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Scanning Electron Microscopy Analysis of Early Floral Development in *Renanthera bella* J. J. Wood, an Endemic Orchid from Sabah

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ABSTRACT

Renanthera bella J. J. Wood is an endemic orchid to Sabah with beautiful bright red petals. Most of the floral development of orchids is significantly affected by seasons and geographical factors. To date, the flower development stages of *R. bella* have never been studied. This study was conducted to observe the morphological changes of flower initiation and early development by apical dissection and scanning electron microscopy (SEM). The floral organs were dissected and fixed in 1.5% glutaraldehyde and dehydrated with alcohol. Characteristic stages of the initial flowering pattern were recorded until the flower was fully open and become senescent. *Renanthera bella* showed a typical acropetal pattern starting with early flowering from the base to the apex, forming a raceme-type inflorescence. Its flower development was divided into ten stages, which started with the flower bud appearance and the initiation of primordia. Next, the flower sepal started to develop within seven days of bud appearance. The final stage occurred after 25 days of observation when the bud opened, with an average bud length and diameter of 1.94 ± 0.56 cm and 0.50 ± 0.29 cm, respectively. The *R. bella* flower maturity stage was achieved between 38 to 40 days after anthesis, with the average length and diameter of petals and sepals increasing to 2.49 ± 0.23 cm and 0.38 ± 0.06 cm, respectively. A capsule successfully formed after one week of pollination. It reached the maturity stage at approximately 15 weeks after pollination. The orchid capsule started to break and expose the seeds inside after 21 weeks. This analysis emphasized the early floral development pattern, which could help estimate the length of flower maturity and pollination.

Keywords: Anther, flower development, pollen, *Renanthera bella*

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INTRODUCTION

Renanthera bella J. J. Wood is an epiphytic orchid endemic to Sabah. This species is distributed from the Mount Kinabalu region to the Lahad Datu district (Chan et al., 1994). According to Pridgeon et al. (2014), *R. bella* can be found at an elevation between 400 to 1200 m and is confined to low-stature hill forests. *Renanthera bella* produces beautiful, attractive blotched-pink to crimson red flowers with a yellowish-cream to apricot yellow base. This species has been widely over-collected and is classified as endangered in Appendix I of the Convention on the International Trade of Endangered Species of Wild Flora and Fauna (Chan et al., 1994). Given the challenges it faces, no studies on the pattern of *R. bella* floral development have been conducted.

Most orchid species are at risk of habitat loss due to various human activities, such as natural habitat destruction and overcollection. Most attempts to culture endangered and rare orchid species *in vitro* have been supported by anatomical and morphological observations of flower development (Arditti & Pridgeon, 1997; Burgeff, 1932). Various reports on molecular genetic analysis of flower development of orchids have been made over the years, such as the floral organ-specific genes (Pan et al., 2014), the role of MADS-box genes in the flower development and evolution (Becker & Theissen, 2003; Tsai et al., 2004; Zahn et al., 2005), and the regulatory mechanisms underlying orchid flower development (Xu et al., 2006). However, the morphological structure of buds and flowers in *R. bella* has not been well-identified.

Scanning electron microscopy (SEM) is used to increase the accuracy of plant and flower surface observations, as well as the development of floral organs. The images provide a more detailed view of the inner floral organs and the leaf surface features of the stomatal guard cells, epidermis, and hairs (if present). SEM was previously used to observe the stoma and micro-level character of the abaxial and adaxial surfaces of *in vitro* and *in vivo Passiflora edulis* leaves (Veeramohan et al., 2013).

Studies on the developmental stages of the inflorescence are essential as they relate to the control of plant reproduction, the pattern of plant growth, the timing of plant production, and how flowers interact with air movement for flowering (Friedman & Harder, 2005). An understanding of plant reproductive biology is essential in plant conservation efforts. In the present study, SEM was used to observe the morphological changes of flower initiation and early development until full bloom by apical dissection. During the observation, it was observed that different flower parts exhibit various patterns of development in their morphological characters. This study can provide basic background information that can be used to predict the maturity of the capsule that is important for seed germination.

MATERIALS AND METHODS

Plant Sources

Three-year-old *R. bella* plants were obtained from the greenhouse of the Institute for Tropical Biology and Conservation (ITBC), Universiti Malaysia Sabah (UMS). The plants were maintained in the greenhouse under natural light, 80% relative humidity, with a temperature of 28/24 ± 2°C day/night. The plants were grown in charcoal and coconut husk medium and fertilized using commercial fertilizer. Measurements for the *R. bella* flowers were recorded for length, diameter, and morphological changes. The *R. bella* flowers were hand-pollinated in September 2017 to form capsules. The plant species were identified by Mr. Johnny Gisil from ITBC and by a botanist from the Poring Orchid Conservation Centre (POCC; 0602′ 20.7″N, 116 06′ 38.2″E). This research was performed at the Tissue Culture Laboratory, ITBC, UMS (06003′ 33″ N, 116 012′ 29″E) under 24 ± 2°C temperature, 80% humidity, and 8/16 hours of light/dark condition.

Observation of Flower Bud Initiation and Development

The flower bud initiation and development of *R. bella* were recorded over two and a half months, starting with bud initiation, to the opening stages, and the formation of the full bloom or mature flower. The method of Smyth et al. (1990) was carried out with minor modifications. The samples were dried in the desiccator (for 1 h) instead of a critical point dryer. Additionally, the concentration of glutaraldehyde used was only 1.5% to prevent damage to the cells and tissues of the flower, as well as the thin surface. Data on bud length, bud width, lip length, lip width, pollen and anther size, and mature petal and sepal length and diameter were recorded weekly. The experiment was conducted using three replicates of plants with a total of eight floral organs per plant. The data was determined by calculating their mean and standard deviation. In addition to these data, the changes in the development of the flowers were recorded from time to time.

Dissection of Floral Organs

The floral organs were dissected into different flower parts or tissue such as bud, anther, pollen, petal, sepal, lip, and column for further observation of their morphology and physiology under the SEM. Both immature and mature floral organs were dissected for the comparison of their development.

Scanning Electron Microscopy (SEM)

The inflorescence and the young buds of *R. bella* were harvested from the greenhouse and dissected immediately into various floral tissues such as bud, anther, pollen, petal, sepal, lip, and column. The bud was held tightly with a pair of forceps and dissected using a blade by cutting the base part of the petal and sepal vertically to reveal the inner tissue (lip, column, anther, and pollen) under the stereomicroscope. The tissues were then fixed in 1.5% glutaraldehyde and incubated at 4°C for 24 h. The samples were then rinsed three times in 0.1 M phosphate buffer with an interval of 10 min. Then, the tissue

materials were dehydrated in a series of ethanol (35%, 50%, 70%, 90%, and 100%) for 30 min at each ethanol concentration. The samples were desiccated for 1 h before mounted on a metal SEM stub and coated with gold in a sputter coater for further observation on the SEM (Zeiss, Germany), and the image was recorded (Smyth et al., 1990).

RESULTS AND DISCUSSIONS

The Morphology of Flower Initiation and Inflorescence Formation

The studies of morphological development of flower bud formation in *R. bella* were based on the methods of physical observation and measurement. To date, the studies and in-depth analysis of *R. bella* flower development are scarce, making it difficult for comparison study.

Initially, the inflorescence started from the interstitial leaf (arrow; Figure 1A) and continue to develop and elongate rapidly after only five days (Figures 1B and 1C). It can grow to 30 cm and longer. Buds and flower organs arise at the peak of the inflorescence in the acropetal chain, where the flower buds begin to mature from the base to the top (Figure 1D). Usually, the inflorescence emerged from the fourth node below the apical leaf (Sakanishi et al., 1980). On the seventh day, cream and brown flower buds with a diameter of 0.02 cm appeared on the apical meristem and formed a raceme type of inflorescence (Figure 1E; stage 1). A raceme is an unbranched,

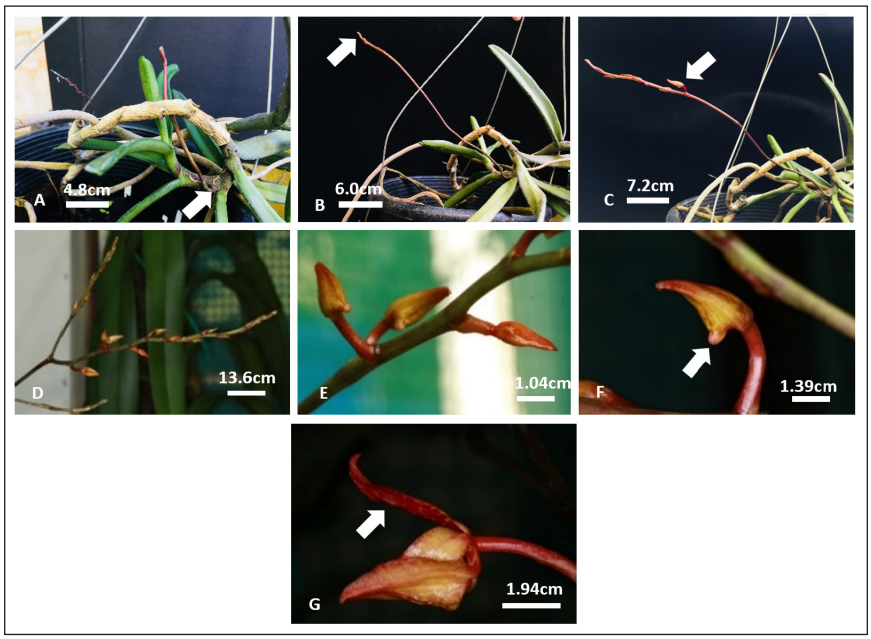

Figure 1. Inflorescence development of *Renanthera bella*. (A) Inflorescence started to arise from the interstitial leaf (arrow); (B) Inflorescence elongated and primordia bud started to appear (arrow); (C) Flower bud started to develop (arrow); (D) *Renanthera bella* flower development with acropetal growth pattern; (E) Formation of raceme inflorescence; (F) Lip started to arise and appear at the bottom of the bud (arrow); (G) Single dorsal sepal started to open (arrow)

elongated inflorescence with pedicellate flowers maturing from the bottom upwards (Harris & Harris, 2004). The lip starts to arise and appear at the bottom of the bud (Figure 1F), whereas the bud begins to open from its dorsal sepal (Figure 1F). There are approximately 7 to 15 flower buds in a single inflorescence. After 25 days, a single dorsal starts to open (Figure 1G).

The description of flower development has been reported in several studies. Previously, the flower development of *Malus* x *domestica* Borkh. (comprising 8 morphological stages) and *Arabidopsis* (comprising 12 stages) were recorded (Foster et al., 2003; Smyth et al., 1990). The overall results indicated that the early development of the inflorescence of *R. bella* followed a typical acropetal development pattern (Harris et al., 1991; Naghiloo & Claen-Bockhoff, 2017), whereby a single dorsal started to open after 25 days (Figure 1G). Xu et al. (2006) reported that most of the angiosperm flowers had four whorls, namely sepals, petals, anthers, and pistils. Based on the observations and measurements from the inflorescences, the process development of *R. bella* flower from its initiation until the bud opening can be separated into ten stages, as shown in Table 1.

Bud Development

The studies of bud development in *R. bella* were based on the methods of physical measurement and SEM analysis, as described by Wang et al. (2014). Continuous observations of early primordial growth and flowering were carried out until the buds reached maturity (bud opening). The flower bud size increased weekly, and the buds started to open after 25 days of observation.

Table 1
Morphological features in Renanthera bella *flower development pattern*

Stages	Development event	Flower development period (days)	Remarks
1	Flower bud started to appear and form a raceme type of inflorescence	7	The flower bud is creamy white, while the inflorescence stalk is light green
2	Flower bud primordia started to develop	7	The flower buds are creamy-white and brown
3	Sepals developed	12	The sepals are creamy red
4	Immature column developed	16	The column is red-brown
5	Lip developed and can be observed between lateral bud sepals.	16	The lip is creamy
6	Stigma papillae appeared	16	-
7	Pollen not fully developed	16	The pollen is light yellow and kidney-shaped
8	Anther cap enclosed the pollen	16	The anther cap is dark brown and has a straight line with a deep curve
9	Sepal enclosed the flower bud	21	The adaxial sepal is pale red
10	Bud started to open	25	Bud is red maroon

Renanthera bella inflorescences will continue to develop under 27 ± 2°C, 10/14 h light/dark with 70% – 80% humidity. Overall measurement on the length and diameter of flower buds was recorded for 5, 12, 18, and 25 days of observation and summarized in Figure 2.

Figure 2A shows the early flower bud that is covered with leaf primordial (arrow). Within seven days of observation, the primordial leaf sheath elongated, and the bud size increased (Figure 2B). Then, the lower part of the lip (Figure 2C) and sepal line (Figure 2D) started to appear and emerged from the bottom of the flower bud on the 16th day of observation. The flower bud developed and elongate then tapered at the apex (Figures 2E–2G). Some differences were noted on the surface character of the epidermal cell for abaxial and adaxial buds, where the abaxial immature bud character showed proximity to the epidermal cell and the presence of several stomata guard cells (Figure 2H), while the adaxial surface of the bud displayed a linear epidermal shape (Figure 2I). The flower induction process involved the triggering of the flowering bud by environmental factors, which result in the change of development patterns, leading to the formation of flowers.

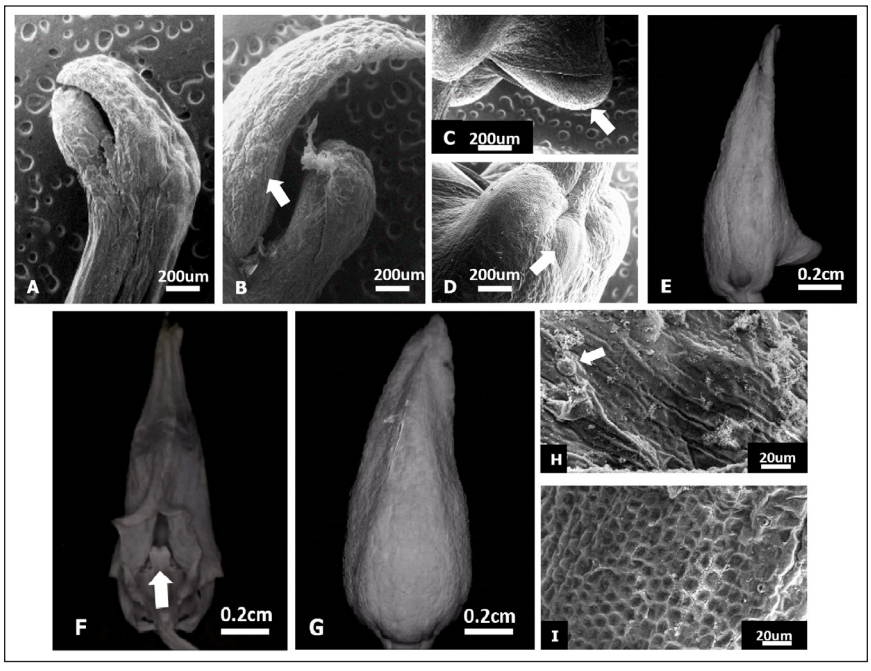

Figure 2. Scanning electron microscope analysis of flower bud development of *Renanthera bella*. (A) Early-stage of epidermal shape; (B) Floral bud with enclosed by young leaf primordium (arrow); (C) Lip appears at the bottom of the bud (arrow); (D) Line of lateral sepal on the immature bud (arrow); (E) Front view of mature flower bud; (F) Lower bud with tapered apex showed the appearance of lip (lateral view) (arrow); (G) Bud reaches a diameter of 0.5 cm and starts to open; (H) Abaxial surface of immature bud showing the proximity of epidermal cell and stomata guard cell (arrow); (I) Adaxial surface of bud with epidermal cell in an irregular shape

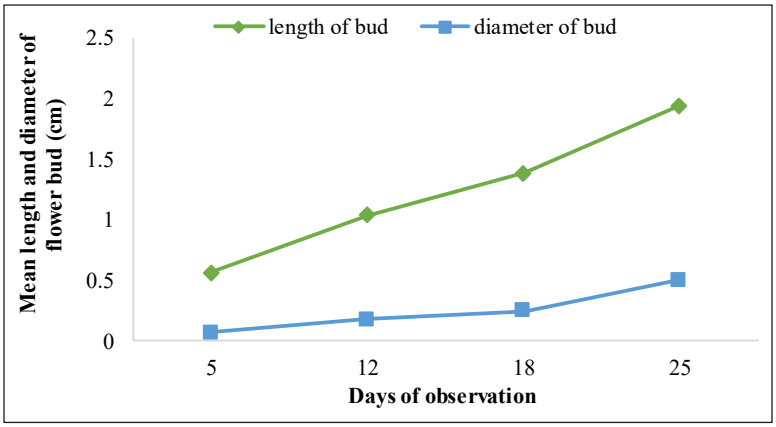

Figure 3. Length and diameter of the flower bud of *Renanthera bella* within 25 days of observation

The flower structure has been studied in various ways. Studies of natural history and evolutionary biology of flowers have emphasized understanding the ultimate (evolutionary) causes of a wide range of variants, such as color, symmetry, meristic arrangements (e.g., flower organ number), size, pollination syndrome and others (Alvarez-Buylla et al., 2010).

Lip and Column Development

The differences between the columns and the lip are shown in Figure 4A to Figure 4D through the front and lateral view. The column consists of the androecium, the male reproductive part, and the modified filaments and styles that fuse in orchid flowers. The immature column appears straight from the sides and has an average length of 0.50 mm (Figure 4A; stage 4), while the mature column reaches 3.5 mm in size and appears curved on the side (Figure 4B). The column contains anther, pollen, and stigmatic papillae that are part of the pollination process (stage 6). The stigmatic papillae have a rostellum character, and their position is below the column (Figure 4C). At its mature level, the column changes to a curve formation (arrow) and has a smooth surface without hair (Figure 4E), while the immature column is straight and has a hairy surface (arrow; Figure 4F).

Lips appeared below the bud between the lateral sepals after 14 days (stage 5). The lip is a modified petal of the orchid flower. The immature *R. bella* lip has an average size of 0.09 mm and is tapered at the apex. The *R. bella* lip is broad and slightly spread at the base (Chan et al., 1994). *Renanthera bella* has gynandrous stamens that fuse with the pistil to form the reproductive part called the column.

Figures 4G–4H show the front and lateral view of the appearance of the mature lip as it tapered to the apex, while Figure 4I shows a vertical view of the lip that forms the hole inward. The nectary primordia initiate under the column and become mature and elongated at the base (Figure 4J). Moreover, the surface of the epidermal cells of the mature column is circular and thick, with wrinkles lining their cell walls (Figure

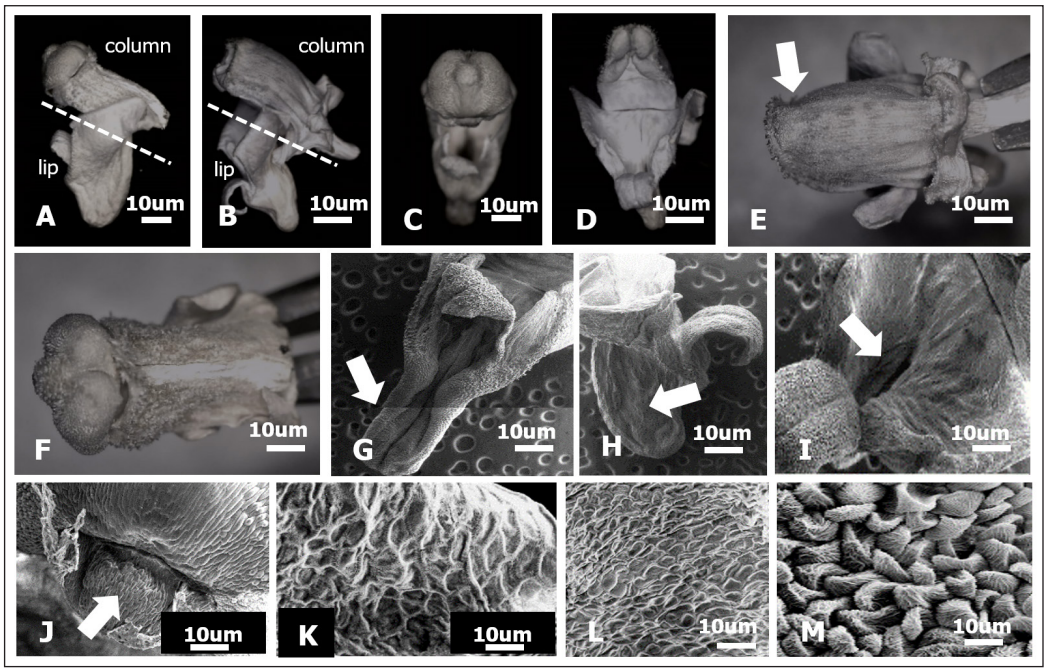

Figure 4. Lip and column development of *Renanthera bella*. (A) Lateral view of immature column and lip; (B) Lateral view of matured column and lip; (C) Front view of immature column and lip; (D) Front view of matured column and lip; (E) Vertical view of mature column change to curve formation (arrow); (F) Vertical view of an immature column; (G) The matured lip is tapered to the apex (front view) (arrow); (H) Lateral view of matured lip (arrow); (I) Vertical view of the mature column forms the hole inward (arrow); (J) Nectary primordia under the column (arrow); (K) The surface of the mature column; (L) The surface of the immature column with thickening circle shape; (M) Epidermal cell from the adaxial surface of mature lip showing dams hemisphere shape (*Note*. Sepal and petal dissected away to reveal inner organ)

4K). An epidermal cell in the middle of the column develops a thick, irregular circular shape that curves inward (Figure 4L). The cell surface of the lip tip has a hemispheric shape (Figure 4M).

Anther and Pollen Development

In stage 7, pollen is formed, while in stage 8, the anther cap develops and encloses the pollen for protection. *R. bella* has an anther cap structure that fuses in front of the column structure from the edge view, while the pollen is located inside the anther cap for protection before pollination at maturity (Figure 5A). The immature anther cap is round and curved with a small circle that rises and forms a line pattern in the middle (Figure 5B). The whole structure of the anther cap has puberulent hair, while the mature anther cap is also hairy on its surface, with a faint line in the middle and a small circle arising in the middle that begins to disappear (Figures 5A–5B). Furthermore, *R. bella* mature pollen has a thick, wrinkled shape (Figure 5C). The anther sac is curved inward to protect the diploid pollen before pollination (Figure 5D), while the pollen stalk in the inner surface of the cell has hexagonal epidermal cells (Figure 5E).

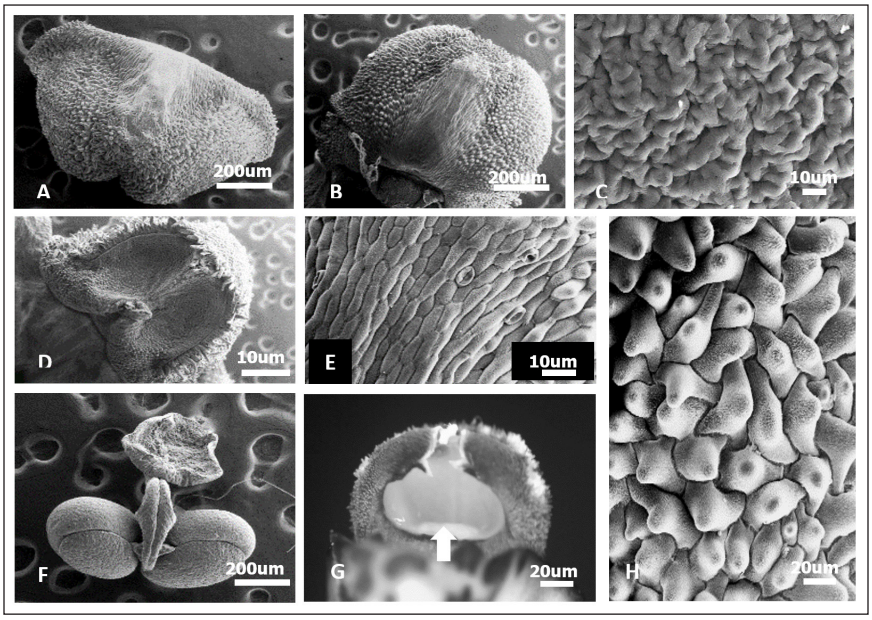

Figure 5. Anther and Pollen Development of *Renanthera bella*. (A) Vertical view of immature anther cap; (B) Matured sepal with pubescent hair; (C) The surface of mature pollen; (D) Vertical view of matured anther sac after pollen removed; (E) The surface of pollen stalk; (F) Mature pollen of *R. bella*; (G) Matured stigmatic papillae (arrow); (H) Vertical view of pollen surface (*Note*. Sepals and petals dissected away to reveal inner organ)

Renanthera bella pollen appears as two masses with kidney-like shapes (Figure 5F). Additionally, the stigma papillae have a sticky hole surface for pollination purposes (Figure 5G). Pollen begins to form together with column formation and develops along with the anther and cap. The pollen size in mature *R. bella* is approximately 0.05 mm. Mature anther of the epidermal cell surface of the epidermis shows a conical shape (Figure 5H).

Petal and Sepal Development

Data on the length and diameter of the outer flower organ (sepal and petal) is recorded in Table 2. The sepal line begins to appear, and curves inward to cover the apex (Figure 6A; stage 9). The primordial petals develop at the same time as the sepals, and they proliferate to enclose the bud (Figure 6B). The sepals on the young flower buds overlap with each other and cover the flower's internal organs (Figure 6C). The basal portion of the petal is broad and extends and tapers into the apex portion. The adaxial cell surface at the tip of the sepals begins to mature and forms a hexagonal cell when mature (Figure 6D). Stomata are observed on the adaxial surface of the sepals. The mature stomata are arranged far away from each other on the surface of the epidermal cell. Each stoma has two thickened guard cells (Figure 6D). Additionally, the mature septic surface of the sepals does not have a definite shape (Figure 6E). At maturity, the average length of the sepals was 0.38 ± 0.06 cm during

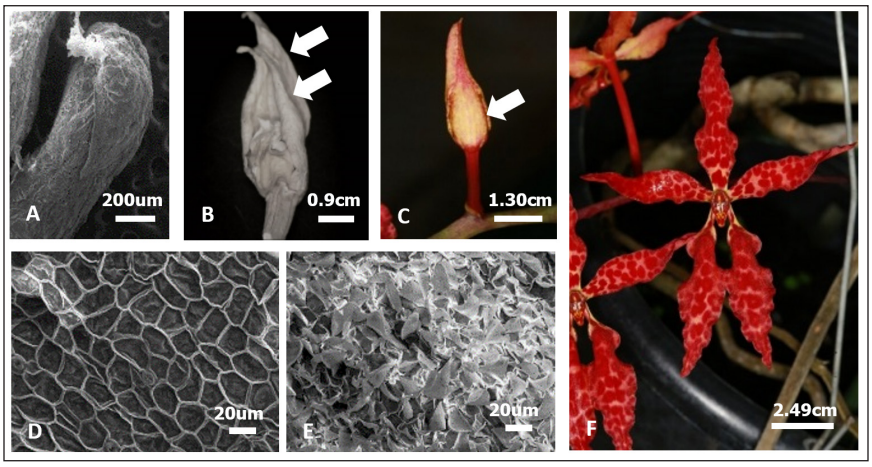

Figure 6. Petal and sepal development of *Renanthera bella*. (A) Sepal line begins to appear and curves inward to cover the apex; (B) Petals also develop an elongated first compare to sepals (arrow); (C) Line of immature sepal appears (arrow); (D) Enlarge the view of the adaxial surface of immature bud; (E) Enlarge the view of abaxial surface of immature bud; (F) Mature flower of *R. bella* (full bloom)

Table 2
Length and diameter of outer flower organ on the primary inflorescence of Renanthera bella *(n=3)*

Days (Age)	27	30	33	35
Length of lateral sepal	2.01 ± 0.16 cm	2.15 ± 0.14 cm	2.28 ± 0.10 cm	2.49 ± 0.23 cm
Diameter of lateral sepal	0.31 ± 0.06 cm	0.34 ± 0.05 cm	0.38 ± 0.07 cm	0.38 ± 0.06 cm
Length of petal	1.70 ± 0.05 cm	2.10 ± 0.08 cm	2.36 ± 0.14 cm	2.39 ± 0.14 cm
Length of stalk	0.81 ± 0.50 cm	0.86 ± 0.49 cm	0.89 ± 0.54 cm	1.01 ± 0.55 cm

growth in this experiment. The epidermal surface of the abaxial and adaxial sections is similar to the sepals. The development of the petal just precedes the anthesis process, and the petals can reach a maximum average length of 2.39 ± 0.14 cm. Like most of the orchid species, *R. bella* flowers consist of four whorls, namely sepals, petals, anthers, and pistils, with a unique feature (Xu et al., 2006). The first whorl of orchid flowers is made up of one dorsal and two lateral petaloid sepals (Figure 6F). Stage 10 occurs when the sepals start to open and elongate rapidly to form the mature flower. The bud begins to open from its dorsal sepal.

Capsule Development in *Renanthera bella*

The formation of the *R. bella* capsule was investigated between September and October. Figure 7 shows the length and diameter of the *R. bella* capsule after 21 weeks of observation. The formation of the capsule was recorded in terms of length and diameter until it matured, starting from the day of hand pollination. Based on the observations, there was a drastic increase in the length of the capsule in the fifth week after pollination, with the diameter of the capsule increasing steadily. For the first six weeks, the diameter of the capsule increased

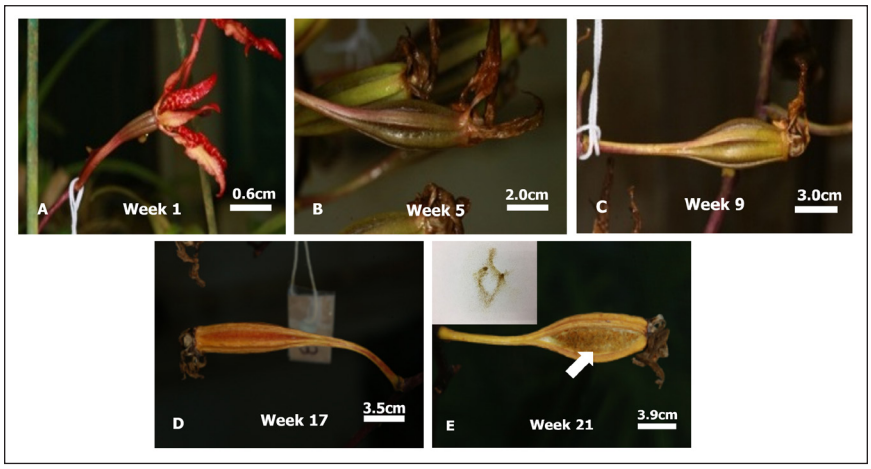

Figure 7. Formation of the *Renanthera bella* capsule after hand pollination. (A) Flower petal starts to shrink and the stalk starts to swollen; (B) Flower petal started to wilt, and the capsule formed; (C) Capsule increase in size; (D) Capsule change its color from green or brown to yellowish in a mature capsule; (E) Over matured capsule with an exposed seed (small figure)

steadily. However, the rate of length of the capsule growth started to decrease after week 5, with only a total of 1 cm growth recorded.

After six weeks, the capsule reached a diameter of 0.6 cm and a length of 3.0 cm. However, the length and diameter of the capsule are not the primary factors in determining the maturity level of seed. According to Malarkodi and Srimathi (2007), the maturity level of a seed can be attributed to the physiological and functional changes that occur from the flower bud formation until full bloom event or the anthesis stage. From the observation, after week 21, the *R. bella* capsule started to break out, showing that it has overreached the mature phase (Figure 7E). The formation and development pattern of the capsules after pollination for week 1 until week 21 are shown in Figures 7A–7E.

CONCLUSIONS

In conclusion, the study demonstrated ten stages of the early flower development pattern of *Renanthera bella*. During the process, flower bud initiation, morphological characteristics of flower events, and capsule formation were revealed. The time required for *R. bella* to produce mature and full bloom flowers from bud inflorescence formation ranged from 7 to 25 days. In addition, approximately after weeks 21, *R. bella* seeds reached maturity. More studies of orchid flower organ identities are required to understand the evolution of the unique structures better. Further work is also needed to identify the essential genes that may be involved and significant for the floral development process.

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