Fertility analyses of interspecific hybrids between *Lagerstroemia indica* and *L. speciosa*

Yi-Qian Ju[1], Xing Hu[1], Yao Jiao, Yuan-Jun Ye, Ming Cai, Tang-Ren Cheng, Jia Wang, Hui-Tang Pan*, Qi-Xiang Zhang

Beijing Key Laboratory of Ornamental Plants Germplasm Innovation & Molecular Breeding, National Engineering Research Center for Floriculture, Beijing Laboratory of Urban and Rural Ecological Environment and College of Landscape Architecture, Beijing Forestry University, Beijing, P.R. China
Corresponding author: htpan@bjfu.edu.cn

Abstract: Interspecific crosses play an important role in gene introgression, plant improvement and speciation. However, poor fertility of F_1 plants was commonly found, which hampered backcrossing and ideal progeny generation. To explore useful materials for further breeding programs, sterile hybrids (DD1, FD1, ZD3) from different cross combinations of *Lagerstroemia indica* and *L. speciosa* and the fertile hybrid (ZD6) were selected. The results showed that pollen grains of sterile hybrids had no germination ability while ZD6 showed 25.90% pollen germination rate. The morphology of stigmas and their papilla cells showed no apparent difference. Normal pollen tubes could be detected in ovaries of ZD6 and ZD3 24 h after pollination. However, the enlarged ovaries of ZD3 began to abscise at 72 h after pollination, which suggested that the barriers occurred during post-fertilization phases. As a consequence, ZD6 can be used as either male or female parent for further *Lagerstroemia* breeding programs, while the sterile hybrids may be used as female parent through embryo rescue culture.

Keywords: crape myrtle; interspecific hybridization; sterility analyses

Crape myrtle is one of the most popular ornamental woody plants around the world considering its diversified plant forms and long-lasting inflorescence. Many elite cultivars of crape myrtle have been bred by interspecific hybridization. It was reported that cultivars Biloxi, Miami and Wichita from crosses between *Lagerstroemia indica* and *L. fauriei* were the first cultivars to combine tree growth habit, mildew resistance and a range of flower colours (Egolf 1987). In 1999, backcrosses between the *L. indica* × *L. fauriei* hybrid cultivar (Tuskegee) and *L. indica* were executed, and cv. Trured was produced with more erected plant-type and longer blooming period compared with Tuskegee (Carroll 2008). Interspecific hybridization can promote the transfer of beneficial alleles between species and thus introduce valuable traits of the parental species into a hybrid (Rieseberg & Willis 2007). However, hybrid sterility of F_1 generation was commonly found, which hinders the use of interspecific hybridization (Chu *et al.* 2014).

L. indica and *L. speciosa*, as the two most popular *Lagerstroemia* species, have different ornamental appeal. *L. speciosa* is a large tree species that exhibits desirable flowering performance and display (Wang *et al.* 2011), however with a more limited range of flower colours and growth habits than *L. indica*

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[1]These authors contributed to the work equally and should be regarded as first co-authors.

(Pounders *et al.* 2007). A combination of desirable traits from these two species would increase genetic variability and introduce new valuable traits into crape myrtle cultivars. Although chromosome counts of *Lagerstroemia* species are often confusing for small chromosome size and dysploidy within the family Lythraceae (Graham & Cavalcanti 2001), the latest results have indicated that both of the two species were diploid ($2n = 2x = 48$) (Yang *et al.* 2012). However, they have divergent genetic backgrounds and taxonomy, *L. indica* is grouped in section Sibia, subsection Sibia, whereas *L. speciosa* is in section Adambea, subsection Adambea (Furtado & Srisuko 1969). Pounders *et al.* (2007) found that all flowered progenies of Tonto × *L. speciosa* were sterile, and the flowers were very uniform among the interspecific progenies, offering limited prospect for selection. Wang *et al.* (2010) found that no viable embryos were generated in the backcrosses of F_1 hybrid as female parent, whereas, some backcrosses with *L. indica* as female parent produced embryos. The sterility of the F_1 hybrids has hampered the utilization of interspecific hybridization and further recombination of genomes of the two species.

To clarify the reason for impaired fertility of F_1 hybrids between the two species and create novel crape myrtle cultivars, we obtained fertile and sterile hybrids successfully and planted them in the same field as materials. Since little information is available about analyses of *L. indica* × *L. speciosa* hybrids with different fertility and the occurrence of pre- or postzygotic barriers in interspecific hybrids, we detected the pollen morphology, pollen vitality and stigma morphology of F_1 hybrids and their parents. Pollen tube behaviours of *L. indica* and *L. speciosa* in the styles and ovaries of the sterile and fertile progenies were also observed. The results laid a foundation for further *Lagerstroemia* breeding programs.

MATERIAL AND METHOD

Plant material. Interspecific crosses were carried out (Table 1) with *L. speciosa* used as pollen donor and *L. indica* Fenjing, Duohuazi and Zixia as maternal parent. In total, five seedlings (DD1-DD6) from *L. indica* Duohuazi × *L. speciosa*, 46 seedlings (FD1-FD46) from Fenjing × *L. speciosa* and 61 seedlings (ZD1-ZD61) from Zixia × *L. speciosa* were generated and cultivated in pots in a greenhouse at Beijing, P.R. China (40°17'N, 116°39'E), and then transplanted to the field at Guangdong, P.R. China (23°03'N, 112°27'E). After blooming for the first time, all F_1 hybrids were infertile except ZD6. We chose three healthy sterile hybrids (DD1, FD1, ZD3) and the fertile hybrid (ZD6) as experiment materials. *L. indica* Creole and *L. speciosa* served as pollen donors for pollen tube behaviour observation.

Identification of hybrids. DNA was extracted from fresh leaves using the FastDNA kit (Tiangen Biotech, Beijing, P.R. China). The Perl script MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html) was used to identify simple sequence repeats (SSRs) of the transcriptome (data not published). Primers were designed using the Primer Premier software (Version 5.0, PREMIER Biosoft) (Table 2). PCR amplification was performed as reported previously by Zhang *et al.* (2012). GeneMapper® *ID* Software(Version 3.2, Applied Biosystems) was used to analyse size peaks.

Sexual hybridization. The progenies were crossed with *L. speciosa* and *L. indica* Creole respectively. Panicles of the maternal plants were emasculated before anthesis in early morning (5:00–7:00 AM). Pollinations were performed after 9:00 AM and the pollinated panicles were isolated with paper bags (Xu 2014). The fruiting rate was evaluated as Cai *et al.* (2010).

Pollen and stigma characteristics. Pollen germinability was measured as described previously (Cai *et al.* 2010). Pollen grains were surveyed under a light microscope (Zeiss Axio Scope A1, Zeiss, Germany) and regarded as germinating when the pollen tube length exceeded the diameter of the pollen grain (Kuligowska *et al.* 2015). Pollens and stigmas were collected and rehydrated (Smyth *et al.* 1990). After critical point drying, the pollens and stigmas were coated with gold and observed under a scanning electron microscope (SEM) (SU8010, Hitachi, Japan). The pollen tubes of *L. indica* Creole and *L. speciosa* were observed

Table 1. Interspecific crosses between *Lagerstroemia indica* and *L. speciosa*

Female parent	Male parent	No. of pollinated flowers	No. of seed pods	No. of F_1 seedlings
L. indica Duohuazi	*L. speciosa*	39	25	5
L. indica Fenjing	*L. speciosa*	35	12	46
L. indica Zixia	*L. speciosa*	24	18	61

Table 2. SSR loci used in hybrid identification

Locus	Repeat motif	Primer sequence (5'-3')	Tm (°C)	Product
SSR967	(GGT)6	F: GGTGAATGGGTACTTGGGGG R: CTCTTAACCTCCACGCTCCG	57.9	171
SSR976	(GAA)6	F: CCACCACCATCTCCCACATC R: GATGACAACTCCTCCACCGG	57.9	208
SSR1022	(TTC)6	F: TAATCAACCAAGCCAGCCGG R: ATCGTGATGGTTGGTGGGAG	55.8	152
SSR1044	(TGC)6	F: TTCTGCTTCTCTTGCTGCGT R: AAGAACATGCCGCGCCTC	53.8	175

Tm – annealing temperature

at 6, 24 and 48 h after pollination of ZD3 and ZD6. Pistils were softened in 8 M NaOH and stained with 0.1% (w/v) aniline blue (Frances & Richards 1990), photographed under a fluorescent microscope (Leica M165 FC, Leica Microsystems, Germany) equipped with a digital camera (Leica DFC450 C, Germany). The parents used as controls were analysed together with the progenies, and comparisons of the pollen germination rate were performed by one-way ANOVA using the SPSS Statistics (Version 20.0, IBM SPSS).

RESULTS

Fruiting rate determination. To confirm the fertility of different F_1 progenies, controlled pollinations were conducted. The results showed that sterile hybrids did not generate any fruit, while their ovaries obviously enlarged three days after pollination. However, all of them abscised seven days after pollination. The fruiting rate of ZD6 showed 67%. When ZD6 served as pollen donor, the fruiting rate was decreased (Table 3).

Hybrid identification by SSR analysis. Seventy-two SSR primer pairs were selected randomly from the transcriptome for PCR amplification, 26 of which were amplified successfully in *L. speciosa*, *L. indica* Zixia and ZD6. Further SSR polymorphism evaluation showed that 23 SSR loci were polymorphic. Of them, four SSR markers were selected to verify interspecific hybridity (Table 2). Based on Mendelian ratios for gene segregation, ZD6 was confirmed to be an interspecific hybrid between *L. indica* Zixia and *L. speciosa*, which showed that all loci of ZD6 were represented by one maternal and one paternal allele (Figure 1, Table 4).

Pollen viability and morphology, stigma morphology. Pollen grains of the parents (Figure 2A–D) had normal sphericity and higher germination rates (63.7%; Table 5). Over 70% pollen grains of these parents (Figure 3A–E) were morphologically normal under SEM. While pollen grains of the sterile plants were irregular in shape (Figure 2E–G), without germination ability (Table 5) and exhibited shrivelled exine patterns (Figure 3F–H). The fertile ZD6 showed

Table 3. Ovary development after controlled pollination with *Lagerstroemia indica* and *L. speciosa*

Female parent[a]	Male parent[b]	No. of pollinated flowers	No. of enlarged ovaries after pollination			No. of seed pods	Fruiting rate (%)
			3 days	5 days	7 days		
DD1		150	63	23	0	0	0
	DD1	86	30	3	0	0	0
FD1		126	39	7	0	0	0
	FD1	101	34	0	0	0	0
ZD3		106	27	16	0	0	0
	ZD3	123	23	4	0	0	0
ZD6		113	87	83	76	76	67
	ZD6	200	181	169	38	38	19

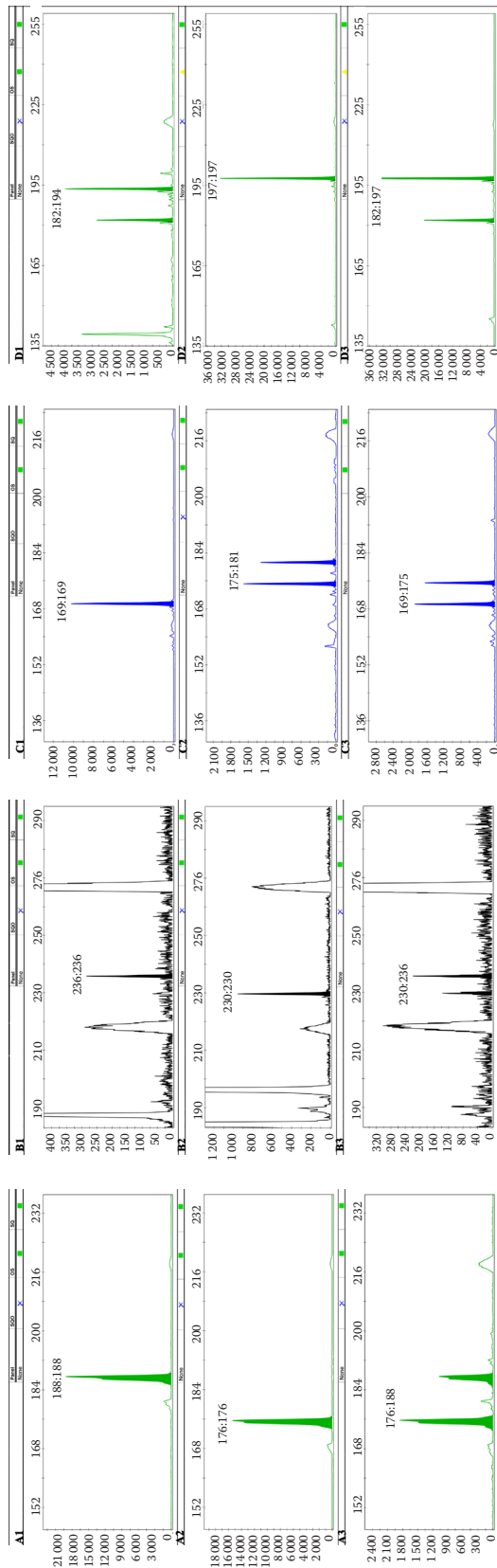[a]*L. indica* Creole, [b]*L. speciosa*

Figure 1. Profiles obtained using four SSR markers: SSR967 (A), SSR976 (B), SSR1022 (C), SSR1044 (D)
1 – paternal (*L. speciosa*) profiles; 2 – maternal (*L. indica* Zixia) profiles; 3 – hybrid (ZD6) profiles; the allele sizes of male and female parents (1 and 2) and hybrid (3) were marked

Table 4. Allele size variation of five SSR markers (all samples are diploid)

Locus	Actual allele sizes (bp)		
	Lagerstroemia speciosa	*L. indica* Zixia	ZD6
SSR967	188:188	176:176	176:188
SSR976	236:236	230:230	230:236
SSR1022	169:169	175:181	169:175
SSR1044	182:194	197:197	182:197

25.9% pollen germination ability (Table 4). Pollen grains of ZD6 were divided into two categories: normal spherical pollen grains with germination ability and distorted and wrinkled pollen grains without germination ability (Figure 2H and 3I). The percentage of normal pollen grains was 23.1%, which was basically in agreement with the pollen germination ability (Table 5).

Stigma diameter and papilla cell length of sterile plants were intermediate between their parents, while the papilla cell length of ZD6 (Figure 4H2) was similar to that of *L. indica*. There were no apparent differences in the morphology of both the stigmas and the papilla cells between the parents (Figure 4A–D), sterile (Figure 4E–G) and fertile plants (Figure 4H) under SEM.

Pollen tube elongation in styles of sterile and fertile progenies. The pollen germination and growth of *L. speciosa* and *L. indica* Creole were normal (Table 5) and large numbers of pollen tubes were detected in the styles of ZD6 and began to penetrate into ovaries 24 h after pollination (Figure 5A–B). The pollen tube behaviour in the style of ZD3 exhibited the same pattern (Figure 5C–D), compared with ZD6.

Table 5. Determination of pollen germination ability (mean ± SE)

Parents and hybrids	Pollen germination rate[a] (%)
Lagerstroemia speciosa	97.41 ± 1.2[a]
L. indica Duohuazi	80.87 ± 2.3[c]
L. indica Fenjing	88.03 ± 3.2[b]
L. indica Zixia	79.55 ± 1.0[c]
L. indica Creole	63.72 ± 3.4[d]
DD1	0[f]
FD1	0[f]
ZD3	0[f]
ZD6	25.90 ± 4.5[e]

[a]Data followed by different letters within a column is statistically different ($P < 0.05$)
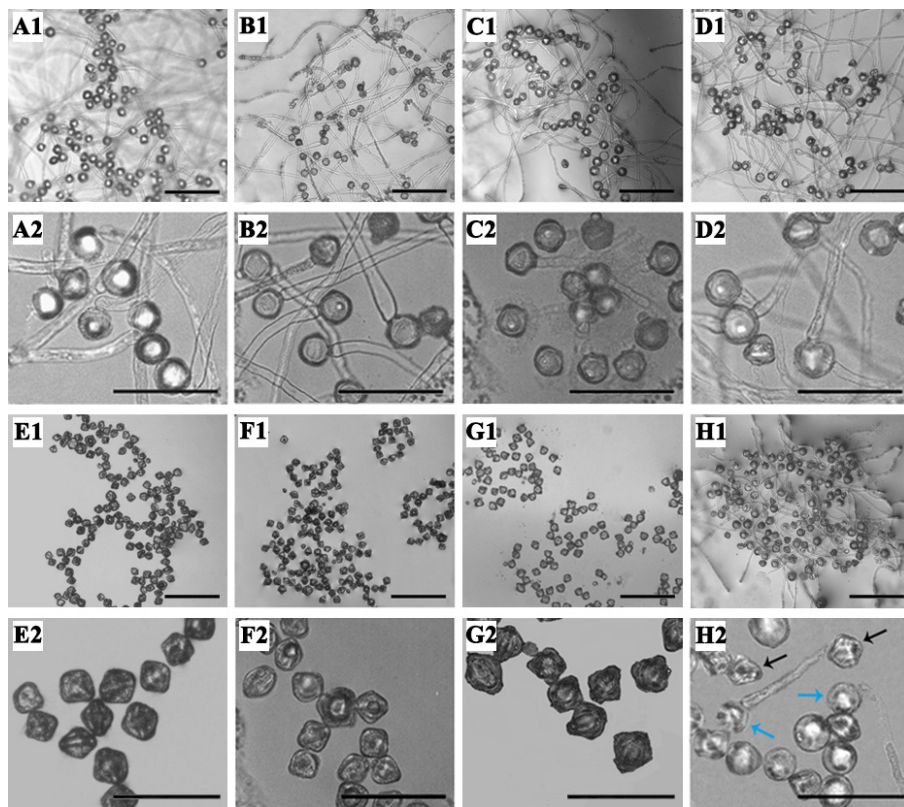
Figure 2. Development of pollen grains observed under a light microscope: *Lagerstroemia speciosa* (A), *L. indica* Duohuazi (B), *L. indica* Zixia (C), *L. indica* Creole (D), DD1 (E), FD1 (F), ZD3 (G), ZD6 (H)
Blue arrows and black arrows represent normal and abnormal pollen grains, respectively; scan bars = 1 mm in (A1–H1) and 500 µm in (A2–H2).
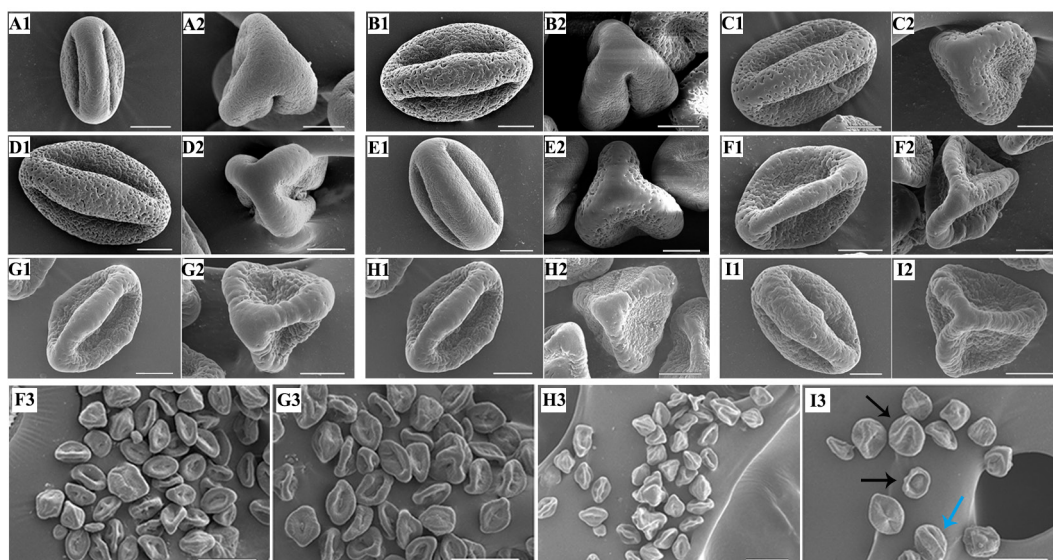

Figure 3. Pollen grains of parents and the progenies under SEM: *L. speciosa* (A), *Lagerstroemia indica* Duohuazi (B), *L. indica* Fenjing (C), *L. indica* Zixia (D), *L. indica* Creole (E), DD1 (F), FD1 (G), ZD3 (H), ZD6 (I)
1 – equatorial view of pollen grain; 2 – polar view of pollen grain; 3 – pollen grain population; blue arrows and black arrows represent normal and abnormal pollen grains, respectively; scan bars = 15 µm in (A1), 10 µm in (A2, B, C, D, E, F1, F2, G1, G2, H1, H2, I1, I2) and 50 µm in (F3, G3, H3, I3)
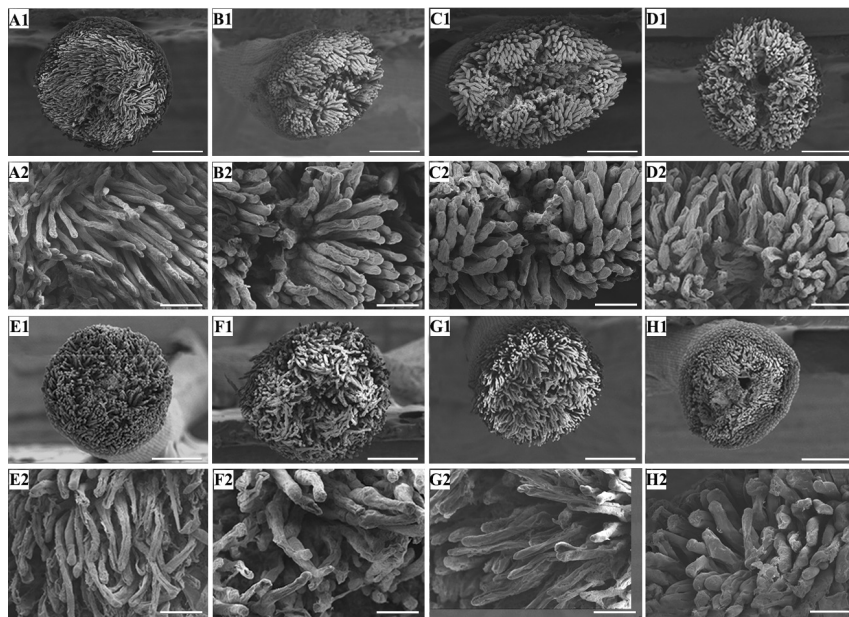
Figure 4. Stigma and papilla cells of parents and the progenies under SEM. *Lagerstroemia speciosa* (A), *L. indica* Duohuazi (B), *L. indica* Fenjing (C), *L. indica* Zixia (D), DD1 (E), FD1 (F), ZD3 (G), ZD6 (H)
1 – stigma; 2 – papilla cells; scan bars = 300 μm in (A1), 210 μm in (B1–D1), 240 μm in (E1–H1), 50 μm in (A2, C2, D2) and 40 μm in (B2, E2–H2)
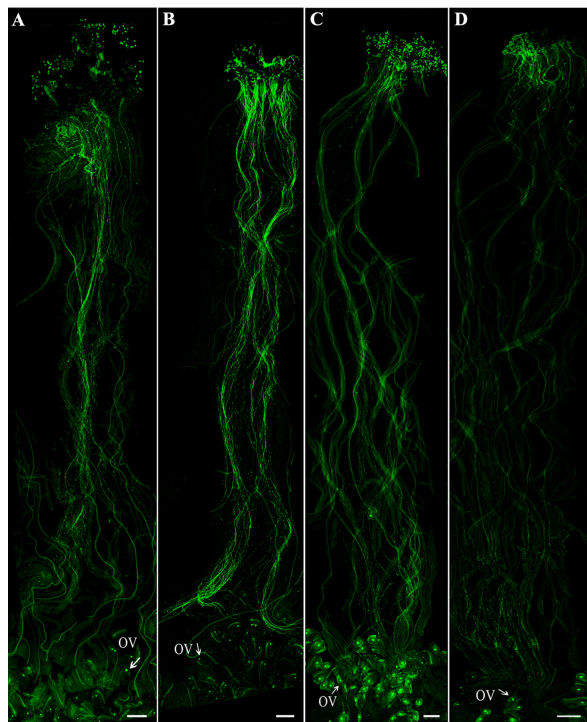

Figure 5. Pollen tube elongation in pistils of ZD6 and ZD3
Pollen tubes of Creole (A) and *Lagerstroemia speciosa* (B) began to penetrate the ovaries (↑) of ZD6 24 h after pollination; pollen tubes of Creole (C) and *L. speciosa* (D) began to penetrate the ovaries (↑) of ZD3 24 h after pollination; OV – ovary; scan bars = 500 μm in (A–D)

DISCUSSION

The divergent genetic backgrounds between *L. indica* and *L. speciosa* may contribute to the sterility or low fertility of female and male gametophytes of hybrids. The sterility of F_1 interspecific hybrids is frequent and was also reported in interspecific hybridization of wheat (Claesson *et al.* 1990) and rice (Naredo *et al.* 2003).

Further fertility analyses showed that the sterility of interspecific hybrids between *L. indica* and *L. speciosa* might be attributed to abnormal pollens. The defects in pollen structure and development might hinder pollen germination. ZD6 had 25.9% pollen fertility, which was in agreement with the proportion of normal pollen grains observed under SEM.

The processes of pollen germination and pollen tube elongation on the stigmas of sterile and fertile progenies were similar, which suggested that backcrosses were not hampered by pre-zygotic barriers since pollen tubes grew through the style to penetrate into the ovaries (Kuligowska *et al.* 2015). However, all the enlarged ovaries of sterile plants aborted seven days after pollination, which suggested that the barriers occurred during post-fertilization phases and embryo rescue might be a way to obtain hybrids. The aim of interspecific hybridization between *L. indica*

and *L. speciosa* was to gain elite crape myrtle cultivars with combinations of complementary traits from the two species. ZD6 obtained in our research will be a useful material for further breeding program. The pollen grains of sterile plants had no germination ability, which suggested that the sterile progenies cannot be used as pollen donors, whereas they can be used as female parents by embryo rescue in the future breeding program. This research lays a foundation for improving crape myrtle ornamental traits.

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