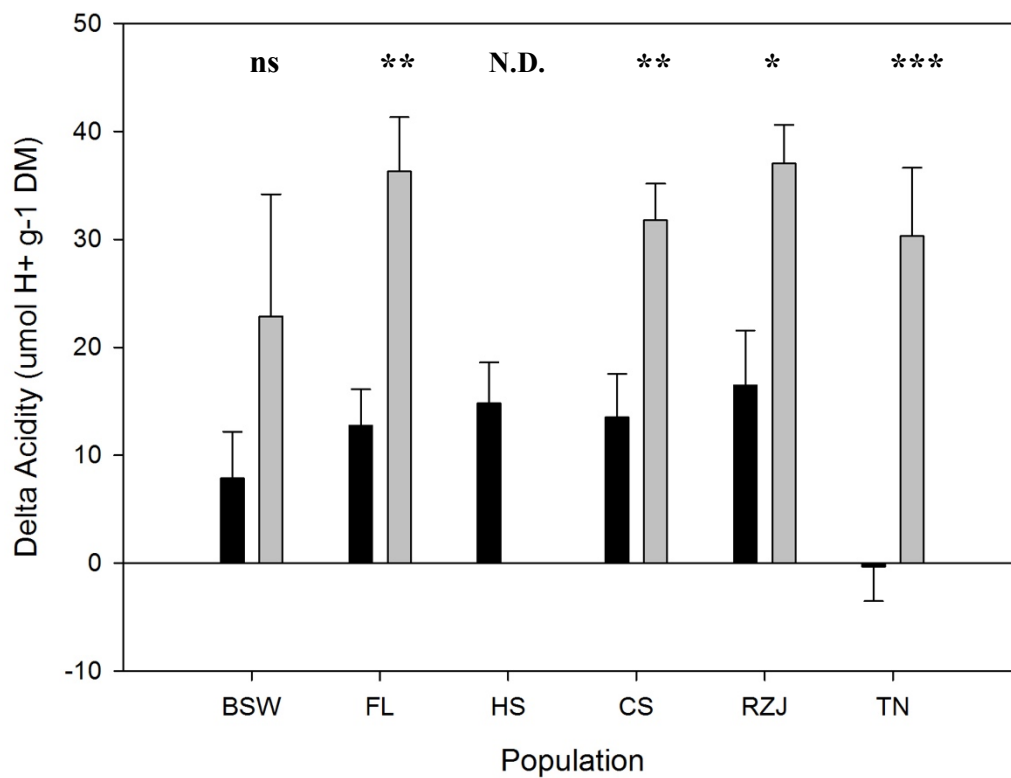


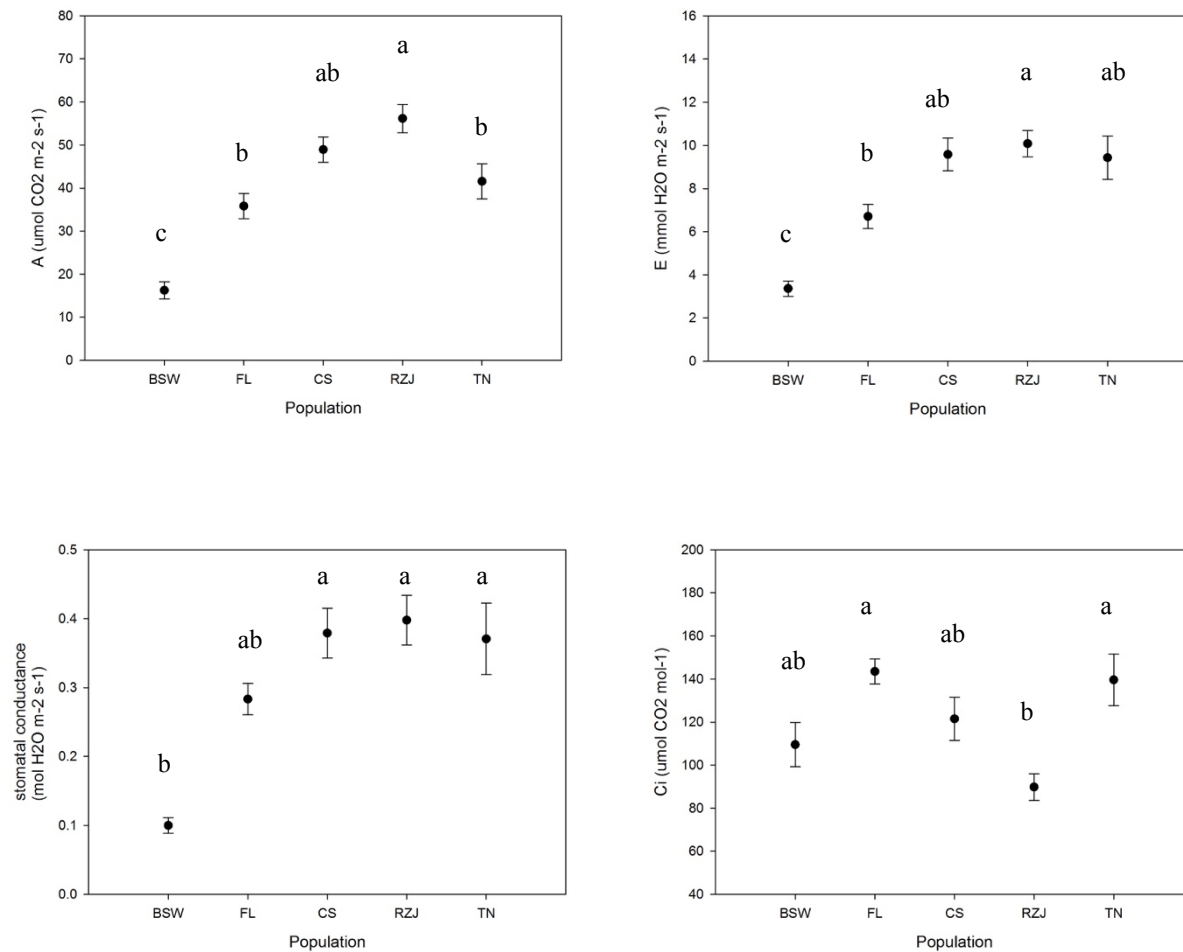


Figure 3. Mean ( $\pm$  SE;  $n = 25-40$  in 2015 and  $n = 8-10$  in 2016) of diurnal acidity fluctuations (delta acidity, AM value minus PM value) in 2015 and 2016 on a dry mass basis. See Table 1 for population abbreviations. Black bars denote values in 2015 whereas grey bars denote values in 2016. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).



ND. Not determined

Figure 4. Mean ( $\pm$  SE;  $n = 8 - 10$ ) of mid-day photosynthetic rates (A), mid-day transpiration rates (E), mid-day stomatal conductance ( $g_s$ ), and mid-day intercellular  $CO_2$  concentration ( $C_i$ ) of *S. littoreus* in six populations in 2016. See Table 1 for population abbreviations. Means among populations are significantly different at  $P < 0.001$ , and letters denote the differences among populations.



## Chapter 4. Ecophysiological Variability in *Spinifex littoreus*, a C4/CAM-Cycling Grass, among Six Populations in Coastal Regions of Taiwan

### Introduction

Coastal sand dunes are considered as one of the most stressful environments for plants, which often face excess light, drought stress, toxic ion concentrations, strong winds, and low nutrient availability (Hesp 1991; Larcher 2001; Maun 2009; Bermúdez and Retuerto 2014). In coastal sand dunes, basic elements are not leached out by rainfall, thus soil is more alkaline and contains fewer nutrients (Larcher 2001).

*Spinifex littoreus* is a perennial halophytic grass that is distributed in the coastal regions throughout Southeast Asia. Based on carbon stable isotope values (Table 1 in Chapter 3), diurnal acidity fluctuation (Table 2, Figure 1, Figure 2 in Chapter 3) and daytime CO<sub>2</sub> uptake (Table 3, Figure 4 in Chapter 3), *S. littoreus* is a C4/CAM-cycling grass. However, the degree of acidity accumulation and photosynthetic parameters differed among years and populations. Diel CAM fluctuation was not shown in Tainan Gold Cost (year 2015) and Baishawan beach (year 2016), two populations of *S. littoreus* in Taiwan, but was found in different years (Figure 1, Figure 2 in Chapter 3). It has been reported that the ability of CAM-cycling photosynthesis is associated with different environmental conditions, especially drought stress, in some *Portulaca* spp. such as *Portulaca grandiflora*, *P. oleracea*, *P. mundla*, (Guralnick *et al.* 2002; Kraybill and Martin 1996, Koch and Kennedy 1980; Sage 2002). *S. littoreus* is widely distributed along coastal regions in Taiwan, the microhabitat including abiotic and biotic factors may also be associated with the nocturnal acid fluctuations. In addition, photosynthetic rates of *S. littoreus* were higher in southern

populations than northern populations. This difference might also be the phenotypic plasticity under different microhabitats.

The aim of this study was to determine whether the CAM-cycling ability in *S. littoreus* is associated with certain ecological factors, and to determine the possible reason causing population differences in their physiological performance.

## **Materials and Methods**

### Sample collection

In 2016, four to five soil samples around different individual's roots of *S. littoreus* (Brum. f.) Merr. (syn. *S. squarrosus*; Poaceae, subfamily Panicoideae; tribe Paniceae) at each location were collected for soil pH measurement. In January 2017, one unshaded leaf from each of ten individuals at each location was collected for osmotic potential measurement (same as the individuals for stomatal density measurement in Chapter 3). Soil samples and leaf samples were immediately placed in zipper plastic bags on ice after collection and transported to the laboratory in Taipei. Soil samples were dried at room temperature whereas leaf samples were stored in the freezer and analyzed no more than two weeks after collection.

### Microhabitat information

CAM acid fluctuation (AM-PM) data were compared with different environmental factors in 2015. Sun/shade was determined whether *S. littoreus* was shaded by any object above the plant. Distance to the sea was roughly estimated by foot-steps. Vegetative/flowering stage was determined whether any of the ramets in the colony was flowering, and gender, if the plant was flowering, was determined by different morphology of male/female inflorescences because *S.*

*littoreus* is a dioecious plant. Presence/absence of interspecific competition was determined whether the co-exist plants were within 1 m of *S. littoreus*. The location of the plants on a sand dune (at top/side/bottom, or growing on flat regions) and the location of plants in a colony (edge/center) were also recorded. In addition, mean temperature, accumulated precipitation (when the experiments were done) at each location was downloaded from Data Bank for Atmospheric and Hydrologic Research.

#### Soil pH

Soil was immediately placed in zipper plastic bags, transported back to the lab, and dried under room temperature for 48 hours. Ten grams of soil and 50 mL of distilled water were placed in a 50 mL plastic tube and shaken for one hour. After soil particle sediment, pH values were measured by a pH meter.

#### Osmotic potential

Liquid from each leaf was removed by pestle of microtube in a 2 mL plastic tube. Ten microliters of liquid was absorbed into a paper disc and analyzed by WESCOR vapor pressure osmometer VAPRO 5520 (ELITech, Logan, UT, USA). Osmolality standards WESCOR OA-010, OA-029 and OA-100 (ELITech, Logan, UT, USA) were used for instrument calibrations, and samples remained in the measurement chamber until water vapor equilibrium (1 min).

#### Statistical analysis

Mean values of diel acidity fluctuations at each of the environmental factors (except the distance to the sea) in each population, soil pH, osmotic potential were compared by a One-Way

Analysis of Variance, or Kruskal-Wallis Test if the data were not normally distributed. The relationship between CAM acidity fluctuation and the distance to the sea was tested by linear regression. All statistical tests were performed using SigmaStat (SystStat, Chicago, IL, USA), and differences among means were considered significant when  $P \leq 0.05$ .

## Results

In 2015, diel acid fluctuation was shown in plants that were located in exposed area and/or at the edge of a colony in five out of six populations (except Tainan Gold Coast). The acid fluctuation was also found in vegetative stage and/or flowering stage in four out of six populations. Plants located at the top of the hill, the center of a colony, and presence/absence of interspecific competition in three out of six populations was also associated with nocturnal acid accumulation (Table 1-6).

Soil pH values were ca. 8.0 - 8.5, and that of the population in Tainan Golden Coast was significantly higher than Fulong Beach (Table 7). Osmotic potential of leaves was lower in Rouzongjiao population than Fulong and Chingshui populations (Table 8).

## Discussion

Although monthly mean temperature (when the experiments were done) from Data Bank for Atmospheric and Hydrologic Research was ca. 28-30 °C for each population, the temperature at the time of *in situ* measurement of gas exchange around the plants was ca. 40-45 °C and was similar among populations (personal observation). Soil pH was significantly different among populations (Table 7), but the effect on plants is not known and requires further investigation. In all populations, the distance to the sea did not correlate with the amount of CAM acid accumulation.

Distance to the sea was assumed to represent a salt gradient of the soil, thus affecting the water potential and ion toxicity. This result indicates either soil water status was similar in all the sample sites at each population, or CAM acid accumulation was not affected by soil water potential.

In addition to tissue succulence as an adaptive trait in the coastal ecosystem, accumulation of solutes is another common phenomenon in coastal halophytes. It has been proposed that plants growing in coastal ecosystem accumulate solutes, such as NaCl, proline, or other amino acids, thus reducing the water potential in the plant tissue, which allows the absorption of water from saline soil (McNaughton 1991; Larcher 2001). Even though the osmotic potential of *S. littoreus* was not as low as sea water (-25 bars; Larcher 2001), it was much more negative than the co-occurring halophyte *Ipomoea pes-caprae* (ca. -10 bars, Sucre and Suárez 2011), although this might be the result of different soil water potentials.

In 2015, different factors contributed to the significant acidity fluctuations of *S. littoreus* at each population. In the populations except Tainan Gold Coast in 2015, CAM acid fluctuation was found when the plants were growing in exposed area, or at peripheral of the colony. Although the acid fluctuation was also observed under different conditions in some populations, these two might be more important factors. Exposed areas on sand dunes often contain with excess light, drought stress and higher temperature conditions, which have been associated with CAM-cycling photosynthesis. In addition, growing in peripheral of a colony may indicate the edge of resources allowing plants to grow, which may also affect the acid accumulation.

In 2016, photosynthetic rates and other parameters were significantly different among populations (Table 3, Figure 4 in Chapter 3). As a result of the low relative humidity at noon in the field, water vapor in the air was not controlled by LI-COR 6400. More specifically, vapor pressure deficit (vpd) was different among populations, and might cause the difference in stomatal

conductance and thus affected photosynthetic rates. In addition, highest leaf water content (Table 2 in Chapter 2) with lowest osmotic potential (Table 8) at Rouzongjiao beach may be the cause of highest photosynthetic rate among populations. By accumulating solutes in the leaf, *S. littoreus* display salt succulence that allows plants absorbing water from the saline soil. Plants at Rouzongjiao beach might face less drought stress, and thus exhibited higher stomatal conductance and higher photosynthetic rates.

In conclusion, based on osmotic potential and microhabitat information, *S. littoreus* exhibits solute accumulation, and CAM-cycling photosynthesis occurs mainly in the exposed area and at the edge of the colony in six different population in Taiwan. The difference in photosynthetic rate might be related to other environmental conditions that were not measured in this study (e.g., soil water potential or wind), or genotypic variability (the ability of accumulating solutes) among populations throughout Taiwan.



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Table 1. Mean ( $\pm$  SE, total n = 29) of morning (AM), late afternoon (PM) leaf acidities and diurnal acidity differences (AM-PM) under different ecological factors at Baishawan beach in June 2015. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).

	n	PM	AM	AM-PM	P
Sun	24	58.53 $\pm$ 4.87	69.30 $\pm$ 5.228	10.77 $\pm$ 4.89	**
Shade	5	68.17 $\pm$ 1.41	62.06 $\pm$ 6.788	-6.11 $\pm$ 6.37	ns
Uphill	8	62.20 $\pm$ 6.18	74.69 $\pm$ 12.250	12.49 $\pm$ 8.81	ns
Slope	21	59.43 $\pm$ 5.20	65.52 $\pm$ 4.179	6.10 $\pm$ 5.02	*
Vegetative	22	56.91 $\pm$ 3.40	71.09 $\pm$ 4.768	14.18 $\pm$ 3.34	***
Flowering	7	70.51 $\pm$ 13.10	58.51 $\pm$ 10.783	-12.01 $\pm$ 12.34	ns
Center	1	41.73	81.490	39.77	-
Edge	28	60.85 $\pm$ 4.17	67.57 $\pm$ 4.606	6.72 $\pm$ 4.32	*
Male	28	59.32 $\pm$ 4.13	66.29 $\pm$ 4.256	6.97 $\pm$ 4.39	*
Female	1	84.63	117.49	32.87	-
Competition	20	61.48 $\pm$ 5.36	65.39 $\pm$ 4.813	3.92 $\pm$ 5.73	ns
No competition	9	57.34 $\pm$ 5.89	73.96 $\pm$ 9.837	16.62 $\pm$ 4.84	**
Distance to the sea	29				ns

Table 2. Mean ( $\pm$  SE, total  $n = 39$ ) of morning (AM), late afternoon (PM) leaf acidities and diurnal acidity differences (AM-PM) under different ecological factors at Fulong Beach in July 2015. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).

	n	PM	AM	AM-PM	<i>P</i>
Sun	29	69.50 $\pm$ 4.43	81.53 $\pm$ 5.05	12.03 $\pm$ 4.09	**
Shade	10	107.88 $\pm$ 12.40	122.60 $\pm$ 10.95	14.72 $\pm$ 6.18	*
Uphill	39	79.34 $\pm$ 5.24	92.06 $\pm$ 5.45	12.72 $\pm$ 3.39	***
Vegetative	30	80.89 $\pm$ 5.29	91.90 $\pm$ 5.54	11.00 $\pm$ 3.85	**
Flowering	9	74.17 $\pm$ 14.94	92.59 $\pm$ 15.55	18.43 $\pm$ 7.24	*
Center	12	87.04 $\pm$ 9.77	93.64 $\pm$ 10.10	6.60 $\pm$ 7.82	ns
Edge	27	75.92 $\pm$ 6.21	91.35 $\pm$ 6.60	15.43 $\pm$ 3.44	***
Male	5	60.96 $\pm$ 13.67	85.78 $\pm$ 11.99	24.82 $\pm$ 4.48	ns
Female	3	98.00 $\pm$ 40.28	97.31 $\pm$ 48.03	0.70 $\pm$ 15.39	ns
Competition	30	82.87 $\pm$ 6.48	96.85 $\pm$ 6.38	13.98 $\pm$ 3.34	***
No competition	9	67.57 $\pm$ 5.98	76.07 $\pm$ 8.84	8.50 $\pm$ 9.94	ns
Distance to the sea	39				ns

Table 3. Mean ( $\pm$  SE, total  $n = 40$ ) of morning (AM), late afternoon (PM) leaf acidities and diurnal acidity differences (AM-PM) under different ecological factors at Haishan Fishing Port in June 2015. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).

	n	PM	AM	AM-PM	<i>P</i>
Sun	30	79.27 $\pm$ 6.09	93.70 $\pm$ 5.35	14.43 $\pm$ 3.03	***
Shade	10	87.23 $\pm$ 7.49	103.17 $\pm$ 10.40	15.94 $\pm$ 12.70	ns
Uphill	20	79.98 $\pm$ 6.87	96.15 $\pm$ 7.15	16.17 $\pm$ 6.56	*
Slope	8	103.28 $\pm$ 11.41	115.45 $\pm$ 8.57	12.17 $\pm$ 3.82	*
Downhill	12	68.72 $\pm$ 7.15	83.02 $\pm$ 7.16	14.29 $\pm$ 6.31	ns
Vegetative	20	75.24 $\pm$ 6.50	95.07 $\pm$ 7.31	19.83 $\pm$ 6.27	***
Flowering	20	87.29 $\pm$ 7.30	97.07 $\pm$ 6.28	9.78 $\pm$ 4.15	*
Center	24	77.34 $\pm$ 6.29	91.32 $\pm$ 5.52	13.98 $\pm$ 3.60	***
Edge	16	87.14 $\pm$ 7.90	103.19 $\pm$ 8.45	16.05 $\pm$ 7.99	*
Male	10	74.50 $\pm$ 9.45	86.51 $\pm$ 8.05	12.01 $\pm$ 6.03	ns
Female	10	100.07 $\pm$ 9.98	107.63 $\pm$ 8.76	7.56 $\pm$ 5.94	ns
Competition	30	76.69 $\pm$ 5.14	93.07 $\pm$ 5.36	16.38 $\pm$ 4.65	***
No competition	10	94.99 $\pm$ 11.67	105.06 $\pm$ 10.13	10.07 $\pm$ 6.16	ns
Distance to the sea	40				ns

Table 4. Mean ( $\pm$  SE, total  $n = 40$ ) of morning (AM), late afternoon (PM) leaf acidities and diurnal acidity differences (AM-PM) under different ecological factors at Chingshui beach in June 2015. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).

	n	PM	AM	AM-PM	<i>P</i>
Sun	30	53.69 $\pm$ 4.36	63.51 $\pm$ 4.53	9.82 $\pm$ 4.39	***
Shade	10	48.41 $\pm$ 6.32	72.96 $\pm$ 9.10	24.55 $\pm$ 8.90	*
Uphill	19	46.83 $\pm$ 3.44	65.40 $\pm$ 5.67	18.57 $\pm$ 5.18	***
Slope	5	70.16 $\pm$ 18.98	55.65 $\pm$ 8.74	-14.51 $\pm$ 21.87	ns
Downhill	16	53.40 $\pm$ 5.37	69.63 $\pm$ 7.26	16.23 $\pm$ 3.08	***
Vegetative	18	58.43 $\pm$ 6.85	72.18 $\pm$ 8.01	13.75 $\pm$ 8.09	**
Flowering	22	47.42 $\pm$ 3.22	60.71 $\pm$ 3.29	13.29 $\pm$ 3.46	***
Center	8	46.03 $\pm$ 3.83	66.03 $\pm$ 6.74	20.00 $\pm$ 4.80	**
Edge	27	54.65 $\pm$ 5.11	66.97 $\pm$ 5.58	12.32 $\pm$ 5.73	**
Only one	5	50.23 $\pm$ 6.05	59.72 $\pm$ 8.07	9.49 $\pm$ 6.19	ns
Male	8	42.44 $\pm$ 3.07	51.48 $\pm$ 3.49	9.04 $\pm$ 4.94	ns
Female	14	50.26 $\pm$ 4.65	65.99 $\pm$ 4.21	15.73 $\pm$ 4.65	**
Competition	30	49.51 $\pm$ 4.08	62.76 $\pm$ 3.98	13.25 $\pm$ 5.18	***
No competition	10	60.97 $\pm$ 7.37	75.22 $\pm$ 11.06	14.25 $\pm$ 4.92	*
Distance to the sea	40				ns

Table 5. Mean ( $\pm$  SE, total  $n = 25$ ) of morning (AM), late afternoon (PM) leaf acidities and diurnal acidity differences (AM-PM) under different ecological factors at Rouzongjiao Beach in July 2015. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).

	n	PM	AM	AM-PM	<i>P</i>
Sun	25	72.20 $\pm$ 5.01	88.65 $\pm$ 5.25	16.45 $\pm$ 5.11	**
Uphill	6	78.73 $\pm$ 10.24	98.99 $\pm$ 8.62	20.26 $\pm$ 11.59	ns
Slope	5	60.39 $\pm$ 17.81	86.94 $\pm$ 18.57	26.56 $\pm$ 12.67	ns
Flat	14	73.62 $\pm$ 5.02	84.82 $\pm$ 5.99	11.20 $\pm$ 6.39	ns
Vegetative	5	84.77 $\pm$ 11.29	111.06 $\pm$ 10.68	26.29 $\pm$ 15.61	ns
Flowering	20	69.06 $\pm$ 5.51	83.04 $\pm$ 5.40	13.98 $\pm$ 5.17	*
Center	12	66.64 $\pm$ 5.79	84.46 $\pm$ 8.08	17.82 $\pm$ 7.84	*
Edge	13	77.33 $\pm$ 7.97	92.51 $\pm$ 6.94	15.17 $\pm$ 6.92	*
Male	6	72.32 $\pm$ 5.86	74.88 $\pm$ 6.56	2.56 $\pm$ 5.57	ns
Female	14	67.66 $\pm$ 7.56	86.54 $\pm$ 7.12	18.88 $\pm$ 6.68	*
Competition	9	80.66 $\pm$ 7.87	87.17 $\pm$ 10.84	6.51 $\pm$ 8.34	ns
No competition	16	67.44 $\pm$ 6.32	89.48 $\pm$ 5.79	22.03 $\pm$ 6.21	**
Distance to the sea	25				ns

Table 6. Mean ( $\pm$  SE, total n = 26) of morning (AM), late afternoon (PM) leaf acidities and diurnal acidity differences (AM-PM) under different ecological factors at Tainan Gold Coast in June 2015. Significant level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or non-significant (ns;  $P > 0.05$ ).

	n	PM	AM	AM-PM	P
Sun	22	50.46 $\pm$ 4.57	52.63 $\pm$ 3.65	2.17 $\pm$ 3.03	ns
Shade	4	70.51 $\pm$ 5.80	56.50 $\pm$ 7.59	-14.01 $\pm$ 11.23	ns
Uphill	2	51.26 $\pm$ 1.09	62.58 $\pm$ 4.99	11.31 $\pm$ 6.09	ns
Slope	8	40.15 $\pm$ 6.84	40.18 $\pm$ 3.09	0.03 $\pm$ 5.46	ns
Downhill	2	35.95 $\pm$ 5.09	49.47 $\pm$ 0.20	13.51 $\pm$ 5.29	ns
Flat	14	64.04 $\pm$ 5.31	59.88 $\pm$ 4.76	4.16 $\pm$ 4.67	ns
Vegetative	17	52.34 $\pm$ 5.83	53.37 $\pm$ 4.63	1.04 $\pm$ 3.31	ns
Flowering	9	55.83 $\pm$ 5.42	52.94 $\pm$ 3.87	-2.89 $\pm$ 7.03	ns
Center	17	53.68 $\pm$ 4.52	53.10 $\pm$ 4.38	-0.57 $\pm$ 3.77	ns
Edge	7	53.16 $\pm$ 10.69	54.15 $\pm$ 6.31	0.99 $\pm$ 6.56	ns
Only one	2	53.80 $\pm$ 22.93	51.01 $\pm$ 1.34	-2.78 $\pm$ 21.59	ns
Male	6	60.55 $\pm$ 6.62	56.97 $\pm$ 4.94	-3.58 $\pm$ 10.56	ns
Female	1	51.68	43.82	-7.86	-
Competition	18	59.40 $\pm$ 5.37	56.46 $\pm$ 4.08	-2.94 $\pm$ 4.11	ns
No competition	8	40.37 $\pm$ 3.29	45.94 $\pm$ 4.61	5.57 $\pm$ 4.34	ns
Distance to the sea	26				ns



Table 7. Mean ( $\pm$  SE,  $n = 4 - 5$ ) of soil pH values of *S. littoreus* in five locations in 2016. See Table 1 for population abbreviations. Means among populations are significantly different at  $P < 0.05$ , and letters denote the differences among populations.

Population	Soil pH
BSW	$8.32 \pm 0.20^{ab}$
FL	$8.07 \pm 0.04^b$
CS	$8.28 \pm 0.10^{ab}$
RZJ	$8.24 \pm 0.10^{ab}$
TN	$8.68 \pm 0.02^a$

Table 8. Mean ( $\pm$  SE,  $n = 10$ ) of osmotic potential values ( $\Psi_{\pi}$ ) of *S. littoreus* in six populations in Taiwan. See Table 1 for population abbreviations. Means among populations are significantly different at  $P < 0.01$ , and letters denote the differences among populations.

Population	$\Psi_{\pi}$ (bars)
BSW	$-19.08 \pm 0.66^{ab}$
FL	$-18.16 \pm 0.74^a$
HS	$-18.67 \pm 0.57^a$
CS	$-19.44 \pm 0.54^{ab}$
RZJ	$-23.59 \pm 1.68^b$
TN	$-20.39 \pm 1.03^{ab}$

Table 9. Mean temperature and accumulated precipitation in six locations in 2015 and 2016. Climate data were collected at the month when samples and *in situ* measurements were taken (BSW, HC, YL, TN in June and FL, LK in July 2015, July 2016 for all populations). See Table 1 for population abbreviations.

Population	2015		2016	
	T °C	Precipitation (mm)	T °C	Precipitation (mm)
BSW	28.70	30.5	29.20	99.0
FL	28.08	189.0	28.76	96.5
HS	28.88	86.5	29.42	7.0
CS	29.22	39.5	29.68	194.5
RZJ	29.21	79.5	29.62	90.0
TN	29.99	23.0	30.24	352.5

## Chapter 5. Conclusions

This study comprises the first report of leaf succulence in the halophytic grass, *Spinifex littoreus*, growing on coastal sand dunes of Taiwan. More than 60 % of the cross-sectional area of the leaf was water-storage parenchyma (hydrenchyma), as well as high water content, high relative water content, and mesophyll succulence confirmed a degree of succulence that compared with other known succulent plants. Tissue succulence is considered as an adaptation to drought stress and to saline environments by diluting tissue salt concentrations, although no halophytic grass has previously been reported with succulent leaves. The osmotic potential of *S. littoreus* also indicated solute accumulation in leaves, which is also a common feature for halophytic plants.

In addition to leaf succulence, this study also is the firstly report of C<sub>4</sub>/CAM-cycling photosynthesis in a grass. Carbon isotope values, Kranz anatomy and previous biochemical studies indicated that *S. littoreus* is a C<sub>4</sub> plant, whereas nighttime stomatal closure and diel acid fluctuations indicated CAM-cycling photosynthesis in *S. littoreus*. Although nocturnal acid increases were exhibited in *S. littoreus*, the ecological significance of this “CAM-cycling” is unclear.

CAM acid fluctuation was not observed in populations of *S. littoreus* at Tainan Gold Coast (year 2015) and Baishawan beach (year 2016). In an attempt to determine which ecological factors might possibly be associated with CAM-cycling in *S. littoreus*, CAM-cycling was frequently found in plants that in unshaded areas and/or at the edge of the colony (except the population at Tainan Gold Coast). In addition, the amount of CAM acid fluctuation did not differ among populations, but varied across the two years of the study. Temperature was similar across years while precipitation varied and was not correlated with the amount of nocturnal acidity increase. The

differences in CAM acidity fluctuations across years may be the result of other environmental factors. Photosynthetic rates were higher in southern populations in Taiwan, which may be the result of other microhabitat differences (e.g., vpd or soil water potential), or genetic variability among the populations.

C<sub>4</sub>/CAM-cycling photosynthesis has only been reported in one family and one genus, *Portulaca* (Portulacaceae) previously. As a result of the current study, the family Poaceae and the genus *Spinifex* can be added to plants with this exceedingly rare form of photosynthetic pathway intermediacy. *Zygochloa* and *Pseudochaetochloa* are sister groups of *Spinifex* (Morrone *et al.* 2012), and have been found mainly distributed in arid regions in Australia (RBG Kew GrassBase 2017). Whether tissue succulence, or even the ability of CAM photosynthesis in these taxa or in other species of *Spinifex* was exhibited in these genus remains unclear and is certainly worthy of investigation. Although PEP carboxylase and other enzymes are both used in C<sub>4</sub> and CAM pathways for CO<sub>2</sub> fixation and other parts of the C<sub>4</sub> cycle, the different timing of enzyme regulation has been proposed to hinder the intermediacy, and thus requires recruitment of unique genes (Christin *et al.* 2014). A potential evolutionary history of *Portulaca* has been proposed, in which a C<sub>4</sub>-specific PEP carboxylase gene was recruited into a CAM plant (Christin *et al.* 2014). Because there are no CAM plants in the Poaceae, this scenario is clearly not possible for the evolution of photosynthetic pathway intermediacy in *Spinifex*. This fascinating difference from *Portulaca* requires further investigation.

*P. oleracea* is viewed as a model organism for understanding regulatory controls between the C<sub>4</sub> and CAM pathways and for studies of the evolution of photosynthetic pathways. Now, *S. littoreus* may offers a new and radically different model for such studies. Furthermore, because of the benefits for leaf succulence and CAM-cycling, as some authors claim, this grass may be

potentially useful for experimental breeding programs to improve drought and salt tolerance in cereal crops (*e.g.* von Caemmerer *et al.* 2012).

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