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## Epiphytic orchids *Stanhopea tigrina* and *Prosthechea cochleata* are differentially affected by drought in a subtropical cloud forest

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### Abstract

We investigated the physiological responses of two epiphytic orchids under three light regimes of 20, 50, and 70% of total daily radiation under drought and rewatering conditions. *Stanhopea tigrina* was the one more affected because it exhibited strong photoinhibition and reduction of both electron transport rate and nocturnal acidity under drought and high radiation. However, this species maintained relatively high relative water content (RWC) values and underwent osmotic adjustment during the drought period and recovered photosynthetic variables during watered period. *Prosthechea cochleata* maintained similar water and photosynthetic responses to light conditions during the drought period and was more tolerant than *S. tigrina*. Principal component analysis provided evidence that water variables, such as RWC and succulence of both leaf and pseudobulb, were the most important variables for both species. Our results suggest that *S. tigrina* is more sensitive to drought than *P. cochleata*, and could be more affected by global warming.

*Additional key words:* chlorophyll fluorescence; Mexico; Orchidaceae; photosynthesis; water stress.

### Introduction

Tropical mountain cloud forests (TMCF) are an important component of regional and global diversity and are one of the most vulnerable to climate change (Foster 2001, Burke 2003). Canopy plants such as epiphytes are conspicuous elements of tropical and subtropical ecosystems that contribute significantly to biodiversity and to carbon, nutrient, and water cycle (Nadkarni 1984, Benzing 1990). Despite high humidity in TMCF, epiphytes are exposed to dry microclimate due to intermittent water pulses, making water shortages the most limiting factor for their establishment and growth (Benzing 1990, Laube and Zotz 2003). Hence, these plants are considered drought susceptible, because they are not soil-rooted but obtain water and nutrients from the atmosphere, on which they are completely dependent (Zotz *et al.* 2010).

Precipitation has a positive effect on epiphyte distribution in tropical and subtropical ecosystems (Wolf and Flamenco 2003, Kreft *et al.* 2004), but it can be highly

variable over space and time scales and thus affecting plant–water relationships (Goldsmith *et al.* 2012). For instance, in the tropical forest of ‘El Triunfo’ Reserve in Chiapas at Southern Mexico, annual precipitation can be up to 5,000 mm (Wolf and Flamenco 2003), while in the most humid forest in ‘El Cielo’ Biosphere Reserve (CBR) in Tamaulipas, in the north of the country, mean annual precipitation is 2,500 mm by year (González-Medrano 2005). Short rainless periods are common in TMCF as occurs in ‘El Cielo’ with 3–4 months of drought (Rzedowski 1996), when epiphytes experience an abrupt change in ecological conditions: solar radiation, temperature, and humidity. To deal with this variation, epiphytes draw on other sources of water such as fog or dew; it is known that fog contributes 20% or more of the total water input in cloud forests (Juvik and Ekern 1978, Stadtmüller 1987), supporting diversity in this ecosystem (Gradstein 2006). While dew is a water source that maintains water balance of epiphytic bromeliads during dry periods in a dry forest, it is not enough to contribute to growth (Andrade 2003).

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*Abbreviations:* ETR – electron transport rate;  $F_v/F_m$  – maximal quantum yield of PSII; NPQ – nonphotochemical quenching; RH – relative humidity; RWC – relative water content; T – temperature; TMCF – tropical mountain cloud forests; VPD – vapor pressure deficit;  $\Delta H^+$  – nocturnal acidification;  $\Psi_s$  – osmotic potential.

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In this scenario, epiphytes may be vulnerable to climate change if it results in quicker onset and more severe and longer droughts (Coumou and Rahmstorf 2012, Trenberth *et al.* 2014), which would affect their physiology and survival and potentially alter their diversity (Nadkarni and Solano 2002, Ellis and Coppins 2007, Zotz *et al.* 2010).

Notwithstanding, light is another important abiotic condition that drives photosynthetic activity and growth. In the epiphytic habitat, plants are exposed directly or indirectly to solar radiation, which is linked to characteristics inherent to host trees, such as size, age, and deciduousness, among others (Benzing 1990, Esseen *et al.* 1996, Callaway *et al.* 2002, Lie *et al.* 2009). Full sunlight can be stressful for epiphytes, causing photoinhibition that results in damage to the photosynthetic apparatus, but low light also limits plant growth and survival (Sultan 2003, Walters 2005, Niinemets 2007). The susceptibility of plants to photoinhibition depends on the species and growth light environments (Anderson and Osmond 1987). It has been considered that shade plants or low-light-grown plants are more susceptible to photoinhibition than sun plants or high-light-grown plants (Osmond 1994). Hence, environmental conditions are determinant in understanding the ecological implications of tolerance to extremes, and particularly important to successful cultivations and preservation of wild orchid species (Lin and Hsu 2004).

Epiphytic species comprise more than 70% of the members of Orchidaceae (Silvera *et al.* 2009), one of the largest and most diverse families of flowering plants, comprising more than 25,000 species (Christenhusz and Byng 2016). Mexico possesses around 1,260 species of orchids (Hágsater *et al.* 2005) from which 40% are endemic, and 15% are categorized as endangered by Mexican environmental laws (SEMARNAT 2010). The epiphytic orchids, *Stanhopea tigrina* and *Prosthechea cochleata*, are considered as threatened and endangered species, respectively. The habitat of most orchids has been negatively impacted by anthropogenic activities (Soto-Arenas and Solano-Gómez 2007, Ray *et al.* 2018). However, a clear gap exists in its physiological requirements to contribute to their conservation; hence, it is necessary to accelerate conservation programs for these species. *S. tigrina* and *P. cochleata* colonize different environments, while the former inhabits the wet sites such as the tropical montane cloud forests in Mexico. The latter is distributed in broad habitat types, from wet to dry (Pridgeon *et al.* 2009) and might be more tolerant to environmental changes.

Epiphyte species respond differentially to abiotic conditions, *e.g.*, the orchids *Miltonia flavescens* and *M. spectabilis* var. *moreliana* exhibit higher values of net photosynthetic rate, dark respiration, leaf transpiration rate, stomatal and intercellular conductance in response to atmospheric CO<sub>2</sub> concentration rate at full sunlight than that at 25% shade, suggesting that these species appear to be adapted to conditions of high irradiance (Pires *et al.* 2012). In counterpart, the orchids *Encyclia nematocaulon* and *Laelia rubescens* strongly diminish their photosynthetic activity and water content because of high radiation during dry periods in a dry forest (de la Rosa-Manzano *et al.* 2014).

Hence, it is important to evaluate how plants respond to changes in water and light availability in order to understand the physiological requirements to establishment, growth, and survival of orchids, in particular, since they constitute one of the most endangered plant families in the world due to fragmentation, destruction, and illegal trading (Nash *et al.* 2003, Kull and Hutchings 2006, Phelps and Webb 2015).

Response to drought has been widely studied in epiphyte species from tropical forests (Griffiths and Smith 1983, Nowak and Martin 1997, Griffiths and Maxwell 1999, Reyes-García and Griffiths 2009, Zotz and Asshoff 2010, Reyes-García *et al.* 2012), but few studies have attempted to study responses to drought of epiphytic orchids in subtropical forest (Martin *et al.* 2004), where short rainless periods are common. Since epiphytic orchids inhabit a wide range of habitats, from very humid to seasonally dry (Dressler 1981), we studied, for the first time, tolerance to drought and light variability of the epiphytic orchids *P. cochleata* and *S. tigrina* from the TMCF in the 'El Cielo' to better understand their response to environmental conditions, with the aim of conserving them. TMCF represents less than 1% of the total area of the country and is one of the most threatened types of vegetation in Mexico (Challenger 1998, Luna-Vega *et al.* 2006). This forest is recognized as one of the ecosystems with the highest diversity per unit of area in Mexico; it presents high endemism of epiphytes and includes 10% of Mexican flora species (Rzedowski 1978, 1996; Luna-Vega 2000, Sánchez-González *et al.* 2008, Sánchez-Ramos *et al.* 2014). In this sense, we proposed the following questions. How do epiphyte orchids tolerate changes in water availability and light intensity? Does water deficit have a greater impact on photosynthetic light-utilization efficiency than excess light? Considering that *P. cochleata* is widely distributed in the 'El Cielo' reserve, we hypothesized that individuals of this species are more water conservative and maintain photosynthetic activity under drought and high-light intensity conditions than *S. tigrina*, which is restricted to cloud forest and could be more vulnerable. Because water is the most limiting abiotic factor for vascular epiphytes (Gentry and Dodson 1987, Zotz and Hietz 2001), we assumed that relative water content, succulence, and osmotic potential of any orchid organ, such as leaf, pseudobulb or root, would be more important than photosynthetic traits, such as nocturnal acidification, electron transport rate, maximum quantum yield of PSII, among others. Finally, we expected that rewatering favors orchid development, displaying a higher relative water content, succulence, and photosynthetic activity, than that of orchids under drought.

## Materials and methods

**Plant material:** We selected the epiphytic orchids *S. tigrina* Bateman ex Lindl and *P. cochleata* (L.) W. E. Higgins due to high abundance in the CBR, although *S. tigrina* inhabits only the TMCF, and *P. cochleata* is also found in the tropical dry forest. *S. tigrina* is endemic to Mexico, while *P. cochleata* is distributed in Colombia, Venezuela, Central

America, Antilles, Mexico, and United States (southern Florida) (Dressler 1981, Pridgeon 1981).

In the TMCF, we collected 15 mature healthy plants, comparable in their leaf number and pseudobulbs of each orchid species. The plants were put in a pot (0.33 m<sup>3</sup>) containing *Quercus germana* Schltdl. & Cham. tree bark as substrate, the most common host tree for epiphytes in the TMCF (Castro-Huerta 2018). Plants were acclimated for 60 d under shade in a common garden in the ejido Alta Cima (where species are distributed). During this time, plants were watered twice a week.

**Experimental design:** After acclimation, five individuals of each orchid species were randomly transferred to one of three light treatments of photosynthetic photon flux density (PPFD). Artificial shading was created using nylon nets and the treatments were light intensities of 20% ( $6.84 \pm 0.57 \text{ mol m}^{-2} \text{ d}^{-1}$ ), 50% ( $12.84 \pm 0.92 \text{ mol m}^{-2} \text{ d}^{-1}$ ), and 70% ( $18.35 \pm 1.27 \text{ mol m}^{-2} \text{ d}^{-1}$ ) of PPFD. PPFD was measured with an *S-LIA-M003* sensor (Onset Computer Corporation, USA) connected to data logger *HOBO H21-002* (Onset Computer Corporation, USA). Air temperature and relative humidity in each treatment were measured with *HOBO pro v2 Temp/RH U23-001* (Onset Computer Corporation, USA). Vapor pressure deficit (VPD) was calculated according to Jones (1992). Minimum data of microenvironment [(minimum temperature ( $T_{\min}$ ), maximum relative humidity ( $RH_{\max}$ ), minimum vapor pressure deficit ( $VPD_{\min}$ )] were extracted from 3:00 to 6:00 h, and maximum data [maximum temperature ( $T_{\max}$ ), minimum relative humidity ( $RH_{\min}$ ), maximum vapor pressure deficit ( $VPD_{\max}$ )] from 13:00 to 16:00 h. Reference sensors were placed outside of shade houses. All sensors registered data each hour during the entire experiment (43 d). Orchid plants were exposed to a drought period of 35 d, when they reached low values of maximal quantum yield of PSII ( $F_v/F_m$ ). Plants were then watered and measured for 8 d. Before the 35-d drought, we measured physiological traits, but they did not show such a strong decline as at 35 d. During the drought period, we evaluated physiological traits that are frequently used to measure environmental stress and determine optimal growth conditions for plants (Lin and Hsu 2004).

**Chlorophyll fluorescence:** The parameters of chlorophyll (Chl) fluorescence were measured on fully expanded orchid leaves; quantum yield was measured at 9:00, 12:00, 15:00, and 18:00 h (here, we presented only data taken at 18:00 h).  $F_v/F_m$  was measured before dawn (6:00 h), and nonphotochemical quenching (NPQ) and electron transport rate (ETR) were assessed at the same hours as quantum yield; here, we presented maximum values of NPQ ( $NPQ_{\max}$ ) and ETR ( $ETR_{\max}$ ), which correspond to 15:00 h. We used predawn maximum fluorescence ( $F_m$ ) to calculate NPQ. All calculations were performed as suggested by Maxwell and Johnson (2000).

**Nocturnal acidification ( $\Delta H^+$ )** was determined with samples (5 cm<sup>2</sup>) from fully expanded leaves collected at dusk and before dawn. After immediate storage in ethanol

(60%, v/v), extraction and titration were performed in the laboratory following the protocol of Zotz and Andrade (1998).

**Relative water content (RWC)** is considered a better indicator of plant water status (Salehi-Lisar and Bakhshayeshan-Agdam 2016). For RWC, we collected leaf, pseudobulb, and root samples of two epiphytic orchids at predawn. Samples were placed in a plastic bag with moist filter paper in an insulated box with ice, transported to the laboratory to measure their fresh mass, placed in distilled water to obtain saturated mass, and then dried at 65°C for 24 h before measuring dry mass. RWC was calculated as: (fresh mass – dry mass)/(saturated mass – dry mass) × 100. For succulence, we considered the same samples used for RWC, but additionally we measured the total area for each sample. Succulence was calculated as: (fresh mass – dry mass)/leaf area.

**Osmotic potential ( $\Psi_s$ )** was measured for leaf and pseudobulb samples collected at predawn (6:00 h). All samples were immediately frozen by immersion in liquid nitrogen and then stored at 5°C in the laboratory. Samples were then ground using a mortar, the tissue liquid was collected with filter paper discs, and osmotic pressure was determined using a vapor pressure osmometer (*VAPRO 5520*, Wescor, Logan, Utah, USA).

**Data analysis:** The experiment was conducted in a completely randomized design, with a 3 × 2 factorial arrangement, three light levels (20, 50, and 70% of PPFD) and two species (*P. cochleata* and *S. tigrina*) with five replicates during drought and rewatering period. Physiological traits were compared using a two-way analysis of variance (ANOVA) followed by the Tukey's test. Normality was checked using the Kolmogorov and Smirnov's test. We used a Kruskal-Wallis test when variables were not normally distributed. In order to determine if water variables are more important than photosynthetic traits, we ran a principal components analysis (PCA, function *prcomp*) to evaluate the physiological characteristics of the species studied under three PPFD treatments. For PCA, we used the variables leaf and pseudobulb RWC, leaf and pseudobulb succulence,  $F_v/F_m$ , leaf and pseudobulb  $\Psi_s$ ,  $\Delta H^+$ , yield and  $ETR_{\max}$  of plants under drought period to understand which variables varied along the axis of the microclimatic gradient; data were centered and scaled. A multivariate analysis of variance (MANOVA) was performed to determine the effect of species and light treatments on the most important variables from the PCA (RWC, succulence, and osmotic potential, all of leaves, and nocturnal acidification). Values of  $P \leq 0.05$  were accepted as significant. All analyses were carried out with the statistical software *R version 3.3.3* (R Core Team 2018).

## Results

**Microenvironment conditions**, such as temperature (T), relative humidity (RH), and vapor pressure deficit (VPD),

presented slight changes among light treatments during 35 d of drought (Table 1).  $T_{\text{mean}}$  was similar between the 20 and 50% PPFD treatments and increased by 1°C at 70% PPFD.  $T_{\text{min}}$  showed values similar to  $T_{\text{mean}}$ , which was around 14°C for the 20 and 50% PPFD treatments, and slightly increased with the 70% PPFD.  $T_{\text{max}}$  was similar among light treatments (*ca.* 28°C).  $RH_{\text{mean}}$  and  $RH_{\text{max}}$  were higher at 20% PPFD than that at the other PPFD treatments.  $RH_{\text{min}}$  decreased nearly by 53%, relative to  $RH_{\text{max}}$ , in each of the light treatments.  $VPD_{\text{mean}}$  showed higher values at 70% PPFD treatment and lower at 20% PPFD treatment.  $VPD_{\text{max}}$  and  $VPD_{\text{min}}$  increased with light treatment intensities, reaching values around 2.12 and 0.17 kPa, respectively (Table 1). During the rewatering period,  $T_{\text{mean}}$  increased 1°C compared to the drought period, but  $T_{\text{max}}$  increased to 3°C at 70% PPFD.  $T_{\text{min}}$  was similar to the drought period under the three light treatments.  $RH_{\text{mean}}$ ,  $RH_{\text{max}}$ , and  $RH_{\text{min}}$  diminished around 10% in the three light treatments compared to the drought period. Values of

$VPD_{\text{mean}}$  (*ca.* 1.22 kPa),  $VPD_{\text{max}}$  (*ca.* 2.73 kPa), and  $VPD_{\text{min}}$  (*ca.* 0.2 kPa) were higher than that of the drought period in the three light treatments.

**Drought period:** Drought and light intensities significantly influenced water relations and photosynthetic activity of the orchids *P. cochleata* and *S. tigrina*. Interaction between species and light levels affected nocturnal acidification ( $\Delta H^+$ ) (Fig. 1A). *P. cochleata* presented a higher mean value of  $\Delta H^+$  [ $27.64 \pm 2.01 \mu\text{mol}(\text{H}^+) \text{g}^{-1}(\text{FM})$ ] than that of *S. tigrina* [ $16.14 \pm 2.01 \mu\text{mol}(\text{H}^+) \text{g}^{-1}(\text{FM})$ ]. Differences in  $\Delta H^+$  between species were notable, especially under 50 and 70% of PPFD. No differences in  $\Delta H^+$  were observed for *P. cochleata* under the three light levels, while the  $\Delta H^+$  of *S. tigrina* decreased under the 50 and 70% PPFD treatments compared to the 20% PPFD treatment. Plants of both epiphytic orchids showed low values of  $F_v/F_m$  ( $< 0.6$ ) under the three light levels but they were notably lower in *S. tigrina* at the 70% PPFD treatment (Fig. 1B).

Table 1. Microclimatic variables in three light treatments (20, 50, and 70% PPFD) during drought and rewatering period. Data of drought period represent register of 35 d, and those of rewatering period are register of 8 d. Data are mean  $\pm$  SE.  $T_{\text{mean}}$  – mean temperature;  $T_{\text{max}}$  – maximum temperature;  $T_{\text{min}}$  – minimum temperature;  $RH_{\text{mean}}$  – mean relative humidity;  $RH_{\text{max}}$  – maximum relative humidity;  $RH_{\text{min}}$  – minimum relative humidity;  $VPD_{\text{mean}}$  – mean vapor pressure deficit;  $VPD_{\text{max}}$  – maximum vapor pressure deficit;  $VPD_{\text{min}}$  – minimum vapor pressure deficit.

	Light level [%]	Drought period	Rewatering period
$T_{\text{mean}}$ [°C]	20	20.70 $\pm$ 0.50	21.37 $\pm$ 0.79
	50	20.77 $\pm$ 0.50	21.38 $\pm$ 0.88
	70	21.13 $\pm$ 0.51	21.58 $\pm$ 0.78
$T_{\text{max}}$ [°C]	20	28.49 $\pm$ 0.99	30.78 $\pm$ 0.78
	50	28.20 $\pm$ 0.97	29.64 $\pm$ 0.98
	70	28.50 $\pm$ 0.99	31.51 $\pm$ 0.95
$T_{\text{min}}$ [°C]	20	14.65 $\pm$ 0.47	14.40 $\pm$ 1.37
	50	14.68 $\pm$ 0.46	14.82 $\pm$ 1.47
	70	15.07 $\pm$ 0.46	14.92 $\pm$ 1.29
$RH_{\text{mean}}$ [%]	20	74.61 $\pm$ 1.44	64.89 $\pm$ 4.86
	50	73.71 $\pm$ 1.41	63.03 $\pm$ 4.71
	70	72.63 $\pm$ 1.33	61.86 $\pm$ 5.20
$RH_{\text{max}}$ [%]	20	91.16 $\pm$ 1.31	87.70 $\pm$ 5.31
	50	90.08 $\pm$ 1.28	84.16 $\pm$ 5.65
	70	86.12 $\pm$ 2.73	84.15 $\pm$ 4.98
$RH_{\text{min}}$ [%]	20	50.45 $\pm$ 2.44	37.50 $\pm$ 4.97
	50	50.66 $\pm$ 2.34	37.01 $\pm$ 5.54
	70	49.11 $\pm$ 2.68	37.55 $\pm$ 4.63
$VPD_{\text{mean}}$ [kPa]	20	0.88 $\pm$ 0.07	1.17 $\pm$ 0.10
	50	0.90 $\pm$ 0.06	1.23 $\pm$ 0.11
	70	0.94 $\pm$ 0.07	1.21 $\pm$ 0.11
$VPD_{\text{max}}$ [kPa]	20	2.08 $\pm$ 0.18	2.79 $\pm$ 0.17
	50	2.11 $\pm$ 0.17	2.65 $\pm$ 0.19
	70	2.17 $\pm$ 0.18	2.75 $\pm$ 0.17
$VPD_{\text{min}}$ [kPa]	20	0.15 $\pm$ 0.02	0.20 $\pm$ 0.09
	50	0.17 $\pm$ 0.02	0.26 $\pm$ 0.09
	70	0.20 $\pm$ 0.02	0.26 $\pm$ 0.08

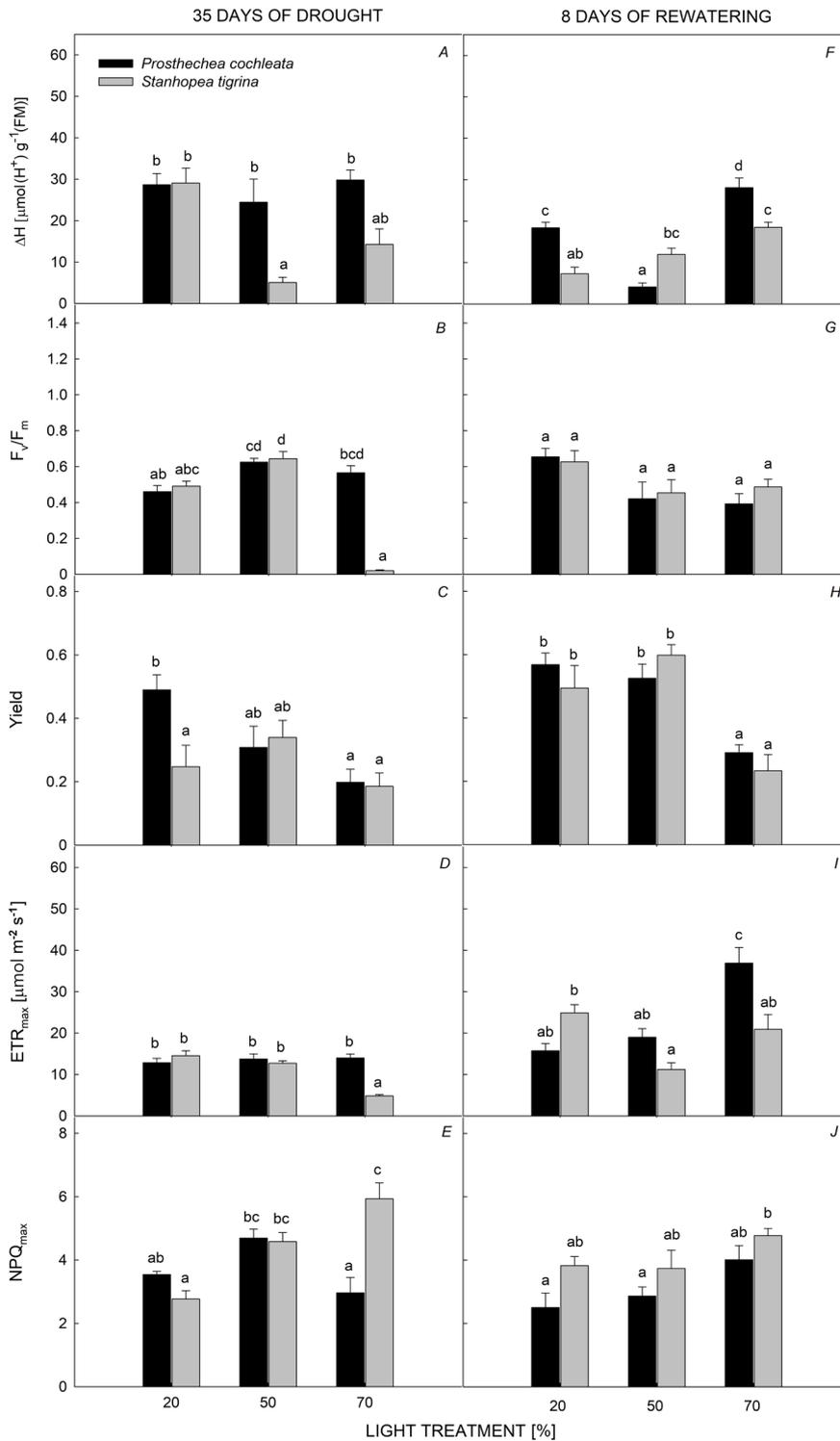


Fig. 1. Nocturnal acidification ( $\Delta H^+$ ), maximal quantum yield of PSII ( $F_v/F_m$ ), yield, maximum electron transport rate ( $ETR_{max}$ ), and maximum nonphotochemical quenching ( $NPQ_{max}$ ) of *Prosthechea cochleata* and *Stanhopea tigrina* at three light treatments during drought and rewatering period. Different letters above bars indicate significant differences,  $P < 0.05$ . Data are mean  $\pm$  SE,  $n = 5$ .

*P. cochleata* plants had slightly higher values of  $F_v/F_m$  ( $0.63 \pm 0.02$ ) under 50% PPFD than those under 20 and 70% PPFD ( $0.46 \pm 0.03$  and  $0.57 \pm 0.04$ , respectively; Fig. 1B). There was a decrease in yield ( $0.20 \pm 0.09$ ) for *P. cochleata* under 70% PPFD compared to values under 20% of PPFD treatment ( $0.49 \pm 0.11$ ; Fig. 1C). No differences in yield were registered for *S. tigrina* under three light intensities (Fig. 1C).

*P. cochleata* plants showed a smaller increase in  $ETR_{max}$  than that of *S. tigrina* ( $14$  vs.  $11 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Although orchids received different light intensities, no differences were observed in  $ETR_{max}$  for *P. cochleata* under the three levels of light (Fig. 1D). While *S. tigrina* had a significant decrease in  $ETR_{max}$  under 70% PPFD compared to plants under 20 and 50% PPFD (Fig. 1D). In addition, there was a significant effect of the interaction

light levels and species on  $NPQ_{max}$ ; *S. tigrina* reached higher mean values of  $NPQ_{max}$  ( $4.43 \pm 0.20$ ) than that of *P. cochleata* ( $3.73 \pm 0.20$ ). Comparisons among light levels showed that *S. tigrina* registered higher values of  $NPQ_{max}$  at 70% PPFD than either the 20 or the 50% PPFD, while *P. cochleata* slightly increased  $NPQ_{max}$  with 50% PPFD compared to the 70% PPFD (Fig. 1E).

Both species showed higher values of RWC in leaves followed by pseudobulbs and roots, and different trends among orchid organs were observed. Interaction of light levels with species affected the RWC of leaves and pseudobulbs. *P. cochleata* had a higher leaf ( $69.6 \pm 2.55\%$ , Fig. 2A) and pseudobulbs ( $54.0 \pm 1.32\%$ , Fig. 2B) RWC than that of *S. tigrina* ( $60.14 \pm 1.41$  and  $45.7 \pm 1.97\%$ , respectively). However, we did not find differences for leaf RWC and pseudobulb RWC between light levels for each species (Fig. 2A,B). Root RWC significantly differed between species, but there was no effect of light levels (Fig. 2C).

Pseudobulbs were the most important organ for succulence in both species, followed by leaves and roots. Succulence of pseudobulbs and leaves was influenced by the interaction of light levels and species (Fig. 3); *P. cochleata* had higher mean values of succulence than that of *S. tigrina* in both organs (Fig. 3A,B). Differences in succulence of pseudobulbs and leaves between light levels were not found for *P. cochleata*, while *S. tigrina* showed higher values of succulence in both pseudobulbs and leaves under the 50 and 70% PPFD, compared to

individuals under the 20% PPFD (Fig. 3A,B). The lowest values of succulence [around  $16 \text{ mol(H}_2\text{O) m}^{-2}$ ] of both orchids were found in roots, compared to other orchid organs. Succulence of roots exhibited significant differences between light levels for both species (Fig. 3C), but no differences were found between species (Fig. 3C).

Osmotic potential ( $\Psi_s$ ) of leaves and pseudobulbs was significantly different between species (Fig. 4A,B). *S. tigrina* showed lower leaf mean  $\Psi_s$  values ( $-0.71 \pm 0.02 \text{ MPa}$ ) than that of *P. cochleata* ( $-0.45 \pm 0.02 \text{ MPa}$ ), but it was inverse for pseudobulbs. Light levels had no effect on leaf  $\Psi_s$  of either species (Fig. 4A).

The PCA showed that components 1, 2, and 3 accounted for 70.2% of the total variation (Table 2, Fig. 5). Component 1 explained 36.5% of the variation and was represented by positive loadings of leaf and pseudobulb succulence and RWC, followed by yield and  $F_v/F_m$  (Table 2, Fig. 5). Component 2 explained 19.1% of the variation and was represented by negative loadings of leaf and pseudobulb  $\Psi_s$  (Table 2, Fig. 5). Component 3, which explained 14.7% of the variation, had positive loading for  $ETR_{max}$  and negative for  $\Delta H^+$  (Table 2, Fig. 5). The MANOVA indicated a significant effect of interaction between species and light treatments on RWC, leaf  $\Psi_s$  and succulence, and  $\Delta H^+$  (Table 3). In post-hoc ANOVAs, there was a significant orchid species effect on all variables, with exception of nocturnal acidification, which was different only between light treatments.

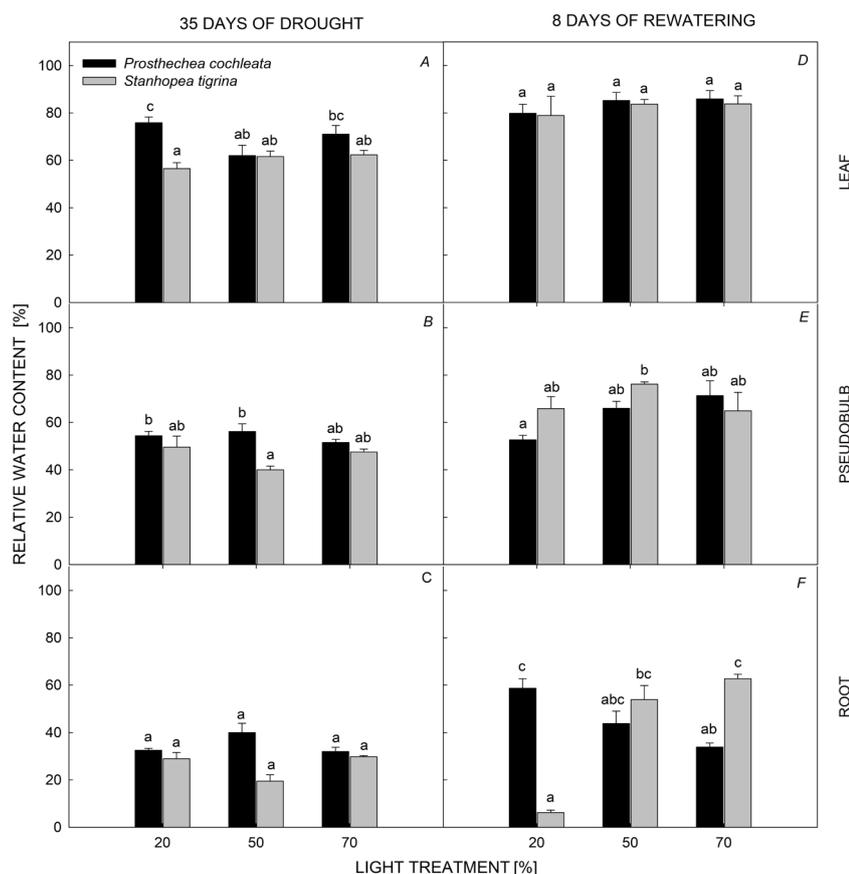


Fig. 2. Relative water content of *Prosthechea cochleata* and *Stanhopea tigrina* at level of root, pseudobulb, and leaf at three light treatments during drought and rewatering period. Different letters above bars indicate significant differences,  $P < 0.05$ . Data are mean  $\pm$  SE,  $n = 5$ .

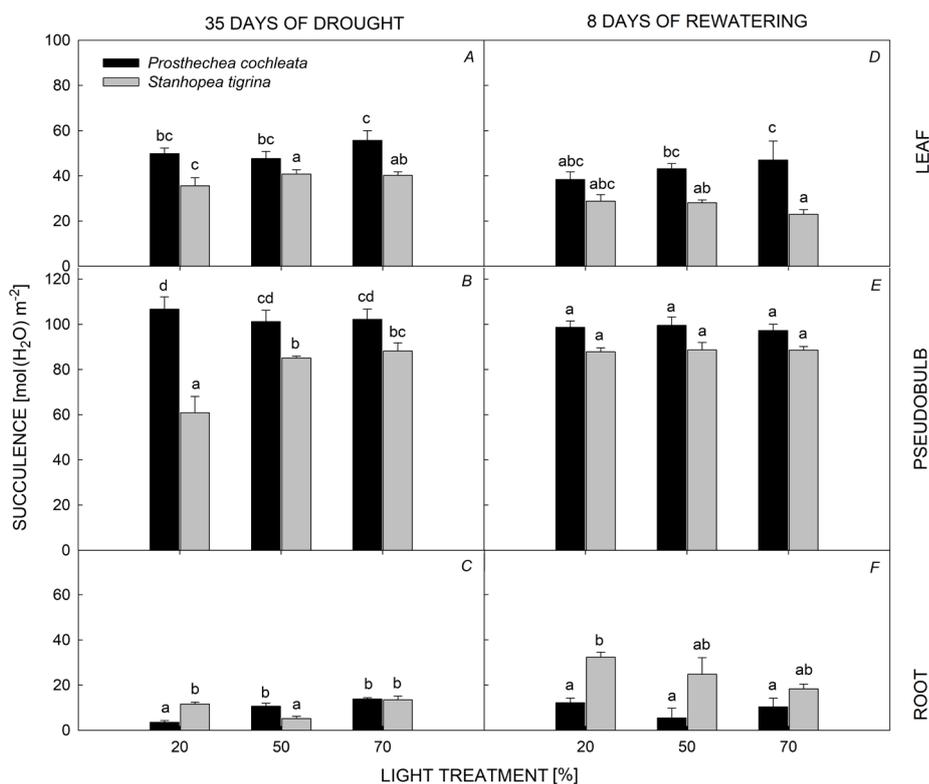


Fig. 3. Succulence of *Prosthechea cochleata* and *Stanhopea tigrina* at level of root, pseudobulb, and leaf at three light treatments during drought and rewatering period. Different letters above bars indicate significant differences,  $P < 0.05$ . Data are mean  $\pm$  SE,  $n = 5$ .

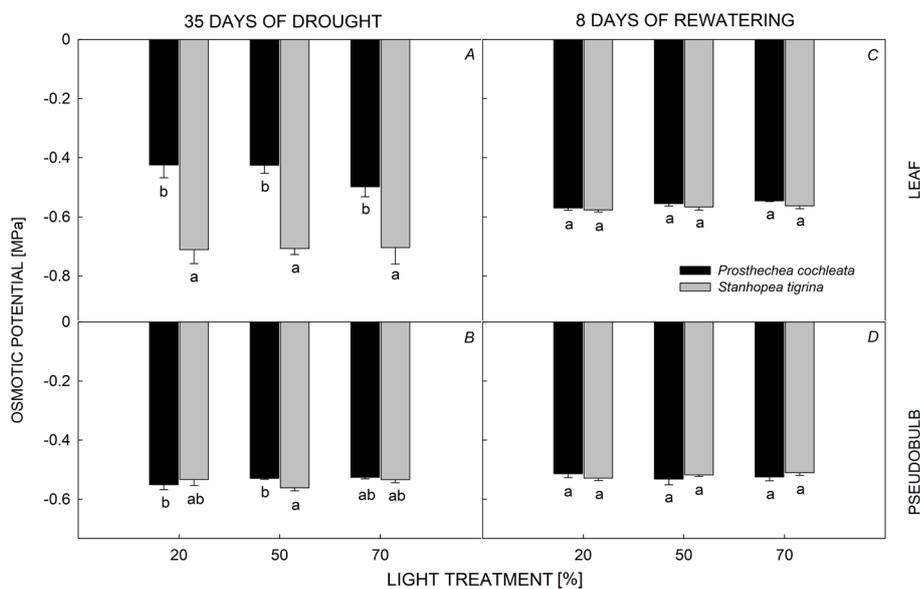


Fig. 4. Osmotic potential of *Prosthechea cochleata* and *Stanhopea tigrina* at level of pseudobulb and leaf at three light treatments during drought and rewatering period. Different letters mean significant differences,  $P < 0.05$ . Data are mean  $\pm$  SE,  $n = 5$ .

**Rewatering period:** After plants were watered, they recovered their photosynthetic activity and water status.  $\Delta H^+$  was affected by the interaction of light levels and species (Fig. 1). *P. cochleata* exhibited significantly higher values of  $\Delta H^+$  [ $16.81 \pm 0.91 \mu\text{mol}(\text{H}^+) \text{g}^{-1}(\text{FM})$ ] than *S. tigrina* [ $12.56 \pm 0.91 \mu\text{mol}(\text{H}^+) \text{g}^{-1}(\text{FM})$ ], although these values were lower than those during the drought period (Fig. 1A–E). Both orchid species presented differences in  $\Delta H^+$  between light levels (Fig. 1F);  $\Delta H^+$  significantly diminished under 50% PPFD and increased under 70%

of PPFD (Fig. 1F). Neither the interactions between light levels and species (Fig. 1G) nor the species (Fig. 1G) had an effect on  $F_v/F_m$ . This variable only was different between light levels, which was higher under the 20% PPFD treatment and lower under the 50 and 70% PPFD treatments. Although *S. tigrina* had the lowest values during drought period under 70% PPFD, plants recovered to  $\sim 0.5$  yield (Fig. 1H).

$\text{ETR}_{\text{max}}$  was affected by the interaction of light treatments and species (Fig. 1I). At the species level, *P. cochleata* had

Table 2. Principal components (PC) obtained from physiological characteristics of *Stanhopea tigrina* and *Prosthechea cochleata* grown under 20, 50, and 70% of PPFD intensities during drought period. RWC – relative water content;  $\Psi_s$  – osmotic potential;  $\Delta H^+$  – nocturnal acidification;  $ETR_{max}$  – maximum electron transport rate;  $F_v/F_m$  – maximal quantum yield of PSII; yield – quantum yield.

Variables	PC1	PC2	PC3
RWC of leaves [%]	0.3617	-	-
RWC of pseudobulbs [%]	0.3966	-	-
Succulence of leaves [ $\text{mol}(\text{H}_2\text{O}) \text{m}^{-2}$ ]	0.4498	-	-
Succulence of pseudobulbs [ $\text{mol}(\text{H}_2\text{O}) \text{m}^{-2}$ ]	0.3183	-	-
$\Psi_s$ of leaves [MPa]	-	-0.5878	-
$\Psi_s$ of pseudobulbs [MPa]	-	-0.5279	-
$\Delta H^+$ [ $\mu\text{mol}(\text{H}^+) \text{g}^{-1}(\text{FM})$ ]	-	-	-0.3452
$ETR_{max}$ [ $\mu\text{mol} \text{m}^{-2} \text{s}^{-1}$ ]	-	-	0.5222
$F_v/F_m$	0.3493	-	-
Yield	0.3740	-	-
Proportion of variance [%]	36.45	19.05	14.66
Cumulative proportion [%]	36.45	55.51	70.17

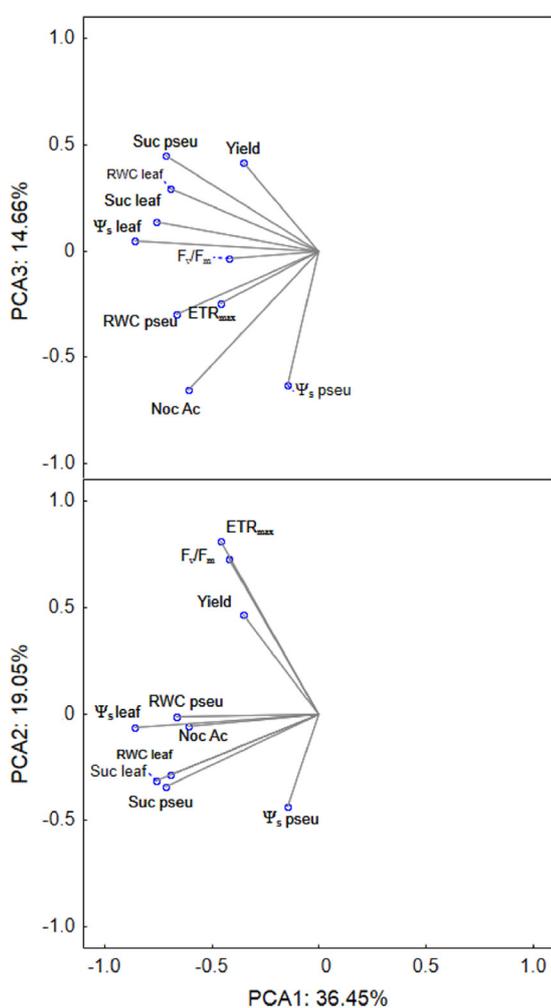


Fig. 5. Principal components analysis (PCA) ordination diagram displaying correlations between physiological characteristics in epiphytes *Prosthechea cochleata* and *Stanhopea tigrina* under 20, 50, and 70% of PPFD treatment during drought period. Physiological variables are listed in Table 2.

higher values of  $ETR_{max}$  than that of *S. tigrina* (Fig. 1I), and values were remarkable under the 70% PPFD. On the other hand,  $NPQ_{max}$  was different between light levels (Fig. 1J) and species (Fig. 1J). *S. tigrina* had significantly higher  $NPQ_{max}$  ( $4.11 \pm 0.23$ ) than that of *P. cochleata* ( $3.13 \pm 0.23$ ).

Different orchid organs recovered RWC, relative to the drought period. Orchids did not have significant differences in leaf RWC ( $\sim 80\%$ ) between light treatments (Fig. 1D). Leaves recovered  $\sim 20\%$  RWC relative to the drought period. Pseudobulbs RWC showed significant differences only between light levels (Fig. 1E), with no differences between species (Fig. 1E); pseudobulbs recovered  $\sim 15\%$  of the RWC lost in the drought period. Orchid roots showed a clear increase of RWC under the three light levels (Fig. 1F); *P. cochleata* significantly increased the RWC of roots under the 20% PPFD ( $59 \pm 8\%$ ), compared to the 50% ( $44 \pm 10\%$ ) and 70% PPFD ( $34 \pm 3\%$ ; Fig. 1F). Roots of *S. tigrina* exhibited the lowest values of RWC under 20% PPFD ( $6 \pm 2\%$ ) compared with the 50% ( $54 \pm 11\%$ ) and 70% PPFD ( $63 \pm 3\%$ ; Fig. 1F).

Neither the interaction of light levels and species nor light levels caused differences in leaf, pseudobulb or root succulence (Fig. 2D–F). *P. cochleata* presented higher values of succulence than that of *S. tigrina*, in both organs, leaves [ $42.81 \pm 2.37$  vs.  $26.72 \pm 2.37 \text{ mol}(\text{H}_2\text{O}) \text{m}^{-2}$ ] and pseudobulbs [ $98.47 \pm 1.60$  vs.  $88.32 \pm 1.60 \text{ mol}(\text{H}_2\text{O}) \text{m}^{-2}$ ]. At the root level, succulence response was reversed between species, *S. tigrina* showed higher values than that of *P. cochleata* [ $25.13 \pm 2.35$  vs.  $9.32 \pm 2.35 \text{ mol}(\text{H}_2\text{O}) \text{m}^{-2}$ , Fig. 2F]. Neither light levels nor species had an effect on  $\Psi_s$  of leaves and pseudobulbs (Fig. 4D–E); mean values of both species were around  $-0.56 \pm 0.01$  and  $0-0.52 \pm 0.01$  MPa, respectively.

## Discussion

Epiphytic orchids responded differentially to drought under different light intensities. *P. cochleata* was more tolerant to temporary drought than *S. tigrina* in agreement

Table 3. Multivariate analysis of variance to estimate the effect of the light and species in physiological traits (relative water content, succulence, and osmotic potential, all of leaves, and nocturnal acidification) of two epiphytic orchids during drought period. Significance codes: 0 ‘\*\*\*’, 0.05 ‘\*\*’.

Sources of variation	Df	F	P
Species	1	50.302	0.0000481***
Light	1	2.668	0.0579819**
Species × Light	1	6.755	0.0009498***
Residuals	26	-	-

with our hypothesis. Despite the increase in NPQ, the interaction of drought and the highest light intensity resulted in a stressful environment for *S. tigrina* photosynthesis, as suggested by the decreases in ETR,  $F_v/F_m$ , yield, and nocturnal acidification. *S. tigrina* is well adapted to intermediate light in the middle zone of the host *Q. germana* in ‘El Cielo’ (Castro-Huerta 2018) but deciduousness of this host during short dry periods can negatively affect their photosynthesis. While *P. cochleata* inhabits more open micro-habitats, such as the tropical dry forest, where this species has the capacity to tolerate both drought and high light intensity as was observed by higher values of ETR,  $F_v/F_m$ , yield, and nocturnal acidification with no differences between light levels. Our results are in agreement with those found for six orchid species of Singapore, where water deficit has a greater impact on photosynthetic light-utilization efficiency than excess light (Tay *et al.* 2015). Other studies indicate that some epiphytic orchids can acclimatize under 100 and 75% of the total irradiance, showing high mean values of net photosynthetic rate (Boardman 1977, Pires *et al.* 2012). Even for the epiphytic orchid *Cypripedium guttatum*, a level of 45% irradiance is optimal for photosynthesis (Zhang *et al.* 2003, 2007).

Low values of  $F_v/F_m$  of *S. tigrina* under the 70% PPFD treatment during the drought period was related to an increase in both light and temperature (Table 1). Leaves exposed to higher light intensity can exacerbate photoinhibition (Mulkey and Percy 1992) and even cause greater vapor pressure deficits leading to reduction of quantum yield (Cornic and Briantais 1991, Crain and Tremblay 2017). The  $F_v/F_m$  seems to diminish when thermal dissipation is not enough to protect the PSII against excessive light (Tognetti *et al.* 1998a,b) as occurred in *S. tigrina*, while *P. cochleata* showed an inverse pattern – a lower dissipation and increased  $F_v/F_m$ . *S. tigrina* could perhaps be considered a shade species, which are known to have reduced photoprotective systems (e.g., xanthophyll pigments) as compared to sun plants (Demmig-Adams *et al.* 1995, Demmig-Adams and Adams 2000, de la Rosa-Manzano *et al.* 2015). When energy dissipation in the antenna of PSII is low, a more active photosynthetic electron transport may help to mitigate the effect of photodamage in shade-grown plants (Kitao *et al.* 2000). However, in our study electron transport of *S. tigrina* was also low and the 70% PPFD treatment

was likely excessive, evidenced by very low yield values at 18:00 h, enhancing risk of photodamage (Demmig-Adams and Adams 2000). High risk of photoinhibition under strong radiation, even in sun-grown individuals, could result from the well-established trade-off between shade tolerance and low growth rate under high irradiance (Grubb *et al.* 1996). Even though *S. tigrina* plants exhibited strong photoinhibition under 70% PPFD, they were able to recover their photosynthetic activity during the rewatering period at 8 d, evidenced by value increases of NPQ, ETR,  $F_v/F_m$ , nocturnal acidification, and yield. Our results are similar to Cui *et al.* (2004) who found that rewatering resulted in sudden revival from the stress in *Doritaenopsis* orchid. Our findings are consistent with the significant role that electron transport capacity and energy dissipation play a role in ameliorating photodamage for shade-grown plants (Kitao *et al.* 2001).

Another plausible explanation for the remarkable differences in  $F_v/F_m$  between orchid species under higher light intensity is the differences in exposed leaf area. Supporting its shade distribution, *S. tigrina* has a larger exposed leaf area (23–45 cm length × 5–13 cm width) than that of *P. cochleata* (15–28 cm length × 1.5–3.5 cm width), which is more vulnerable to photoinhibition than those grown in full sun, because shade leaves have a higher light-capturing capacity as a result of larger antenna size of PSII, and lower rates of light-saturated photosynthesis due to lower amounts of photosynthetic enzymes (Björkman 1981, Anderson and Osmond 1987, Osmond 1994).

Lower values of nocturnal acidification of *S. tigrina* under high light intensity during the drought period are in agreement with decreased functioning of their photosynthetic apparatus. This species was not able to use high light intensities, unlike *P. cochleata*, which improved nocturnal environmental conditions, such as low VPD (~ 0.17 kPa) to maintain higher nocturnal acidification, although similar values under the three light conditions without water were exhibited. Values of nocturnal acidification of both orchid species are comparable to epiphyte bromeliads from dry forest during the dry season in the field (Reyes-García *et al.* 2008) and under drought in greenhouse conditions (Reyes-García *et al.* 2012). It is well known that nocturnal acidification depends directly on PPFD preceding day (Andrade *et al.* 2007) and PPFD values around 13 and 18 mol m<sup>-2</sup> d<sup>-1</sup> resulted in a negative effect on photosynthesis of orchids from TMCF. The time (35 d) of drought imposed on both orchids was longer (especially for *S. tigrina*) than that imposed on epiphytic orchids from tropical dry forest (15 d, de la Rosa-Manzano *et al.* 2014), which has harsher environmental conditions, such as higher nocturnal and diurnal VPD (0.6 and 5.0 kPa, respectively), resulting in a deep decrease of photosynthetic activity. In our study, *P. cochleata* maintained similar nocturnal acidification under the three light treatments during the drought period, suggesting that low stomata aperture could be parallel to recycling of respiratory CO<sub>2</sub> via CAM (Lüttge 2002), such as in *Tillandsia ionantha* in which 22% of the malic acid accumulated was derived from internal CO<sub>2</sub> after 50 d without water (Nowak and Martin 1997). Similar response

was found in other species of orchids (Lüttge 1987, Griffiths *et al.* 1989) and the *Tillandsia* genus (Griffiths *et al.* 1986, 1989; Martin and Adams 1987, Loeschen *et al.* 1993). Presumably recycling respiratory CO<sub>2</sub> constitutes an adaptation that contributes to the apparent tolerance of orchids to long periods of drought; however, it is necessary to realize measurements in the field.

The decrease of leaf osmotic potential observed in both epiphytic orchids after 35 d is a characteristic response of many CAM plants to drought (Griffiths *et al.* 1986, Lüttge 1987, Smith 1989, Martin 1994). The lower leaf osmotic potential of *S. tigrina* (around -0.70 MPa) was in agreement with the decline in leaf RWC and the increase in nocturnal acidification, which had lower values under the 70% PPFD treatment. More negative leaf osmotic potential is a common adaptation of plants to drought (Lambers *et al.* 1998) to maintain nonzero cell turgor pressure and allows plants to harvest water (Martin *et al.* 2004). Hence, differences in osmotic potential between pseudobulbs and leaves of *S. tigrina* may allow translocating water from high concentration sites to low concentration sites. During the drought period, osmotic potential for the epiphytic orchids increases *S. tigrina* resistance to drought, by osmotic adjustment, a mechanism that helps plants acclimatize to dry conditions (Sanders and Arndt 2012), and not a simple concentration of solutes due to tissue desiccation. Similar phenomena occur in other epiphytes species that accumulate osmolytes (Griffiths *et al.* 1986, Smith 1989, Martin 1994, Stiles and Martin 1996, Nowak and Martin 1997). However, it is necessary to study the role of solutes, such as glycine betaine or proline, in epiphytes because their content varies among species (Ashraf and Bashir 2003).

Apparently, *P. cochleata* plants are highly resistant to drought because leaf osmotic potential remained similar during both the drought and rewatering periods, and leaf relative water content declined by only 20% at 35 d without water. In contrast, the RWC of the orchid *Eria velutina* decreased from 98% to a minimum of 65% at 20 d of drought in the field in southeastern Australia (Sinclair 1983a). Also, it can be compared with declines in relative water content of 70% for leaves of the fern *Pyrrosia angustata* (Sinclair 1983a,b). Leaf relative water content was relatively high in epiphytic orchids during the drought period, supporting the idea that perhaps stomata were slightly closed to conserve water and diminish nocturnal CO<sub>2</sub> uptake and probably to recycle internal CO<sub>2</sub> (Goh and Kluge 1989). *P. cochleata* was more water conservative than *S. tigrina*, even at 70% PPFD, which could represent an advantage for dealing with long drought periods in the TMCF.

Relative water content of leaf, pseudobulb, and root of each orchid studied remained constant among light treatments during the drought period, and the principal components analysis suggests that responses in terms of water related traits, such as RWC and succulence, were more evident during the drought period, supporting our prediction. *P. cochleata* appeared to be relatively more resistant to water depletion than *S. tigrina* because *P. cochleata* leaf, pseudobulb, and root RWC decreased by around 15%, while *S. tigrina* RWC decreased by 30%

during the drought period, reinforcing the idea that *S. tigrina* was more vulnerable to drought. Apparently, pseudobulbs of both species were more susceptible to drought than leaves because they lost more water (Fig. 1). Stancato *et al.* (2001) also found that the decrease in RWC of drought-stressed plants (45 d) was greater in pseudobulbs than that in leaves of the epiphytic orchid *Cattleya forbesii* Lind., indicating that pseudobulbs facilitate a slow reduction of RWC in the leaf. Even though the epiphytic orchids were under stress for 35 d, this did not represent severe hydric stress for plants, because leaf RWC was above 40% (Cockerham and Leinauer 2011). Leaf RWC was around 70% for *P. cochleata* and 60% for *S. tigrina*, suggesting that, among other things, high nocturnal humidity (~90%) and low nocturnal VPD (~0.17 kPa) may have contributed to water conservation during the drought period. This was similar to what occurred in *Cattleya* orchid plants after three weeks of stress: they maintained leaf RWC above 70%, because the pseudobulbs were capable of delaying excessive water loss during drought. Pseudobulbs consist of water-storing tissue that orchids use to adjust to water deficit (Zheng *et al.* 1992, Pires *et al.* 2012). Water lost from leaves through transpiration may be rapidly replaced by water previously stored in the pseudobulbs, which perform as water suppliers, as likely occurred with the epiphytic orchids under stress conditions in this study, evidencing that pseudobulbs are effective reservoirs for buffering plant stress (Goh and Kluge 1989, Yang *et al.* 2016).

Succulence is an adaptive characteristic by which epiphytes store water in large, water-rich parenchyma cells. Notably, *P. cochleata* was more succulent than *S. tigrina* considering the three organs. Root was the least succulent, similar to findings for the orchid *Epidendrum secundum* under field conditions (Moreira *et al.* 2009). Less succulence in roots was related to presence of an exoderm with dead cells and thick suberized walls that prevent apoplastic transport (Fahn 1990). Roots of *S. tigrina* clearly increase succulence during the rewatering period, while those of *P. cochleata* maintained similar succulence through the drought period. Orchids are characterized by the presence of velamen roots, capable of absorbing and storing water (Benzing *et al.* 1982, Benzing 1987). *S. tigrina* could be more efficient than *P. cochleata*, because it recovered around 35% of their root RWC.

We conclude that the two epiphytic orchids were differentially affected by interaction of drought and light, reinforcing the view of separation of niches in the CBR, *S. tigrina* is more shade restricted in the TMCF, while *P. cochleata* colonizes more open sites including the tropical dry forest. *P. cochleata* was able to conserve more water; its RWC remained around 70% under the three PPFD treatments. Also, it is probably more water-use efficient than *S. tigrina*, giving it an advantage in subsequent stressful environmental conditions, such as high temperature and water deficit in TMCF (Nadkarni and Solano 2002) and may be more resilient than drought-resistant bromeliads (Wolf and Flamenco 2006, Rosado-Calderón *et al.* 2018). *S. tigrina* appeared to be more vulnerable to drought under high light; however, this species makes an osmotic adjustment to adapt to

stress conditions, where high relative water content and succulence of pseudobulbs play an important role in their water balance. Also, for both species, favorable nocturnal microenvironment with maximum relative humidity and minimum VPD might help to mitigate the drought period. Nevertheless, further studies focused on responses to drought in the field would be useful for conservation of epiphytic orchids.

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