

Article

# Floral Structure and Breeding Systems of *Manglietia conifera* Dandy (Magnoliaceae)

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**Abstract:** *Manglietia conifera* Dandy is a fast-growing tree species that has been introduced to China from Vietnam, which has great potential for commercial planting. However, plantation development is hindered by a lack of seed material, due to low natural seed-set in locally grown trees. Thus, we investigated the morphological characteristics of male and female flower organs, and conducted controlled pollination to understand the breeding systems of the species. The individual flower of *M. conifera* is bisexual, and the stamen group is polymerized at the base of the receptacle. Pollen is symmetrically distributed on both sides. Controlled pollination suggests that apomixis does not occur in *M. conifera*. Results from the flower structure, pollen–ovule ratio, outcrossing index, and controlled pollination indicated that the breeding system in *M. conifera* was outcrossing (partially self-compatible, pollinators required), and self-incompatibility occurred in a later stage of embryonic development. Moreover, the self-incompatibility phenomenon was revealed by the abnormal germination of pollen on the stigma. This paper provides a basis for controlled pollination programs of *M. conifera*.

**Keywords:** flower biology; breeding systems; controlled pollination; seed production; floral characteristics; *Manglietia conifera*

## 1. Introduction

*Manglietia conifera* Dandy (Magnoliaceae), formerly known as *M. glauca*, is an evergreen broad-leaved tree species distributed naturally in northern Vietnam (Figure 1). It is fast-growing, predominantly with a straight single bole, and has good natural pruning, making it a popular plantation species for wood production in the northern provinces of Vietnam [1]. The species was first introduced to China in the 1960s and, to date, 110,000 hectares of plantations have been established in the tropical and subtropical regions in the southern provinces of Guangdong, Guangxi, Fujian and Hainan (Figure 1) [2]. Under suitable site conditions [3], the average diameter at breast height can reach 3 cm per annum, which is fast-growing for a high-quality timber species in southern China [4].

*Manglietia conifera* trees growing in China flower annually with moderate intensity, but fruit-set is extremely low and totally absent in some years [5]. This poor fruit-set and low seed yield seriously hamper the expansion of plantation development in China [4,6]. To date, research on *M. conifera* in China has mainly focused on cultivation techniques [7], early growth adaptability [3] and wood properties [8]. Study on the reproductive biology and breeding systems is still in its infancy. The flower of *M. conifera* is solitary and bisexual. The main flowering period is from early March to mid-May [9].

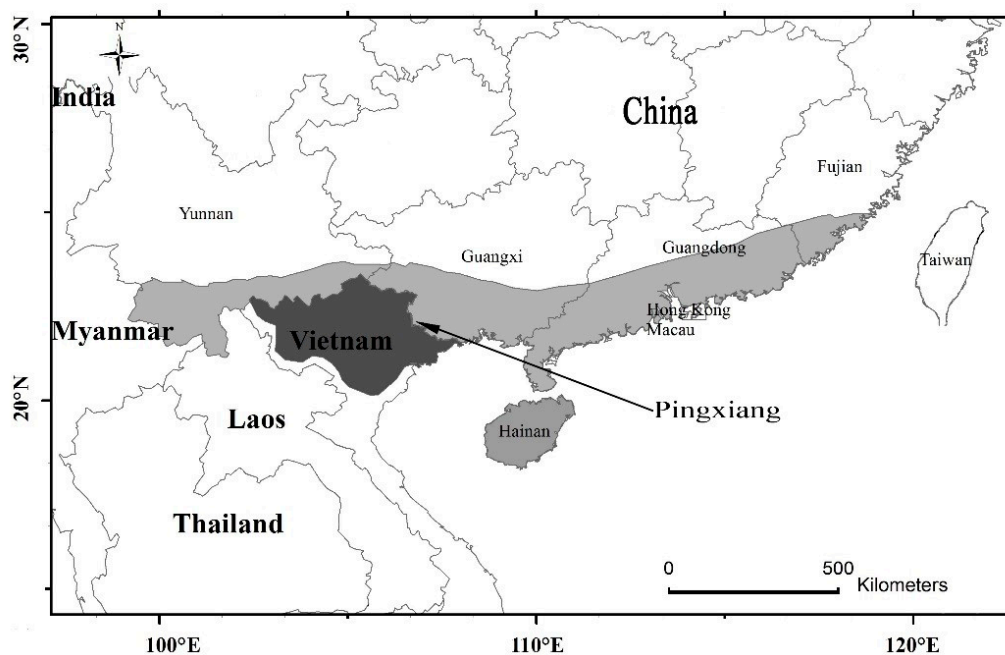
Breeding systems play a decisive role in the success of reproduction in plants [10–12], and research in this field mainly includes floral characteristics, pollination and mating systems [13]. Flowering pattern and flower longevity directly affect the type of breeding systems [14,15]. Flower arrangement is also an important factor that influences mating success [16]. Understanding the floral structure is the basis for studying breeding systems [17–19]. Murawski [20] divided mating systems into selfing, mixed mating and outcrossing. Individuals with hermaphrodite flowers may resort to self-pollination, which leads to minimum genetic variation in their offspring [21]. However, geitonogamy may also mitigate pollen limitation in self-compatible plants [22]. In addition, morphological characteristics of the flower and breeding systems of a plant are useful to research in order to explore the causes of plant abortion. The breeding systems and abortion mechanism of plants are discussed by means of the hybridization index, pollen–ovule ratio, bagging test and other methods and indicators [23,24]. Therefore, floral syndrome and breeding system investigations are not only conducive to understanding the pollination mechanism and mating patterns, but also have scientific significance for further investigating the evolution and environmental compatibility of plants [25].

Thus, we analyzed the floral structure, breeding system and controlled pollination of *M. conifera*: (1) What are the floral structure characteristics of *M. conifera*? (2) What are the breeding systems, results from flower structure, pollen–ovule ratio, outcrossing index, and controlled pollination? (3) What are the results of controlled pollination, and what are the barriers to successful pollination, and fruit and seed-set? The answers to these questions can contribute to artificial pollination, the development of crossbreeding and variety improvement, as well as promote seed production.

## 2. Materials and Methods

### 2.1. Research Area and Study Materials

The study was conducted at the Experimental Center of Tropical Forestry, Chinese Academy of Forestry in Pingxiang, Guangxi Zhuang Autonomous Region (21°57′47″–22°19′27″ N, 106°39′50″–106°59′30″ E) (Figure 1). The area has a subtropical monsoon climate with a mean annual temperature of 21.5 °C. The mean annual rainfall is 1310 mm, mainly concentrated between mid-April and August, with average of 1260 annual sunshine hours.



**Figure 1.** Natural distribution of *Manglietia conifera* in Vietnam (dark shade) and planted area in China (light shade).

*Manglietia confiera* plantation stands located at Central Arboretum (CA), Baiyun Experimental Field (BY) and Fubo Experimental Field (FB) were used for the study. Basic stand characteristics of the plantations are given in Table 1.

**Table 1.** Basic characteristics of *M. confiera* plantations in Guangxi.

Site	Altitude (m)	Year Planted	Mean Tree Height (m)	Mean DBH (cm)	Density (trees·ha <sup>-1</sup> )	Flowering Period
CA	240	2002	16.7	26.1	800	Early March to early May
BY	540	1998	18.5	28.7	1200	Late March to early May
FB	630	2003	24.7	32.4	1000	Late March to mid-May

Note: CA = Central Arboretum; BY = Baiyun Experimental Field; FB: Fubo Experimental Field; DBH: diameter at breast height.

## 2.2. Methods

### 2.2.1. Flower Structure

*Manglietia confiera* flowers open at approximately 6 p.m., followed by pollen shedding soon after [9]. Therefore, at 4.30 p.m., before anthesis and anthers presenting pollen, 30 individual flowers were marked from three randomly selected trees (10 flowers of every tree) at each stand (three stands total nine trees), and the morphology of the flower structure was observed using a stereomicroscope (Olympus SZX10 Research Stereomicroscope System, Tokyo, Japan). Length and width of the floral organs, (stamens and pistils) were measured using a scanning electron microscope (Hitachi S-3000N, Tokyo, Japan).

### 2.2.2. Outcrossing Index and Pollen–Ovule Ratio

At each stand, 30 mature flowers that had not yet dispersed pollen were marked from three randomly selected trees (10 flowers of every tree) at each stand (three stands total nine trees). The total number of anthers and ovaries in each flower was counted. Then, 10 anthers from each flower were randomly selected to calculate the average amount of pollen in a single flower. The number of pollen grains was estimated using a hemocytometer [26]. The average number of ovules per flower was calculated using a stereomicroscope from 10 ovaries from each flower [27]. The breeding system was determined based on the ratio of pollen grains to ovules, as described in Cruden [28].

The outcrossing index (OCI) was estimated from the flower structure data, following Cruden's method described by Dafni [26] (Table 2).

**Table 2.** Scoring standards of outcrossing index.

Observation	Expression	Score
Flower diameter	<1 mm	0
	1–2 mm	1
	2–6 mm	2
	>6 mm	3
Temporal separation of stigma receptivity and anther dehiscence	Anther and stigma matured at the same time or protogyny (stigma matured first)	0
	Protandry (anther matured before stigma)	1
Spatial positioning between stigma and anthers	At the same height	0
	Spatial separation	1

Based on Dafni [26], the sum of the scores in Table 2 is the outcrossing index (OCI) which determines the breeding systems (Table 3).

**Table 3.** Breeding systems as determined by Dafni’s outcrossing index value.

Outcrossing Index Value	Breeding Systems
0	Cleistogamy
1	Obligate autogamy
2	Facultative autogamy
3	Self-compatible and sometimes requires pollinators
4	Outcrossing but partially self-compatible, requires pollinators

### 2.2.3. Controlled Pollination

To determine the breeding system, we carried out seven pollination treatments on three trees with a large number of flowers at each stand (three stands in total):

- Natural pollination (free pollination), non-treatment, testing the fruiting under natural conditions;
- Emasculation before flowering, bagging, and non-pollination, detecting whether the flower has spontaneous apomixis.
- Bagging before flowering, no emasculation and non-pollination, detection of spontaneous self-pollination.
- Emasculation before flowering, no bagging and non-pollination, checking whether the fruiting is restricted by the pollinator.
- Xenogamy, emasculation before flowering, bagging, artificial cross-pollination with pollens from different plants, testing the compatibility of cross-sex.
- Geitonogamy, emasculation before flowering, bagging, artificial cross-pollination with pollens from different flowers on the same plant, detecting whether the same plant can be fertilized.
- Self-pollination, bagging before flowering, artificial self-pollination with pollens from the same flower, testing whether self-fertilization can be viable.

In each tree, 210 individual flowers at the green bud stage were tagged on ten branches, each consisting of 21 flowers. The number of flower buds for each controlled pollination treatment was 30 in each tree. Therefore, a total of 270 flowers marked from 9 trees were used for treatment. During initial opening, controlled pollination was carried out.

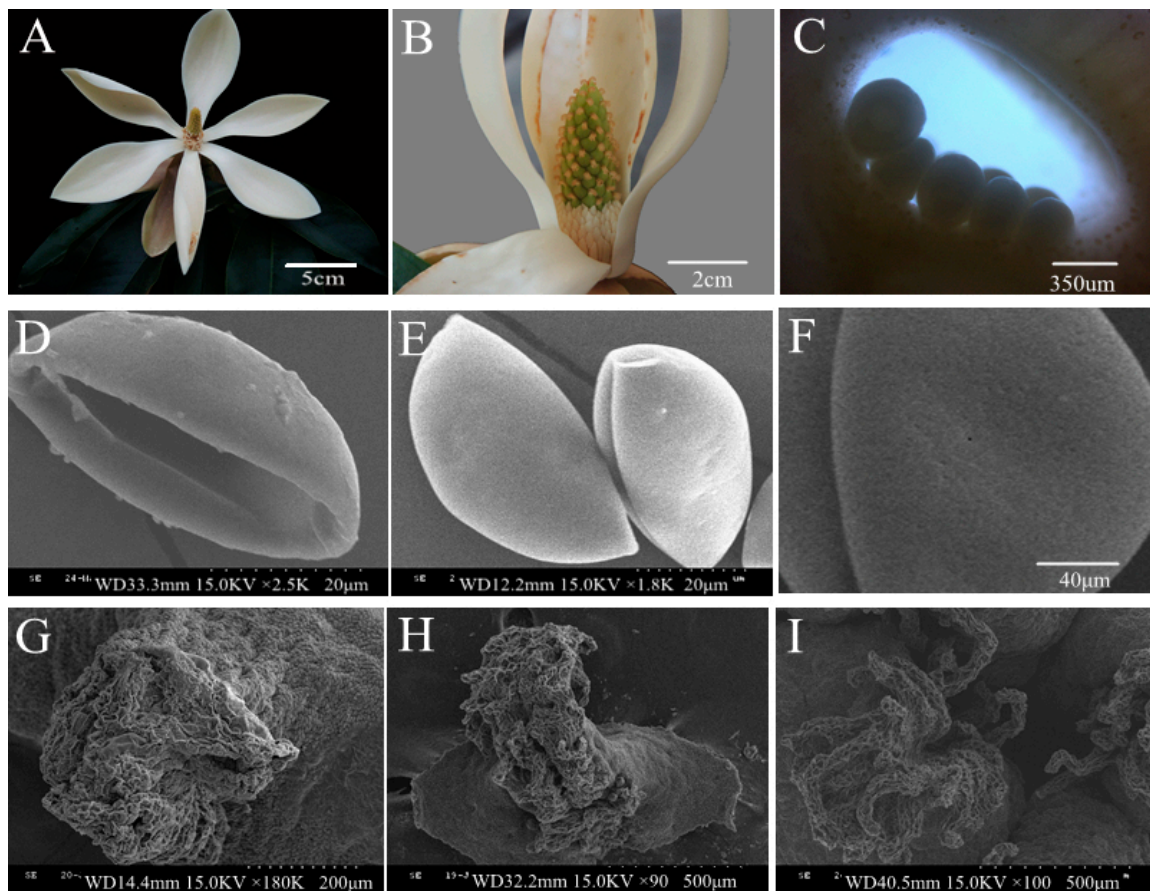
At 4 and 12 h after pollination, five flowers in each pollination treatment were observed for pollen germination on the stigma using a scanning electron microscope.

Successful pollination was monitored after 35 days, which was defined as >50% of distinctly enlarged ovaries in a single fruit. Fruit-set was determined three months after pollination treatments and the number of seeds in each fruit was counted five months after pollination. Analysis of variance (ANOVA) was performed to evaluate the difference of successful pollination, fruit-set, mean number of seeds per fruit for different treatments followed by LSD (Least Significant Difference) test using DPS 14.50 (Data processing system) software [29].

## 3. Results

### 3.1. Flower Structure

The diameter of individual flowers varied from 15 cm to 25 cm when fully opened and the perianth was composed of nine (occasionally 11) tepals which were thick, fleshy spoon-shaped 6–10 cm long and 5–7 cm wide (Figure 2A). The stamens were inserted at the base of the lower receptacle, and the height of the stamens only reached the lower edge of the gynoecium or pistil, 15–25 mm from the top of the gynoecium (Figure 2B). The gynoecium was cone-shaped, 4–6 mm long, and contained 45–85 carpels with 8–12 ovules per carpel (Figure 2C). The androecium consisted of 140–185 stamens, each 7–11 mm long and 0.8–1.5 mm wide. The anthers were milky or hazel in color, and the two pairs of slender anthers were parallel to the two sides of the stamens.



**Figure 2.** Flower structure and male and female organs of *M. confiera* flower. Note: (A) Full bloom; (B) Inner configuration of the flower; (C) Double row of ovules in the carpel; (D,E) Boat-shaped pollen grain; (F) Porous texture of the pollen wall; (G–I) Different stigma types.

Pollen was bilaterally symmetrical, heteropolar, oval or oblong in the polar view and boat-shaped in the equatorial view, with a single groove at the far pole, and an abrupt end either closed or not closed. The polar axis was approximately 62.3 µm and the equatorial axis was approximately 25.5 µm (Figure 2D). The pollen wall was smooth, and the polar axis mask was sparse with concave ornamentation (Figure 2E,F). There were three types of stigma morphology: short stigma (Figure 2G), outward turning (Figure 2H) and octopus claw (Figure 2I). The short stigma was symmetrical and the stigma mastoid was short. The stigma elongated outwards and the mastoid surface was symmetrically distributed on both sides, with a furrow forming in the middle. The octopus claw stigma was turned outwards, with several elongated mastoid surfaces extending to the periphery, forming the most receptive of the three designs for pollen reception.

### 3.2. Outcrossing Index and Pollen–Ovule Ratio in *M. confiera*

The mean number of pollen grains per flower was 737,128 and the mean number of ovules was 556, giving a pollen–ovule (P/O) ratio of 1146 (Table 4). Based on Cruden [28], the breeding system of *M. confiera* was facultative outcrossing.

We estimated the OCI with the method presented in Table 2. The diameter of a single flower of *M. confiera* was >6 mm, therefore giving an index score of 3. The stigma and anther were separated, which is a score of 1, and the pollen was available after the stigma is receptive, which is a score of 0. Thus, the total OCI value was 4 (Table 5). Based on Dafni [26], the breeding systems were considered to be outcrossing, partly self-compatible, and needs pollinators.

**Table 4.** Floral characteristics of *M. conifera*.

Items	Flower Diameter (mm)	Pollen Grains per Flower	Ovules per Flower	Pollen–Ovule Ratio (P/O)
Mean sample size	198.86 ± 43.93	737,128 ± 51,812	556 ± 169	1146

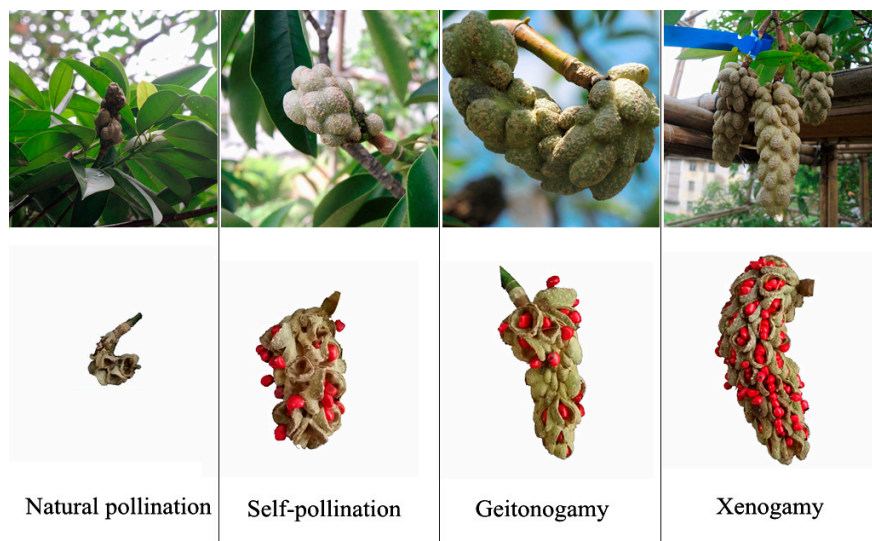
Note: Data is the mean ± standard deviation.

**Table 5.** Outcrossing index of *M. conifera*.

Characteristics	Value	Outcrossing Index (OCI)	Breeding Systems
Flower diameter	15–5 cm >6 mm = 3	4	Outcrossing, partially self-compatible, pollinators required
Temporal separation of stigma receptivity and anther dehiscence	Protogyny = 0		
Spatial position between anthers and stigma	Gynoecea upper = 1		

### 3.3. Controlled Pollination in *M. Conifera*

Fructification and number of seeds per fruit of *M. conifera* after pollination are shown in Figure 3 and Table 6. The pollination efficiency, fruit-set, and mean number of seeds per fruit of emasculation and bagging, emasculation and no bagging, no emasculation and bagging were all zero, suggesting that apomixis does not occur in *M. conifera*.

**Figure 3.** Fructification and fruit of *M. conifera* after pollination.

Successful pollination was defined as having >50% of swollen ovaries within a single fruit. The pollination efficiency calculated 30 days after pollination treatments showed that three pollination treatments (emasculation and bagged; emasculation and no bagging, no emasculation and bagging) did not produce swollen ovaries, and that these flowers were subsequently shed naturally after 20–25 days. The control, namely the natural pollination treatment, produced a small number of swollen ovaries (5–20 from 270 flowers), and did not meet the criterion of successful pollination. Cross-pollination resulted in significantly higher pollination efficiency (60.3%) than the other treatments ( $p < 0.05$ ).

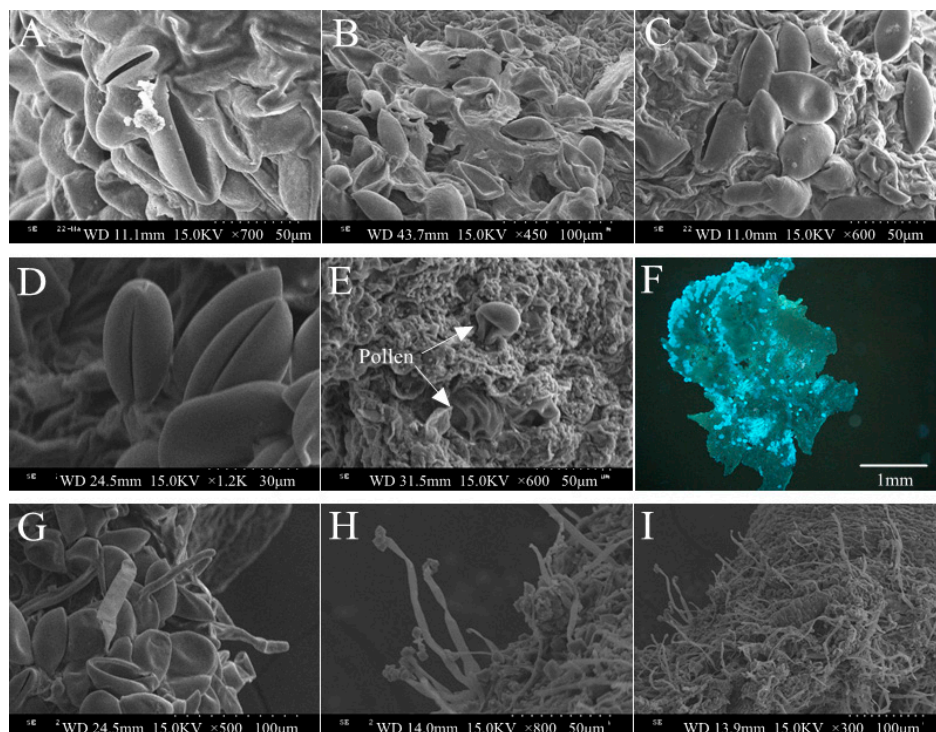
There were significant differences in the fruit-set rate among pollination treatments (Table 6). There were also significant differences ( $p < 0.05$ ) in the number of seeds per fruit among different treatments, with the value being greatest (235) in the xenogamy treatment. Under natural conditions, the mating pattern was mainly outcrossing and partly self-compatible.

**Table 6.** Seed-set in *M. conifera* following different pollination treatments.

Treatment	Number of Trees	Number of Flowers	Successful Pollination (%)	Fruit-Set (%)	Mean Number of Seeds per Fruit
Natural pollination	9	270	0	6.7 d	23 ± 2 c
Emasculation, bagged	9	270	0	0	0
Emasculation, not bagged	9	270	0	0	0
No emasculation, bagged	9	270	0	0	0
Xenogamy	9	270	60.3 a	47.7 a	235 ± 53 a
Geitonogamy	9	270	43.6 b	20.2 b	106 ± 32 b
Self-pollination	9	270	7.7 c	3.3 c	73 ± 28 b

Note: Data is the mean ± standard deviation. The letters in the table refer to the results of multiple comparison of LSD. The different alphabet of small letter shows the discrepancy on 0.05 levels notable.

When stigma receptivity was the strongest, stigma secretion completely covered the mastoid surface, and pollen that fell on the stigma quickly adhered to these exudates. The pollens were covered in mucus, blending the pollen grains into a mass that could not be easily shed (Figure 4A). When the stigma gradually turned brown, the secretory fluid layer on the stigma gradually thinned to dry, and the pollen grains were dispersed between blocks (Figure 4B). Pollen grains adhered to the stigma, and pollen tubes could germinate on the stigma after approximately 2 h (Figure 4C). Pollen germination was observed using a scanning electron microscope 4 and 12 h after pollination on self-pollinated, geitonogamous, and xenogamous. Pollen of xenogamy could germinate normally on the stigma, and grow inwards into the stigma (Figure 4D,E). At 12 h, several germinated pollen tubes entered the stigma vascular tissue (Figure 4F), pollen on self-pollinated stigmas grew in the direction of the stigma, and pollen tubes grew in the opposite direction of the stigma or elongated without entering the vascular tissue of the stigma (Figure 4G–I).



**Figure 4.** Pollen germination on the stigma. Note: (A). Rapid adhesion of pollen and stigma secretion after contact; (B). Dried, cracked stigma secretion, about 30 min after pollination; (C). Pollen germination on the stigma by xenogamy after 2 h; (D). Pollen germination on the stigma by xenogamy after 4 h; (E). Pollen germination on the stigma after 12 h following geitonogamy treatment; (F). Pollen tube piercing stigma vascular tissue; (G–I). Abnormal pollen germination on stigma after self-pollination.

#### 4. Discussion

This study clearly showed poor fruit-set and low seed yield in *M. conifera* especially under open pollination. Based on the floral morphology and the outcrossing index (Tables 4 and 5), the breeding system of *M. conifera* is considered facultative outcrossing (partially self-compatible, pollinators required).

The flower comprehensive characteristics were closely related to the plant reproduction fitness [30]. Many insect-borne plants tend to adapt to insect visits through morphological, flower-design, and display variations of flowers [31], such as *Cynanchum otophyllum* Schneid. through the floral structure [32], and *Sinocalycanthus chinensis* Cheng et S. Y. Chang through pollen [33]. The flowers of *Magnoliaceae* plants are large, colorful, scented, and have significant characteristics of insect-borne pollination [34]. *M. conifera* has these characteristics and shows the adaptability to insect pollination. From the structure of the flower, the perianth of *M. conifera* grows outwards. The pistil and the stamen are isolated, which likely makes it more difficult for pollen to fall naturally on the stigma. Therefore, pollinators are required for pollination. Morphological characteristics of the stigma affect pollination efficiency [35]. Of the three types of stigma found in this study, i.e., short flower, outward turning, and octopus claw, the short-flower stigma was not conducive to pollen reception. The mastoid surface of the outward turning stigma received more pollen. The octopus claw stigma was the most favorable receptacle for pollen transfer among the three stigma types.

The results of controlled pollination suggest that apomixis does not occur in *M. conifera* due to the limited amount of pollen. Open pollination leads to low fruit and seed-set, and given that manual cross-pollination greatly improves seed-set, the low fruit-set from open pollination is likely due to the lack of the native pollinator. Therefore, pollinators were required to enhance fruit-set. The fruit-set rate and seeds of a single fruit of xenogamy and geitonogamy were significantly higher than autogamy, the seed-set for geitonogamy was much lower than its pollination success rate. Seed-set for xenogamy was higher than that for geitonogamy. Jiang [4] also reported similar results of artificial pollination in *M. conifera*. However, results of artificial pollination for *M. conifera* obtained by Zhao [6] were different in that the seed-set for geitonogamy (30%) was higher than that for xenogamy (20%). This study revealed that the breeding system of the species is outcrossing and partly self-compatible. The pollen by xenogamy and geitonogamy could be inserted into the stigma, but the growth of the pollen tube of self-pollinated plants showed no visible direction on the stigma (Figure 4G–I). Therefore, the self-incompatibility phenomenon of *M. conifera* is first manifested in the abnormal germination of pollen on the stigma. The much lower seed-set than pollination success rate of geitonogamy indicated that self-incompatibility occurred in a later stage of embryonic development. This is a lagging performance of self-incompatibility, which is similar to the geitonogamy study in *M. alba* [36].

#### 5. Conclusions

The breeding system of *M. conifera* is outcrossing (partially self-compatible, pollinators required). This provides important information for artificial pollination to promote fruit-set and seed yield. Outward turning stigma and octopus claw stigma provided more favorable receptacle for pollen transfer. This feature can be used as a criterion for selection of mother trees. Additional studies are recommended to further understand the pollination mechanism in order to improve fruit-set and seed yield.

**Author Contributions:** J.H., H.J. and Q.J. designed the experiment, prepared samples and drafted the manuscript. L.P. and Q.P. performed the experiment. K.P. and A.K. analyzed the data and drafted the manuscript. All authors contributed to and approved the final manuscript.

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