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Floral biology and pistil receptivity of the drumstick tree (*Moringa oleifera* Lam.)

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Abstract: Drumstick (*Moringa oleifera* Lam.) has a wide range of uses due to its high nutritional value and the high oil content of its seeds. Many aspects of its reproductive biology remain poorly understood. We investigated the floral morphology of drumstick, its stigma receptivity and the structural and cytochemical features of the stigma and style at different developmental stages. The inflorescences are panicles of hermaphroditic flowers, with a pistil consisting of one open-type stigma and a hollow stylar canal. Stigma receptivity was assayed based on pollen germination, pollen tube growth and fruit set following artificial pollination. Flowers at later developmental stages exhibited greater stigma receptivity, higher percentages of pollen germination and a higher fruit set than those in earlier stages. Enhanced stigma receptivity was associated with increased amounts of insoluble polysaccharides, lipids and proteins in the canal cells at later developmental stages. An ultrastructural study of the cells lining the canal indicated that they were secretory cells containing an enlarged endoplasmic reticulum, dictyosomes, mitochondria, plastids and ribosomes. Post-anthesis, these organelles exhibited degeneration at the end of the secretory phase. This study provides an important contribution to current knowledge of the anatomy and ultrastructure of the style and stigma in drumstick.

Key words: *Moringa oleifera* Lam.; stigma; style; stigmatic receptivity; ultrastructure

INTRODUCTION

The drumstick tree (*Moringa oleifera* Lam., family Moringaceae) is broadly known and widely distributed because of its many uses [1-3]. This rapidly-growing, small- to medium-sized tree is indigenous to sub-Himalayan tracts of India and tropical-African countries [4,5] and has spread to many tropical and subtropical regions. Because drumstick is an economically important species, it has attracted much attention in recent years. Despite extensive planting programs and research on its agronomic [6,7], nutritional [8,9] and pharmacological properties [10,11], relatively little is known about its reproductive system.

Drumstick fruit production tends to be low in comparison with its abundant floral display; the reasons for its low fruit set are unclear. Previous studies have shown that drumstick flowers throughout the year, with two peaks in flower production per year, and that anthesis peaks occur within one day [13-15]. The drumstick tree is a mixed mating species adapted to outcrossing, although selfing is also possible [16]. It is insect-pollinated, with large numbers of insects required for pollination [13,14]. Fruit set via open pollination is typically 11-15%, while hand pollination yields 62-100% [15].

Flower receptivity plays a crucial role in pollination dynamics, reproductive success and plant productivity. The stigma is the first pistil surface to intercept pollen grains; pollen adhesion to the stigma is followed by hydration and germination. The drumstick stigma is perforated and hollow and is more recep-

tive during the third day post-anthesis; this receptivity lasts 2-3 days [14]. However, many anatomical and ultrastructural features of the drumstick flower stigma and style are largely unexplored. We investigated stigma and style structure and stigma receptivity during flower development in drumstick. The findings of this study should improve our understanding of the factors affecting the effective pollination period in drumstick, and could provide opportunities to optimize pollination and increase the fruit set.

MATERIALS AND METHODS

Material and study site

This study was conducted at Guangzhou (23°8'N, 113°17'E; 43.4 m a.s.l.) in southern China at the beginning of July 2014. The daily mean maximum and minimum temperatures and mean temperatures per month were 28.5°C, 13.3°C and 22°C, respectively. The relative humidity averaged 77% and the annual rainfall was 1700-2000 mm. Five 3-year-old drumstick trees were selected from a drumstick germplasm conservation farm based on flowering performance and accessibility.

Fruit set field studies

The fruit set of flowers at different developmental stages was assessed following artificial pollination. We defined anthesis as the time when the perianth is fully open. The flower stages assessed were: -2 days pre-anthesis, -1 day pre-anthesis, 0 days or anthesis, and 1, 2, 3 and 4 days post-anthesis. Flowers were emasculated approximately 2 days pre-anthesis and isolated immediately using polyester pollination bags. Flowers manipulated at -2 days and -1 day pre-anthesis and on the day of anthesis had their own pollen removed and immediately crosspollinated artificially. At other developmental stages, the pollination time was referred to as the emasculation day, and flowers were pollinated using pollen from fresh, 0-day flowers from different trees. Pollen was visible on the stigmas once applied. Flowers that were younger or older than the stage under study were manually removed from the branches at the time of pollination. Fruit set was surveyed 3 weeks later as it became more stable.

Pollen behavior on stigma surfaces following artificial pollination

To determine the floral stage that corresponds to optimum stigma receptivity, pollen germination on the stigma and subsequent pollen tube growth were assessed at various floral stages. One panicle per tree was tagged at each stigma developmental stage and artificial pollination was performed. To observe pollen tube germination on the stigma, flower samples were harvested 8 h after pollination and fixed in formalin-acetic acid-alcohol (FAA). Fixed stigma material was cleared by immersion in 1 M NaOH at room temperature until most tissues became transparent. They were then rinsed thoroughly in distilled water three times and stained with 0.1% aniline blue. Using a fluorescence microscope, germinating pollen grains on the stigma surface were observed under UV irradiation as bright yellow-green fluorescences. The outcome of each pollination event was judged according to the levels of pollen germination and pollen tube growth on the stigma. A pollen grain was considered germinated when its tube length was greater than the diameter of the pollen grain [17]. Fluorescing pollen grains without a pollen tube were considered ungerminated. We examined 30 flowers per treatment.

Microscopy

Fresh pre-anthesis and post-anthesis flowers were observed under an Olympus SZX10 stereomicroscope following preparation for microscopic evaluation. Pistils for light microscopic examination were dissected and fixed in FAA, dehydrated in a series of ethanol solutions and infiltrated and embedded in LR White resin. Sections (thickness: 2 μm) were cut using a Leica RM2155 rotary microtome. Periodic acid-Schiff (PAS) was used to detect insoluble polysaccharides, Sudan Black B for lipids and Coomassie Brilliant Blue for proteins. The preparations were examined and photographed with a Zeiss Standard microscope. For scanning electron microscopy (SEM), fixed stigmas were dehydrated in a series of ethanol solutions, critical-point dried using CO_2 as the substitution liquid and coated with gold. The material was viewed with an XL-30-ESEM scanning electron microscope. For transmission electron microscopy (TEM), stigmas were fixed in 2% glutaraldehyde with 0.2% caffeine

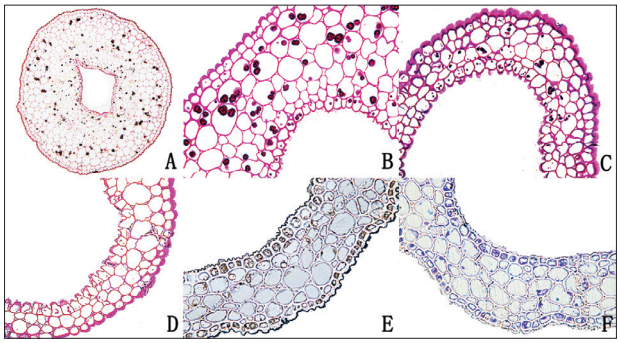

Fig. 1. Morphological characters of inflorescence and flowers of *Moringa oleifera* Lam. **A** – inflorescence; **B**-**G** – flowers at different development stages; **B** – globular stage, buds were greenish and inconspicuous; **C** – elongated stage, the bud enlarged and the color became greenish-white; **D** – two days before anthesis, the color was yellowish-white all over and center portion bulged out; **E** – anthesis, the bud split open while the anthers remained closed; **F** – two days after anthesis, sepals and petals were yellowish-white and the anthers were yellow; **G** – flattened stage, sepals and petals were yellow and withered.

in 0.05 M of phosphate buffer. The material was post-fixed in 0.5% osmium tetroxide for 30 min, dehydrated in a series of ethanol solutions and embedded in Spurr resin. Sections were observed with a Tecnai 12 transmission electron microscope.

Data collection and analysis

The means±standard error for fruit set and pollen germination values were calculated using SPSS 19.0 software for Windows and compared using analysis of variance (ANOVA) and Duncan's new multiple range test (significance was determined at $P<0.05$).

RESULTS

Morphology

The drumstick tree is hermaphroditic, with zygomorphic gullet flowers arranged in large panicles (Fig. 1A). There was a highly significant difference in flower production per inflorescence between trees. Only 1-3% of the flowers in an inflorescence bloomed each day; thus, it took 1-2 months for the entire inflorescence to bloom, depending on its size. Flowers have one pistil and five unequal stamens. An average of 25 days was required from bud initiation (Fig. 1B) to anthesis (Fig. 1E), presumably depending on the temperature. The flower stayed open for about 7 days.

Table 1. Percentage of fruit set at different stages of stigma development after controlled pollination.

Development stage (days)	% Fruit set
-2	13.3±6.3 d*
-1	26.7±8.2 d
0	53.3±9.3 c
1	93.3±4.6 a
2	86.7±6.3 ab
3	66.7±8.8 bc
4	16.7±6.9 d

*Means followed by the same letter in the same column are not significantly different from each other at $P\leq0.05$ according to Duncan's multiple range test.

Fruit set at different stages of stigmatic development

The results of the controlled pollination experiment (Table 1) indicate that fruit set was possible at all stages of stigma development, even though in some cases a low frequency was obtained. Rarely, fruit set occurred in flowers pollinated 2 days pre-anthesis or 4 days post-anthesis, suggesting nonreceptivity of the stigma during these periods. However, because deposited pollen may remain viable after the stigma becomes receptive, it was not possible to determine the exact timing of stigma receptivity in this study. For example, fruit set may occur even if pollen was captured 1 day pre-anthesis, when the stigma was not receptive.

Pollen germination at different stages of stigma development

The percentage of receptive stigmas, defined by pollen germination, at different stigmatic stages was varied significantly between treatments. Pollen germination was significantly higher in more developed flowers than in younger flowers. Almost no pollen germination was observed on stigmas 2 days or 1 day pre-anthesis (Table 2). On the day of anthesis, the germinated pollen grains had very short pollen tubes (Fig. 3A). Peak drumstick stigma receptivity was recorded 1 day and 2 days post-anthesis, with more than 93% of observed stigmas being receptive and pollen grains having long tubes (Fig. 3B). Stigmas observed 3 days post-anthesis exhibited high pollen germination, but

Table 2. Percentage of receptive stigma (pollen-germinated stigma) at different stigma development stages after controlled pollination.

Development stage (days)	% Receptive stigma
-2	3.3±3.3 d*
-1	10.0±5.6 d
0	63.3±8.9 b
1	93.3±4.6 a
2	96.7±3.3 a
3	60.0±9.1 b
4	36.7±8.9 c

*Means followed by the same letter in the same column are not significantly different from each other at P≤0.05 according to Duncan's multiple range test.

had short pollen tubes and an untidy aspect, with the pollen tubes lacking clarity of appearance and growth direction.

Stigma and style structure

The drumstick stigma is perforated and hollow, with thick, elongated walled cells arranged linearly into compact masses, forming a rim-like structure at the stigma head. The cells are small near the stigma head with minute spaces that are larger below the stigma head. The linear arrangement of cells narrows towards the stigma head (Fig. 3C and D).

The hollow style is comprised of an epidermis, ground tissue and a central canal coated by canal cells with a strongly undulated cuticle (Fig. 2A and B). In most style cells of very young flowers (Fig. 1D), PAS-positive substances were abundant (Fig. 2B). On the second day of anthesis (Fig. 1F), PAS-positive substances were mainly distributed in canal cells and filled the intercellular spaces between loosely arranged subepidermal canal cells with a fibrillar consistency (Fig. 2C). During the same period, the content of subepidermal canal cells also included lipids and proteins (Figs. 2E and F). In the flattened stage (Fig. 1G), fewer polysaccharides, lipids and proteins were detected in all style cells (Fig. 2D).

The inner layers of ground tissue and canal cells were aerenchymatic. The canal cells were approximately isodiametric in transverse section, with a thicker cell wall covered by cuticle consisting of a slightly reticulate cuticle layer and the cuticle proper bordering the canal (Fig. 3G). The cytoplasm of the epithelial cells con-

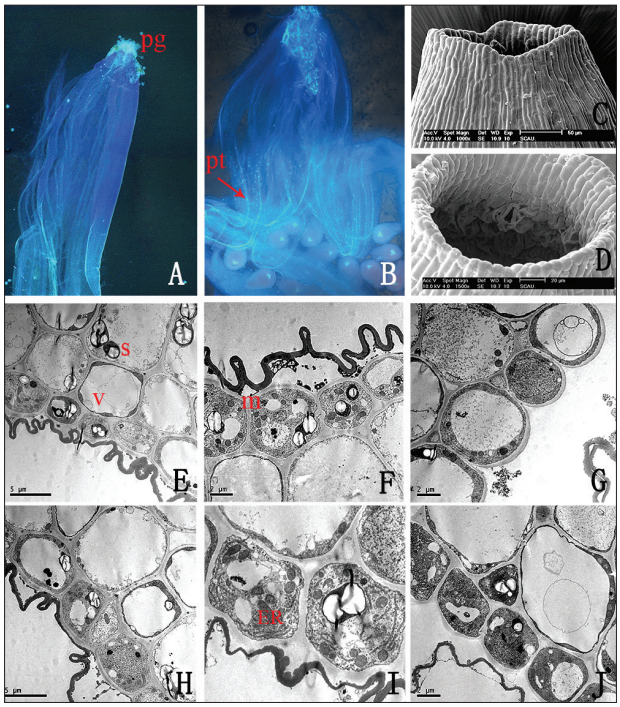

Fig. 2. Semithin transverse section of style *Moringa oleifera* Lam. **A-D** – PAS staining; **A** – elongated stage; **B** – two days before anthesis; **C** – anthesis; **D** – two days after anthesis; **E** – Sudan black B staining; **F** – Coomassie brilliant blue staining. EP, epidermis; GT, ground tissue; CC, canal cell.

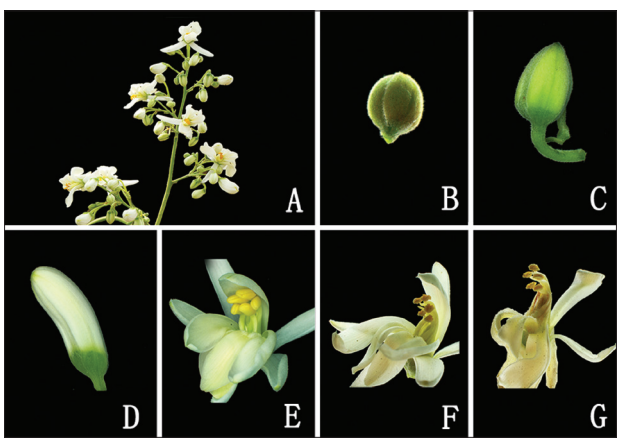

Fig. 3. Structure of stigma and style of *Moringa oleifera* Lam. **A** – pollen grain germination on stigma of anthesis; **B** – pollen grain germination on stigma at two days post anthesis; **C-D** – SEM of stigma; **E-J** – TEM of style transverse section; **E-F** – two days pre anthesis; **G-I** – two days post anthesis; **J** – flattened stage. Pg, pollen grains; pt, pollen tube; er, endoplasmic reticulum; v, vesicles; p, plastid; m, mitochondria; d, dictyosome; tl, tannin-like; cl, cuticle layer.

tained abundant mitochondria and plastids with starch grains, free ribosomes and an endoplasmic reticulum (Fig. 3E-I). The endoplasmic reticulum and vesicles were abundant (Figs. 3H and I), with some vesicles in close contact with the plasmalemma, indicating that they are secretory cells. In very young flowers (Fig.1D), the canal cells were lined with a dense cuticle layer adhering closely to the canal cells. The epithelial cells were metabolically active, with numerous mitochondria and plastids of different sizes containing starch grains (Fig. 3E and F). The epidermis cells were characterized by the presence of a large vacuole, filled with tannin-like material, and several plastids rich in starch grains were observed. On the second day of anthesis (Fig. 1F) the canal cell cytoplasm began to atrophy and diminish (Fig. 3G). The large vacuoles became divided, forming smaller vacuoles (Figs. 3H and I). The cuticle was disrupted in places and cuticle particles could be discerned on the surface of the wall and inside the canal. Further, the plastids contained no starch; the fibrillar and granular substances formed in the canal cells passed into the canal, stretching the cuticle as development proceeded. In the flattened stage (Fig. 1G) the canal cells were loosely arranged, with a denser cytoplasm and distorted cells (Fig. 3J).

DISCUSSION

The drumstick tree has a hermaphroditic flower with little spatial and temporal separation between male and female functions. The anthers begin to dehisce before the stigma becomes most receptive, and the style slightly exceeds the length of the anthers. This phenomenon of simultaneous ripening of the pistil and stamen is common, and protandry occurs frequently in outcrossing angiosperms such as *Tectona grandis* [18], *Grevillea robusta* [17] and *Collinsia heterophylla* [19]. In drumstick, however, the protandrous flowers still permit self-pollination. Muluvi et al. [16] studied drumstick outcrossing rates using amplified fragment length polymorphism markers and found it to have a mixed mating system with an outcrossing rate of 0.74.

The stigma is reported to be receptive at the time of anthesis in many tree species such as *Malus domestica* [20], *Armeniaca vulgaris* [21] and *Cerasus avium* [22]. We found that stigma receptivity in drumstick flowers was delayed, with higher percentages of pollen germination and fruit set observed in older than in younger flowers. There have been conflicting findings with respect to the optimal timing of drumstick stigma receptivity. Bhattacharya and Mandal [14] reported delayed stigma receptivity favoring cross-pollination, with which our results are consistent. However, Kanthaswamy [15] found that the stigma was receptive 1 day pre-anthesis. The receptive period can vary among species and cultivars. In drumstick, stigma receptivity lasts only 3-5 days. Flowers of high mountain plants are generally long-lived; stigma receptivity can be maintained over longer periods in such plants [23]. In pear, the surfaces of immature stigmas can support pollen adhesion but cannot provide a proper hydration substrate [24]; this is also true in drumstick. Its perforated stigma facilitates pollen adhesion, but only mature stigmas can support pollen germination and pollen tube growth. We observed that drumstick pollen grains remain viable for 2-4 days, depending on the cultivar and environment (data not shown). If pollen grains are loaded 2 days pre-anthesis drumstick stigmas, even though these stigmas are not receptive at that time, as the pollen grains viability lasts 4 days they could still germinate. This may explain why 2 days pre-anthesis drumstick stigmas supported little pollen germination. Before the flowers reached 1 day post-anthesis, insufficient exudates were produced and little secretion occurred, suggesting that the acquisition of competence to support pollen hydration and germination defines the transition of the stigma from an immature to a mature stage.

Owens [25] distinguished between two different stigma types among 40 caesalpinioid genera. Wet, papillate stigmas were usually captitate, while wet, non-papillate stigmas were all crateriform or chambered. The drumstick stigma, which is perforated, is of the wet, non-papillate sort. Plant styles have been classified into three types: hollow, closed and semi-closed. The drumstick style is hollow, with a canal lined with a glandular epidermis. Hollow styles are less common in angiosperms, having been documented among monocotyledonous species such as *Cyphia stenopetala* [26] and *Ornithogalum sigmoideum* [27], as well as dicotyledonous species such as *Tectona grandis* [18], *Cyclamen persicum* [28] and *Polygala vayredae* Costa [29]. In drumstick, the liquid-filled internal canal of the hollow style is surrounded by a layer of specialized

inner epidermal cells that ensure its liquid content is withdrawn once the pistil has reached maturity. Hollow styles containing secretions have been reported in *Colophospermum mopane* [30], *Chrysanthemum multicaule* [31] and *Ornithogalum sigmoideum* [27]. This secretion can contain lipids, polysaccharides and proteins, varying in chemical composition between species. Stigma and transmitting tissue secretions appear to have many functions, including facilitating pollen adhesion to stigmas [32], pollen-stigma recognition [33], pollen tube growth and ovule penetration [34], attraction and nourishment of floral visitors [35].

Our findings indicate significant differences in the fine structure of canal cells pre- and post-anthesis. Pre-anthesis, drumstick canal cells are rich in plastids, dictyosomes, mitochondria, ribosomes and ER; post-anthesis, the amount of cytoplasm and the number of organelles decrease. These data suggest that metabolic activity in drumstick pre-anthesis is very high, when secretory synthesis and secretory transfer are intense. Low activity post-anthesis indicates low secretory activity in the cell at that stage. These changes are similar to those that occur in *Ornithogalum sigmoideum* [27], but opposite to those in *Lilium longiflorum* [36].

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