Floral micromorphology of the genus *Ensete* Bruce ex Horan. (Musaceae) in Thailand

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ABSTRACT: To fulfil scarce and incomplete information on floral micromorphology of ensets (*Ensete* Bruce ex Horan.) in the banana family (Musaceae), a comparative anatomical study of two species: *E. glaucum* (Roxb.) Cheesman and *E. superbum* (Roxb.) Cheesman, native to Thailand was conducted. It was found that, apart from five fertile stamens presented in other members of the Musaceae family, both ensets possess a short staminode. It is suggested from this investigation that six is the basic number of *Ensete* androecial whorl and the taxa could secure the most primitive status within the family and the Zingiberales order, of which stamen numbers are reduced. The results also indicated that the vascular bundle position in compound tepal, the vascular patterns in vascular zone of ovary and cell shapes of stigma epidermis and the ovary cortex are of systematic significance in conjunction with pollen size and exine ornamentation. These useful micromorphological characters can be further applied for identification of other *Ensete* species distribute elsewhere in the world.

KEY WORDS: Amphicribal vascular bundle, Anatomical characters, Carpellary dorsal bundle, Floral anatomy, Free tepal, Ginger order, Monocotyledons, Staminal strands, Sterile stamen.

INTRODUCTION

The *Ensete* Bruce ex Horan., a small genus of eight to nine species, is distributed in tropical regions of Asia, Africa and Madagascar (Horaninow, 1962; Cheesman, 1947a; Baker and Simmonds, 1953). Two species, *E. glaucum* (Roxb.) Cheesman and *E. superbum* (Roxb.) Cheesman were found in Thailand. *Ensete glaucum* has a relatively wide distribution from Burma (Myanmar) to the Philippine Island and Java, while *E. superbum* distributes in southern part of India, West India, Assam to northern Thailand (Roxburgh, 1820; Baker, 1893; Cheesman, 1947b; Simmonds, 1962). The *Ensete* was separated from *Musa* L. based on several morphological characters such as T-shaped embryos and papillose pollen grains (Lane, 1955; Bekele and Shigeta, 2011) and genetic status supported the separation (Ude *et al.*, 2002; Wong *et al.*, 2002; Liu *et al.*, 2010; Li *et al.*, 2010). *Ensete*, as well as other members in the Musaceae, possesses unique floral characters, i.e. compound tepal which is the fusion of three outer and two apiculate inner tepals; one free tepal; five fertile stamens and one pistil (Cheesman, 1947a & b; Bartlett and Specht, 2010). The two ensets, *E. glaucum* and *E. superbum*, can be distinguished by their morphology. *Ensete glaucum* possesses cylindrical stem, sheathing petioled leave and ovate to lanceolate bud, whereas *E. superbum* short conical stem, petioled leave without sheathing, and cordate bud. However,

floral structures of *E. glaucum* are very similar to those of *E. superbum* (Roxburgh, 1820; Baker, 1893; Cheesman, 1947b).

Anatomical character is an important taxonomic information in many plant groups, particularly in monocotyledons (Zarrei *et al.*, 2010). Though anatomy of Musaceae of both vegetative and reproductive parts such as leaves, rhizomes, ovaries, and flowers, have been generally studied (Tilak and Pai, 1974; Triplett and Kirchoff, 1991; Sumardi and Wulandari, 2010), floral anatomy of *Ensete* species have not been compared in detail. The aim of this study was, therefore, to describe the floral organs including compound tepal, free tepal, stamen, stigma and pollen of the two *Ensete* found in Thailand in order to support the identification.

MATERIALS AND METHODS

Plant collection

Two populations each of two *Ensete* species; *E. glaucum* and *E. superbum*, were collected from the field in Thailand as shown in Table 1.

Table 1 List of plant materials used in this study.

Species	Vouchers	Locality
Ensete glaucum (Roxb.) Cheesman	SS & JS 244	Kanchanaburi, Thong Pha Phum
	SS & JS 317	Tak, Umphang
Ensete superbum (Roxb.) Cheesman	SS & JS 242	Kanchanaburi, Thong Pha Phum
	SS & JS 391	Kanchanaburi, Sangkhla Buri
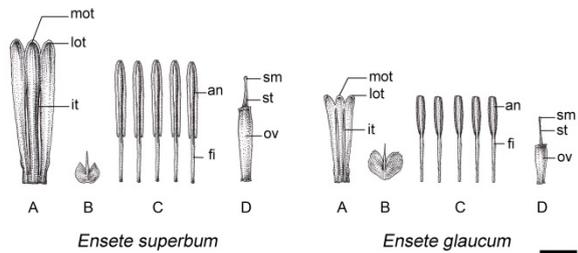
Fig. 1. Floral parts of the two *Ensete* species, *E. superbum* and *E. glaucum*, showing four organs. A: Compound tepals. B: Free tepals. C: Stamens. D: Pistils. Scale bar = 1 cm. Abbreviations: mot, median outer tepal; lot, lateral outer tepal; it, inner tepal; an, anther; fi, filament; sm, stigma; st, style; ov, ovary.

Fig. 2. Flower of *Ensete* showing positions of microtome transverse sections (TS, horizontal lines). Four organs of the *Ensete* flower. A: Compound tepal. B: Free tepal. C: Stamen. D: Pistil. Seven parts of TS namely, 1: upper part; 2: middle and 3: base of compound tepal; 4: middle part of free tepal; 5: middle part of anther; 6: middle part of stigma and 7: middle part of ovary. Scale bar = 1 cm.

Transverse section method and epidermal preparation

Compound tepals, free tepals, anthers, stigmas and ovaries (Fig. 1) from two accessions of each *Ensete* species were taken from three mature male flowers before anthesis. Base, middle and upper parts of the compound tepals were investigated, while only middle part were taken from the free tepals, anthers, stigmas and ovaries (Fig. 2). All materials were fixed overnight in 70% ethanol. Samples were then dehydrated in a series of tertiary butyl alcohol, embedded in paraplast, and sectioned using Leica SM 2000R sliding microtome. Sections were stained with Safranin-O and Fast Green, finally mounted with DePeX.

Free tepal were soaked in 5% KOH for 1 h, washed with water three times then dipped in 50% hypochlorite solution until it cleared. The samples were then dehydrated in ethanol series (50%, 70% and 95%), stained with 1% Chalazal Black E in 95% ethanol and mounted with DePeX on slides.

All anatomical characters were investigated under light microscopy (Olympus CX3 light microscope, U.S.A.) and photographed (Olympus BX43, U.S.A. compound microscope with an Olympus DP11 digital camera, U.S.A., attached).

Pollen morphology

Pollen were collected before anthesis, cleaned and dehydrated with 95% ethanol, mounted on stubs using carbon tape, coated with gold palladium using Hitachi E-102 Ion Sputter and photographed using Hitachi S2500 scanning electron microscopy. Terminology used to describe anatomical features of flower and pollen was from Tomlinson (1933), Tilak and Pai (1974), Kirchoff (1992), Xue *et al*. (2005) and Simao *et al*. (2007).

RESULTS

Compound tepal

Compound tepal of *Ensete* and other members of Musaceae is composed of three outer tepals and two

inner tepals united at base (Fig.1A). Transverse section at basal, middle and upper parts of the compound tepal possess connective tissue between the two whorls, three outer tepals fuse together on abaxial side and two inner tepals adnate at the margins of the median outer tepal on adaxial side (Fig. 3). Adaxial epidermis of basal part of the compound tepal is consisted of one layer of small elliptic cells, whereas one layer of pentagonal cells was found on abaxial side (Figs. 4A & 4F). The mesophyll at basal part is composed of three to four spongy parenchyma and two short palisade parenchyma cell layers (Figs. 4A & 4F), while the middle and upper parts are consisted of one to two spongy parenchyma
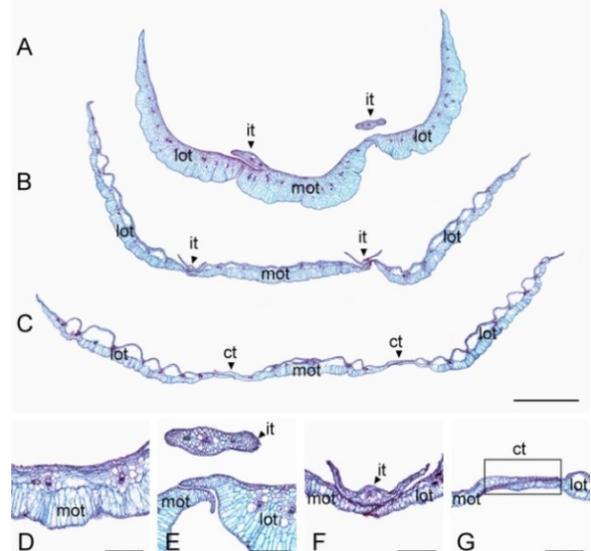
Fig. 3. (A-G) Transverse section of compound tepal of *E. glaucum*. A: Basal, B: Middle and C: Upper parts. Scale bar = 1,000 μm. D: Middle part of compound tepal transverse section showing median outer tepals. E: Inner tepal at basal. F: Middle parts showing one median strand of tepal. G: Connective tissue between median and outer tepals at upper part. Scale bar = 200 μm. Abbreviations: ct, connective tissue; mot, median outer tepal; it, inner tepal; lot, lateral outer tepal; pm, palisade mesophyll; sm, spongy mesophyll; vb, vascular bundle.

and one rectangular palisade parenchyma cell layers (Figs. 4B-C & 4G-H). The spongy parenchyma cell sizes at the middle part of the compound tepal of *E. superbum* are larger than those of *E. glaucum* (Table 2). The numbers of vascular bundle ring at basal part of the compound tepals of the two *Ensete* are different; two rings were found in *E. superbum* (Fig. 4A), while only one ring in *E. glaucum* (Fig. 4F). One row of vascular bundle situates on the adaxial side was found at the middle and upper parts of the compound tepals in both species, but at different position. The vascular bundles of *E. glaucum* locate between tepal ribs, whereas, at center of the tepal rib in *E. superbum* (Figs. 4B-C & 4G-H). Collateral bundles surrounded by three to five laticiferous cells were found in both *Ensete* species. Sclerenchyma or fibrous cells of *E. glaucum* situate above the phloem, however, are not well developed in *E. superbum* (Figs. 4D-E & 4I-J).
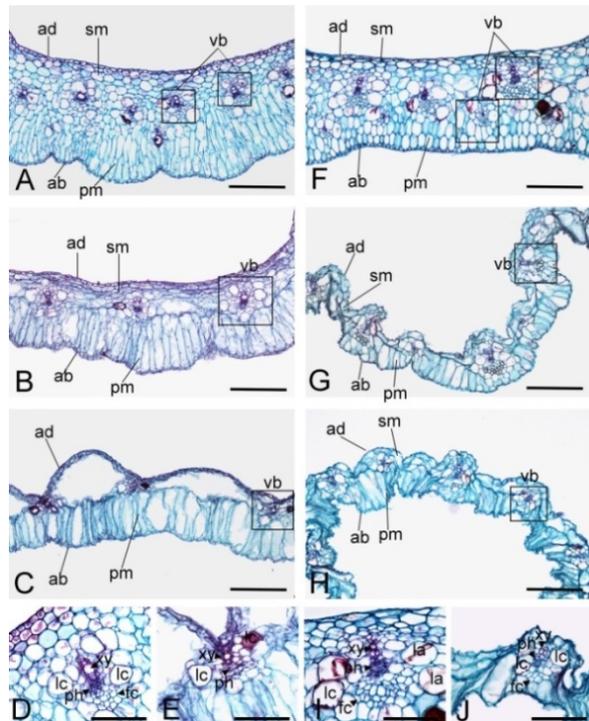
Fig. 4. *Ensete glaucum* (A-E) and *E. superbum* (F-J). Transverse section of compound tepal of *E. glaucum*. A: Basal, B: Middle and C: Upper parts. Transverse section of compound tepal of *E. superbum*. F: Basal, G: Middle and H: Upper parts. Scale bar = 200 μm. D: Vascular bundle at basal and E: Middle parts of *E. glaucum*. I: Vascular bundle at basal and J: Middle parts of *E. superbum*. Scale bar = 50 μm. Abbreviations: ab, abaxial epidermis; ad, adaxial epidermis; fc, fibrous cell; lc, laticifer cell; ph, phloem; pm, palisade mesophyll; sm, spongy mesophyll; vb, vascular bundle; xy, xylem.

Free tepal

Free tepals of the two species are bilateral and quite similar in anatomical characters. In surface view, the epidermal cells on both sides elongate with straight walls (Figs. 5A-B & 5D-E). Transverse section of the free tepal showed one layer of epidermis with thickened cell walls of square-to-rounded shape covered by thin cuticle. One hypodermal layer presents underneath both sides of the epidermis of *E. glaucum*, meanwhile, absents in *E. superbum*. Mesophyll is composed of parenchyma cells. Amphicribal type of vascular bundles was found in the two *Ensete*. The vascular positions in the free tepal are different, i.e. those of *E. superbum* situates closer to the adaxial side, whereas centrally locates in *E. glaucum*. Vascular bundles are surrounded by parenchyma cells and arrange in a single horizontal row (Figs. 5C & 5F).
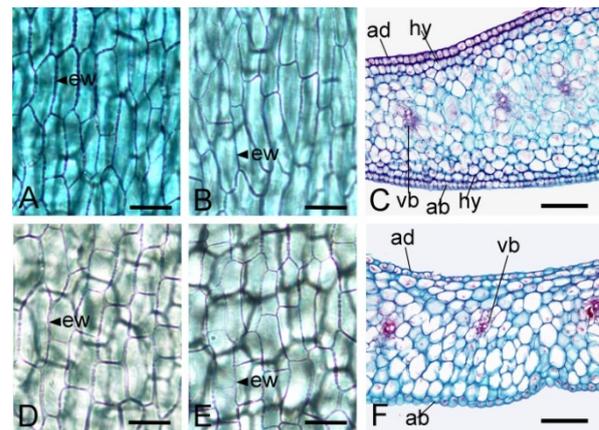
Fig. 5. *Ensete glaucum* (A-C) and *E. superbum* (D-F). A: Adaxial and B: Abaxial epidermis of free tepal in *E. glaucum*. D: Adaxial and E: Abaxial epidermis of free tepal in *E. superbum*. Scale bar = 50 μm. C: Transverse section of free tepal of *E. glaucum* and F: *E. superbum*. Scale bar = 100 μm. Abbreviations: ab, abaxial epidermis; ad, adaxial epidermis; hy, hypodermis; vb, vascular bundle; ew, epidermal cell wall.

Androecium

Ensete androecium is consisted of five fertile and one reduced stamens (Bartlett and Specht, 2010) (Fig. 6). The anther is comprised of two microsporangia which adnated on the connective tissue. Each microsporangia is separated into two locules by septum and split at longitudinal section. Mature anther wall includes one layer of thin-walled swelling epidermal cells and one layer of thin-walled endothecium with annular or helical thickening that shrink and dehiscence. The epidermis of connective tissue is thin-walled cell and spindle-like shaped with helical thickening. The connective tissue is composed of parenchyma cells, amphicribal bundles which locate in the center and a connective tissue cavity forms on both sides of the bundles (Figs. 7A & 7D). Pollens are spheroidal with radial symmetry (Figs. 7B & 7E). The *E. glaucum* pollen is verrucate, but *E. superbum* is perforate (Figs. 7B-C & 7E-F). The sizes and thickness of pollen walls of two *Ensete* are slightly different (Table 2). The

pollen size of *E. glaucum* is 113.9±5.2 µm, whereas *E. superbum* is 124.3±5.9 µm. The wall thickness of *E. glaucum* is 6.8±1.4 µm, whereas *E. superbum* is 7.0±1.7 µm.
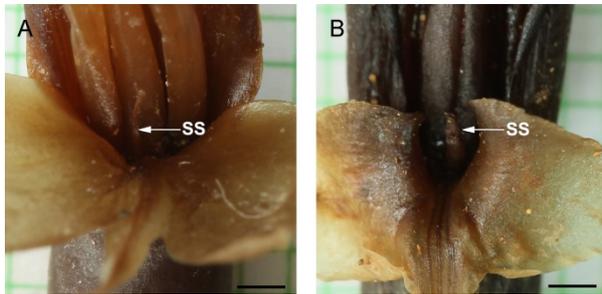
Fig. 6. The sterile stamens. A: *E. glaucum* and B: *E. superbum* as seen from adaxial side of the flowers. Scale bar = 1 mm. Abbreviations: ss, sterile stamen.
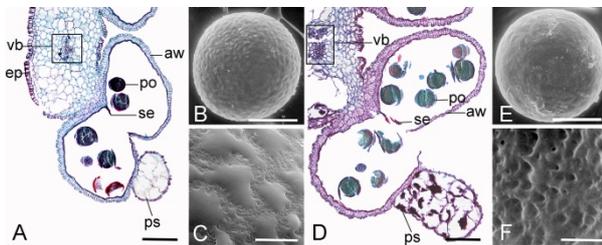
Fig. 7. The Androecium details of *E. glaucum* and *E. superbum*. Transverse sections of anthers. A: *E. glaucum* and D: *E. superbum*. Scale bar = 200 µm. Scanning electron micrograph showing pollen morphology. B: *E. glaucum* and E: *E. superbum.* Scale bar = 50 µm. Scanning electron micrograph showing pollen exine ornamentation of C: *E. glaucum* and F: *E. superbum*. Scale bar = 6 µm. Abbreviations: aw, anther wall; ep, epidermis; po, pollen; ps, pendulum-like structure; se, septum; vb, vascular bundle.

Gynoecium

Ensete stigma is zygomorphic and triangular in shape with three lobes (Figs. 8G & 8I). The stigmatic surface is smooth and composed of one epidermal layer. The epidermal cells on the stigma of the two species are different in shape; *E. glaucum* has square shape, while elliptic shape in *E. superbum* (Figs. 8H & 8J). Three collateral bundles are surrounded by polygonal parenchyma cells which are located near the style canal in each lobe (Figs. 8G & 8I). The ovary is inferior with 3-carpellate fuse into one gynophore. The epidermal layer of the ovary is composed of smooth or convex epidermal cells which were thick-walled cell. Cortex comprises elliptic parenchyma cells in *E. glaucum*, but rounded in *E. superbum* (Figs. 8C & 8F). Collateral bundle with fibrous cells is surrounded by rounded or elliptic parenchyma cells. One ring of collateral bundles was found in *E. glaucum*, whereas two rings in *E. superbum*. Amphicribal bundles were found near nectary ducts of both species. Idioblasts with raphide

were found in both cortex and vascular zone of the two *Ensete*, particularly in *E. glaucum* which possess many idioblast in the ovary (Figs. 8A & 8D).

All discriminating characters of the two enset species are presented in Table 2.
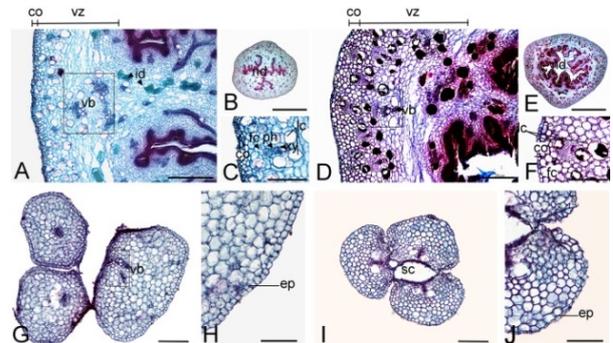
Fig. 8. The gynoecium details of *E. glaucum* (A-C & G-H) and *E. superbum* (D-F & I-J). Ovary transverse sections of *E. glaucum* (A-C) and *E. superbum* (D-F). Scale bar = 300 µm (A & D); 1,500 µm (B-E); 150 µm (C-F). Stigma transverse sections of *E. glaucum* (G-H) and *E. superbum* (I-J). Scale bar = 100 µm (G & I); 30 µm (H & J). Abbreviations: co, cortex; ep, epidermis; fc, fibrous cell; id, idioblast; lc, laticifer cell; nd, nectary duct; ph, phloem; vb, vascular bundle; vz, vascular zone.

DISCUSSION

The floral structure of bananas is important and useful for the generic and species classification. Floral evolution of Zingiberales involved modifications in perianth, androecial and gynoecial whorls and the Musaceae is the most primitive in this order with one compound tepal, one free tepal, five fertile stamens and one pistil (Kress, 1990; Rudall and Bateman, 2004).

The important character of Musaceae is their compound tepal which is a fused structure of two inner and three outer tepals. Investigation of the transverse sections of these organs revealed that there are connective tissue between the inner and outer tepals; the three outer tepals fused together in abaxial side and inner tepals adnate at margin of median outer tepals in adaxial side (Figs. 3A-C). The outer tepal structures are rather like leaf structures which spongy and palisade parenchyma cells can be found (Fig. 3D). Meanwhile, the inner tepals are much reduced (Figs. 3E-F), e.g. with only one median strand vascular bundle and without fibrous cell as in the outer tepals. The structure of the inner tepals hinted the relatedness to the free tepal and possibly changed from petal structure (Tilak and Pai, 1974).

The floral transverse sections of *E. glaucum* and *E. superbum* are quite similar with several anatomical characters. The ovaries of *E. glaucum* and *E. superbum* share basic characters, but bear some differences in cell shapes and vascular bundle patterns. The epidermis

Table 2 Floral anatomical characters of *E. glaucum* and *E. superbum*.

Characters	E. glaucum	E. superbum
Compound tepal at basal part (TS)		
Adaxial epidermal cell shape	Elliptic	Elliptic
Abaxial epidermal cell shape	Pentagonal	Pentagonal
Number of spongy parenchyma layer	Four	Three
Spongy parenchyma cell shape	Elliptic	Elliptic
Number of palisade parenchyma layer	Two	Two
Palisade parenchyma cell shape	Hexagonal	Hexagonal
Number of vascular bundle ring	One	Two
Vascular bundle type	Collateral bundle	Collateral bundle
Sclerenchyma cell above vascular bundle	Not well developed	Well developed
Compound tepal at middle and upper part (TS)		
Adaxial epidermal cell shape	Elliptic	Flat
Abaxial epidermal cell shape	Heart-like	Heart-like
Number of spongy parenchyma layer	One to two	One to two
Spongy parenchyma cell shape	Horizontal rectangle	Horizontal rectangle
Spongy parenchyma cell size at middle part	14.15±4.07 µm	23.58±7.59 µm
Number of palisade parenchyma layer	One to two	One
Palisade parenchyma cell shape	Long hexagonal	Long hexagonal
Vascular bundle position	Between the tepal ribs	Center of the tepal ribs
Number of vascular bundle row	One	One
Vascular bundle type	Collateral bundle	Collateral bundle
Fibrous cells above vascular bundle	Not well developed	Well developed
Free tepal		
Epidermal cells with straight tangential walls	Present	Present
Epidermal cell shape	Square to rounded	Square to rounded
Epidermal cell walls	Thick	Thick
Hypodermal layer	Present	Absent
Parenchyma cell shape in mesophyll	Rounded	Rounded
Vascular bundle position	Center of the tepal	Closed to adaxial side
Vascular bundle type	Amphicribal bundle	Amphicribal bundle
Stigma		
Epidermal cell shape	Rounded	Elliptic
Parenchyma cell shape	Polygonal	Polygonal
Vascular bundle type	Collateral bundle	Collateral bundle
Ovary		
Epidermal cell shape	Rounded	Elliptic or rounded
Cortical cell shape	Elliptic	Rounded
Median vascular strand of outer tepal ring number	One	Two
Median vascular strand of outer tepal vascular type	Collateral vascular bundles with fibrous cells	Collateral vascular bundle with fibrous cells
Carpellary dorsal/out androecium vascular type	Amphicribal vascular	Amphicribal vascular
Nectary duct	Tri-radiate	Tri-radiate
Anther		
Epidermal cells of anther wall	Without annular	Without annular
Endothecium cells	Helical thickening	Helical thickening
Vascular bundle type	Amphicribal bundle	Amphicribal bundle
Pollen		
Shape	Spheroidal	Spheroidal
Exine ornamentation	Verrucate	Perforate
Size (µm) ± SD	113.9 ± 5.2 µm	124.3 ± 5.9 µm
Wall thickness (µm) ± SD	6.8 ± 1.4 µm	7.0 ± 1.7 µm

layer consists of cuboidal to convex cells (Figs. 8C & 8F). Ram *et al.* (1962) studied on ovary structure and development in *M. acuminata* 'Pisang Jilin' and *M. acuminata* subsp. *burmannica* and reported the presence of hypodermis at outer cortex layer. In our result, two to three layers of elliptic cells in *E. glaucum* (Fig. 8C) and rounded cells in *E. superbum* (Fig. 8F) can be found in cortex of the ovaries. These layers later become fruit peel. It was found that most of the cells in vascular zone are irregular and there were laticifer cells

surrounding vascular bundles. Kirchoff (1992) stated that the collateral bundle with fibrous cells in ovary is the same one called median vascular strand in perianth members. These median vascular strand numbers can be used to distinguish the two species which two vascular bundle rings with fibrous cells locate at outermost ring were found in *E. glaucum*, whereas three were found in *E. superbum*.

Tilak and Pai (1974) studied on vascular anatomy of *E. superbum* and proposed that the carpellary dorsal and the stamina strands are united up at the top of the ovary. Also, in our study, the amphicribal vascular ring including six bundle pairs was found on dorsal side of *E. superbum* ovary. On *E. glaucum* ovary, however, the six amphicribal vascular bundle pairs were separated into five fused and one isolated. The fused pair vascular forms located on the same ring of the ovary is similar to that of the connective tissue on fertile stamens and suggested that the sixth stamens were reduced, left only five found in banana flowers (Kress, 1990). The fact agreed with that reported by Tilak and Pai (1974) that in *E. superbum*, four to six tiny strand pairs in connective tissue of the stamens reduce into one pair and end at the tip of each anther.

The nectaries of *E. superbum* and some *Musa* were studied by Tilak and Pai (1974) and Kirchoff (1992), they reported that the nectary consists of glandular cells in tri-radiate positions locate in axile tissue near the top of the locules; the three grooves locate in the septa. The nectary of *E. glaucum* shares common characters which were also found in other species in Musaceae.

The five fertile stamens is the primitive character found in Musaceae, while in other members of the Zingiberales, a single fertile stamen was found in Zingiberaceae and Costaceae, only half fertile stamen in Cannaceae and Marantaceae and a sterile stamen modify into a staminode in Zingiberaceae and Costaceae (Kirchoff, 1991; Kirchoff, 1992; Simao, 2007). The phylogenetic study based on morphological and molecular data (Kress *et al.*, 2001; Kress and Specht, 2005; Liu *et al.*, 2010; Bekele *et al.*, 2011) suggested that *Ensete* is the most primitive genus in Musaceae. Agreed to those reports, the flower of *Ensete* shows its primitiveness by having five fertile stamens and one much reduced sterile stamen on adaxial side of the free tepal (Figs. 6A & 6B). The fact was supported by the presence of six pairs of staminal strands in the ovary which continue, in fused pairs, into five fertile stamens and two separated, poorly developed into the position of the sterile stamen. Meanwhile, this staminode was not found in *Musa* and *Musella* (Franch.) H.W. Li investigated by the authors (data not shown).

The fertile stamens of the two *Ensete* are similar. The anther wall is composed of epidermal cells and annular or helical thickening endothecium, whereas *Musella* anther wall which was studied by Xue *et al.*

(2005) is composed of epidermis with annular and helical thickening and reduced endothecium. When the anther released pollen, the epidermis and endothecium cells became shorten. Moreover, the pendulum-like structure was found on the anther wall, located near stomium region in each lateral pollen sac. The structure possesses thin-walled epidermal cells enclosing compound parenchyma cells (Figs. 7A & 7D). This structure may act in defense mechanism as found in *Heliconia* which raphides fending off insect from feeding on pollen were observed in idioblast septa (Simao *et al.*, 2007). Idioplasts containing raphides was found in all organs of both *Ensete* flowers especially in ovary and stigma of *E. glaucum* and believed to act in defense to animal feed of the flowers.

The *Ensete* is bat pollinated, possesses nocturnal and odoriferous flowers, gelatinous nectar and pendent inflorescence orientation (Nur, 1976; Endress, 1995). Liu *et al.* (2002) stated that the bat pollination is the most primitive phenomenon in this family. The sticky pollen character supported that *Ensete* were pollinated by animals that can cause pollen aggregation and attached to visitors (Hesse, 1981). Moreover, *Ensete* pollen wall is thin and acetolysis preparation of the pollen is not quite possible.

Pollen grains of the two *Ensete* were of different in exine ornamentation. That of *E. glaucum* pollen is verrucate, but of *E. superbum* is perforate. Moreover, pollen exine of *E. glaucum* is quite similar to that of *Musella lasiocarpa* (Franch.) C. Y. Wu ex H. W. Li var. *rubribracteata* Zhenghong Li & H. Ma (Hong *et al.*, 2011) and supported the close relationship between the two genera. In agreement, molecular phylogenetic studies (Liu *et al.*, 2010; Li *et al.*, 2011; Bekele *et al.*, 2011) suggested that *Musella* should be a member of *Ensete*. Pollen morphology may provide information about relationships between these species in Musaceae.

Several anatomical features supported the fact Cheesman (1947b) mentioned that several *Ensete* species tolerate cool and dry environments and can be grown in grassy and rocky habitats. The thicken cell walls of epidermal layers may help to survive in drought (Oertil *et al.*, 1990; Coutinho *et al.*, 2013). Moreover, the hypodermal layer found in both *Ensete* species studied, especially the hypodermis sometimes found under both adaxial and abaxial epidermal layers of the free tepals in *E. glaucum*, is an important character of the plant adaptation to low humidity.

In conclusion, the anatomical characters of *E. glaucum* and *E. superbum* flower can be differentiated based on the vascular bundle positions in the tepals in connection to those in the vascular zone of the ovary. The adaxial cell sizes and reduced fibrous cells in the compound tepals, the presence of hypodermis, the pollen exine ornamentations and the cell shapes in stigma epidermis and in the cortex of the ovaries are

additional distinguish characters. The floral vascular anatomy should be further studied in more detail in order to understand vascular development in comparison to that of other Musaceae species.

ACKNOWLEDGEMENTS

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