

Physiological responses of orchid pseudobulbs to drought stress are related to their age and plant life form

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Received: 3 August 2018 / Accepted: 17 December 2018 / Published online: 2 January 2019
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Abstract As storage organs for water and nutrients, pseudobulbs play an important role in the survival of orchids. However, the differences in morphological and physiological responses of pseudobulbs to drought stress between epiphytic and terrestrial orchids remain undefined, and little is known about the physiological imparity of different-aged pseudobulbs. We investigated the anatomy and changes in physiology of pseudobulbs in an epiphytic orchid (*Cymbidium tracyanum*) and a terrestrial orchid (*C. sinense*) and compared their responses and recovery during and after periods of drought stress. In particular, “ramets

severance treatment” and “multiple leafless pseudobulbs treatment” were applied for *C. tracyanum* to verify the utilization strategy for the stored water. When compared with *C. sinense*, the pseudobulbs of *C. tracyanum* have larger water-storage cells and higher water content, and the enhanced water storage of *C. tracyanum* can be used and recovered more rapidly. And they had more flexibility in shifting stored nonstructural carbohydrates. The remarkably high concentration and different change trends of abscisic acid (ABA) between different-aged pseudobulbs under drought stress were only found in *C. tracyanum*. When pseudobulb age was considered, the stored water and carbohydrate changed more rapidly in the youngest pseudobulbs of both species in response to stress. Our results indicated that the pseudobulbs differ in their means for coping with drought conditions according to their life form and age. These findings contribute to our understanding about the functional diversification of pseudobulbs and their strategies for ecological adaptations.

Communicated by Timothy Bell.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11258-018-00904-x>) contains supplementary material, which is available to authorized users.

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Keywords Pseudobulb · Orchid · Drought · Epiphytic · Terrestrial

Introduction

As renowned horticultural plants, numerous species in the genus *Cymbidium* (Orchidaceae) have been

cultivated in worldwide. While some of them are now endangered in wild because of climate change and the destruction of natural habitats (Luo et al. 2002; Liu et al. 2009). Both epiphytic and terrestrial life forms can be found in *Cymbidium*. Compared with terrestrial species, epiphytes benefit from high light intensity and relatively low competition, but are challenged by restricted supplies of nutrients, and especially, water (Zotz and Bader 2009; Zotz and Hietz 2001). And may due to divergent life forms, closely related species in *Cymbidium* develop different strategies for coping with drought stress (Li et al. 2017). Therefore, it is beneficial for utilization and conservation of *Cymbidium* species by studying the different responses of epiphytic and terrestrial members to drought stress.

The study of water-related anatomy of leaves has shown that, compared with terrestrial species of *Cymbidium*, the epiphytic species in that genus have higher values for leaf mass per unit area (LMA), leaf thickness, epidermal thickness, saturated water content (SWC), and the time required to dry saturated leaves to 70% relative water content (T_{70}). Those adaptive traits give epiphytes greater ability to tolerate drought (Zhang et al. 2015). Our recent physiological and proteomic analyses of leaves from epiphytic and terrestrial orchids demonstrate that, under drought stress, the former type has higher capacity for photoprotection and maintenance of the carbon balance. Furthermore, the epiphytic species respond more effectively to leaf-applied ABA (Li et al. 2017).

The pseudobulb, an adaptively unique stem of many orchids, serves as a buffer against drought stress because of its ability to store water and carbon. During periods of drought, the presence of pseudobulbs may help to slow the reduction in leaf water content and water potential (He et al. 2013). Leafless pseudobulbs enable epiphytic *Dimerandra emarginata* to sustain leaf water content for nearly a month in the absence of rain fall (Zotz 1999). To maintain carbohydrate balance, the pseudobulb functions mainly as a sink (Hew and Yong 1994). However, the pseudobulb can also act as a source when the level of carbohydrates decreases due to drought stress (He et al. 2011; Stancato et al. 2001). Although studies have continued to focus on the function of stored water and carbohydrates in pseudobulbs, quantitative research on the physiological response of pseudobulbs to drought stress is still limited, especially when comparing between terrestrial and epiphytic orchids.

Clonal reproduction is a widespread phenomenon in the Orchidaceae, with tubers or rhizomes forming in terrestrial species, and rhizomes in epiphytic species (Cribb and Gasson 1982). Clonal integration enables plants to exchange resources between ramets efficiently (de Kroon et al. 1996; Liu et al. 2007; Lu et al. 2015; Mondragón et al. 2004). Having resource-storing pseudobulbs may cause clonal-integrated orchids to exhibit different functions compared with typical clonal-integrated plants, such as *Catasetum viridiflavum*, the stores in plants with 1 to 3 pseudobulbs appear to be important for both vegetative growth, whereas for a plant with more pseudobulbs, their number is not correlated to vegetative growth (Zimmerman 1990). Therefore, we designed our study to investigate the possible relationship between different-aged pseudobulbs and the resource sharing caused by physiological integration, especially water exchange.

Mature plants of both epiphytic and terrestrial life forms within *Cymbidium* (Orchidaceae) usually possess more than four annually produced pseudobulbs. The traits make the genus suitable for investigating the functional differences of pseudobulbs between epiphyte and terrestrial species and at different pseudobulb ages. In this study, we used epiphytic *Cymbidium tracyanum* and terrestrial *C. sinense* grown under the same environmental conditions, with each tested plant having four pseudobulbs. Our main objectives were to (1) characterize the structural and physiological responses to drought by pseudobulbs from epiphytic and terrestrial species; and (2) quantitate the differences in physiological functions by different-aged pseudobulbs under drought conditions. The goal was to expand our understanding of the functional diversification of pseudobulbs and ecological adaptations by orchids.

Methods

Plant materials

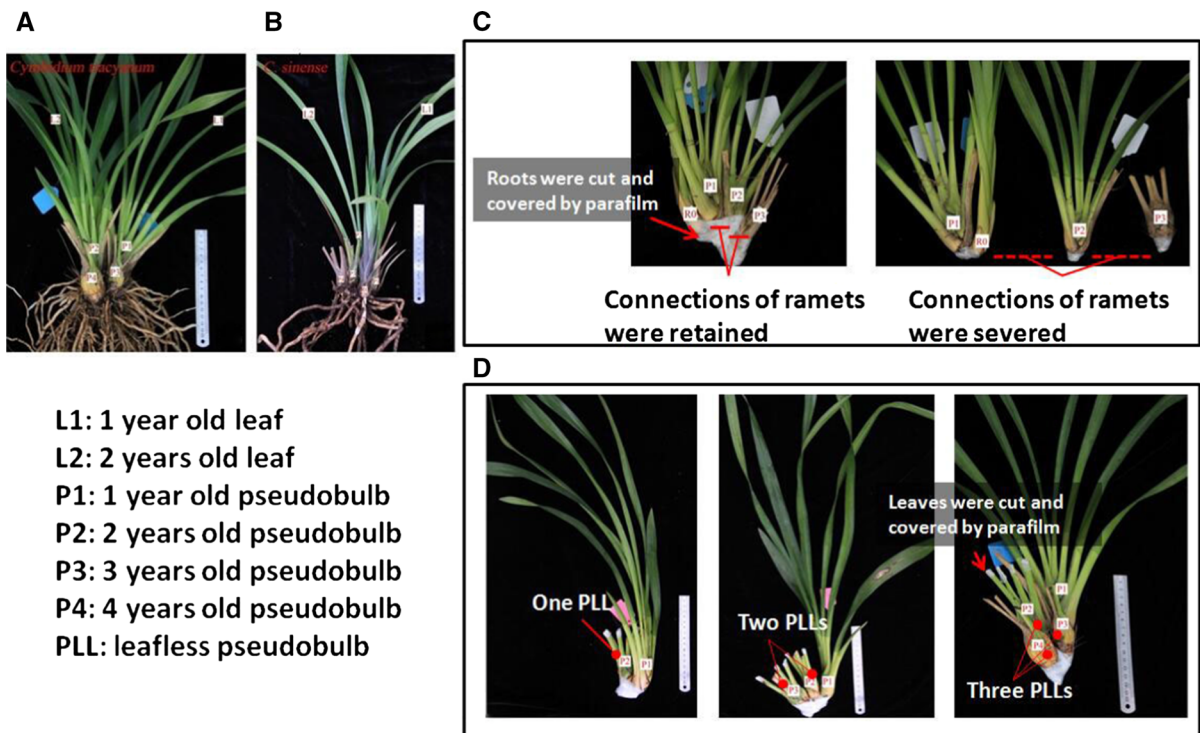
Cymbidium tracyanum and *C. sinense* were selected to compare differences in the functional diversification of their pseudobulbs in response to drought stress. The former is a typical epiphytic orchid grown on tree trunks in subtropical forests of southeastern China at elevations from 1200 to 2000 m, while the latter is a

terrestrial orchid always grown on forest floors in well-drained, shaded thickets of subtropical and tropical forests in southeastern China, at elevations of 300 to 1500 m. Both species are capable of clonal growth, but their rhizomes are short and ramets grow as a cluster. The mean distance between adjacent ramets along the rhizomes is approximately 1 cm. The pseudobulbs can remain connected to the ramets even if the leaves drop (called leafless pseudobulbs). In this research, we used 26 mature individuals of fairly uniform size and examined four ramets per species after removing any redundant leafless pseudobulbs (Fig. 1b, c). They were planted in plastic pots containing a bark mixture and placed in a greenhouse at the Kunming Institute of Botany, Kunming, China. Growing conditions included 20% full sunlight (300 to 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$), temperature of 18 to 24 °C, and relative humidity of 50 to 70%. Before the experiments began, the plants were watered to maintain soil water content (WC) of 65 to 75%. The soil WC was calculated for each individual according to the weight of the soil at the time of watering (initial weight) and the weight of the soil after being dried at 100 °C for

48 h (final weight). Therefore, soil WC = (initial weight – final weight)/initial weight \times 100.

Soil drought treatments

We compared the responses of *C. tracyanum* and *C. sinense* to gradually anabatic drought and recovery. Prior to treatment, anatomical details about the newly mature pseudobulbs were recorded, net photosynthesis (A_n) were measured from 1- and 2-year-old leaves for two species from 09:00 to 11:30 a.m.in the morning, total soluble sugar and starch were measured from 1-, 2-, 3- and 4-year-old pseudobulbs for two species, and the concentrations of ABA were measured from 1- and 2- year-old leaves and 1-, 2-, 3- and 4-year-old pseudobulbs and roots in each species. Afterward, the plants were arranged in a completely randomized design in which half of them were watered to maintained soil WC of 65 to 75% (control), while irrigation was halted for the other half to allow the soil to dry. When the soil WC was reduced to 45 to 55%, 25 to 35%, or 5 to 15%, the parameters mentioned above were measured again in same organs at each of



- L1: 1 year old leaf
- L2: 2 years old leaf
- P1: 1 year old pseudobulb
- P2: 2 years old pseudobulb
- P3: 3 years old pseudobulb
- P4: 4 years old pseudobulb
- PLL: leafless pseudobulb

Fig. 1 Plant with four ramets from *Cymbidium tracyanum* (a) and *C. sinense* (b). Example of “ramets severance treatment” (c) and “multiple leafless pseudobulbs treatment” (d) in *Cymbidium tracyanum*

those moisture levels. After those measurements were made for plants exposed to 5% to 15% WC, water was again applied so that the soil could recover to 60% to 75% WC. During that recovery phase, leaf gas exchange was measured daily until the values for A_n returned to the untreated control level. Meanwhile, post-recovery values were also determined for those parameters mentioned above.

We found that water stored in pseudobulbs can be used during drought stress in *C. tracyanum*, but not in *C. sinense*, so we did “Ramets severance treatment” (de Kroon et al. 1996; Zhang et al. 2012) and “Multiple leafless pseudobulbs treatment” (Zimmerman 1990) to clarify water use strategy in different ages of *C. tracyanum* during drought stress.

Ramets severance treatment

After removing the redundant, old leafless ramets from *C. tracyanum* plants, only the three most recently formed ramets were kept for exploring their physiological functions at different ages in response to drought stress. All roots of these plants were also cut subject plants to sever drought, and the incisions were sealed with parafilm. For half of the plants, the connections of ramets between pseudobulbs were severed with knife, while those connections were retained for the other half of the plants (Fig. 1c).

Values for A_n were measured from 09:00 to 11:30 a.m. in the morning daily from 1- and 2-year-old leaves. When those values dropped below zero for one of the plants, we collected all pseudobulbs and determined their WC and ABA concentrations.

Multiple leafless pseudobulbs treatment

We used plants of *C. tracyanum* that had four ramets of different ages. The roots and all of the leaves except the youngest one (1 year old) were removed, and the incisions were sealed with parafilm. This design provided us with plants that had one, two or three leafless pseudobulbs after the superfluous ramets were trimmed away (which means that leaves of 2-year-old ramet and pseudobulbs of 3- and 4-year old ramets were trimmed to let the plants had one leafless pseudobulb, leaves of 2-year-old ramet and pseudobulb of 3-year-old ramets were trimmed to let the plants had two leafless pseudobulbs, leaves of 2-year-old

ramet were trimmed to let the plants had three leafless pseudobulbs) (Fig. 1d).

Decreases in A_n values for each plant in the three groups were monitored from 09:00 to 11:30 a.m. in the morning daily. Measurements continued until the A_n values dropped below zero, at which time we made note of duration over which A_n had remained positive. We then collected all of the pseudobulbs for determining WC and ABA concentrations.

Examinations of pseudobulbs anatomy and water content

Transverse sections of the middle part of newly mature pseudobulbs were examined and photographed with a digital camera mounted on a Leica DM2500 microscope (Leica Microsystems Vertrieb GmbH, Wetzlar, Germany) and a Leica S8APO dissecting microscope (Leica Microsystems Vertrieb GmbH, Wetzlar, Germany). Six fresh pseudobulbs from different individuals and randomly selected images (36 per species) were used. Samples were photographed at $\times 5$ magnification to observe the area of the water-storage cell, and were photographed at $\times 10$ magnification to observe the area of the xylem and vascular bundle lengths under the microscope. Samples were photographed at $\times 4$ magnification to observe vascular bundle density using the dissecting microscope. These parameters were measured with the Image J software (National Institutes of Health, USA).

Six 1- and 2-year-old leaves, 1-, 2-, 3- and 4-year-old pseudobulbs or roots were sampled from different plants, and immediately weighed to obtain their fresh (FW) before 09:00 a.m. in the morning. The samples were then oven-dried at 60 °C for 48 h before determining dry weight (DW). Water content was calculated as $(FW - DW)/FW \times 100$.

Measurements of leaf gas exchange

An open gas exchange system incorporating infrared CO_2 and water vapor analyzers (Li-6400, Li-Cor Inc, Lincoln, NE, USA) was used to determine the values of A_n for six mature leaves. During the measurements period, the light level was set at a saturation intensity of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ (based on photosynthetic light response curves, data not shown), the relative air humidity was 60%, the air temperature was 22 °C, and the CO_2 concentration was maintained at

400 mol mol⁻¹. All measurements were made from 09:00 to 11:30 a.m., when CO₂ uptake was maximal.

Analysis of ABA accumulation

After each leaf sample was ground to fine power under liquid nitrogen, an approximately 200 mg sample was collected in a centrifuge tube. To each tube, we added 1 mL of ethyl acetate spiked with 5 ng of D₄-ABA to be used as the internal standard. The samples were then vortexed for 10 min. After centrifugation at 13,000×g for 10 min at 4 °C, the supernatants were transferred to fresh 2-mL tubes, and then evaporated to dryness on a vacuum concentrator (Eppendorf, Hamburg, Germany). Each residue was resuspended in 0.5 mL of 70% methanol (v/v), vortexed for 10 min, and centrifuged at 13000×g for 10 min at 4 °C to clarify the phases. The supernatants were pipetted to glass vials and then analyzed by HPLC–MS/MS (LCMS-8040 system, Shimadzu, Kyoto, Japan). Measurements were conducted on a 1200-L liquid chromatography–mass spectrometry system (Varian). At a flow rate of 0.1 mL min⁻¹, 15 mL of each sample was injected onto a Pursuit C8 column (3 m, 150 × 2 mm²) (Varian). A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) was used in a gradient mode for separation.

Analyses of total soluble sugars and starch

After the dried leaves were ground to pass through a 1-mm sieve, 0.1 g of powdered materials was placed in 10-mL centrifuge tube, to which 5 mL of 80% ethanol was added. The mixture was incubated in an 80 °C water bath for 30 min, and then centrifuged at 4000 rpm for 15 min. The pellets were re-extracted with 80% ethanol. Supernatants were combined for determining the total soluble sugars in the collected extracts, based on the anthrone method (Seifter et al. 1949). An aliquot of the extract was hydrolyzed in 5 mL of 0.4% anthrone solution (4 g of anthrone in 1000 mL 95% of H₂SO₄) in a boiling water bath for 15 min. After cooling, the total soluble sugar concentration was determined using a UV-2500 spectrophotometer (Shimadzu, Kyoto, Japan) at 620 nm. Glucose was used as a standard. The total soluble sugar concentration was calculated on a dry matter basis (% d.m.).

The ethanol-insoluble pellets were used for starch extractions. Starch in the residue was released in 2 mL of distilled water for 15 min in a boiling water bath. After cooling to room temperature, 2 mL of 9.2 mol L⁻¹ HClO₄ was added. Starch was hydrolyzed for 15 min before 4 mL distilled water was added. The samples were then centrifuged at 4000 rpm for 10 min. After the pellets were re-extracted with 2 mL of 4.6 mol L⁻¹ HClO₄, the supernatants were combined and made up to 20 mL. Starch concentrations were measured spectrophotometrically at 620 nm using anthrone reagent, and were calculated by multiplying glucose concentrations by the conversion factor of 0.9 (Li et al. 2008).

Statistical analysis

Statistical analysis was performed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The data were subjected to analysis of variance (ANOVA) and independent-sample *t* tests. Tukey's multiple comparison tests were used at the $\alpha = 0.05$ level to determine whether significant differences existed between drought treatments.

Results

Differences in anatomical and morphological traits of pseudobulbs between *C. tracyanum* and *C. sinense*

The pseudobulb transverse sections were anatomically similar between species, showing vascular bundles scattered throughout parenchymatous ground tissue that comprised smaller assimilatory cells and larger water-storage cells (Fig. 2). However, *C. tracyanum* had larger water-storage cells (Fig. 2c, d; Table 1), *C. sinense* had higher density of vascular bundle (Fig. 2a, b; Table 1).

Compared with *C. sinense*, the pseudobulbs WC and the ratio of total leaf areas to pseudobulbs dry weight were higher for *C. tracyanum* (Table 1).

Physiological responses of *C. tracyanum* and *C. sinense* to drought treatments

As the soil WC declined, values for A_n in different-aged leaves (1- vs. 2-year-old) decreased gradually in

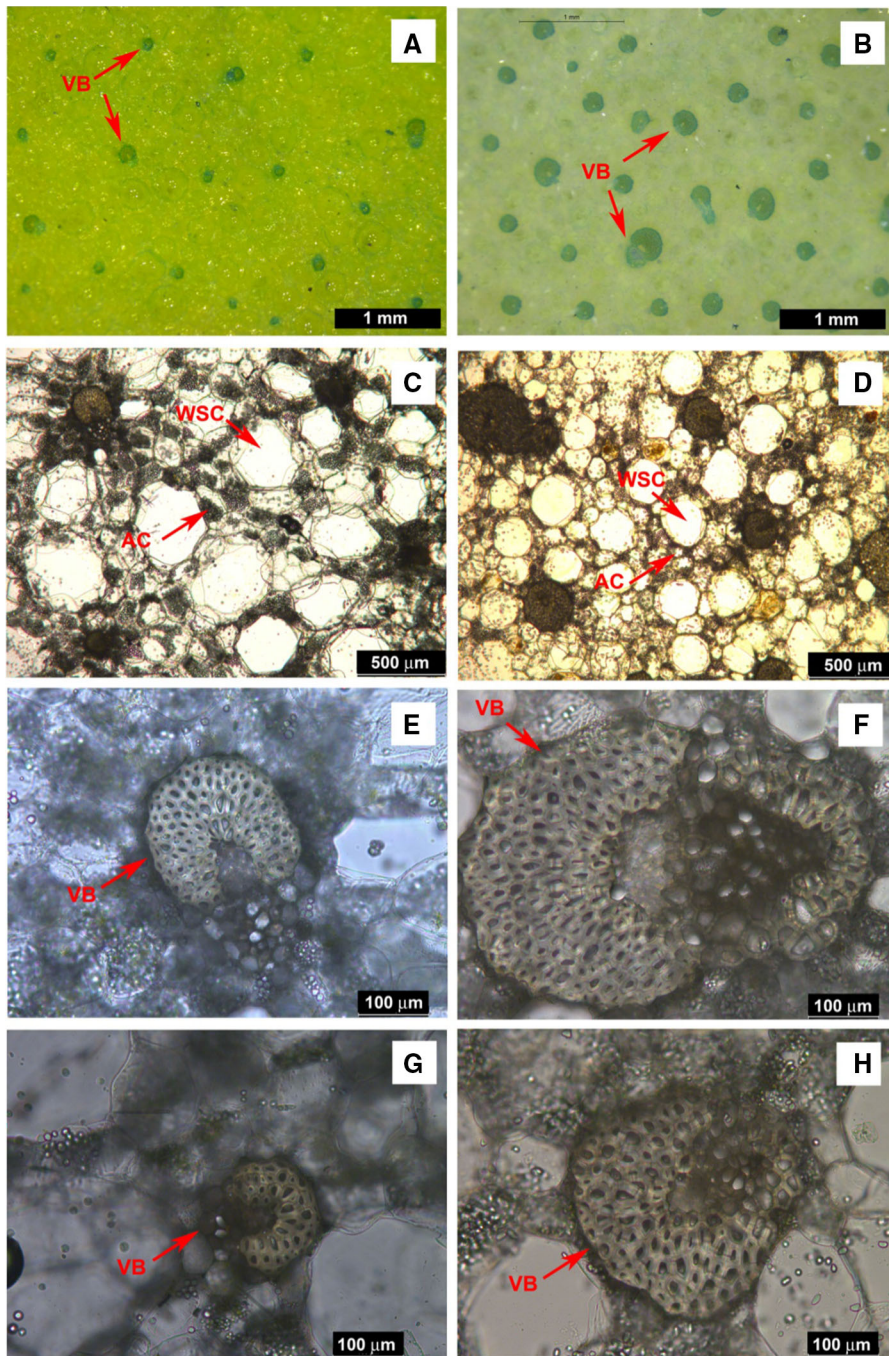


Fig. 2 Anatomical observations of pseudobulbs from *Cymbidium tracyanum* and *C. sinense*. **a, c** pseudobulb cross section from *C. tracyanum*; **b, d** pseudobulb cross section from *C.*

sinense; **e, g**, vascular bundle cross section from *C. tracyanum*; **f, h** vascular bundle cross section from *C. sinense*. *VB* vascular bundle, *WSC* water stored cells, *AC* cells

both *C. tracyanum* and *C. sinense*, but those differences were not significant between species at each age. Meanwhile, A_n remained significantly higher

($p < 0.05$) in *C. tracyanum* than in *C. sinense* after the soil WC dropped to 15 to 25% in 1-year-old leaves, to 25 to 35% for 2-year-old leaves. When soil WC was

Table 1 Functional traits of pseudobulbs in *Cymbidium tracyanum* and *C. sinense*

	<i>Cymbidium tracyanum</i>	<i>C. sinense</i>	Significance
Relative water content (%)	0.88 ± 0.01	0.76 ± 0.02	0.00
Leaf area/pseudobulb dry weight (mm ² g ⁻¹)	147.25 ± 14.71	73.22 ± 16.21	0.015
Area of water-storage cell (mm ²)	134,801.78 ± 19,832.43	65,042.69 ± 6074.64	0.025
Area of xylem (mm ²)	451.05 ± 14.97	479.82 ± 27.94	0.632
Vascular bundle density (number mm ⁻²)	5.56 ± 0.51	8.03 ± 0.52	0.023
Vascular bundle length (mm)	0.12 ± 0.04	0.26 ± 0.08	0.218

Data are mean ± SE ($n = 4-6$), statistical differences (p values) were determined with independent-sample t tests

reduced to 5 to 15% (30 days treatment), A_n dropped to a negative value. However, after soil WC rose to 60 to 75% for 3 days, A_n returned to the level found under well-watered conditions (Fig. S1).

During the drought-stressed and recovery processes, values for WC did not change significantly in the leaves and roots of either species, or in the *C. sinense* pseudobulbs. However, the WC for pseudobulbs from *C. tracyanum* decreased gradually, with the rate of decline being faster for 1-year-old pseudobulbs that were attached to immature ramets (Fig. 3a). However, when the soil WC restored, the pseudobulbs WC recovered immediately for *C. tracyanum* (Fig. 3).

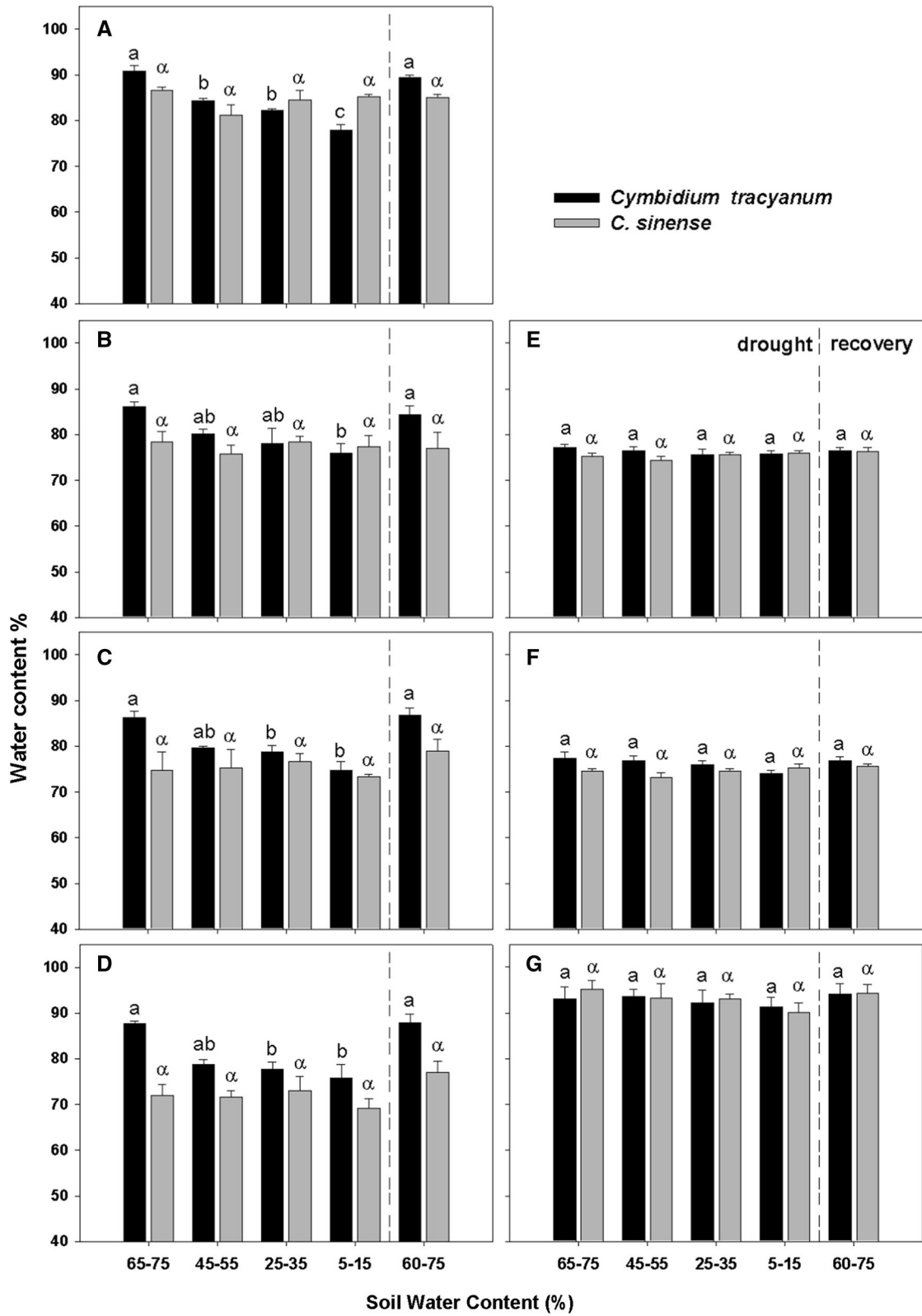
Under well-watered conditions, the ABA concentration was significantly higher in pseudobulbs from *C. tracyanum* than in leaves and roots of that species. In contrast, ABA concentrations were not markedly different among the leaves, roots, and pseudobulbs from *C. sinense* (Fig. 4). As the degree of drought intensified, ABA concentration increased in 1-, 2- and 3-year-old pseudobulbs of *C. tracyanum*, but not in its 4-year-old pseudobulbs (Fig. 4d). For *C. tracyanum*, the ABA concentration in pseudobulbs increased significantly when soil WC dropped to 15% (drought sustained for approximately 30 days), while the same response was found in the leaves and roots when soil WC dropped to 45 to 55% (drought sustained for approximately 5 days). However, the significant increase in ABA concentrations for *C. sinense* pseudobulbs and roots occurred when soil WC dropped to 25 to 35% (drought sustained about 20 days), a trend that was the same as noted for the leaves of *C. tracyanum*. After rehydration, the values of ABA concentrations were restored to normal levels for all organs tested in both species (Fig. 4).

Before the drought treatments began, total soluble sugar and starch concentrations did not differ significantly among pseudobulbs of different ages from *C. tracyanum*. However, for *C. sinense*, the level of total soluble sugar decreased as pseudobulbs age increased while the opposite pattern was observed for starch concentrations (Fig. 5). Under drought conditions, total soluble sugar concentrations in the pseudobulbs of any age were not markedly changed in either species. After recovery for 3 d, however, total soluble sugar concentrations were significantly lower in the 1- and 2-year-old pseudobulbs from *C. sinense*. For *C. tracyanum*, starch concentrations in 1-year-old pseudobulbs declined noticeably when soil WC dropped to 5 to 15%, and decreased in pseudobulbs of all ages during the recovery period. By comparison, the starch concentration in *C. sinense* decreased significantly when soil WC dropped to 5 to 15% and during recovery period only in 1-year-old pseudobulbs (Fig. S2).

Physiological responses of *C. tracyanum* to “ramets severance treatment” and “multiple leafless pseudobulbs treatment”

When roots were removed from *C. tracyanum*, A_n values gradually decreased as the treatment period was extended. However, the decline in A_n values in 2-year-old leaves was slower in the nonsevered ramets than in the severed ramets (Fig. S3).

Compared with the untreated group, the final WC for both 1- and 2-year-old pseudobulbs was markedly reduced in the severed and nonsevered treatments. The final WC for 2-year-old pseudobulbs was lower in the severed treatment than in the nonsevered treatment. Values for WC in the leafless pseudobulbs (3-year-



◀ **Fig. 3** Changes in water content (WC) of 1-year-old leaves (e), 2-year-old leaves (f), 1-year-old pseudobulbs (a), 2-year-old pseudobulbs (b), 3-year-old pseudobulbs (c), 4-year-old pseudobulbs (d) and roots (g) for *Cymbidium tracyanum* and *C. sinense* plants during drought-stress treatment. Each vertical bar represents mean \pm SE for six measurements from individual plants. Different letters above bars indicate significant differences in each parameter between drought treatments ($p < 0.05$, based on ANOVA, followed by Tukey's post hoc tests for comparison)

old) hardly changed when they were detached from clonal integration, but were significantly decreased when the pseudobulbs were still connected to the other ramets (Fig. 6a).

At the end of the drought treatment, ABA concentrations in the 1- and 2-year-old pseudobulbs were similar between the severed and nonsevered groups, but were significantly higher than in the unstressed group. However, the ABA concentration was significantly lower in the severed leafless pseudobulbs than in either the nonsevered or untreated groups (Fig. 6b).

When the roots were removed from the multiple leafless pseudobulbs of *C. tracyanum*, leaving no water supply to the plants, plants with more leafless pseudobulbs maintained leaf photosynthesis for longer time, i.e., Leaf photosynthesis maintained about 6 days for plants with one leafless pseudobulb, 11 days for the plants with two leafless pseudobulbs and 25 days for the plants with three leafless pseudobulbs (Fig. S4A). The daily decline in WC for 1-year-old pseudobulbs was slower when the plants had more leafless pseudobulbs (Fig. S4B). At the end of the treatment period, the final WC was similar of leafless pseudobulbs with different age (Fig. S4B inset).

Discussion

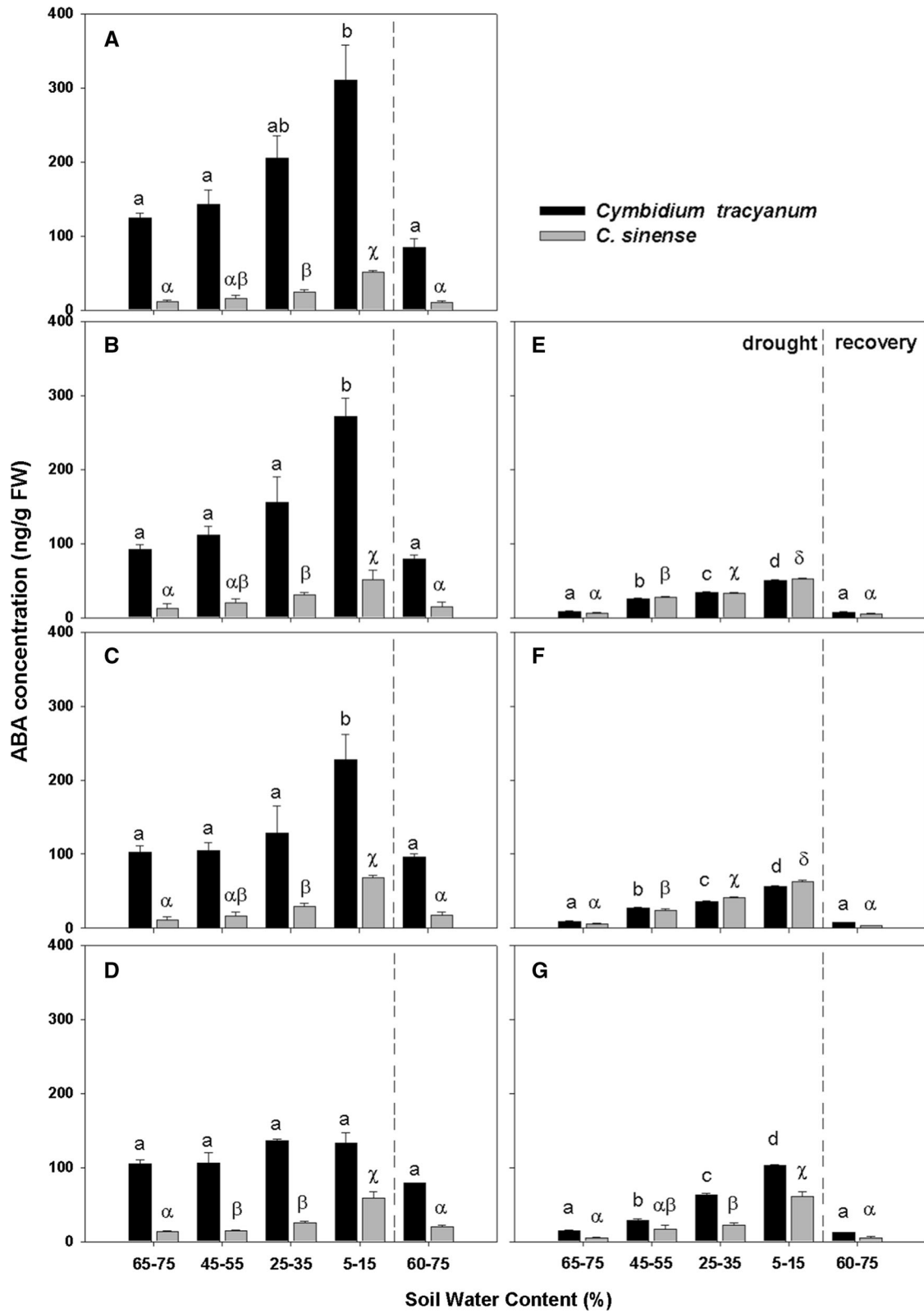
Different utilization strategies for storing water in pseudobulbs of *C. tracyanum* and *C. sinense*

Pseudobulbs are common water-storage organs in both epiphytic and terrestrial types of orchids (Ng and Hew 2000). Here, we found significant differences in water-related traits and physiological responses of pseudobulbs to drought between epiphytic and terrestrial orchids. Compared with *C. sinense* (terrestrial), *C. tracyanum* (epiphytic) have larger water-storage cells

and higher WC (Fig. 2c, d; Table 1), which might enable the latter to maintain normal physiological functions over a longer time under water deficit condition.

As expected, *C. tracyanum* was able to utilize the water stored in pseudobulbs quickly during the drought-stressed treatments (Fig. 3a–d), and leafless pseudobulbs helped support photosynthesis for a long time (Fig. S4A). Sinclair (1983) showed that various epiphytic orchids can buffer transpiration to extend stomatal conductance and photosynthesis over 20 days when plants are exposed to low levels of soil moisture. Furthermore, *Dimerandra emarginata*, a typically epiphytic orchid in tropical moist forest, maintains normal leaf water content for 23 days without rain (Zotz 1999). Other drought-avoiding succulent plants that generally have high-capacity water-storage tissues utilize a similar strategy that uses stored water to buffer the transpiration stream and extend carbon uptake during periods of drought (Ogburn and Edwards 2010). These include *Tillandsia schiedeana* (Bromeliaceae), which can buffer transpiration for 34 days (Martin 1994); *Opuntia ficus-indica* (Cactaceae), for 20 days (Acevedo et al. 1983); *Ferocactus acanthodes* (Cactaceae), for 40 days (Nobel 1977); and *Agave deserti* (Agavaceae), for 8 days (Nobel 1976).

When compared with *C. tracyanum* and the above-mentioned species, water stored in pseudobulbs of terrestrial *C. sinense* was rarely used under drought stress—maybe just similar to caudiciform succulent trees which exhibit a different way to use stored water. For example, although baobab (*Adansonia* spp.) trees have large trunks with highly parenchymatous, water-storing wood, they do not use stem water stores to extend their diurnal opening of stomata (Chapotin et al. 2006c). Instead, those stem water stores can be used for flushing new leaves before the onset of the rainy season, with a concomitant decrease in stem volume of up to 12% (Chapotin et al. 2006a). It is hypothesized that baobab stem water is largely unavailable to the transpiration stream, the effect of stem-stored water occurs on a seasonal rather than a diurnal scale (Chapotin et al. 2006b). We guess that the water stored in pseudobulbs of *C. sinense* may be used to meet seasonal needs, rather than short-term demands.



◀ **Fig. 4** Changes in concentrations abscisic acid (ABA) of 1-year-old leaves (e), 2-year-old leaves (f), 1-year-old pseudobulbs (a), 2-year-old pseudobulbs (b), 3-year-old pseudobulbs (c), 4-year-old pseudobulbs (d) and roots (g) for *Cymbidium tracyanum* and *C. sinense* plants during drought-stress treatment. Each vertical bar represents mean \pm SE for 6 measurements from individual plants. Different letters above bars indicate significant differences in each parameter between drought treatments ($p < 0.05$, based on ANOVA, followed by Tukey's post hoc tests for comparison)

Different utilization strategies for carbohydrate storage in pseudobulbs of *C. tracyanum* and *C. sinense*

We previously reported that *C. tracyanum* and *C. sinense* are isohydric species (Li et al. 2017), closing their stomata and maintaining nearly constant leaf water potential. However, it may induce reducing of CO₂ diffusion into the leaves early during a drought period. Under such stress, isohydric species that rely upon carbon reserves may be more susceptible to carbon starvation (Tardieu and Simonneau 1998).

Under well-water conditions, we found here that the total concentrations of sugar and starch were higher in *C. tracyanum* than in *C. sinense*, and the opposing trends, with concentrations of sugar and starch rising as pseudobulbs ages increased for *C. sinense*, but not for *C. tracyanum* (Fig. 5). The sum of soluble sugars and starch is the pool of nonstructural carbohydrates (NSCs). These NSCs can provide a temporary source of carbon when current photosynthesis cannot meet the immediate demands (Gessler and Treydte 2016; Hartmann and Trumbore 2016; von Arx et al. 2017), our finding means that *C. tracyanum* has greater potential to survive an eventual stress.

Previous studies with ABA have found that it acts as a growth inhibitor (Barrero et al. 2005; Nagel et al. 1994). During drought treatment, the ABA concentration in most pseudobulbs of *C. tracyanum* increased gradually as the stress intensified, with the exception being the oldest leafless pseudobulb (Fig. 4d), and ABA concentration significantly decreased in leafless pseudobulbs when they were separated from plants (Fig. 6b). Together with the relatively stable NSC of different-aged pseudobulbs in *C. tracyanum*, we speculate that the leafless pseudobulbs own the ability of reconstruction of plant clonal integration during disadvantageous environment. That is, the relatively

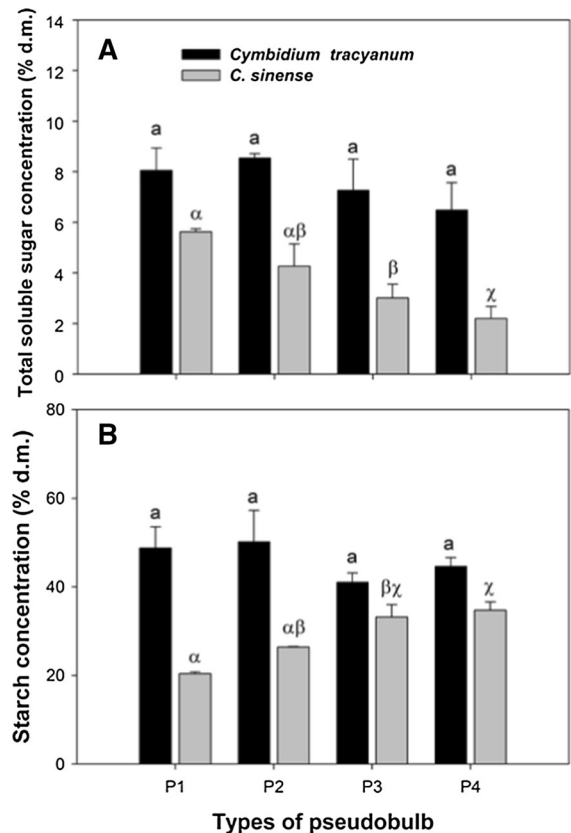


Fig. 5 Changes in total soluble sugar (a) and starch concentration (b) for pseudobulbs of *Cymbidium tracyanum* and *C. sinense* in different ages (P1–P4 refers to caption for Fig. 1). Each vertical bar represents mean \pm SE for six measurements from individual plants. Different letters above bars indicate significant differences in each parameter between ages ($p < 0.05$, based on ANOVA, followed by Tukey's post hoc tests for comparison)

lower ABA concentrations when plants were exposed to drought stress and higher NSC concentration in leafless pseudobulbs offer the buds a chance to germinate.

Compared with *C. sinense*, *C. tracyanum* can make better use of stored starch as a mechanism for drought resistance and to recover normal physiological functions more quickly (Fig. S2). In most plants, starch serves as the main carbohydrate reserve in most plants, and can be rapidly mobilized to provide soluble sugars. Its metabolism is very sensitive to environmental changes (Krasensky and Jonak 2012). Sugars that accumulate in response to stress can function as an osmolyte to maintain cell turgor and protect membranes and proteins from stress damage (Kaplan and

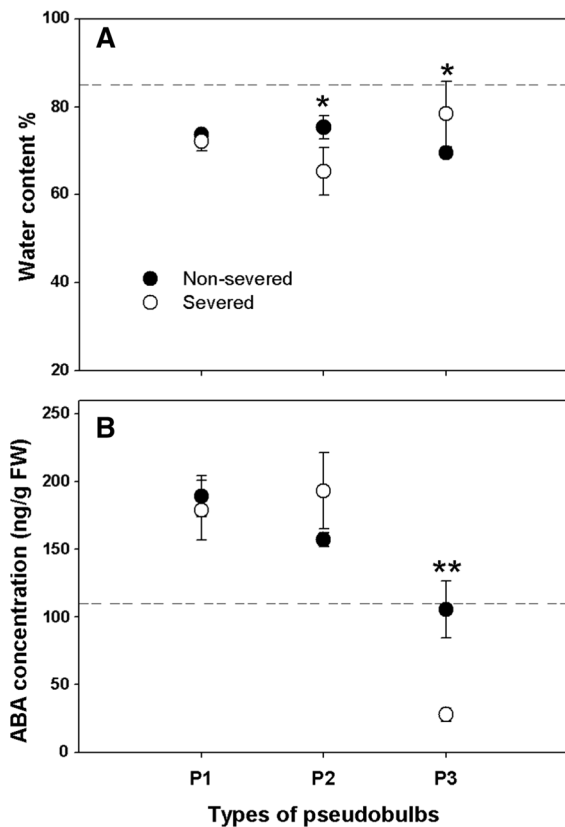


Fig. 6 Final relative water content (WC) (a) and abscisic acid (ABA) concentrations (b) after “ramets severance treatment” (P1–P3 refers to caption for Fig. 1). Horizontal dashed lines represent mean values of WC or ABA concentration of untreated pseudobulbs. Statistical differences (p values) between the two species at different severed treatment were determined with independent-sample t tests. ($p > 0.05$; * $p < 0.05$; ** $p < 0.01$)

Guy 2004). The transition between starch and sugar is important for plants tolerance to drought. Drought generally leads to a depletion of starch and the accumulation of soluble sugars (Todaka et al. 2000), so that the leaves can maintain an adequate level of soluble sugars under drought conditions (Chaves et al. 2003). This flexibility in converting starch to sugar may suggest that epiphytic *C. tracyanum* can adapt to longer periods of drought stress compared with *C. sinense*.

Different accumulate patterns of ABA in different organs of *C. tracyanum* and *C. sinense*

ABA functions as a plant stress hormone. It is assumed that drought stress induces a rise in ABA level in the

root and leaf as result of transportation or biosynthesis (Tardieu and Davies 1993; Zhang et al. 2018). Stomatal opening was highly sensitive to endogenous levels of foliar ABA and that drought stress in *C. tracyanum* and *C. sinense* both caused an rise in foliar ABA levels (Fig. 4e, f; Fig. S1). While due to the rapid decrease of ABA in the leaves after water recovery, stomata were able to completely reopen for 3 days of the two species (Fig. 4e, f; Fig. S1), this means that the plants can immediately benefit from improved moisture condition (Brodrribb and McAdam 2013).

We found that *C. tracyanum* plants have a higher concentration of ABA in pseudobulbs (Fig. 4a–d). Although, possible interactions between ABA and aquaporins (AQPs) which can facilitate water transport across biological membranes (Kaldenhoff et al. 2008) are not yet well understood, several studies have demonstrated that AQPs are upregulated by ABA. For example, this phytohormone induces transcription factors that regulate the expression of PIP AQPs in *Arabidopsis* (Kaldenhoff et al. 1996; Shinozaki et al. 1998). Exogenous ABA can increase the expression of 12 PIPs in both roots and the above ground parts of *Arabidopsis* plant (Jang et al. 2004). For *Zea mays* (maize), ABA increases gene expression and the protein content of most PIP isoforms (Parent et al. 2009). If one considers the rapid utilization and recovery of storage water and lower-density vascular bundles in pseudobulbs of *C. tracyanum* (Fig. 3a–d), it is possible that the interaction between ABA and AQPs was involved in physiological response of pseudobulbs to drought stress.

In the field, roots of epiphytes may partially experience drought stress due to heterogenous environment of their habitat (Li et al. 2017; Lu et al. 2015). And previous studies have found that ABA can be rapidly generated by partially dried root systems which will decrease stomatal conductance in the absence of changes in leaf water status (Dodd et al. 2010; Stoll et al. 2000). For epiphytic *C. tracyanum*, compared with roots, ABA concentration changed less sensitively in pseudobulbs during drought stress which increased significantly when soil water content drops by 5% to 15% (Fig. 4a–c). However, ABA concentration increased just as soil water content drop to 45% to 55% in roots (Fig. 4g). This indicates that pseudobulb can weaken the effect of the frequently increased ABA induced by roots during drought stress in the field.

In summary, our results revealed the differences in the morphology and physiology of different-aged pseudobulbs from epiphytic and terrestrial within *Cymbidiums* orchids in response to drought stress. Pseudobulb from the epiphytic *C. tracyanum* featured more drought-tolerance traits compared with the terrestrial *C. sinense*. The former was also more flexible in its ability to utilize stored water and carbohydrates from the pseudobulb when plants were responding to drought stress. And higher ABA concentration in pseudobulbs may help *C. tracyanum* to sustain survival in epiphytic habitats. These findings improve our current understanding about the functional diversification of pseudobulbs in epiphytic and terrestrial life forms and at different ages. Further research, based on long-term observations and controlled experiments, is needed to investigate the function of pseudobulbs.

Acknowledgements The authors are grateful to Dr Jia-lin Huang (Key Laboratory for Economic Plants and Biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China) for his help in the preparation of our experimental materials. And our research was financially supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDB3101), the National Natural Science Foundation of China (31670342) and the CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science (09KF001B04).

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