Temporal and Spatial Characteristics Endogenous Hormone Regulation During Male Cone Development of Dacrydium pectinatum

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Abstract

Background: *Dacrydiumpectinatum* de Laubenfels is a perennial gymnosperm dominant in tropical montane rain forests. Due to severe damages by excessive deforestation, typhoons, and other external forces, the population of the species has been significantly reduced. Furthermore, natural regeneration is poor. In order to better understand the reproductive process in *D. pectinatum*, we examined the morphological and anatomical changes during the development of male cone and analyzed the endogenous hormone dynamics.

Results: Our study indicates that *D. pectinatum* male buds become distinguishable in April in tropical montane rain forests, while microspore sac forms in September and pollen mother cell forms and divides in December. Pollen grains mature and disperse in the following February. A mature male cone averages 8.5 mm in length. Level of GA, IAA, ABA and JA and their ratios fluctuated during late August to late November when sporogenous tissues were actively differentiated.

Conclusions: The differentiation of sporogenous tissues is accompanied by variations in levels of endogenous hormones (GA, ABA, IAA, and JA) and their balances. The new insights about the cone development in *D. pectinatum* lay the foundation for future cone induction with hormones and study of factors contributing to the species' low rate of seed germination.

Background

D. pectinatum de Laubenfels is a perennial gymnosperm in the family of Podocarpaceae. This evergreen dioecious tree is a dominant species in tropical montane rain forests and native to China (Hainan province), Malaysia (Billiton), Borneo, and Philippines (Luzon and Mindanao) (de Laubenfels 1988). An adult tree can live for more than 2000 years and grow up to 30 m tall and 3 m in diameter at breast height. The wood of *D. pectinatum* is valuable for constructing buildings and high-grade handcrafting such as ships (http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=210000447). However, due to excessive logging and shifting cultivation, there is a significant decrease in the species' natural forest resources, and it is now listed as endangered (IUCN 3.1) by the International Union for Conservation of Nature. On the coastal plains of Sabah, Sarawak and Kalimantan, 80% of *D. pectinatum* occupancy area has lost during the massive conversion of lowland forest to oil palm plantations (https://www.iucnredlist.org/). Similarly, *D. pectinatum* natural forests have been significantly reduced in China since 1960s due to severe damages by excessive deforestation, typhoons, and other external forces. According to a report published in 2016 (China Forestry Bureau 2016), only 14,484 hectares are available in China, accounting for 3,473,575 square meter of forest volume.

A survey of *D. pectinatum* found that 78% of trees in natural forests had a large diameter at breast height (> 5 cm) and natural regeneration was poor in the montane rain forests (Ding et al. 2012). A study reports that *D. pectinatum*'s natural forests have a long childhood, few flowering individuals, and low seed setting rates (Wu et al. 2018). Currently, the exact length of juvenile phase is unknown for the species, but is

suspected to be long, since none of the trees with a DBH of 10 cm or smaller produced reproductive structures (Li et al. 2015). Among the 180,032 *D. pectinatum* seeds collected from a natural stand in Hainan province, China, the viability and germination rate was found to be 3.11% and 0.02%, respectively (Chen et al. 2016). These factors have seriously hindered artificial cultivation and efficient use of resources.

Efforts have been undertaken to protect *D. pectinatum* and conserve its biodiversity. However, research on this endangered species is still in its infancy. Currently, most of the published studies are focused on the activity of medicinal ingredients, seedling growth, forest community structure, population genetic diversity, and origin of evolution (Huang et al. 2014; Su et al. 2010; Yang et al. 2008; Wu et al. 2018; Keppel et al. 2011). Limited information is available on reproduction. According to the descriptions in de Laubenfels (1988) and Flora of China (Fu et al. 1999), the reproductive structures of *D. pectinatum* occur on the terminals. Pollen cones (male, microsporangiate strobili) are 6–12 mm long and in clusters of 1–3. The seed-bearing structure (female cone, macrosporangiate strobili) is subtended by a short zone of small leaves which are ~ 2 mm long, while the cone bracts themselves may be up to 3 mm long. Seeds are ovoid and 4-4.5 mm long.

Reproductive structure formation is a significant process in a plant's life because of its important role in producing offspring and evolution. In this study, we investigated male cone development in *D. pectinatum.* We focused on the morphological and anatomical changes that occur during the development of the male cone, microsporangia and microsporangium wall, and analyzed the endogenous hormone changes. Our study provides further insights into male cone development of this important species.

Materials And Methods

Materials

Male reproductive structures of *D. pectinatum* were collected monthly from early April 2018 to February 2020 at Bawanglin Forest Reserve (Between 18°53'~19°30' north latitude, between 108°38'~109°17' east longitude) of Changjiang County, Hainan Province, China. Changjiang County is in a typical tropical monsoon climate zone. The annual average temperature is 24.3 °C, with 39.8 °C as the highest and 0 °C as the lowest. There is no winter throughout the year, and the four seasons are like spring. The annual accumulated temperature is 8400 ~ 9100 °C, while the total solar radiation is 135 kcal / cm^2, Rainfall is abundant with average annual precipitation of 1676 mm. Male buds were collected from north, south, east and west sides of mature trees that are over 100 years old. Bracts were dissected and removed under a SMZ-168 stereomicroscope before buds were stored at 4°C in 2.5% glutaraldehyde or FAA fixative solution (formalin: acetic acid: 70% alcohol = 1:1:18). Photos were taken of male shoots at different stages of development with a Nikon digital camera.

Semi-thin Sectioning

Male buds fixed in FAA were dehydrated and embedded in paraffin as described in Mazur et al (Mazur et al. 2016). Sections (~ 4 μm) were cut with a microtome (RM2016, Shanghai, China) and mounted on slides. After rehydration, specimens were stained with 1% saffron and 0.5% solid green and observed under a Nikon Eclipse Ci light microscope (Japan). Digital images were taken with a Nikon digital camera.

Observation of Morphological Development of Male Cones by the Scanning Electron Microscope (SEM)

Male buds originally fixed in 2.5% glutaraldehyde were further treated with 1% osmium acid. After dehydration with a series of increasing concentration of ethanol and dipping into isoamyl acetate, dried samples were sputter-coated with gold prior to scanning electron microscopy examination (SEM, U8010, HITACHI, Japan), according to Kang (Kang et al. 2014).

Changes of Endogenous Hormones(IAA□ABA□GA, and JA) in Male Buds of Dacrydium pierrei

Sample preparation

Samples preserved at ultra-low temperature were ground (30 Hz, 1 min) to fine powders with a grinding machine. Ground samples of 50 mg was accurately weighed and dissolved in a 0.5 mL-extract solution, containing methanol, water, and formic acid (v:v:v = 15:4:1). After 10 minutes of extraction, supernatant was obtained by centrifugation for 5 min at 14 000 rpm. The extraction and centrifugation steps were repeated twice. All supernatants were combined and dried at 35℃ under nitrogen gas. The extracts were then resuspended with 100 μL of 80% methanol-water solution and sonicated for 1 min, followed by filtration through a 0.22 micron PTFE membrane.

Acquisition Conditions of Chromatographic Mass Spectrometry

Extracts obtained from above were subjected to an Ultra-High-Performance Liquid Chromatography (UPLC) (Shim-pack UFLC SHIMADZU CBM3OA, http//www.shimadzu.com.cn) and a Tandem Mass Spectrometry (Ms/ms) (Applied Biosystems 6500 Quadrupole Trap, http://www.applied biosystems.com.cn/). The detailed procedures described in(Ma et al. 2008) were followed.

The liquid phase conditions were:

(1) Chromatographic column: Waters ACQUITY UPLC HSS T3 C18 1.8 um, 2.1 m × 100 mm;

(2) Mobile phase: water phase was ultrapure water (0.04% formic acid added) in the water phase, acetonitrile (0.04% formic acid added) in the organic phase;

(3) Elution gradient: 0 min water/acetonitrile (90:10V/V), 5.0 min 40:60V/V, 70 min 40:60V/V, 7.01 min 90:10V/V and 10.0 min 90:10V/V;

(4) The flow rate of 0.35 mL/min, column temperature of 40°C, and injection volume of 2 micron.

Mass spectrometry conditions were as followed: electrospray ionization (ESI) temperature was 500°C, mass spectrometry voltage was 5500V, curtain gas was 35 psi, the collision-activated dissociation parameter was set to medium in the dissociation, and each ion pair was scanned according to the optimized cluster voltage and collision energy. The hormonal content obtained in the analysis was expressed as mg/g fresh weight.

Statistical analysis

Data were analyzed by using the IMB® SPSS® version 22 and presented as mean values with their respective standard deviations (mean ± SD, n = 3). At 95% confidence level, Student's t-test and Fisher's Least Significant Difference (LSD) multiple comparison were used for statistical analyses. The statistical differences are mentioned in the text or considered as * $p < 0.05$ and ** $p < 0.01$.

Results

Arrangement and phenology of male cones

As described by de Laubenfels (1988), *D. pectinatum* male reproductive structures appear on the top of current-year branches. Two or three microspore bulbs cluster together, showing a V-shaped distribution, subtended by decussate bud-scales (bracts) (Fig. 1a). In Bawanglin Forest Reserve, male reproductive structures were first observed in early April, with a light green color and a diameter of 1–2 mm. From the beginning of May to the end of August, the cones gradually elongated and enlarged, and the green color deepened (Fig. 1b-e). The cones reached their maximum length of the year in September, while the width continued to growth (Fig. 2). By October, the outer scales of the cone appeared brown on the margin, and the scales became sharp (Fig. 1e). During November to the following January, the outer scales were greatly elongated and evenly thickened and became yellowish-brown (Fig. 1f-g). Furthermore, the whole structure of the male cone became more compact. Cones enlarged rapidly in January and February. By late February, cones gradually expanded and became yellow-brown, with the outer scales cracked and mature microspore sacs dispersing pollens (Fig. 1g). Males cones were mainly found in the well sunlit parts of the outer crown, and there were approximately 45 male cones in each mature *D. pectinatum* tree. The SEM images show that spirally-arranged microsporophylls were formed by April and underwent differentiation and enlargement through December (Fig. 4). Microsporophylls were tightly arranged around the main axis.

Microscopic anatomy of D. pectinatum male cone

In April, microsporophyll primordia started to become visible, which were formed in the order of from the base of the bud to the top (Fig. 3a-b). The bud was wrapped by phylloclades, with the left and right phylloclades along the central axis forming a U shape close to the central axis. By September, sporogenous tissues in sac-like microsporangia became condense due to the further differentiation and

accelerated cell division of the microsporophyll primordia (Fig. 3c-e). Microsporangia seemed to derive from a group of hypodermal cells of the microsporophyll. The formation and division of pollen mother cells were found in December. Mother cells were actively dividing, producing four microspores per mother cell through meiosis (Fig. 3e). Numerous pollen grains were formed in late January of the following year (Fig. 3f).

Male cone had a central axis on which 15 to 20 microsporophylls were spirally arranged. Each mcrosporophyll bored one or two microsporangia on the abaxial surface. A mature microsporangium consisted of one layer of epidermis, a multilayered endothecium, tapetum, and microspore mother cells (Fig. 3e-f). Tapetum separated from endothecium as microsporangia developed.

Dynamic of Endogenous Hormones during Male cone Development

During male cone development, GA content decreased during September and October, then recovered by late November (Fig. 5a). IAA content did not change during August and September, while peaked in late October and then decreased to its lowest level in late November (Fig. 5b). ABA exhibited the most dynamic change, with significant difference among each time point (Fig. 5c). ABA content was lowest in early September and highest in late September, declining after the plateau. There was no significant change in JA content, with an exception in late October when a large increase occurred (Fig. 5d).

In terms of ratios among endogenous hormones, ABA/IAA was found the lowest in early September and highest in late September (Fig. 6a). ABA/GA peaked in late September and October, while no difference existed among the other timepoints (Fig. 6b). IAA/GA plateaued in late October and were the lowest in late August and late November (Fig. 6c). Mirroring the dramatic increase of JA content in late October, JA/IAA ratio in late October was much higher than the other periods (Fig. 6d), while ratios of ABA/JA and GA/JA were much lower (Fig. 6e-f).

Discussion

There are 21 species in the genus *Dacrydium* (https://www.conifers.org/po/Dacrydium.php). Their natural distribution ranges from New Zealand, New Caledonia, Fiji and the Solomon Islands through New Guinea, Indonesia, Malaysia and the Philippines, to Thailand and southern China (de Laubenfels 1969; Quinn 1982). Currently no information is available about when reproductive cones start to initiate for *Dacrydium* species. In New Zealand, *D. cupressinum* cone initiation is suggested to occur in late summer or autumn with pollination occurring in spring (Norton et al. 1988). *D. pectinatum* male cones initiate before April in Hainan Island, China, because male buds are distinguishable by early April (Fig. 1a). Different species and climates can be the contributing factors for the discrepancy observed. Buds will be collected in March and February for examination in order to determine the initiation period for *D. pectinatum* male strobili in the future. *D. pectinatum* male cones average 8.5 mm in length (Fig. 2), similar to de Laubenfels's report for the same species (6–12 mm) (de Laubenfels 1988), while longer than *D. Bidwillii*'s (2 to 6 mm) (Young 1907). When more information become available for other *Dacrydium*

species, it will be interesting to compare their reproductive buds and cones' morphology and phenology. This is important for the diversity conservation.

Similar to other coniferous species such as *Metasequoia glyptostroboides* (also known as the dawn redwood, Chinese redwood and water fir), *D. pectinatum* has microsporophylls spirally arranged around a main axis, and each microsporophyll consists of a phylloclade at the apex and one or two microsporangia at the base (Fig. 3). Like *M. glyptostroboides* (Jin et al. 2012), *D. pectinatum* male cones are mainly located around the outer and sunlit parts of crown. This is advantageous for pollen dispersal by wind, which is a common in conifers (Leslie 2011a, Leslie 2011b). Our study shows that the development of *D. pectinatum* microspore can be divided into four stages: initiation and differentiation of microsporophyll primordia, microspore sac formation, division of pollen mother cells, and pollen grain formation. This process lasts for about 12 months. A similar study will be conducted on female buds and cones.

Plant hormones and their interplay have important roles in various aspects of plant growth, development, and reproductive processes. Major phytohormones include auxins, abscisic acid, cytokinins, ethylene gibberellin, brassinosteroids, jasmonates, and strigolactones (Davis 2009; Kazan and Lyons 2016). Effects of hormones and their balance on reproductive bud initiation and development seem to depend on species and sex. In female *Gnetum parvifolium* buds, level of GA_3, zeatin riboside (ZR), and ABA declines, and IAA increases as development progresses. In contrast, these endogenous hormones have the opposite trends in male buds. As for ratios, female and male buds share similar trends for ABA/GA_3 and ZR/GA_3, and differ in ABA/IAA and ZR/IAA (Lan et al. 2018). High levels of GA_3 are reported to be beneficial for male cone formation in *G. parvifolium* (Lan et al. 2018) and other conifers, such as *Pinus* (Niu et al. 2014), Douglas-fir (Kong et al. 2008), and white spruce (Greenwood et al. 2011). In our previous study with *Metasequoia*, higher levels of GA_{1+3} and lower levels of IAA and ABA were beneficial to male primordium initiation, while higher levels of IAA and GA_{1+3} and a lower level of ABA were favorable to female cone initiation (Liang and Yin 1994). In *D. pectinatum*, level of GA, IAA, ABA and JA and their ratios fluctuated during late August to late November when sporogenous tissues were actively differentiated, suggesting their involvement in make cone development.

It is noteworthy that there was a dramatic increase of JA in male buds collected in late October after microspore sac was formed (Fig. 5d). JA is well known for its roles in plant's biotic responses, such as drought, salt stress, low temperatures (Piotrowska et al. 2009; Wasternack et al. 2014). More recently, there is increasing evidence indicating JA's involvement in plant development and reproduction. According to the review by Huang et al and Yuan and Zhang (Huang et al. 2017; Yuan and Zhang 2015), JA is found in control of stamen development in Arabidopsis, inhibition of petal expansion in Arabidopsis, sex determination in maize, and control of stamen and spikelet development in rice, as well as regulate embryo/seed development and induction of leaf senescence. The actions of JA can be channeled through its signaling pathway. To our knowledge, no reports of similar information are available in a gymnosperm species. Therefore, the JA surge in *D. pectinatum* shall be further investigated.

In summary, *D. pectinatum* male buds become distinguishable in April in tropical montane rain forests and continue to differentiate and develop until the following February. The dynamic change of endogenous hormones suggests their roles in cone development. Cone induction with hormones may provide an alternate approach to address the seed shortage issue due to the species' long juvenile phase. It is suggested that treatments for male cone induction should be applied no later than April before differentiation of vegetative and reproductive buds. This is the first report on the anatomical and endogenous hormone changes that occur during the development of *D. pectinatum* the male cone. Combining morphological analyses of reproductive development with transcriptome studies in the future may lead to the understanding of molecular mechanisms behind reproductive development in *D. pectinatum*.

Abbreviations

Ms
Microspore; Sg:sporangia; Ph:phylloclade; Mc:mother cell; Pg:pollen grain; T:tapetum; En:endothecium; E:epidermis.

Declarations

Authors' contribution statement

SX and WJ designed the experiments. LW, LZ, and HS performed the experiments. RM and YD analyzed the data. YZand LH wrote the paper.

Availability of data and materials

Research data are not shared.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of Interest

The authors declare that there are no conflicts of interest in this research article.

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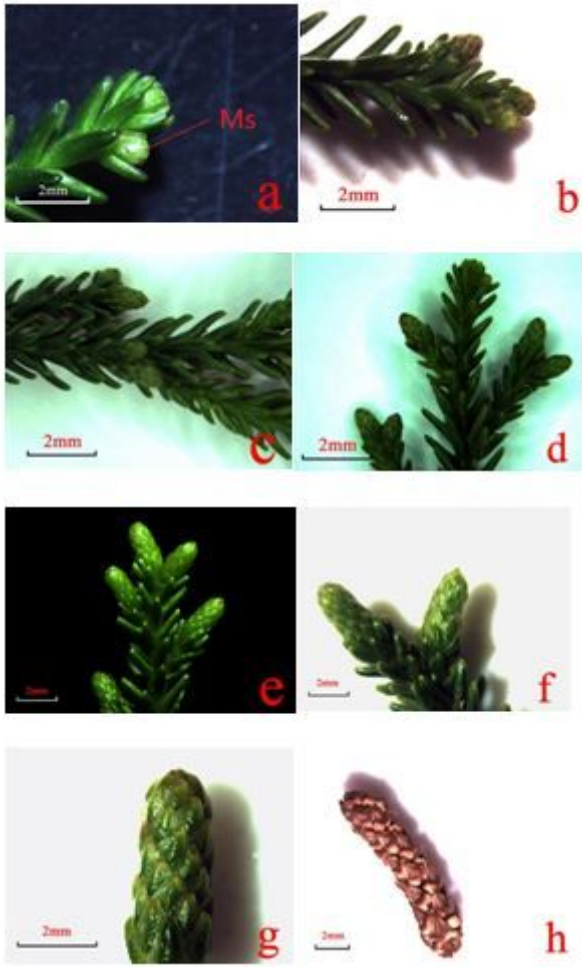
Figures

Figure 1

External morphological images of male cones of D. pectinatumat different developmental stages Note:(a) Early April 2018; (b) May 2018; (c) July 2018: (d) August 2018; (e) October 2018; (f) November 2018; (g) January 2019; (h) February 2019. (Ms: Microspore; Bars = 2 mm)
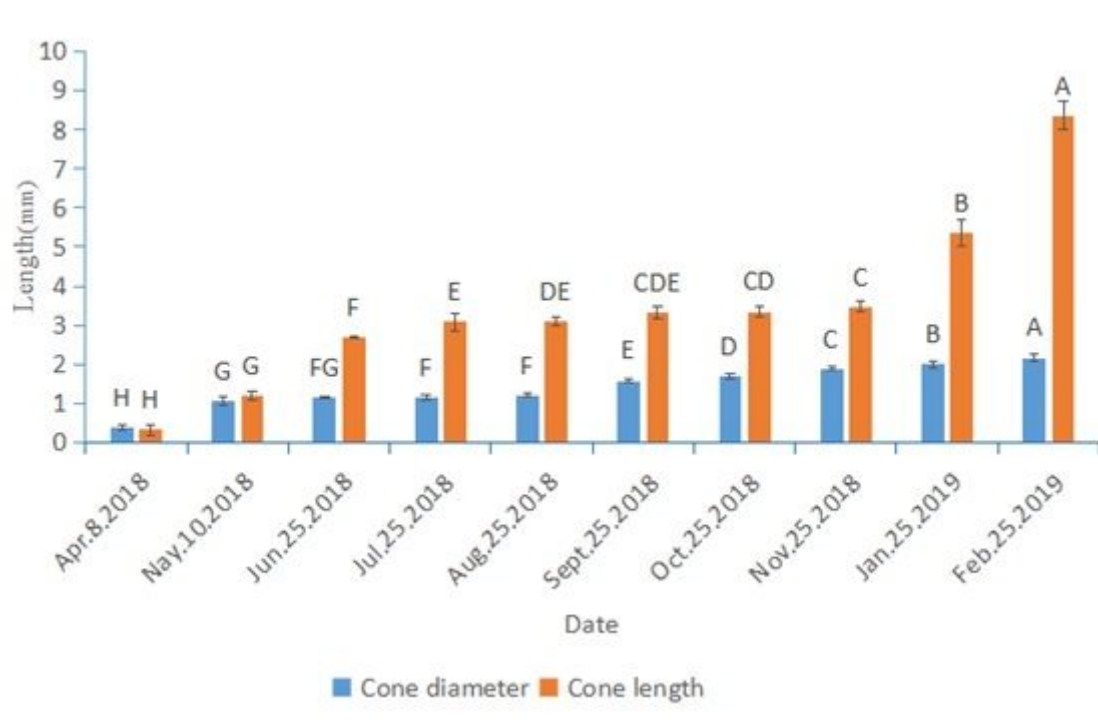
Figure 2

Length and diameter of male cones of D. pectinatumat different developmental stages Note: Different letters among length or diameter indicate significant difference at p<0.01
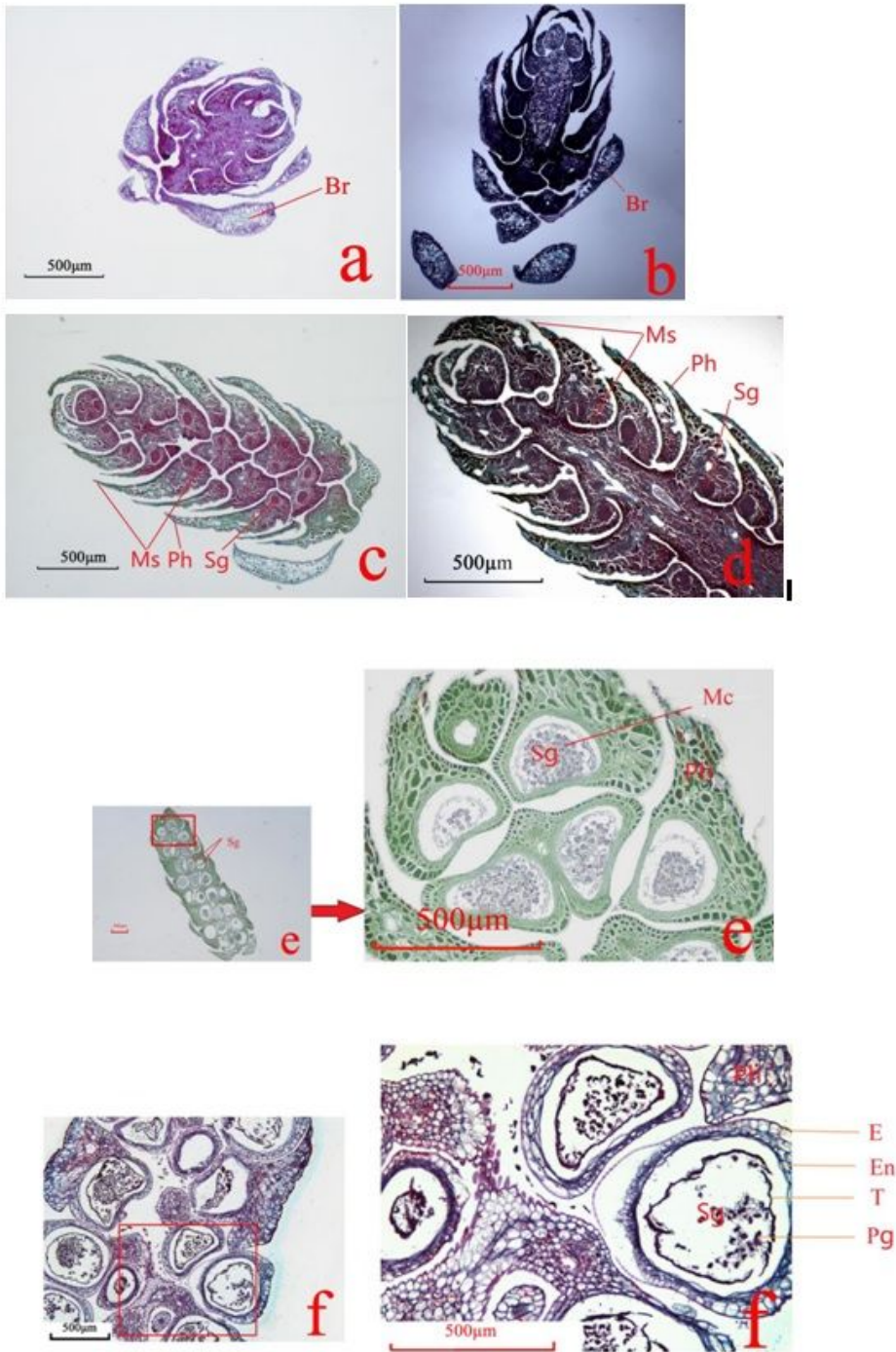
Figure 3

Microscopic images of male cones of D. pectinatum at different developmental stages. Note: a-b: microspore primordium formation and development stage, April 2018; c-d: microspore sac formation stage, September 2018; e: pollen mother cell formation and division, December 2018; f: pollen grain formation, late January 2019. Sg: sporangia; Ph: phylloclade; Mc: mother cell; Pg: pollen grain; T: tapetum; En: endothecium; E: epidermis; Bars=500 μm.
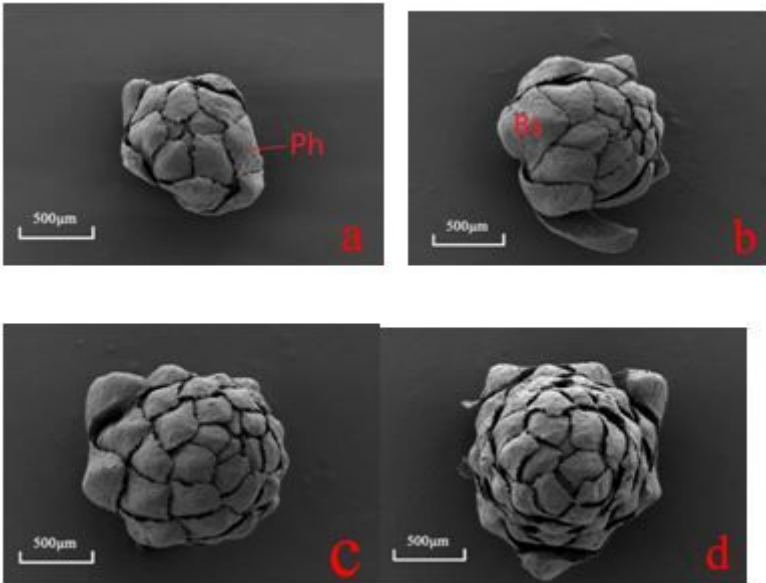
Figure 4

SEM images of male cones ofD. pectinatum at different developmental stages Note: (a) April 2018; (b) June 2018; (c) September 2018; (d) December 2018. Ph: phylloclade; Bars=500 μm.
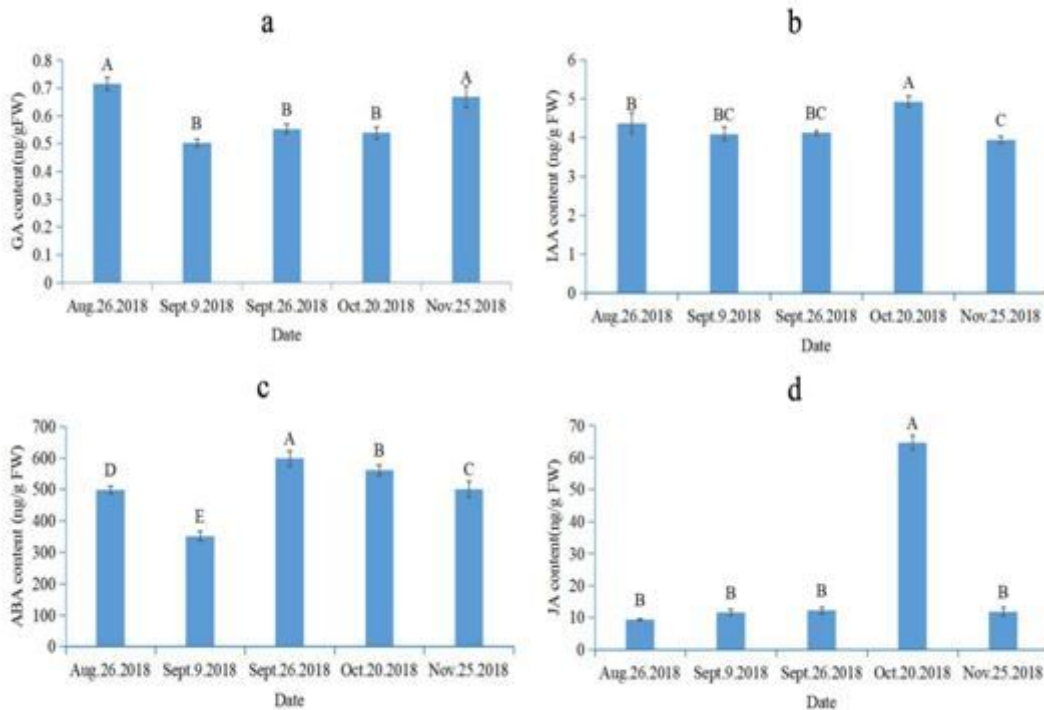

Figure 5

Endogenous hormone changes in male cones of D. pectinatum at different developmental stages Note: The error bars represent standard deviation, and different letters indicate significant difference at p<0.01
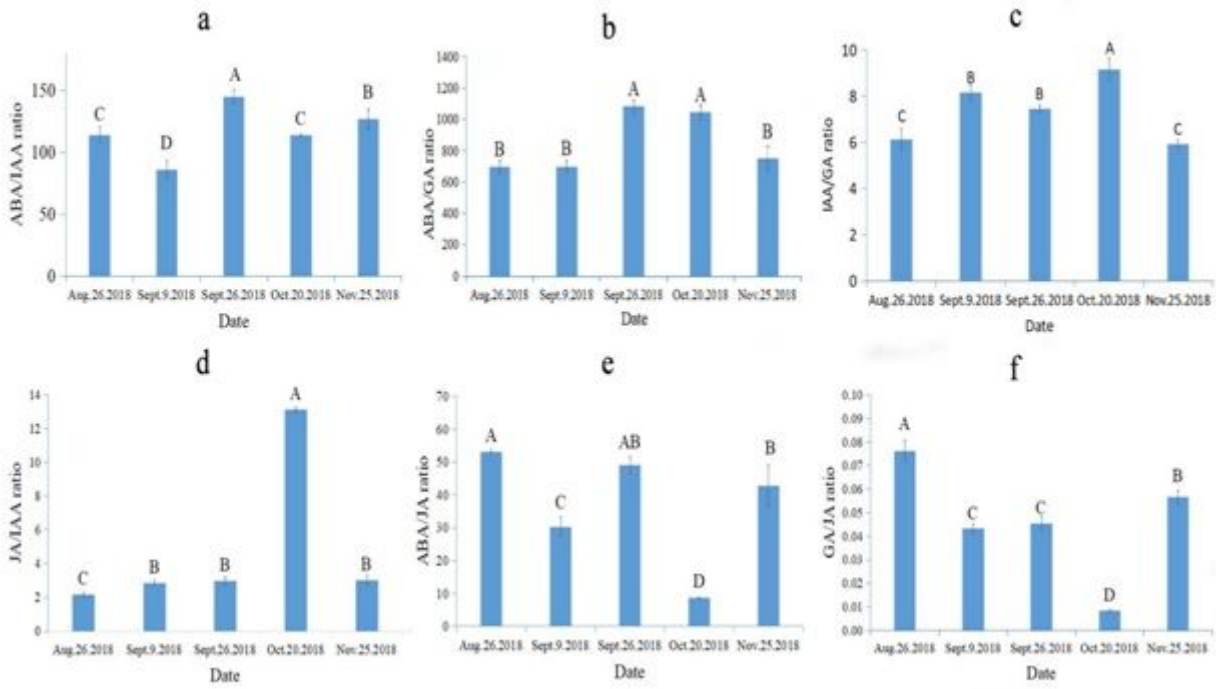
Figure 6

Ratios of endogenous hormones in male cones ofD. pectinatum at different developmental stages. Note: Error bars represent standard deviation, and different letters indicate significant difference at p<0.01。