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Evaluation of pollen viability, stigma receptivity and fertilization success in *Lagerstroemia indica* L.

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To provide theoretical basis for artificial pollination in *Lagerstroemia indica* L., pollen viability and stigma receptivity were tested and the morphological change of stigma was observed. Pollen viability tested by *in vitro* culture, stigma receptivity examined by benzidine-H_2O_2 testing and fruit set estimated by field artificial pollination were analyzed in this study. The maximum pollen viability was observed at 10:00 am one day of anthesis (DA), of which 'Hong Wei' (46.2%) was significantly lower than that of 'Yin Wei' (56.8%) and 'Zi Wei' (62.5%). The stigma receptivity of the three crape myrtle cultivars was sustained for eight days, which was 95.7 to 96.9% at 1 DA to two days after anthesis (DAA), then declined to 75.5 to 79.9% at 3 to 4 DAA and 50.6 to 59.7% at 5 to 6 DAA, and only 29.5% at 7 DAA. Higher stigma receptivity was associated with columnar style, upward stigma, green and wet papillae and copious exudates at stage 1 (1 DA to 2 DAA). Frequencies of fruit set at stage 1 (74.4%) and stage 2 (3 to 4 DAA) (78.9%) were significantly higher than that at stage 3 (5 to 6 DAA) (21.9%). So, selecting pollen at 10:00 am 1 DA and stigma of 1 DA to 4 DAA was a strategy to enhance fruit set in the future artificial hybridizations for crape myrtle.

Key words: *Lagerstroemia indica* L., dimorphic pollen, pollen viability, stigma receptivity, fruit set.

INTRODUCTION

Lagerstroemia indica (commonly known as crape myrtle) is a deciduous shrub or small woody species native to China, having been cultivated for 1500 years (Zhang, 1991; Chen, 2001). Due to its excellent traits of long-lasting bloom in mid- to late- summer, striking flower color, sinuate trunks, good drought tolerance, and ease of production and cultivation, crape myrtle has been widely introduced to many regions in Australia (Egolf and Andrick, 1978), America (Egolf, 1985), Europe (Attorre et al., 2000) and Turkey (Göre, 2009) for serving as an ornamental plant.In the sexual reproduction of higher plants, doubl fertilization success is determined by a Number of factors including pollen viability, female receptivity and environmental influences, in which pollen and stigma fulfill important functions (Russell, 1992). Any success in breeding experiments or artificial pollination procedures should be accompanied by tests on pollen viability and timing and duration of the stigma receptivity (Lavithis and Bhalla, 1995; Stone et al., 1995; Nautiyal et al., 2009). Currently, systematic studies on pollen viability and stigma receptivity have been reported in a varity of genera, for example, pollen longevity of *Tectona grandis* (Tangmitcharoen and Owens, 1997a, b) and *Grevillea robusta* (Kalinganire et al., 2000) remained for 3 and 4

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Abbreviations: DA, Day of anthesis; **DAA,** day after anthesis.

days, and their viability reached a maximum of 92.2% at 1 day of anthesis (DA) and 95% at 1 day after anthesis (DAA), respectively. Stigma receptivity of *G. robusta* (Kalinganire et al., 2000) and *Zinnia elegans* (Ye et al., 2007) which exhibited peak value at 2 DAA with the morphological characters of taller papillae, abundant exudates and increased stigmatic groove was maintained for 4 and 10 days, and at 2 to 3 DAA with yellow stigma and two lobes on the tip of stigma like 丫, respectively. However, integrated study on pollen viability and stigma receptivity in *L. indica* has not been reported previously. There were only a few reports on pollen viability in *L. indica* (Nepi,et al., 2003; Zhang et al., 2008; Ye et al., 2010). The purpose of the present study was to provide theoretical basis for enhancing fruit set and developing new varieties in *L. indica* crossbreeding, concentrating on the pollen viability, pollen longevity, timing and duration of stigma receptivity and fruit set under defined dimorphic pollen and stigmatic developmental stages.

MATERIALS AND METHODS

Plant materials

The 3-4-year-old plants of three crape myrtle cultivars ('Hong Wei', 'Zi Wei' and 'Yin Wei') were obtained from the experimental field site of Huazhong Agricultural University, Wuhan, China. Field trials were carried out during 2007 to 2008 in the experimental field site of Huazhong Agricultural University. Laboratory tests were carried out with the facilities in the Laboratory of Silivculture and Tree Breeding, Ministry of Education, Huazhong Agricultural University, China.

In vitro pollen viability

As *L. indica* came into bloom, inflorescences at early flowering stage were excised from the tested plants and kept temporarily in small vials full of tap water. At nightfall before testing, opened florets were manually removed to avoid mixing with the tested florets. During 1 DA to 2 DAA, pollen grains were collected 11 times from 7:00 to 16:00 at 3 h intervals except 16:00 of 2 DAA. Pollen viability was tested on *in vitro* germination medium ME_3 (Leduc et al., 1990) supplemented with 16% (w/v) PEG4000 and 12% (w/v) sucrose (Sinopharm Chemical Reagent Co., Shanghai) for 0.5 h (preliminary experiments showed that this was the optimal medium for pollen germination in this species, data not shown). For each sample, germinations of five no-overlapped views were recorded with at least 50 pollen grains per view. Pollen was viewed as germinated when the length of the pollen tube exceeded the diameter of its pollen grain. The experiments were replicated three times. Pollen viability was estimated by pollen germination rate which was calculated as the ratio of the number of germinated pollen grains to the total number of pollen grains.

Fruit set of different pollen types by artificial pollination

Using two-factor randomized design, 3 cultivars ('Hong Wei', 'Zi Wei' and 'Yin Wei') × 3 pollen types (real pollen, feed pollen and mixture pollen), 9 cross combinations were studied. When inflorescences came into bloom, all florets to be pollinated were emasculated and isolated by transparent sulfuric acid paper bag on the day before anthesis to avoid self-pollination. Subsequently, between 8:00 and 10:00 am, florets were hand pollinated with fresh pollen of the corresponding cultivar using a small paintbrush, enclosed within transparent sulfuric acid paper bags, and the number of the pollinated florets in every combination with 10 to 15 replicates were labeled and recorded. To facilitate the development of capsules, the bags were removed a week later and the capsules were harvested after a month. Fruit set was expressed as the ratio of harvested capsules to pollinated florets.

Observation of stigmatic morphological characters

Samples of the three plants were randomly collected for each of the three crape myrtle cultivars for conducting the observation. On the day before anthesis, a total of 120 buds for three cultivars (at least 40 buds per cultivar) were selected to emasculate and isolated by transparent sulfuric acid paper bag individually. At 8:00 am of each day in the following eight days, five pistils per cultivar were excised to observe the morphological characters with the style performance and stigma performance, including the bent degree and color of style, the orientation, color and papillae of stigma and the amount of exudates.

Stigma receptivity by benzidine-H_2O_2 test

Florets were isolated by transparent sulfuric acid paper bag at 1 DA to 7 DAA. The pistils of the three cultivars were collected at 8:00 am and tested by benzidine-H_2O_2 (1% benzidine: 3% H_2O_2 hydrogen peroxide: water = 4:11:22, v/v) (Sinopharm Chemical Reagent Co., Shanghai) respectively (Dafni and Motte, 1998). The collected pistils were stained for 10 to 15 min at 28°C and a pistil was regarded as receptive when more than 2/3 of the stigmatic area were stained dark blue and associated with some amount of bubbles. Each treatment was carried out with ten pistils in three replicates. Stigma receptivity was evaluated by dyeing rate which was calculated as the ratio of the number of stained stigmas to the total number of tested stigmas.

Fruit set at different stigma stages by artificial pollination

Using two-factor randomized design, 3 cultivars ('Hong Wei', 'Zi Wei' and 'Yin Wei') × 3 stigma stages (1DA to 2 DAA, 3 to 4 DAA and five to six DAA classified by the results of benzidine-H_2O_2 examination), a total of 9 cross combinations were studied. The process of pollination and the calculation of fruit set were as previously stated.

Statistical analysis

Percentage data as pollen viability, stigma receptivity and fruit set were transformed via arcsine before analysis. Statistical analysis was performed by SAS software followed by Duncan's multiple comparisons at the level of 0.05 and 0.01 (SAS, 2000).

RESULTS

Pollen viability

Results of single factor variance analysis showed that pollen-collecting time had a very significant (p=0.01) effect on viability of real pollen or feed pollen in all three crape myrtle cultivars (Figure 1).

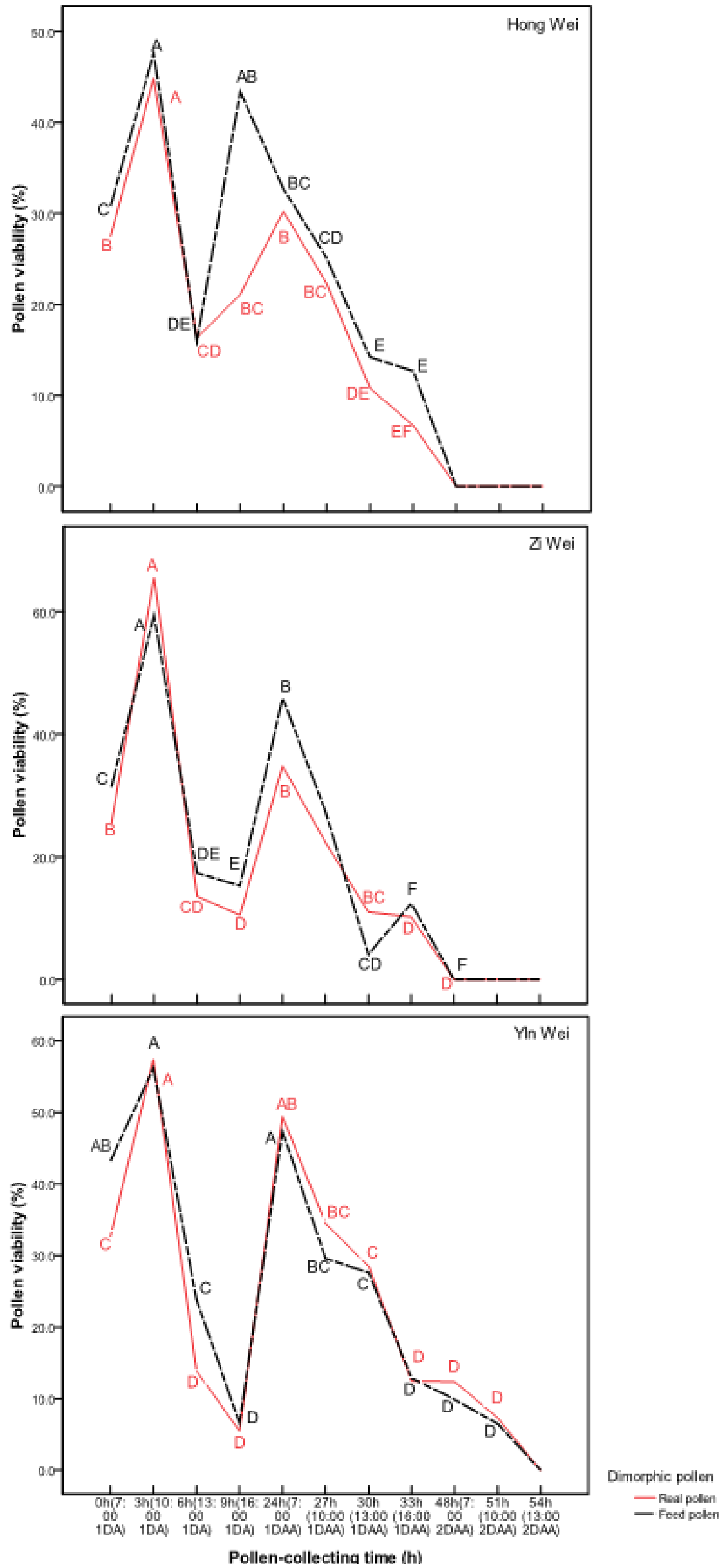

Figure 1. The changing trend of pollen viability with collecting time in three *L. indica* cultivars. *0 h stands for the pollen-collecting time of 7:00 am 1 DA, and other times equal to the time of subtracting 7:00. The black capital letters stand for the results of multi-comparison of feed pollen (p=0.01), while the red capital letters for the real pollen (p=0.01).

Table 1. The relationship between cultivar, dimorphic pollen type and pollen viability (10:00 am 1 DA) of *L. indica*.

Dimorphic pollen type	Germination rate (%)	Cultivar	Germination rate (%)
Real pollen	55.9±3.7^{A}	Hong Wei	46.2±2.1^{bB}
Feed pollen	54.4±2.5^{A}	Zi Wei	62.5±3.2^{aA}
		Yin Wei	56.8±2.5^{aAB}

Table 2. Multi-comparison of fruit set among different combinations of *L. indica* cultivars and three pollen types.

Combination	Feed pollen(% of fruit set)	Real pollen(% of fruit set)	Mixture pollen(% of fruit set)
Hong Wei	72. 9 ± 7.0^{abAB}	56.9 ± 11.8^{bcAB}	49.4 ± 5.3^{bcAB}
Zi Wei	35.5 ± 2.9^{cB}	84.1 ± 4.7^{aA}	34.7 ± 2.8^{cB}
Yin Wei	56.9 ±14.5^{bcAB}	57.8 ± 13.4^{bcAB}	59.7 ± 9.3^{bcAB}

Figure 2. Morphological characters of stigma.

The pollen viability of three cultivars were maintained for 33 h in 'Hong Wei', 33 h in 'Zi Wei' and 51 h in 'Yin Wei'. In the natural environment, the pollen viability was less than 10% (data not shown) at 5:00 am when the corolla just surpassed the calyx. In 'Hong Wei', the real pollen viability was 27.5% at 7:00 am 1 DA, which increased very significantly to the peak (44.8%) at 10:00 am and decreased to 16.3% till 13:00 pm, 21.1% at 16:00 pm of 1 DA. It later increased significantly to 30.2% at 7:00 am of 1 DAA, then decreased to 22.3% at 10:00 am of 1 DAA and decreased very significantly to 10.8% at 13:00 pm of 1 DAA, before it lost vigor at 16:00 pm of 1 DAA.

Two-factor analysis of variance showed that cultivar and the interaction of cultivar and dimorphic pollen type had highly significant effect on the peak pollen viability (10:00 am 1 DA). Further multi-comparison on cultivars (Table 1) indicated that the pollen viability of 'Zi Wei' (62.5%) was significantly higher than that of 'Hong Wei' (46.2%), while that of 'Yin Wei' (56.8%) had no significant difference with them.

Fruit set of different pollen types

Two-factor analysis of variance showed that cultivar and pollen type had no significant effect, but their interaction had highly significant effect on the fruit set. The dimorphic pollen in three cultivars all had fertility. Further multi-comparison (Table 2) revealed the fruit set of three pollen types showed no difference either in 'Hong Wei' or 'Yin Wei'. However, the fruit set of real pollen (84.1%), to a great extent, was significantly higher than that of feed pollen (35.5%) and mixture pollen (34.7%) in 'Zi Wei'.

Morphological characters of stigma

The stigmatic morphological characters during the period of 1 DA to 7 DAA greatly changed in the developmental process (Figure 2). At 1 DA to 2 DAA, the style was columnar or slightly-bent about 30° at the front end, light green, and the stigma was characterized by upward,

Table 3. The relationship between cultivar, growth day of stigma and stigma receptivity of *L. indica*.

Cultivar	Dyeing rate (%)	Growth day of stigma	Dyeing rate (%)
Hong Wei	73.8 ± 4.9^{A}	1 DA	97.9 ± 1.1^{A}
Zi Wei	68.9 ± 6.2^{A}	1 DAA	97.4 ± 1.5^{A}
Yin Wei	77.1 ± 4.1^{A}	2 DAA	95.7 ± 1.7^{A}
		3 DAA	79.9 ± 2.9^{B}
		4 DAA	75.5 ± 3.6^{B}
		5 DAA	59.7 ± 3.6^{C}
		6 DAA	50.6 ± 2.3^{C}
		7 DAA	29.5 ± 4.8^{D}

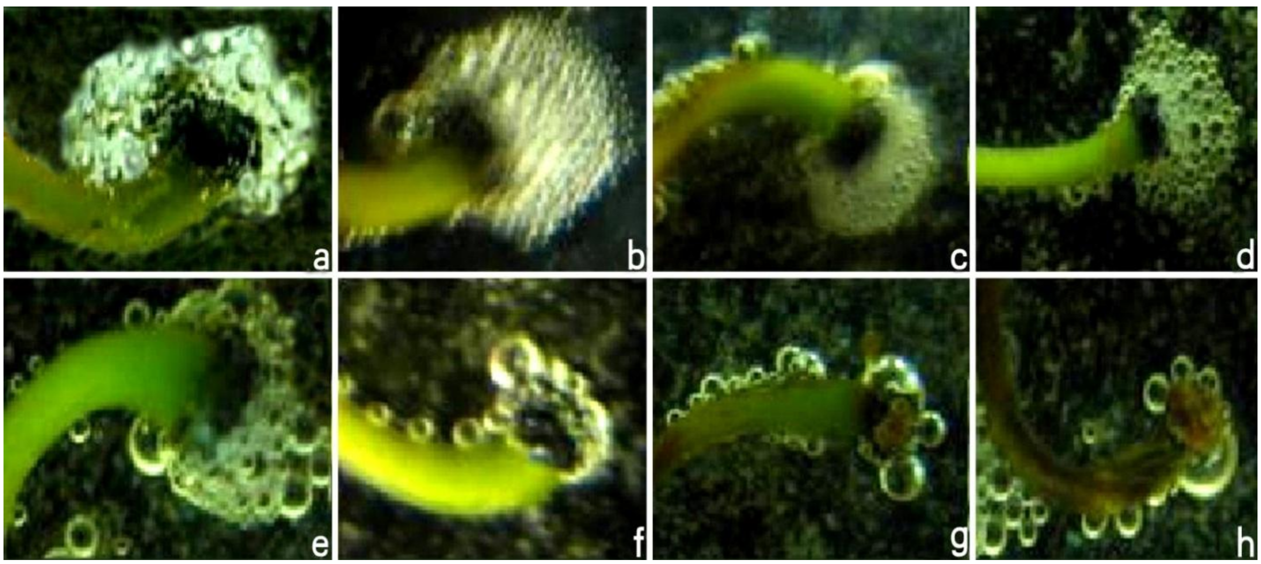

Figure 3. Stigmatic reaction to benzidine-H_2O_2 (a to h stands for the 1 DA to 7 DAA).

green, wet papillae and accumulative copious exudates. At 3 to 4 DAA, the front end of style became slightly curled at about 45°, dark green, and the stigma was characterized by obliquely downward, light green, wet papillae and a few exudates. At 5 to 6 DAA, the style bended at about 90° from the upper middle part in a semicircle-like, wilted from the base and brown, and the stigma was downward, yellow green, slightly wet papillae and few remnants of exudates, suggesting decrease in the support to pollen adhesion. Up to 7 DAA, the style further curled at the front end withered at the base, the stigma looked introflexed, wilted and collapsed as the brown papillae.

Stigma receptivity

Stigma receptivity had no significant difference among the three *L. indica* cultivars which were 73.8% in 'Hong Wei', 68.9% in 'Zi Wei' and 77.1% in 'Yin Wei', while significant differences were observed among growth days (Table 3, Figure3). At 1 DA, the stigmatic surface was not only inundated by more bubbles around its peripheral areas, but completely stained blue, with the stigma receptivity of 97.9%, indicating that the stigma was extremely receptive to pollen (Table 3, Figure 3a). At 1 to 2 DAA, the stigmatic surface accumulated many small and compact bubbles, and was still entirely stained with the stigma receptivity of 97.4 and 95.7% (Table 3; Figure 3b, c). As the stigma developed into 3 to 4 DAA, the bubbles became significantly decreased in amount and density; the staining extent weakened and the stigma receptivity obviously decreased to 75.5 to 79.9% (Table 3; Figure 3d, e). At 5 to 6 DAA, the bubbles further decreased, the staining extent further weakened, and the stigma receptivity significantly declined to 50.6 to 59.7% (Table 3; Figure 3f, g). Until 7 DAA, only few visual bubbles and scarcely staining area were observed and the stigma receptivity was only 29.5% (Table 3; Figure 3h).

Based on the detailed observation of stigmatic morphological character and examination of stigma receptivity on

Table 4. The relationship between cultivar, stigma stage and fruit set of *L. indica*.

Cultivar	Fruit set (%)	Stigma stage	Fruit set (%)
Hong Wei	46.9 ± 17.7^{b}	Stage 1 (1 DA-2 DAA)	74.4 ± 12.5^{A}
Zi Wei	60.9 ± 20.2^{a}	Stage 2 (3-4 DAA)	78.9 ± 8.9^{A}
Yin Wei	53.4 ± 18.6^{a}	Stage 3 (5-6 DAA)	21.9 ± 5.9^{B}

a subset of stigmas, four developmental stages could be classified in the stigmatic developmental process, that is, stage 1 (1 DA to 2 DAA), stage 2 (3 to 4 DAA), stage 3 (5 to 6 DAA) and stage 4 (7 DAA).

Fruit set at different stigma stages

Results of field pollination showed that stigma stage as well as cultivars had highly significant effect on fruit set, but their interaction had no significant effect. Further multiple comparisons (Table 4) revealed that fruit set of 'Zi Wei' (60.9%) and 'Yin Wei' (53.4%) had no significant difference, but that of these two cultivars were significantly higher than that of 'Hong Wei' (46.9%). Fruit set of stage 1 (74.4%) and stage 2 (78.9%) had no significant difference, but that of these two stages were highly significantly higher than that of stage 3 (21.9%).

DISCUSSION

Pollen viability

The pollen viability for three cultivars exhibited similar trend of fluctuant decline with prolonged flowering time (Figure 1). This fluctuant trend of pollen viability with prolonged flowering time was caused by its physiological change as well as the real-time temperature and relative humidity (RH). On a sunny day in August, the environment conditions at 7:00 am were of relatively low temperature and high RH. The temperature gradually rose from 10:00 am, reached the peak till 13:00 pm, gradually decreased after 16:00 pm and maintained this low temperature at night, while RH went up at night. So, the pollen viability was greatly decreased at 13:00 pm 1 DA under the conditions of relative high temperature and low RH, while it increased to sub-peak at 7:00 am 1 DAA under the conditions of relative temperature and higher RH. The performance of pollen viability being affected by different conditions has been clearly reported in many plants. Pollen viabilities of *Oryza sativa* and *Zea mays* when exposed to higher temperature and lower RH, >35°C/65 to 70% and 27.2°C/42%, were maintained for 30 and 43 min, while when subjected to lower temperature and higher RH, 25 to 28°C/70 to 80% and 22.3°C/54.3%, they were prolonged to 60 and 72 min, respectively (Li et al., 2002; Fonseca and Westgate, 2005). Pollen viability of *Rosmarinus officinalis* when treated with 38°C/48% were maintained for only 10%, but significantly increased to 20% after being stored at 4°C/100% for 1 h (Aronne et al., 2006). Thus, loss of pollen viability seems to be a general response due to higher temperature and lower RH. Hence, the fluctuant trend of pollen viability in *L. indica*, which decreased at 13:00 pm 1 DA and increased at 7:00 am 1 DAA, is ascribed partly to the real-time temperature and RH in the study.

The pollen viability of three cultivars 'Zi Wei' (62.5%), 'Hong Wei' (46.2%) and 'Yin Wei' (56.8%) (Table 1) was higher than our previous report of 38.9, 30.1 and 42.8%, respectively (Ye et al., 2010). These inconsistent results might be because the experimental seedlings sown in two different years were different in genotype and plant physiology and growth. Additionally, the pollen grain tested in this study underwent no treatment, while that of Ye et al. (2010) were collected from the dried bud at 25°C for 2 h in homoeothermic incubator. These previous results may demonstrate that the pollen viability of crape myrtle has close relationship to genotype, pollen-collecting time, pollen grain treatment and medium components, and even the real-time and site-specific environmental conditions may play a key role in the pollen viability.

The present study shows that all the dimorphic pollen had viability, and there was no significant difference in the pollen viability (10:00 am 1 DA) and longevity for each crape myrtle cultivar (Figure 1; Table 1). Similar results were reported in two crape myrtle varieties where the dimorphic pollen viability for each one had no significant difference (Zhang et al., 2008). However, Nepi et al., (2003) presented that at anthesis, the viability of real pollen (90%) was higher than that of feed pollen (65%) tested by fluorochromic reaction (FCR) assay for crape myrtle. According to the present results, the dimorphic pollen viabilities varied with crape myrtle materials (genotypes). This trait also existed in genus *Senna* of *Leguminosae* family; the dimorphic pollen viabilities for either *Senna alata* or *Senna bicapsularis* had high significant difference, but no significant difference for *Senna surattensis* (Luo et al., 2009).

Stigmatic morphological traits, stigma receptivity and fruit set

Morphological traits of stage 1 (columnar style, upward and green stigma, wet papillae and copious exudates) and stage 2 (slightly curled style, obliquely downward and yellow green stigma, wet papillae and a few exudates) were associated with higher stigma receptivity of above 95 and 75.0%, respectively (Figure 2; Table 3). This positive correlation between stigmatic morphological traits and higher stigma receptivity has been reported in many plants, such as straight style, enlarged stigma and turgid stigmatic papillae in teak (Tangmitcharoen and Owens, 1997a), widely-opened stigmatic grooves, taller stigmatic papillae and abundant secretion in silky oak (Kalinganire et al., 2000), and elongation of stigmatic papillae and increased amounts of stigmatic exudate in almond (Yi et al., 2006). These reports indicated that we could primarily judge the stigma receptivity by the stigmatic morphological traits, which would provide some reference for the judgment of the effective pollination period (EPP) (Williams, 1965; Galen and Plowright, 1987).

The results of Table 4 suggest choosing the stigma of two previous stages (stage 1 and stage 2) to be pollinated could obtain higher fruit set. The stigma with the morphological characters of columnar or slightly curled style, upward and green stigma, wet papillae and copious exudate was the preferred choice for pollination. Furthermore, the descending order of stigma receptivity was different with varying examination methods, that is, stage 1 > stage 2 > stage 3 (dyeing rate) by benzidine-H_2O_2 testing and stage 1 and stage 2 > stage 3 (fruit set) by field artificial pollination. To sum up, fertilization success does not merely depend on the high stigma receptivity, but also the pollen viability, pollen-stigma interaction, the fertilization process, the seed development process, the seasonal factors and the environment conditions. Further investigations are required to expound this observation.

Meanwhile, three different methods of stigmatic morphological observation, benzidine-H_2O_2 testing and field artificial pollination were adopted and successfully examined the stigma receptivity of three crape myrtle cultivars. Comprehensive methods which detect stigma receptivity have been reported in many plants, such as stigma morphology and pollination in *Prunus dulcis* (Yi et al., 2006), MTT staining and pollination in *Nolana humifusa* and *Nolana laxa* (Amy and Rosanna, 2010) and three integrated methods in *Zinnia elegans* (Ye et al., 2007).

In these examination methods, benzidine-H_2O_2 staining was destructive to stigma, was artificial pollination time-consuming, unrepeatable during same period and hard to handle the reference standards, while the stigmatic morphological observation was intuitive, simple and rapid. So, determining the stigma receptivity with stigmatic morphological observation is a feasible technique to promote the artificial pollination efficiency, which has important practical significance in *L. indica* crossbreeding.

Conclusion

Under field conditions, the maximum pollen viability were observed at 10:00 am 1 DA. The dimorphic pollen of three cultivars all had viability and fertility. The stigma receptivity of three cultivars could sustain 8 d and be subdivided into four developmental stages: stage 1 (1 DA to 2 DAA), stage 2 (3 to 4 DAA), stage 3 (5 to 6 DAA) and stage 4 (7 DAA). Higher stigma receptivity was associated with columnar or slightly curled style, upward or obliquely downward stigma, green and wet papillae and copious exudates at stage 1. Frequencies of fruit set at stage 1 (74.4%) and stage 2 (78.9%) were significantly higher than that at stage 3 (21.9%). So, in view of our study, strategies to enhance fruit set in the artificial pollination should select the pollen of 10:00 am 1 DA and the stigma of 1 DA to 4 DAA. This information is especially valuable for breeders to developing new varieties in *L. indica* crossbreeding.

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