

# Age-related differences in physiological and metabolic responses of *Pleione aurita* (Orchidaceae) pseudobulbs to drought stress and recovery

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## ABSTRACT

The pseudobulb is a storage organ for water and nutrients that plays a crucial role in the growth and survival of epiphytic orchids. However, the role of water and metabolites in pseudobulb during adaptation to environmental stress are rarely detected through control experiments. In the present study, water-related physiological traits and metabolite changes in the pseudobulbs at the flowering stage and full leaf expansion stage for *Pleione aurita* were investigated after drought stress and recovery treatments. We found that the composition of non-structural carbohydrates (starch vs. soluble sugar) varied over the lifetime of pseudobulbs, and older pseudobulbs stored more water, whereas younger pseudobulbs stored more dry matter. When plants were subjected to drought stress and subsequent recovery, multiple metabolites in the pseudobulbs including non-structural carbohydrates, flavonoids, phenolic acids, as well as amino acids and their derivatives responded positively to these water level fluctuations. For those metabolites that differently accumulated in both stress and recovery processes, old pseudobulbs contained a higher number of these key metabolites than did the connected younger pseudobulbs. In addition, young and old pseudobulbs use different metabolic pathways to both respond and recover to drought. These results indicate that orchid pseudobulbs cope with water level fluctuations by mobilizing metabolite reserves and that pseudobulbs of different ages exhibit different physiological and metabolic responses to drought stress. These findings broaden our understanding of the role pseudobulbs play in the survival of orchids growing in epiphytic habitats.

## 1. Introduction

Epiphytic plants germinate and root on other plants (usually a tree) but do not parasitize their hosts (Benzing, 1990). Represented by more than 27,000 species, vascular epiphytes account for approximately 9% of extant vascular plant diversity (Zotz, 2013). Unlike terrestrial plants, which obtain a stable supply of water and nutrients from the soil, epiphytic plants rely largely on intermittent water and nutrient supply from the atmosphere (Benzing, 1990, 1998). Different plant taxa therefore have evolved various adaptations to cope with the intermittent supply of water and nutrient in the epiphytic habitat. For example, orchids thicken and enlarge their stems to form succulent organs. The

specialized stems of orchids, i.e., the pseudobulbs, occur in roughly half of all orchid genera (Zotz, 2016) and play a crucial role in the growth and survival of orchids (He, 2018; Ng and Hew, 2000).

The storage of nutrients and water is essential for epiphytic plants to survive harsh environments. As mineral and carbohydrate storage organs, pseudobulbs are reserves of photosynthetic products and recycled minerals from leaves, and these reserves are important in the initiation of new growth during the next growing season (Wang et al., 2008; Yong and Hew, 1995a; Zhang et al., 2022; Zimmerman, 1990). Among abiotic factors, water is the key limitation to the growth of epiphytic plants (Laube and Zotz, 2003; Zotz et al., 2010). Pseudobulbs act as water reserves, both storing water to mitigate water loss from leaves during

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drought stress and contributing to the fast recovery of plants when water is available (He et al., 2013; Li and Zhang, 2019; Yang et al., 2016). Pseudobulbs also cope with drought stress by mobilizing multiple stored components. One class of stored components that play an important role in the maintenance of cellular osmotic potential is sugar (Morgan, 1984), in orchid pseudobulbs particularly, non-structural carbohydrates (NSC, including starch and soluble sugars). The role of NSCs in the response of pseudobulbs to drought stress has been studied extensively (Li et al., 2018; Li and Zhang, 2019; Stancato et al., 2001; Zotz, 1999), largely because NSCs can be converted to increase concentrations of osmotically active solutes that moderate the effects of drought. Other metabolites, such as free amino acids, organic acids, phenols and flavonoids, have attracted less attention in orchid pseudobulbs (Tay et al., 2019), although they have been shown to participate in adjustments during adaptation to water deficit in many other species (Niinemets, 2016; Rangani et al., 2020; Vijayaraghavareddy et al., 2021; Zhao and Ren, 2021).

An orchid plant usually possesses several pseudobulbs of different ages, and these young and old pseudobulbs have been shown to have divergent physiological responses to drought stress (Li and Zhang, 2019). Research has also indicated that both the water and metabolite content of pseudobulbs differ at various developmental stages or seasons (Jin et al., 2016; Wang et al., 2008; Yong and Hew, 1995a; Zimmerman, 1990). However, little is known about specific metabolite changes during development or in response to drought stress and recovery. One possible approach to addressing this gap in our knowledge is metabolomics, which has been used to investigate plant responses to abiotic stresses (Arbona et al., 2013; Sawada et al., 2009), as metabolites can serve as proxies for gene expression, protein interactions and other regulatory processes.

Previous studies have suggested that the water and nutrients stored in pseudobulbs plays a key role in the growth and survival of epiphytic orchids, but the role of water and metabolites in pseudobulb during adaptation to environmental stress are rarely detected through control experiments. To understand the role of nutrients in pseudobulb under drought stress, we measured water-related physiological traits and used a widely targeted metabolomics technology based on ultra-performance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS) to analyze metabolite changes throughout the development of *P. aurita* pseudobulbs and during two drought and recovery events. We hypothesize that *Pleione aurita* pseudobulbs at distinct stages of development respond differently to drought stress and recovery.

## 2. Materials and methods

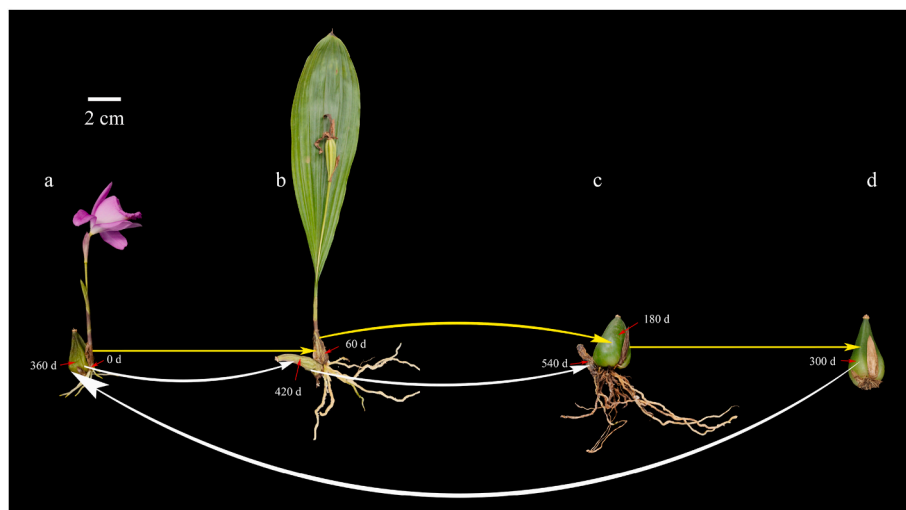
### 2.1. Plant material and the determination of pseudobulb age

The life history of *P. aurita* has been previously described (Zhang et al., 2017b, 2022). Briefly, new shoots with a flower are produced at the side of a pseudobulb in Spring (Fig. 1a). The base of the shoot swells during the expansion of the leaf, producing a new pseudobulb at around 60 d, or at full leaf expansion, two months after flowering (Fig. 1b). At 6 months, or 180 d, the plant sheds its leaf, the old pseudobulb and roots die, and the connected newly produced pseudobulb reaches its full size (Fig. 1c). The newly produced pseudobulb is dormant for four months (Fig. 1d), then produces flowers within two more months in the next growing season (Fig. 1a). The old pseudobulbs shown in Fig. 1a, b and c are 360 d, 420 d and 540 d, respectively.

### 2.2. Drought stress and recovery

A total of 300 dormant pseudobulbs were grown in plastic nursery trays (54 × 27 × 10 cm) containing a mixture of 70% bark, 20% moss, and 10% humus. These nursery trays were then placed in a greenhouse before the onset of the growing season in 2019. Environmental conditions inside the greenhouse included a 50% shading by shade nets and temperature of 20–30 °C throughout the growing season controlled by a humidification and ventilation system. Plants were fertilized with slow-release fertilizer (N–P–K ratio as 1:1:1) at the onset of the growing season and watered weekly, and drought stress was imposed by stopping irrigation for 20 d. Plants were either stressed at flowering (Fig. 1a) or at leaf full expansion (Fig. 1b) in the present study. Recovery treatment was to resume normal water for three days.

For plants at leaf full expansion, photosynthetic rate was monitored with a portable photosynthesis system (LI-6400, Li-Cor, USA) during and after drought treatment between 9:00 and 12:00 a.m.. During the recordings, a saturate light intensity (600  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was used, the atmospheric  $\text{CO}_2$  concentration was maintained at 400  $\mu\text{mol mol}^{-1}$  by a  $\text{CO}_2$  injector system (LI-6400-01; Li-Cor, USA), and relative air humidity in the leaf chamber and leaf temperature were maintained at approximately 80% and 25 °C, respectively. For pseudobulbs from each group (control, drought-stressed, recovery), water content was quantified and metabolite content analyzed. Water content of a pseudobulb was calculated as (fresh weight-dry weight)/fresh weight × 100%, and at least six plants were used for the measurements of net photosynthetic rate and water content.



**Fig. 1.** Development of *Pleione aurita* over a completely growing season and the age of pseudobulbs (modified from Zhang et al., 2022). a, flowering stage; b, leaf full expansion; c, leaf shedding; and d, dormant pseudobulb.

## 2.3. Metabolite analysis

### 2.3.1. Determination of non-structural carbohydrates

We identified metabolites accumulated in pseudobulbs treated independently to two drought and re-watering experiments. To investigate non-structural carbohydrate polymers in the pseudobulb, total water soluble sugars and starch were determined. Total water soluble sugars were extracted from 0.25 g pseudobulb samples with 5% acetic acid solution and then analysed with a continuous flow analyzer (AA3, Seal Analytical, Germany). Subsequently, starch was extracted from the residual material and hydrolyzed. Glucose served as a standard to determine the starch concentration using a spectrophotometer (UV-2500, Shimadzu, Japan) following the methods of Li and Zhang (2019). Three replicates, each of which was a mix from three plant individuals, were involved in the determination of non-structural carbohydrates.

### 2.3.2. Widely targeted metabolomic analysis

Pseudobulb samples were harvested and stored at  $-80^{\circ}\text{C}$ . Samples were freeze-dried, crushed and extracted following the method of Wang et al. (2021). Briefly, 0.6 ml 70% aqueous methanol was used to extract metabolites from 100 mg of powdered pseudobulb overnight at  $4^{\circ}\text{C}$ . Following centrifugation at 10,000g for 10 min, the extracts were absorbed and filtrated before analyzing by a UPLC-ESI-MS/MS system (UPLC, Shim-pack UPLC SHIMADZU CBM30A system; MS, Applied Biosystems 4500 QTRAP) at Metware Biotechnology Co., Ltd (Wuhan, China). Three replicates, each of which was a mix from three plant individuals, were involved in metabolomic analysis.

The UPLC conditions were as follows: column, Waters ACQUITY UPLC HSS T3 C18 (1.8  $\mu\text{m}$ , 2.1 mm\*100 mm); the mobile phase consisted of pure water with 0.04% acetic acid (solvent A) and acetonitrile with 0.04% acetic acid (solvent B). Sample measurements were performed with a gradient program that employed the starting conditions of 95% A and 5% B. Within 10 min, a linear gradient to 5% A, 95% B was programmed and maintained for 1 min. Subsequently, a composition of 95% A, 5% B was reached within 0.10 min and maintained for 2.9 min. The column oven was set to  $40^{\circ}\text{C}$ ; the injection volume was 4  $\mu\text{l}$ . The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

Linear ion trap (LIT) and triple quadrupole (QQQ) scans were conducted on a triple quadrupole-linear ion trap mass spectrometer (QTRAP, API 4500 QTRAP UPLC-MS/MS System) equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst software (ABSciex). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature  $550^{\circ}\text{C}$ ; ion spray voltage 5500 V/-4500 V; ion source gas I, gas II, curtain gas were set at 50, 60, and 30 psi, respectively; the collision gas was high. Instrument tuning and mass calibration were performed with 10 and 100  $\mu\text{mol/L}$  polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as multiple reaction monitoring (MRM) experiments with collision gas (nitrogen) set to 5 psi. Declustering potential (DP) and collision energy (CE) for individual MRM transitions was done with further DP and CE optimization. A specific set of MRM transitions was monitored for each period according to the metabolites eluted within this period. Metabolites were identified based on a self-constructed database (MWDB, MetWare database) and the public metabolite information database.

## 2.4. Statistical analysis

One-way ANOVA was used to examine differences between treatments, with means discriminated by LSD multiple comparison tests at  $p < 0.05$ . Hierarchical cluster analysis (HCA) was carried out in R package (v.3.6.1). Orthogonal partial least squares-discriminant analysis (OPLS-DA) was used to compare the metabolic profiles among different treatments. Differentially accumulated metabolites (DAM) were determined by variable importance in projection (VIP)  $\geq 1$  and differential multiple

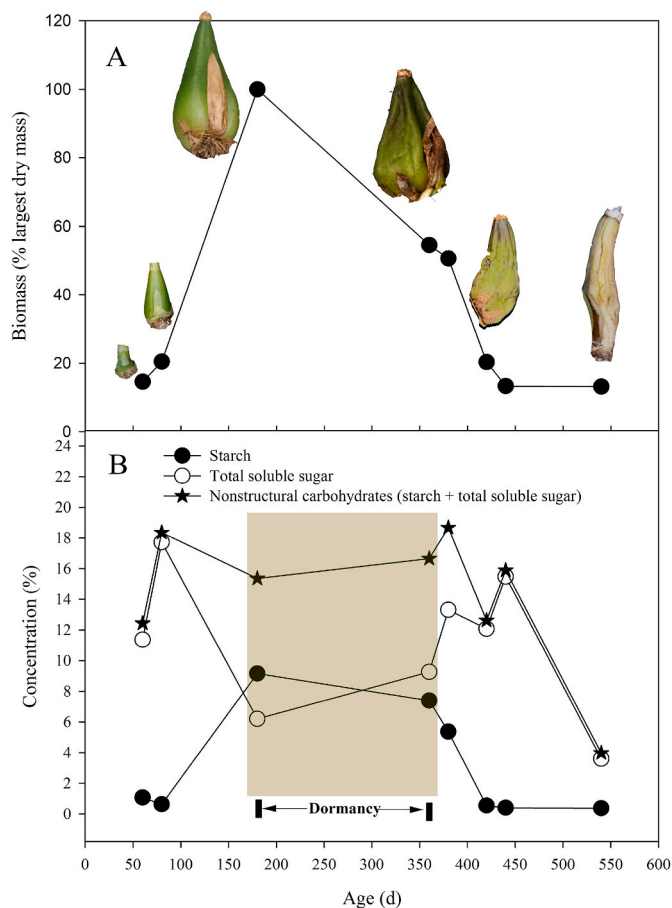
value (fold changes)  $\geq 2$  or  $\leq 0.5$ . The intersection set of DAMs between both the control group and drought-stressed group and between drought-stressed group and recovered group was defined as “key metabolite”, which indicates these metabolites respond positively to both the drought and recovery process. DAMs were further mapped to the KEGG Pathway database ([www.kegg.jp/kegg/pathway](http://www.kegg.jp/kegg/pathway)).

## 3. Results

### 3.1. Developmental changes in pseudobulb biomass and nonstructural carbohydrate content

In the first growing season (0 d–180 d), the biomass of newly produced pseudobulbs increases, swelling at the base of the single leaf. At the end of the first growing season (180 d), when the single leaf drops and roots die, the pseudobulb reaches its largest biomass before going dormant. After about 6 months of dormancy, the biomass of pseudobulbs decreased in order to support the development of new shoots and inflorescences. By the end of the second growing season, pseudobulbs lose more than 80% of their biomass, then die (540 d, Fig. 2A).

Starch content in pseudobulb was the highest at the end of the first growing season and decreased with the production of new shoots and inflorescences. This trend is consistent with changes in pseudobulb biomass. Total soluble sugar content in pseudobulbs increased between 60 d and 80 d and remained relatively low during dormancy. From the beginning of the second growing season, total soluble sugar content increased gradually in pseudobulbs and dropped to the lowest level before pseudobulbs died (540 d). Owing to higher content of the soluble sugar than starch, these results indicate that changes in NSCs during



**Fig. 2.** Developmental changes in biomass (A) and nonstructural carbohydrates (B) of pseudobulbs. Values are expressed as means of at least three replicates.

development are largely represented by changes in soluble sugar content (Fig. 2B).

### 3.2. Physiological response of plants to the drought-recovery event at flowering stage

At the flowering stage, there is only one pseudobulb per individual plant. The drought stress significantly decreased water content of pseudobulbs, and there was a recovery of water content after re-watering (Fig. 3A). Drought stress also inhibited the growth of new leaves and roots. Specifically, the leaves and roots of drought-stressed plants were 38% and 47% shorter than those of control plants. Growth resumed rapidly after watering began as indicated by the significant higher leaf and root length of recovered plants than those of drought-stressed plants (Fig. 3B).

Starch content was higher in the pseudobulbs of drought-stressed plants than in those of control plants. In contrast, total soluble sugar content was lower in drought-stressed plants than in controls plants. After three days of re-watering, starch content decreased, although not in a statistically significant manner, whereas total soluble sugar increased. As a result, NSCs, which is the combination of starch and total soluble sugar, did not change significantly during drought or recovery treatments (Fig. 3C).

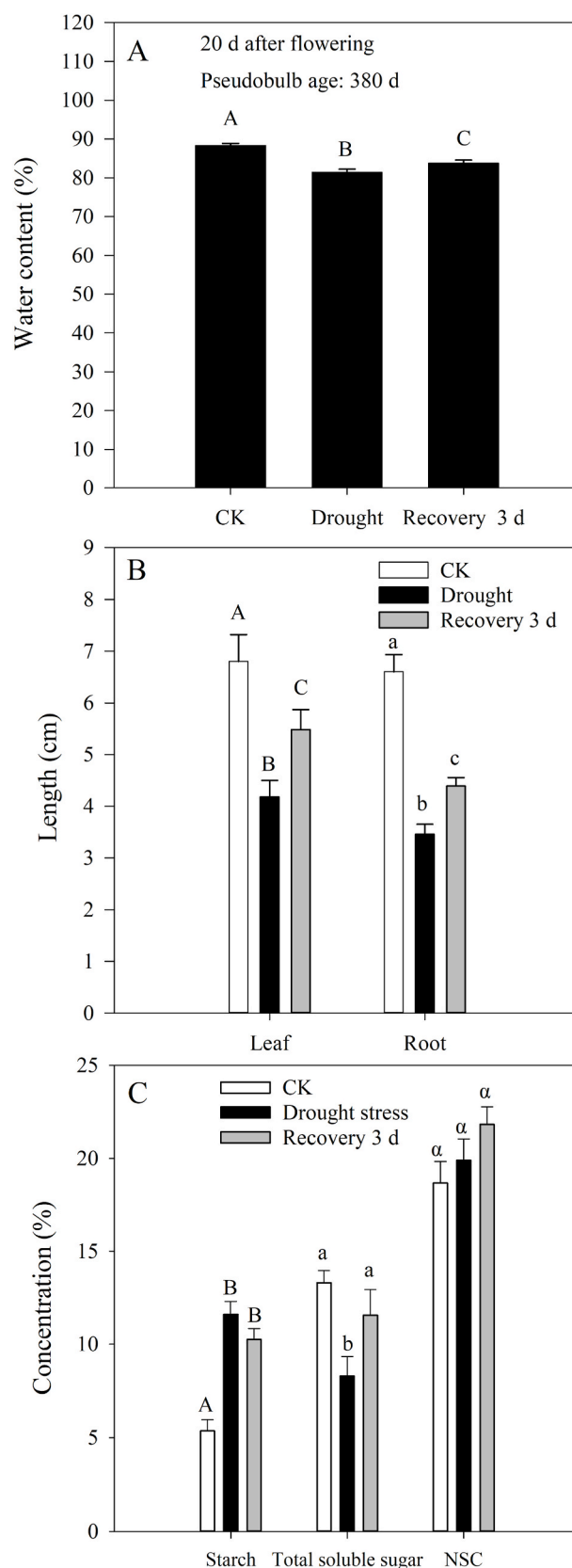
### 3.3. Physiological response of plants to the drought-recovery event at leaf full expansion

Before and after drought stress, water content was higher in old (440 d) pseudobulbs than in young (80 d) pseudobulbs (Fig. 4A). During drought stress, water content of both young and old pseudobulbs decreased, and it was significant lower in old pseudobulbs. In both young and old pseudobulbs, water content recovered after watering resumed. Net photosynthetic rate and stomatal conductance of plants decreased throughout drought stress, reaching close to 0 after 20 d without watering. Photosynthesis and stomatal conductance recovered rapidly but was still lower than the control group 10 days after re-watering (Fig. 4B). Starch concentration was very low in both young and old pseudobulbs at the leaf full expansion stage, and NSC concentration was therefore determined by total soluble sugar. Total soluble sugar content in both young and old pseudobulbs decreased in response to drought stress and recovered after re-watering, although the recovery was not significant for the young pseudobulbs (Fig. 4C and D).

### 3.4. Variation of metabolites in pseudobulb during drought stress and recovery

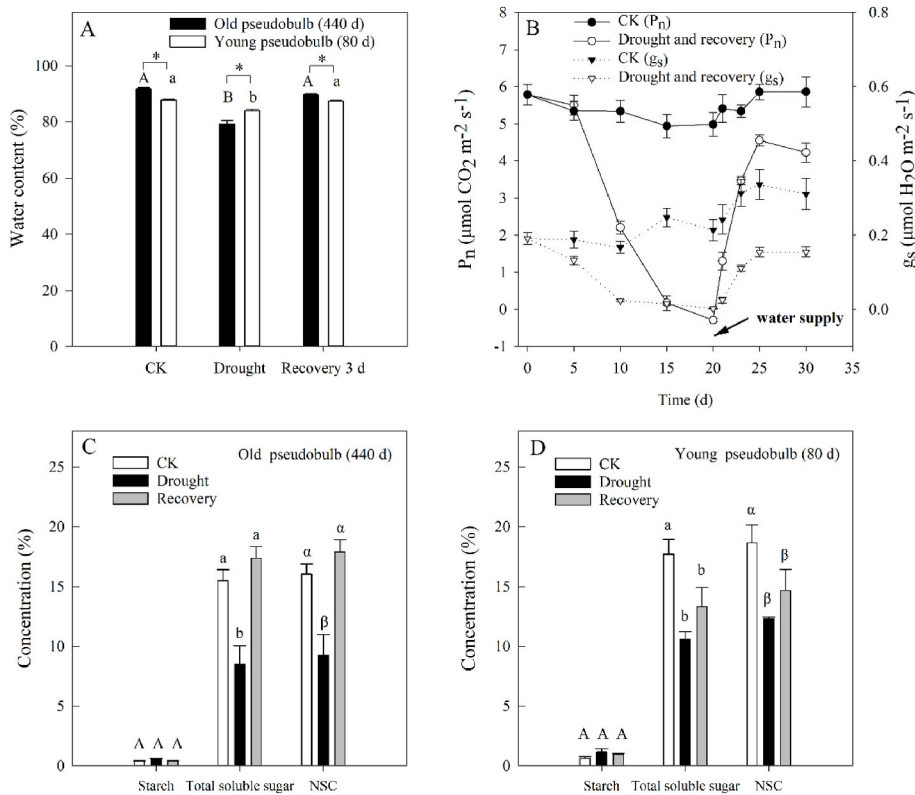
Drought stress and re-watering dramatically altered the metabolome of pseudobulbs at the flowering stage. Metabolite profiling identified 107 differentially accumulated metabolites between drought-stressed pseudobulbs and controls. In addition, 44 metabolites were differentially accumulated between drought-stressed and recovered pseudobulbs, of which 22 were shared during the drought-recovery process. We used hierarchical cluster analysis to evaluate the relative abundance of these 22 key metabolites (Fig. 5). Two main groups were identified, representing two different patterns of metabolite abundance under drought and recovery. The first group included metabolites that decreased during drought stress and increased after re-watering (V-shaped recovery). Six metabolites showed this V-shaped pattern: salidroside, glucosyloxybenzoic acid, isosalicylic acid O-glycoside, isofraxidin, peonidin 3,5-O-diglucoside chloride, 3,4,5-trimethoxyphenyl-β-D-glucopyranoside. The second group included metabolites that increased during drought stress and decreased after re-watering (inverted V-shaped). A total of 16 metabolites, showed this inverted V-shape pattern: morin, L-tyramine, pipelicolic acid, 1-methylhistidine, DL-2-aminoadipic acid, γ-aminobutyric acid, etc (Fig. 5).

At the leaf full expansion stage, a plant usually has two connected

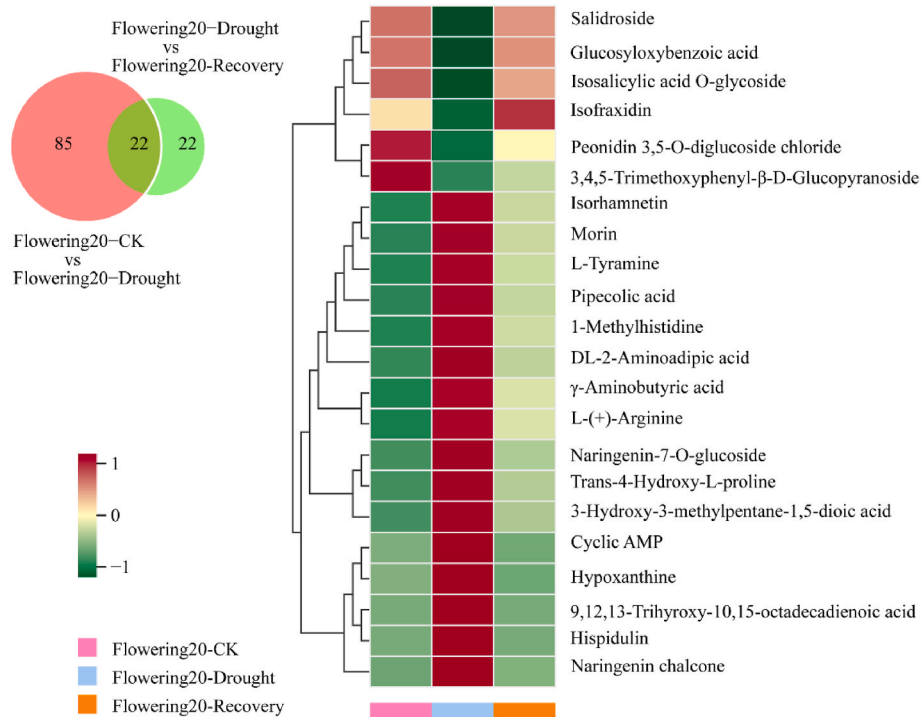


**Fig. 3.** Response of plants to the drought-recovery event at flowering stage. Values are expressed as mean  $\pm$  SE ( $n = 6$  for water content and leaf and root length, and  $n = 3$  for non-structural carbohydrate concentration). Different letters indicate significance ( $p < 0.05$ ) from the LSD multiple comparison tests.





**Fig. 4.** Response of plants to the drought-recovery event at leaf full expansion. Values are expressed as mean  $\pm$  SE. For water content, net photosynthetic rate ( $P_n$ ) and stomatal conductance ( $g_s$ ), six plants were used, and for non-structural carbohydrate (NSC, including starch and total soluble sugar), three samples, each of which was a mix from three different plant individuals, were involved. Different letters indicate significance ( $p < 0.05$ ) from the LSD multiple comparison tests. \*, a significant difference between different ages of pseudobulbs.



**Fig. 5.** Key metabolites of the pseudobulb (age of 380 d) involved in the drought-recovery response at flowering stage (Flowering20, 20 d after plant flowering).

pseudobulbs, one young (60 d) and one old (420 d) (Fig. 1). Metabolite profiling of young pseudobulbs identified 89 DAMs between drought-stressed and control plants, and 54 DAMs between drought-stressed and recovered plants. A total of 34 metabolites changed significantly during both drought and recovery. A total of 21 metabolites (e.g.,

ephemeranthoquinone B, allopurinol, N, N, N-p-coumaroyl-cinnamoyl-caffeoyl spermidine) showed a V-shaped recovery during the drought-recovery process, decreasing during drought stress then increases during recovery. An additional 13 metabolites showed an inverted V-shaped accumulation pattern, including feruloyltartaric acid, 3-O-(E)-p-

coumaroyl quinic acid, and trans-3-O-p-coumaric quinic acid, etc (Fig. 6).

More number of metabolites were differentially accumulated in old pseudobulbs than in the connected young pseudobulb. Specifically, we identified 123 metabolites that were differentially accumulated between drought-stressed and control pseudobulbs, and 61 between drought-stressed and recovered pseudobulbs. Forty key metabolites participated in both the drought and recovery responses. Of these, 6 metabolites showed a V-shaped pattern, decreasing during drought stress and then increasing during recovery. These included uridine 5'-diphospho-D-glucose, genistin, D-glucose 6-phosphate, senkyunolide K, oxidized glutathione, and neohesperidin. Another 34 metabolites showed an inverted V-shaped pattern, increasing during drought stress and decreasing during recovery. These included  $\alpha$ -aminocaproic acid, pyrocatechol, and trans-4-hydroxy-L-proline, etc (Fig. 7).

The summary of metabolomic analysis also revealed that different ages of pseudobulbs accumulated different numbers of metabolites in response to drought stress and recovery. For example, the most abundant set of key metabolites (40) were accumulated in the oldest pseudobulbs (440 d), whereas only 22 key metabolites were identified in pseudobulbs at 380 d. There was also aged-related variation among pseudobulbs regarding patterns of metabolite accumulation. During the drought-recovery process, more metabolites (21 vs. 13) showed a V-shaped pattern in young pseudobulbs (80 d), whereas more metabolites (16 vs. 6 and 34 vs. 6) showed an inverted V-shaped pattern in older pseudobulbs (380 d and 440 d). The content of most amino acids and derivatives showed a inverted V-shaped pattern during the drought-recovery process. For other classes of metabolites, such as phenolic acids and flavonoids, metabolites had both V-shaped and inverted V-shaped patterns during the drought-recovery process (Fig. 8).

Metabolite profiling identified a total of 225 metabolites differentially accumulated between the old pseudobulb and the connected young pseudobulb of an individual plant at leaf full expansion. Secondary metabolites (e.g., phenolic acids, flavonoids, alkaloids, lignans and comarins) were present at significantly higher levels in the old

pseudobulb than in the newly produced young pseudobulb, whereas amino acids and their derivatives were present at lower levels in the old pseudobulb (Fig. 9). Old pseudobulbs and their young connected pseudobulbs shared only 9 DAMs during drought stress, whereas a large number of DAMs were limited either to old pseudobulbs (52 DAMs) or to their young connected pseudobulbs (45 DAMs). Similarly, during recovery from drought, old pseudobulbs and their young connected pseudobulbs shared 34 DAMs, whereas 89 DAMs were specific to old pseudobulbs and 55 to young connected pseudobulbs (Fig. 9).

### 3.5. Metabolic pathways in pseudobulbs of an individual plant

For a certain individual plant, KEGG enrichment analysis of the DAMs identified in pseudobulbs during drought stress and recovery were mainly associated with metabolic pathways and biosynthesis of secondary metabolites. Specifically, metabolic pathways involved in the response of the old pseudobulb (440 d) to drought stress included the biosynthesis of phenylalanine, tyrosine and tryptophan, glucosinolate biosynthesis, and biosynthesis of amino acids, etc. In the young connected pseudobulb (80 d), metabolic pathways involved in responses to drought stress included purine metabolism, flavonoid biosynthesis, as well as the biosynthesis of flavone and flavanol, etc. Metabolic pathways involved in the recovery of old pseudobulb (440 d) included pyrimidine metabolism, purine metabolism, and the metabolism of ABC transporters, etc. Metabolic pathways involved in the recovery of young connected pseudobulb (80 d) included phenylpropanoid biosynthesis, flavonoid biosynthesis, the biosynthesis of flavone and flavanol, etc (Fig. 10).

## 4. Discussion

### 4.1. Metabolites in the pseudobulb under drought stress and recovery

In this study, we examined whether pseudobulbs of *Pleione aurita* at distinct developmental stages responded differently to drought stress

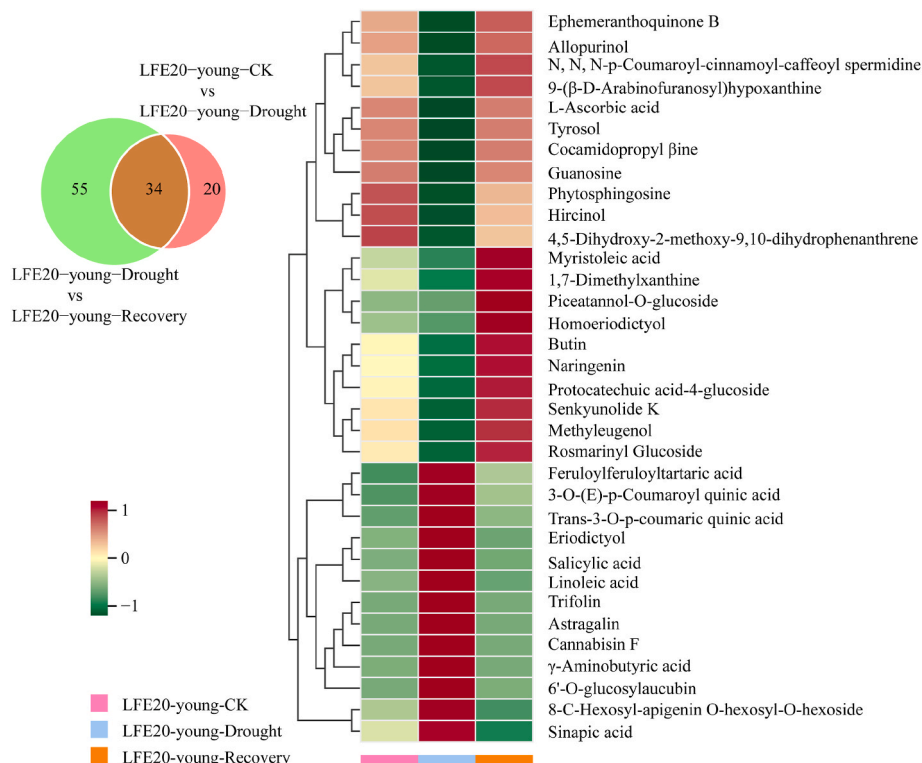


Fig. 6. Key metabolites of young pseudobulbs (age of 80 d) involved in the drought-recovery response at leaf full expansion (LFE20, 20 d after leaf full expansion).

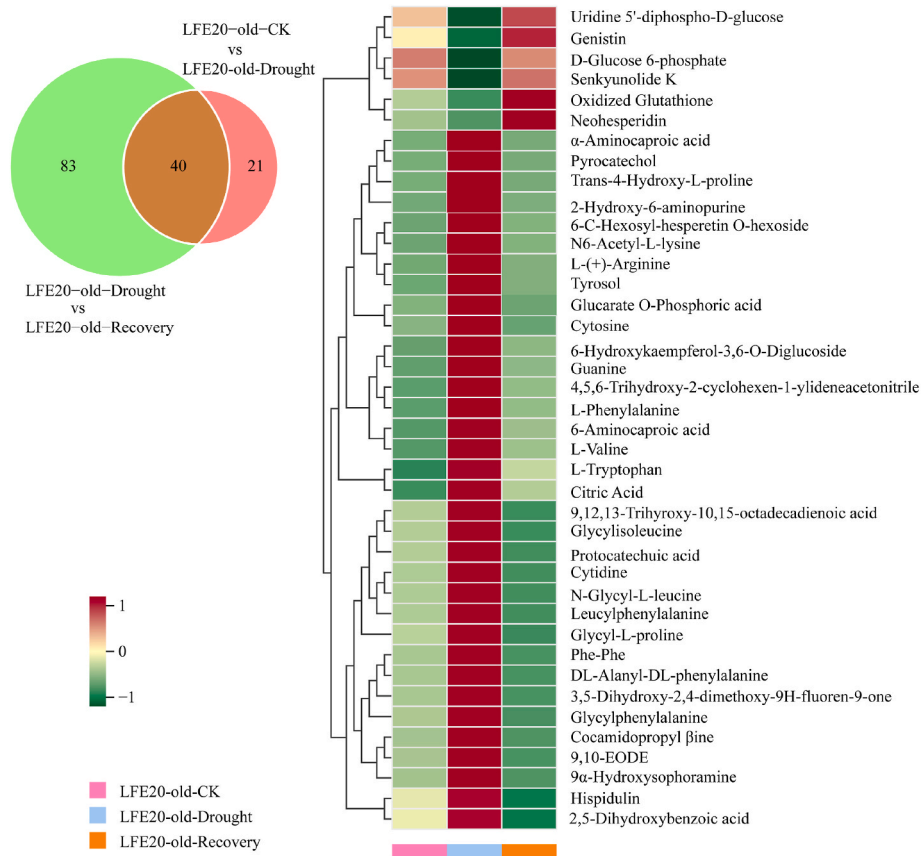


Fig. 7. Key metabolites of old pseudobulbs (age of 440 d) involved in the drought-recovery response at leaf full expansion (LFE20, 20 d after leaf full expansion).

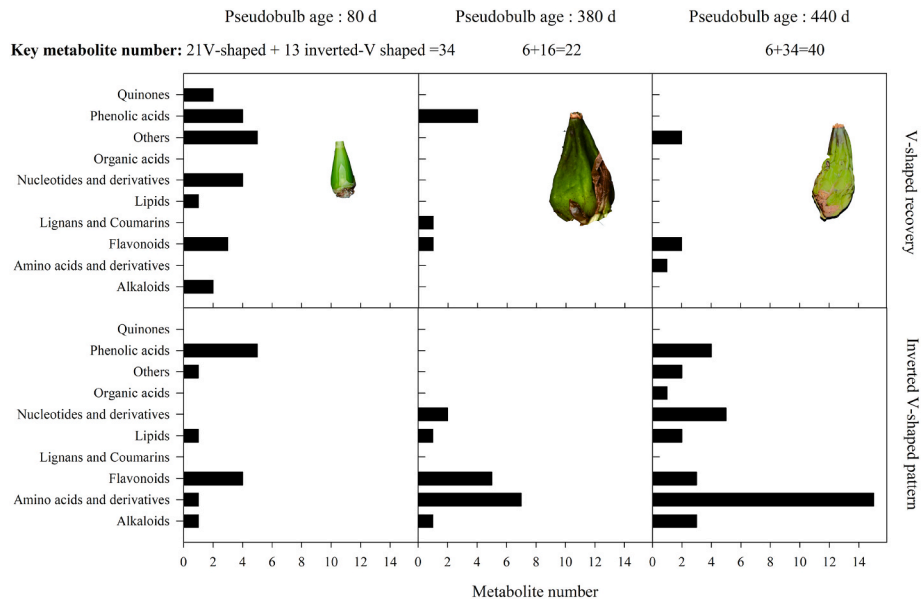


Fig. 8. Summary of key metabolites in pseudobulbs following two independent drought-recovery experiments (Flowering stage with the pseudobulb age of 380 d and full leaf expansion stage with the pseudobulb age of 80 d or 440 d). V-shaped recovery indicates metabolite content decreased during drought stress and increased after re-watering, while the inverted V-shaped pattern indicates metabolite content increased during drought stress and decreased after re-watering.

and recovery. We determined how drought stress affects various factors in pseudobulbs, including the concentration of non-structural carbohydrates and other metabolite content. Non-structural carbohydrates represent a reserve of sugars and can provide a temporary source of carbon during stresses when current photosynthesis cannot meet the

immediate demands of plants (Hartmann and Trumbore, 2016). Therefore, changes in NSC concentration and composition have frequently been reported in plants under stress conditions (Épron and Dreyer, 1996; Hare et al., 1998). However, changes in NSC concentration and composition in response to drought stress have yet to be

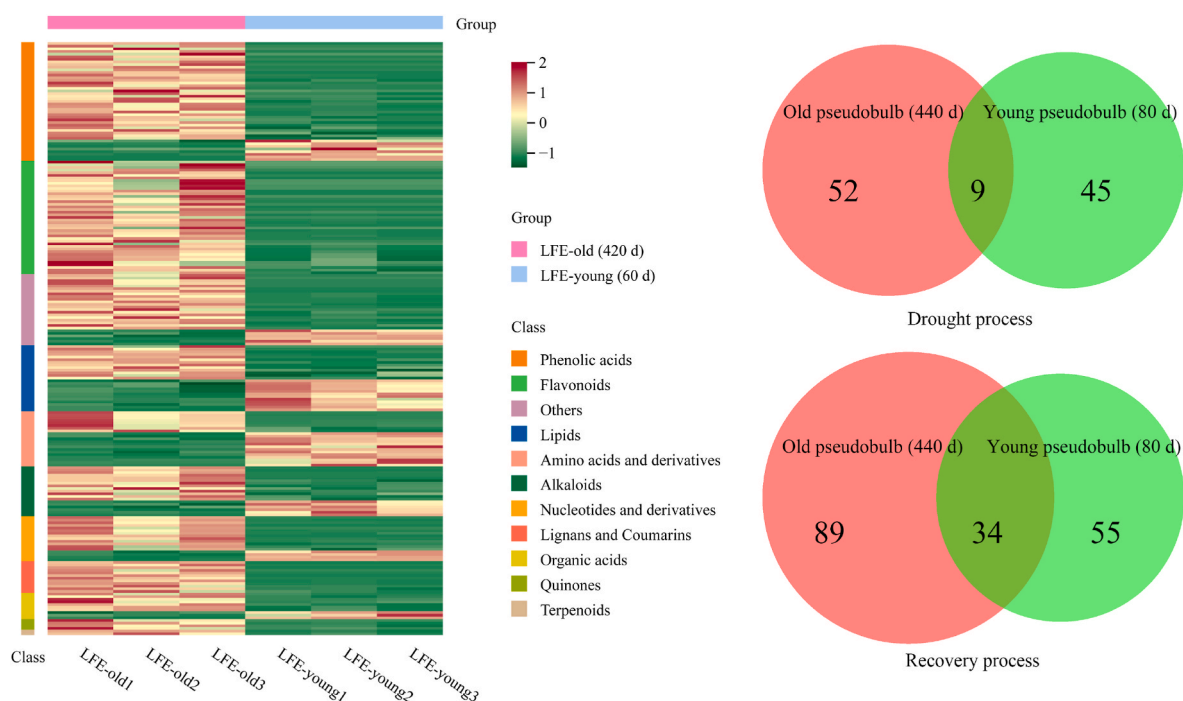


Fig. 9. Heat map showing differentially accumulated metabolites between old pseudobulb and the young connected pseudobulb at leaf full expansion (LFE).

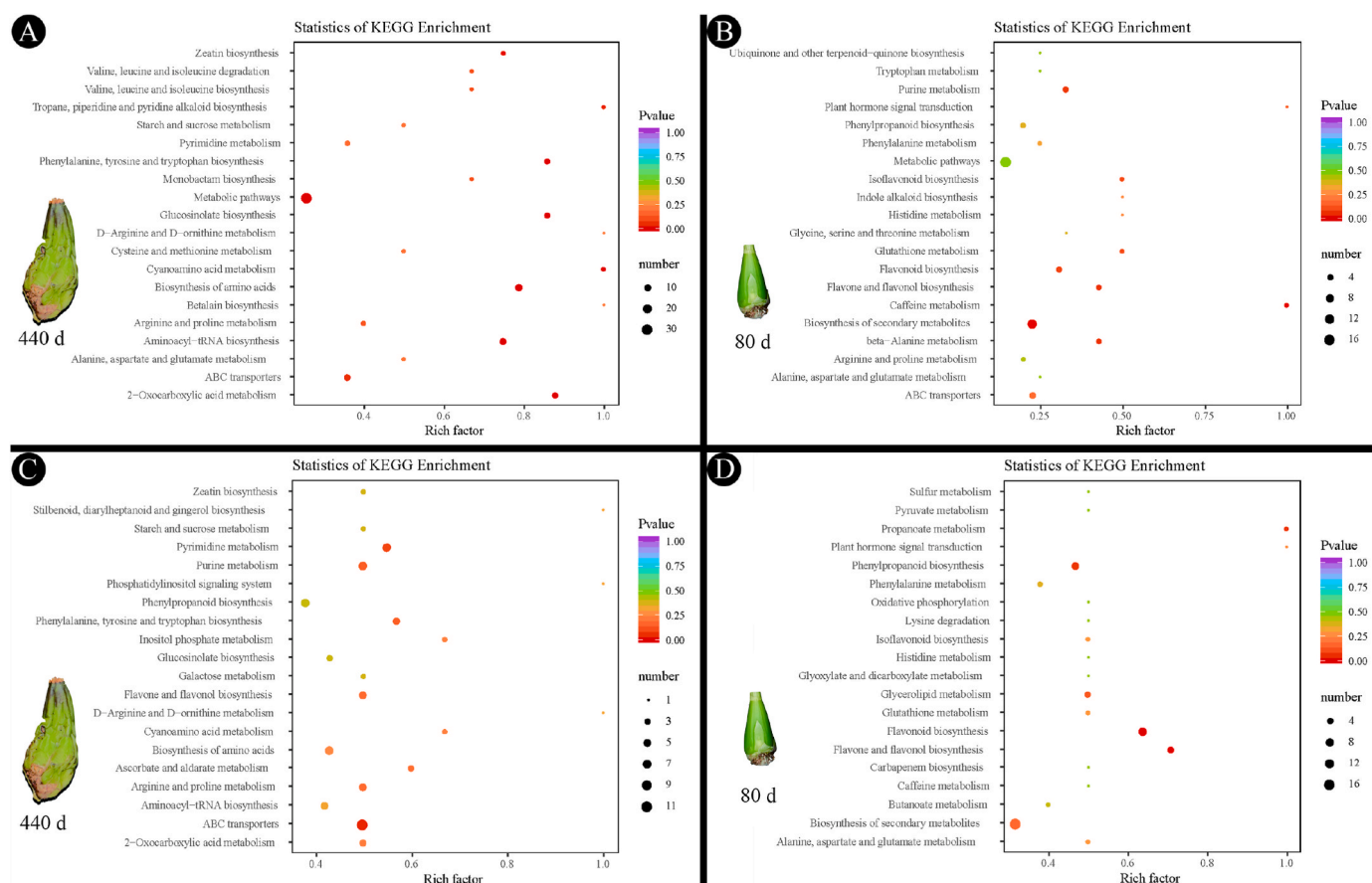


Fig. 10. KEGG enrichment of differentially accumulated metabolites in drought-stressed (A, old pseudobulb at full leaf expansion; B, young connected pseudobulb) and recovered (C, old pseudobulb at full leaf expansion; D, young connected pseudobulb) plants.



observed conclusively, either vary between organs (Pinheiro et al., 2001; Zhang et al., 2017a) or have been found to be insensitive to water deficit (Li and Zhang, 2019).

In the present study, net photosynthetic rate and stomatal conductance decreased throughout drought stress, and reached close to 0 or even negative values after 20 d without watering (Fig. 4B), indicating the closure of stomata and stop of production of carbohydrates by plants during drought stress. We found that drought stress decreased concentrations of total soluble sugars at all ages. In addition, NSC concentration was unaffected by drought stress and recovery at the flowering stage, but decreased in response to drought stress at leaf full expansion (Figs. 3C and 4C, D). This decrease in total soluble sugar, or NSC, of pseudobulbs during drought stress may indicate the less carbohydrate income from leaf photosynthesis and more importantly, the catabolism of pseudobulb-stored starch and polysaccharides followed by synthesis and transport of monosaccharides or oligosaccharides to the leaf for osmoregulation (Stancato et al., 2001). Since concentration and composition of NSC varied over the lifetime of pseudobulbs (Fig. 2B), what and how much can be mobilized probably contribute to the age-related differences in responses to drought stress.

Widely targeted metabolic profiling of pseudobulbs under drought and recovery failed to identify many specific monosaccharides, but did identify a large number of flavonoids, phenolic acids, as well as amino acids and their derivatives. Specifically, we found that some flavonoids, such as hispidulin, accumulate in pseudobulbs during drought stress and return to previous levels once watering is resumed, indicating that these metabolites have an important role in plant responses to drought stress. This supposition is consistent with previous findings that showed flavonoids and phenolic acids participate in plant biotic interactions and responses to environmental stresses (Dong and Lin, 2021; Mierziak et al., 2014; Sharma et al., 2019; Winkel-Shirley, 2002). In fact, many flavonoids, commonly present as glycosides, scavenge superoxide radicals, screen UV light, and maintain liposome stability (Popova and Hinch, 2016; Tattini et al., 2004), which alleviate damage to plants caused by drought stress.

Amino acids and their derivatives were also a class of main DAMs between the pseudobulbs of drought stressed plants and those of control or recovered plants. For example,  $\gamma$ -aminobutyric acid is frequently detected in the responses of plants to drought stress. This non-protein amino acid has been shown to have multiple prominent roles in plant responses to environmental stress (Seifikhah et al., 2019). Furthermore, amino acids and their derivatives, especially free amino acids, are considered important osmolytes as sugars in plants (Good and Zaplachinski, 1994; Hildebrandt et al., 2015).

Previous research has suggested that purine metabolism and phenylpropanoid biosynthesis play important roles in drought response in *Dendrobium sinense* (Zhang et al., 2021). Our KEGG enrichment analysis provides support for these metabolic pathways playing roles in drought response. In general, our finding that *P. aurita* pseudobulbs accumulate specific sets of metabolites in response to drought stress supports findings in studies of other orchid species (Wu et al., 2016; Zhang et al., 2021), further indicating that drought-induced accumulation of metabolites may improve drought tolerance in orchids.

#### 4.2. Pseudobulbs of different ages respond differently to drought stress

Plants generally show developmental differences when responding to drought stress. This is because plants at distinct developmental stages differ greatly in anatomy and individual size; thus, when suffering from water deficit, plants perform according to age-related physiological traits, metabolism, and gene expression (Bangar et al., 2019; González-Villagra et al., 2018; Wang et al., 2011; Zotz et al., 2001). *Pleione aurita* flowers at the end of the dry season, before the single leaf is fully constructed (Fig. 1). We found that drought stress at the flowering stage reduced the consumption of starch and slowed the production of leaves and roots (Fig. 3B and C). These findings may reflect plant

strategies to minimize water loss by transpiration in newly-produced organs. Our study also confirms that metabolite content varies in pseudobulbs of different ages (Figs. 2B and 9 and see also Jin et al., 2016). Specifically, there were fewer metabolites in pseudobulbs at the flowering stage (380 d) than at leaf full expansion (pseudobulbs of 80 d and 440 d; Fig. 8). This lower number of key metabolites at the flowering pseudobulbs may be because a 20 day's water withholding provided less severe drought stress at the flowering stage than at the leaf full expansion stage. Due to the much larger area of leaf transpiration, previous research has shown that *Pleione* species are vulnerable to drought stress after leaves are fully expanded (Zhang et al., 2016). Thus, two drought events have different effects on changes in plant biomass after a growing season. We found that the biomass of both control and drought-stressed plants at flowering increased, whereas those of stressed at leaf full expansion decreased by approximately 30% after a growing season (Fig. S1).

Previous studies have documented age-related differences in the physiological and metabolic responses of plant organs to drought stress (González-Villagra et al., 2018, 2022). Furthermore, pseudobulbs have been shown to function in an age-dependent manner, with younger pseudobulbs having higher sink activities for both photosynthetic products (Yong and Hew, 1995b) and exogenous nitrogen (Zhang et al., 2022). Here, we found water content differed in young and old pseudobulbs, and identified several metabolites that are differentially accumulated in pseudobulbs at different ages. This big difference in chemical composition of pseudobulbs at different ages leads us to speculate that there is functional differentiation for different-aged pseudobulbs when responding to stress.

KEGG enrichment analysis also indicated that different pathways are involved in pseudobulb drought-recovery responses at different ages (Fig. 10), which further supports the functional differentiation for different-aged pseudobulbs. In general, pseudobulbs are physiologically integrated, exchanging water and other resources between individuals of different ages (Li and Zhang, 2019). This type of physiological integration is a widespread phenomenon in plants and helps plants to ameliorate the adverse effects of stress (Li et al., 2015; Zhang et al., 2012). Thus, the differentiated response to drought stress may facilitate coordination by pseudobulbs of different ages to achieve better physiological integration.

#### 4.3. Frequent drought stress and the presence of pseudobulbs in epiphytic orchids

Orchids have adapted to the intermittent water supply of epiphytic habitats by evolving important biological characteristics that increase water uptake and storage capacity as well as decrease water loss. The water stored within pseudobulbs is key to maintain the whole-plant water balance during drought periods, while the thick impermeable epidermis of the pseudobulb slows the loss of stored water (Zhang et al., 2018). In some cases, the pseudobulb is also involved in Crassulacean Acid Metabolism (CAM), a water-conserving photosynthetic pathway of orchids (Rodrigues et al., 2013). However, a recent study indicates that pseudobulbs are equally important structures in both  $C_3$  and CAM orchid taxa (Fu et al., 2022), suggesting a complicated relationship between plant metabolism and the presence of pseudobulbs. Our finding that both water storage and loss by the old pseudobulb under drought stress was more than that of the younger connected pseudobulb agrees with previous studies that old stems are important for the survivorship of epiphytic orchids and plants in other water-limiting ecosystems (Zotz, 1999; Raphael and Nobel, 1986). In addition, our results indicated that old pseudobulbs mobilized more secondary metabolites than young connected pseudobulbs when responding to water deficits. For some primary metabolites such as amino acids, although the content in old pseudobulbs was generally lower than that of the younger connected pseudobulbs, more number of this class of metabolites were found participating the drought-recovery response in the old pseudobulb

(Fig. 9). These results suggest that pseudobulbs are not only water reserves (He et al., 2013; Li and Zhang, 2019; Yang et al., 2016) or nutrient reserves (Wang et al., 2008; Yong and Hew, 1995a; Zhang et al., 2022; Zimmerman, 1990), but also metabolite reserves when coping with water deficit. This finding expands our understanding of the role of pseudobulbs in the survival of orchids at epiphytic habitats with intermittent water supply.

## 5. Conclusions

In this study, we investigated physiological and metabolic responses of orchid pseudobulbs at different ages to drought stress and recovery. We found that non-structural carbohydrate composition varied between pseudobulbs of different ages, and old pseudobulbs stored more water, whereas the young connected pseudobulbs stored more dry matter. Drought stress and subsequent recovery mobilized multiple metabolites in the pseudobulb, including non-structural carbohydrates, flavonoids, phenolic acids, as well as amino acids and their derivatives. Mobilization of these metabolites indicates that several pathways (e.g., purine and phenylpropanoid metabolism) play roles in pseudobulb's drought response. Old pseudobulbs store more water and mobilize more key metabolites than the young connected pseudobulbs implies that pseudobulbs adopt different strategies at different ages to respond to drought stress. This study provides evidence that orchid pseudobulbs function as metabolite reserves during drought stress and that this function varies according to the age of pseudobulb.

## Author contributions

Ze-Xin Fan and Shi-Bao Zhang conceived this study and designed the experiments. Wei Zhang performed the experiments and wrote the manuscript. Xiu-Mei Dong and Yu-Wen Zhang help to analyze the data. All authors discussed the results and revised the manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2023.107655>.

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