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Identification of Male Fertility of Longquan No. 5 Lines in Loquat (*Eriobotrya japonica* Lindl)

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Abstract: Five methods were used to identify the male fertility, including paraffin section to observe anther and pollen structure during balloon stage, stereomicroscope to observe anther configuration and pollen dispersal situation, scanning electron microscope to observe pollen submicroscopic morphology, TTC method to determine pollen viability and *in vitro* culture method to determine pollen germination rate. The results showed that there were great differences in pollen quantity and morphology between 'Longquan No. 5' loquat and its offspring lines obtained from the moderately degenerated seeds. While, 'Longquan No. 5' was male fertile, its offspring lines from the degraded-seeds had poor pollens, most anthers dehisced abnormally or delayed to dehisced after blossoming, there were many abnormal and poorly developed pollens and the pollen exine ornamentation was very different from their maternal plant. The pollen viabilities and germination rates in *in vitro* culture of the lines from the degraded seeds were obviously lower than their maternal plant (3.67-16.83 and 0.89-15.67%, respectively). The lines from the degraded seeds showed pollen abortion, belonging to sporogenesis male sterility. There were some differences in the male sterility degree among the materials.

Key words: Male fertility, identification, loquat

INTRODUCTION

Loquat (*Eriobotrya japonica* Lindl.) belongs to family *Rosaceae*, subfamily *Maloideae* and is originated from China. As an important economic fruit crop, loquat is widely cultivated between 20 and 35° latitude including China, Japan, India, Pakistan, Madagascar, Reunion Island, Mauritius Island, the Mediterranean countries (Spain, Turkey, Italy, Greece, Israel), the United States (mainlyCalifornia and Florida), Brazil, Venezuela and Australia (Li *et al*., 2008). There are 5 ventricles in the ovary of loquat and 2 ovules in each ventricle. In the process of fruit growth, most ovules degenerated for some reasons and just 3 or 4 ovules can mature to seeds (Deng *et al*., 2007).

In the research on embryo abortion and seed degradation in loquat, we had established many germplasm resources, which came from moderately degradated seeds of 'Longqun No. 5'. We had found that these plants from moderately degradated seeds had abundant genetic diversity and some of them had the potential to become elite varieties. Some lines were found to show the typical characteristics of male sterility e.g., stamen with slim and degenerative anthers. Meanwhile, some lines were found to show low seediness (for example, there were 1.13 seeds in 'Chuannong No. 1' loquat) or seedless, white flesh and so on. And these characteristics are very different from their maternal plant, showing great value in breeding. Male sterility is a very common phenomenon in plants (Schnable and Wise, 1998), which is the basis of using heterosis to increase quality and yield (Chase, 2006; Wu *et al*., 2007). Therefore, it is very important to investigate the male fertility of these materials for breeding and production. Recently, Yamamoto *et al*. (1997), Lillecrapp *et al*. (1999), Guo *et al*. (2007) and Luo *et al*. (2000) carried out a lot of researches on male sterility in *Citrus*, apricot, pear and peach, respectively. But there has been no report about male fertility in loquat till now. So, the authors used 5 methods to examine the male fertility of 'Longqun No. 5' and its offspring lines from the moderately degradated seeds.

The purposes of the present study were to offer a theoretical basis for the analysis of genetic background of sterile materials, to determine the male fertility of 'Longquan No. 5' and its offspring lines from the moderately degradated seeds and to provide with guidance for production.

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MATERIALS AND METHODS

Materials: The 15 years old 'Longquan No. 5' plants and 8 years moderate-degraded-seeds plants (degenerated-seeds plants for short), including 'Chuannong No. 1' (there were 1.13 seeds in each fruit and the average fruit weight was 24.9 g), 'Chuannong No. 3' (the fruits of cross-pollination were seedless) and No. 209, were used as experimental materials. The experimental materials were planted in the loquat orchard of Biotechnology Research Center for Horticulture of Sichuan Agricultural University.

Anther's structure observation: At balloon stage, the well developed side-flowers were sampled and shucked off the perianths. Then the anthers were taken down and placed in FAA (38%formaldehyde: acetic acid: 70% ethanol = 5: 5: 90). The method of Paraffin section was used to make permanent slices. The sections were 10 μm thick, stained with 1% safranine and color separated with 0.5% fast-green. These materials were observed with OLYMPUSBX51 optical microscope.

Anther morphology observation: Tested materials were the flowers which were from healthy branches at peripheral crown of 'Longquan No. 5'lines loquat. The blooming flowers with anthers just at dehiscence were sampled at 2 pm on the blooming day or the next day. Five flowers in the upper, middle and bottom canopy per material were sampled respectively and randomly. Then the characteristics of anthers and the pollens just after pollen dispersal were observed with LEICA-S8APO stereomicroscope.

Pollen submicroscopic morphology observation: At balloon stage, the flowers were sampled, then the pollens were collected after disseminating pollen in room (some materials, whose pollens were very poor in natural conditions, were artificially ruptured to obtain pollens). The pollens were scattered uniformly on sample stage with a double-side adhesive tape, then the pollen submicroscopic morphology was observed with JSM-5900 scanning electron microscope at a voltage of 20KV after the sample stage with pollens was treated by spray-gold about 200-300 Å in vacuum condition. Screen out the representative visual field to take photos and count the rate of abnormality pollens, at 600× (groups of pollen grains), 3000× (individual pollen grains, polar region), 10000× (the central part of equator region, in the observation of the exine ornamentation of pollen surface). Analyze statistically the abnormal pollen rate and measure Poleaxis length (P), Equatorial length (E), perforations density and size of 20 pollens. The taxonomy of pollen morphology was according to Erdtman's method and standard (1978).

Examining pollen viability: The tested materials were sampled from balloon stage to browning period. At midday of some days at 16-18°C in blooming stage, 20 flowers per material were sampled. And the materials were brought to laboratory with ice pot, then all the anthers were taken off with forceps, forty anthers were selected and put into 1.5 mL centrifuge tube randomly after well mixing, the pollen viability were examined after cultivation for 2 h at a constant temperature of 25°C. The method was according to the description by Ji *et al.* (2007), but some steps of the method were altered. Put the disseminating pollens into the 1.5 mL centrifuge tube and added 500 μL phosphoric acid buffer with 0.5% TTC. Then cultured the tested materials at 37°C in dark condition for 2.5 h after well mixing. 20 μL pollen suspension per material was sampled to squash. Count the rate of red pollens in total pollens (pollen viability) and took photos with OLYMPUSBX51 optical microscope (10×10).

Pollen culture *in vitro*: At balloon stage, 20 flowers per material were sampled. Anthers were collected, dried at 25°C, triturated to collect pollens (some materials, whose pollens were very poor in natural conditions, were artificially ruptured to obtain pollens). The method applied to culture pollens was according to the description by Wang *et al.* (2007), but some steps of the method were altered. The medium included 12% sucrose+100 mg L^{-1} boric acid+10% polyethylene glycol (PEG_{8000})+0.6% agar. Then pollens were inoculated in the medium at 25°C for 8 h with 3 replicates. The pollen germination was observed for 5 fields of microscopy (OLYMPUSBX51) at random. The average rate of pollen germination was analyzed statistically.

Investigation on fruit setting percentage in open pollination: When a majority of flowers were at balloon stage, the flowers of same period were labeled after extirpation of the opened flowers and low-development stage flower buds, then these flowers were bagged with sulfuric paper bags. And these sulfuric paper bags were removed when the styles became wilting and brown. The fruit setting percentage was counted after 8 weeks.

RESULTS

Comparison of anther transverse section: The paraffin sections indicated that the transverse section configuration of the anthers of 'Longquan No. 5' and its offspring lines from the moderately degradated seeds were not obviously different, but there was great difference in the pollen number and pollen morphology in anther chamber (Plate, 1). The anther transverse section of all tested materials showed butterfly shape. When the anthers came to maturity, four microsporangiums

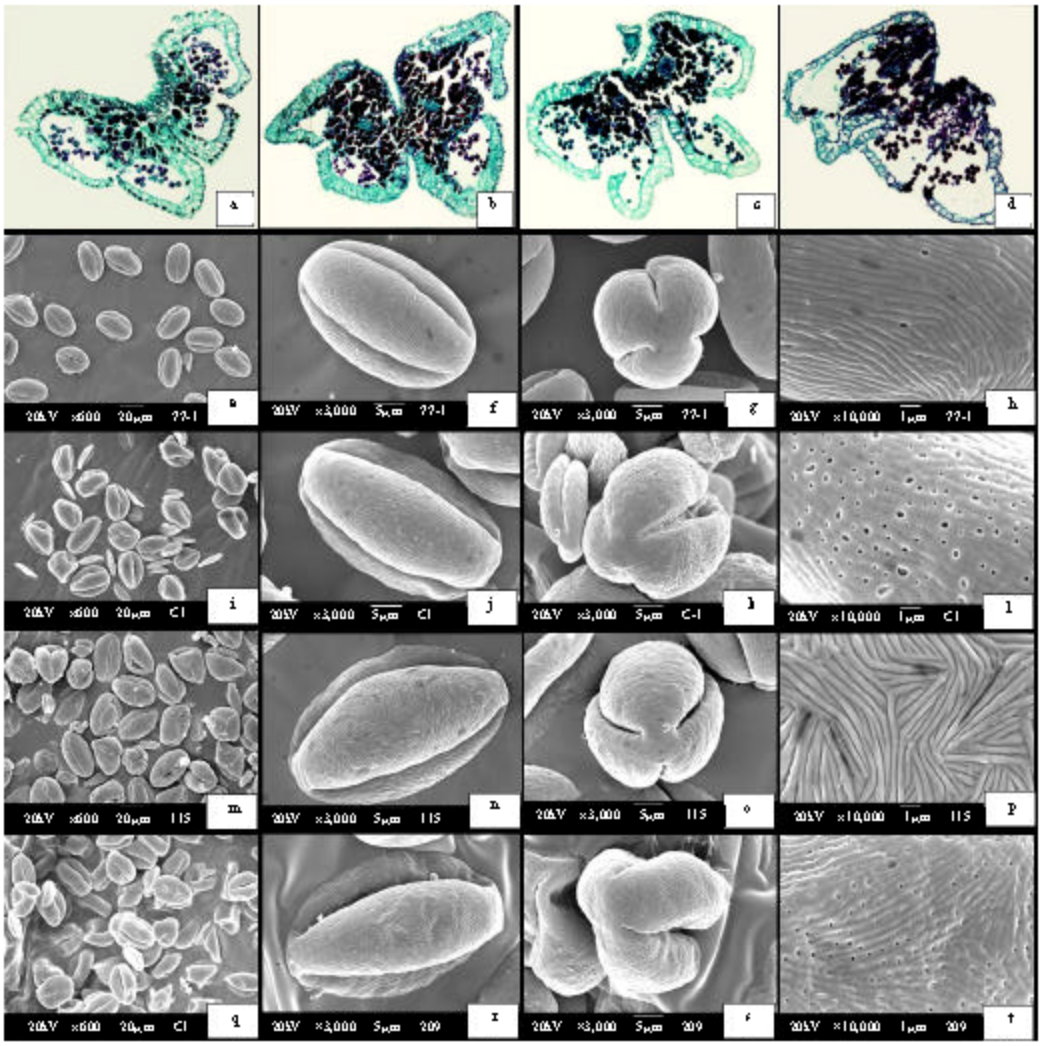

Plate 1: a-d) Anther paraffin sectioning in the big bud period (×100) (1. Longquan No. 5; 2. Chuannong No. 1; 3. Chuannong No. 3; 4. No. 209); e-t. Pollen submicroscopic morphology of Longquan No. 5 and its progeny lines from degenerated seeds (5. Longquan No. 5; 6. Chuannong No. 1; 7. Chuannong No. 3; 9. No. 209; a. Pollen grains in group; b. Equatorial view of pollen; c. Polar view of pollen; d. The exine ornamentation of pollen)

combined to 2 anther chambers which were separated by connective with vascular bundle from filament. In 'Longquan No. 5' loquat, the microspores developed to male gametophyte namely pollens and the anther chambers were filled with pollens uniformly. But there were a large number of abnormal pollens and only few normal pollens in the lines from the degraded seeds. The connective parenchyma cells of 'Chuannong No. 3' and 'Chuannong No. 1' were obviously more than the other two materials, so their anther chambers were obviously less than other two materials. Although, the anther chamber of 'No. 209' was big, the pollens were few and many of them were abnormal.

Comparison of the characteristics of anther morphology and disseminating pollen: The anthers were observed and analyzed with stereomicroscope. The results showed that there were great differences among 'Longquan No. 5' and its offspring lines from the moderately degradated seeds in the characteristics of anther morphology and anther dehiscence. The tested materials could be divided into 3 kinds. The first was 'Longquan No. 5', which had many big and plump anthers. Most anthers could disseminate pollens by longitudinal crack on sunny day. A large number of pollens scattered in cracked anther chamber. The second included 'Chuannong No. 1' and 'No. 209', many anthers of which were slim and degenerative and

Table 1: Comparisons of pollen morphological characters among 'Longquan No. 5' and its progeny lines from degenerated seeds

	Pollen size (μm)		Exine ornamentation				
Cultivars (Lines)	P×E	P/E	Type	Character	Perforations density (Number μm^{-2})/ Diameter (μm)	Abnormal pollen rate (%)	Plate
							1e
							1f
				Striation distinct with a few			1g
Lonquan No. 5	30.47×19.22	1.59	Striate	perforations near polar area	0.48/0.183	4.79	1h
							1i
							1j
							1k
Chuannong No. 1	30.26×20.61	1.47	Striate-perforate	Striation indistinct with many large perforations in equatorial area	1.74/0.265	30.45	1l
							1m
							1n
				Striation thick and dense,			1o
Chuannong No. 3	32.43×21.11	1.54	Striate	arranged in a spiral way	0.08/0.187	71.83	1p
							1q
							1r
				Striation thin and sparse with			1s
No. 209	28.16×18.93	1.49	Striate-perforate	many small perforations	1.64/0.159	48.71	1t

Table 2: Male sterility of 'Longquan No. 5' and its progeny lines from the degenerated seeds

Cultivas (Lines)	Pollen viability (%)	Pollen density (grain/visual field)	Pollen germination rate (%)	Fruit setting rate in self-pollination (%)
Lonquan No. 5	82.48a	274.0a	73.28a	9.47a
No. 209	16.83b	30.4c	15.67b	0.00c
Chuannong No. 1	3.67c	38.2c	14.58b	3.57b
Chuannong No. 3	14.29b	92.4b	0.89c	0.00c

Different letters following the data within each column mean significant difference at 0.05 level (SSR analysis)

the anthers could not dehisce normally or delayed to dehisce and the pollens' quantity was small. The third was 'Chuannong No. 3', whose flower buds and petals were wide (the anthers and petals were increased by 22.98% compared with its parent 'Longquan No. 5') and the anthers looked hypertrophy and plump. However, there were similar liquid transparent materials in most anthers. Most anthers dehisced abnormally or delayed to dehisce after blossoming. The anthers quickly became brown after dehiscence. The pollens' quantity was very small.

Comparisons of pollen morphological characters

Shape of pollens: Observed with SEM, 'Longquan No. 5' not only had plentiful pollens, but also the pollen grains were plump and the incompletely developed or abnormal pollens were very few (Plate 1 e). 'Chuannong No. 1', 'Chuannong No. 3' and 'No. 209', lines from degenerated seeds, had lots of incompletely developed small or very small pollen grains and abnormal ones with medium size (Plate 1i, m and q). The rate of abnormal pollens of 'Chuannong No. 3' reached 71.83% and 'Chuannong No. 1' and 'No. 209' were 30.45 and 48.71%, respectively (Table 1).

The equatorial side views of the normal pollens of four materials were all oblong (Plate 1f, j, n, r), while the polar views of the pollen grains were 3-split round, with three annular grooves which did not converge in the polar plane (Plate 1g, k, o, s). The whole shape of normal pollens of 'Longquan No. 5' and its progeny from degenerated seeds was prolate (P/E = 1.47-1.59).

Size of pollens: The pollens of 'Longquan No. 5' and its progeny from degenerated seeds were in medium size, with the Polar diameter (P) 28.16-32.43 μm, the equatorial diameter (E) 18.93-21.11 μm. Compared with 'Longquan No. 5', the sizes of pollens of the lines from degenerated seeds differed greatly. The pollens of 'Chuannong No. 3', 'No. 209'and 'Chuannong No. 1' were bigger, smaller and similar to the ones of the maternal plant respectively (Table 1).

Exine ornamentation of pollens: The exine ornamentation of pollens differed obviously among 'Longquan No. 5' and its degenerated-seed plants and could be classified into 2 types: striate type and striate-perforate type. But the characters of exine ornamentation of pollens were greatly different in the same type. The distinct striation arranged regularly with a few perforations near polar area in 'Longquan No. 5' (Plate 1f, h). The thick and dense striation arranged in a spiral way in 'Chuannong No. 3' (Plate 1n, p). The striation was indistinct, with many large perforations in equatorial area in 'Chuannong No. 1' (Plate 1j, l). And the striation was thin and sparse, with many small perforations in equatorial area in 'No. 209' (Plate 1r, t).

Comparison of the pollen viability and density: The results indicated that all the pollen viability reached the highest on the 3rd day after flowering. And on that day the anthers were completely mature, but the pollen viability and pollen density were very different among 'Longquan No. 5' and its progeny from degenerated seeds (Table 2). Of all tested materials, the pollen viability of 'Longquan No. 5' was the highest (82.48%), while the pollen viabilities of 'No. 209', 'Chuannong No. 1' and 'Chuannong No. 3' were significantly lower (only 3.67, 16.83 and 14.29%, respectively). The pollen density of 'Longquan No. 5' was higher than that of 'Chuannong No. 3', 'Chuannong No. 1' and 'No. 209' and the latter 3 lines' pollen densities were only 13.94, 11.09 and 33.72% of the maternal plant, respectively.

Comparison of the rate of pollen germination *in vitro* and fruit setting percentage: The pollen germination rates were significantly different after culture *in vitro* for 8 h among 'Longquan No. 5' and its progeny from degenerated seeds (Table 2). The data showed that the rate of pollen germination of degraded seed plants ranged from 0.89-15.67%, which was significantly lower than that of maternal plant, in particularly the rate of pollen germination of 'Chuannong No. 3' was only 0.89%. These were consistent with the morphology of anthers and pollens of the maternal cultivar ('Longquan No. 5') and its offspring lines from the degenerative seeds ('Chuannong No. 1', 'Chuannong No. 3' and 'No. 209').

Most loquats are self-fruitful and the fruit setting percentage in self-pollination is 8-12% in most cultivars. Therefore, people have never paid attention to the cross-pollination in loquat, although there is a theory that cross-pollination may increase fruit setting percentage and the result of field pollination experiment of loquat was basically similar with it (Freihat *et al*., 2008). So, the application of male sterility and low-degree sterility loquat materials was greatly ignored. The data indicated that the fruit setting percentages of degraded-seed plants were significantly lower than that of the parent cultivar. In self- pollination, the fruit setting rate of 'Chuannong No. 1' was only 3.57%, while 'Chuannong No. 3' and 'No. 209' even could not set fruit. The results showed that rarely seeded fruits could be gained in self pollination in 'Chuannong No. 1', without the need of fruit thinning. This unique advantage made 'Chuannong No. 1' have a great value in research and production, while deploying pollination trees or artificial pollination were necessary in the commercial production for 'Chuannong No. 3' and 'No. 209'.

DISCUSSION

Division of male fertility: The male sterility of plants is believed to be related to the fertility gene which expresses in certain time and space and the expression is influenced by internal and external factors (Yuan *et al*., 2007). The results of the present experiments were basically in agreement with this theory. In order to use in production and breeding, based on these results in differences of pollen fertility, the four materials could be divided into 3 classes referring to the standard on soybean fertility (Zhao *et al*., 2004): 'Longquan No. 5' was male fertile (pollen fertility was higher than 40%), 'Chuannong No. 1'and 'No. 209' were low-degree male sterility (pollen fertility was 10-20%), 'Chuannong No. 3' was deep-degree male sterility. The characteristic of male fertility of the tested materials were stable among years, suggesting that it was not caused by external factors. So, the male fertility was determined by genetic gene and the degraded-seed plants were valuable germplasm materials of low-degree male sterility or deep-degree male sterility.

Comparison of methods to identify pollen fertility: At present, there are many methods to identify pollen fertility. Generally, the pollen fertility is identified by the colors in dyeing, such as TTC dyeing method and I_2-IK dyeing method. But the colors of pollen dyeing are not only influenced by the development stage of pollen, but also by the density of dye and time of dyeing and there is no reliable judgment standard of dyeing colors, so the result is prone to causing a great error. In the present study, scanning electron microscope technique combined with paraffin section technique could observe well not only the slight changes of structure of inner anther sac, but also the pollen submicroscopic morphology structure and pollen exine ornamentation. And to some extent, it could help to know the reason of male fertility. Meanwhile, the lines from degenerated seeds could be used as pollination materials to identify their own pollen fertility. Hereby, using 'preliminary observation → micro-scale analysis → practical test' as steps to judge male sterility and multiways would be more scientific for identifying male fertility.

Anther and pollen submicroscopic morphology in relation to pollen abortion: The degenerated pollen of the male-sterile kiwifruit plant showed a poorly sculptured sexine, an anomalous nexine and an intine consisting of a single stratum only (Biasi *et al*., 2001). In the present study, the 3 lines from the degraded seeds were low-degree or deep-degree male sterile, which was shown as fewer pollens, large number of abnormal pollens,

unique pollen exine ornamentation such as thick and dense striation with swirl or thin and scattered striation with many perforations. And the large number of abnormal pollens could result from lack or conjugate of inherent substances in process of pollen development, such as the alterations in polyamine, carbohydrate, nutrient element and endogenous hormones (Aribaud and Martin-Tanguy, 1994; Huo *et al*., 2000; Li *et al*., 2006). The degraded-seeds plants were low-degree or deep-degree male sterile, which could be used as valuable materials to research male sterility in loquat and find out the cause of changes of male fertility and related genes in molecular biology through comparison of the genes related to pollen fertility between sterility and fertility materials in the same cultivar according to the methods of molecular marker on pollen sterility in citrus (Cao *et al*., 2006) and peach (Yu *et al*., 2006).

The male sterility has been studied widely on cytology, physiology and biochemistry, genetics and molecular biology, which is attributed to the important theory and application value of male sterility on heterosis seed production in crops, vegetables and so on. The superior character of fruit germplasm resources with male sterility can be preserved by vegetative propagation. But the researches on male sterility of fruit trees are very few, in particular, there has been no report on ways and mechanism of pollen sterility in loquat. The authors found that the mature seed number of a fruit was closely related to the male fertility degree in loquat. Therefore, the genetic characters and molecular mechanism of loquat germplasm resources of different degree male fertility should be paid more attention and their exploitation and utilization value should be used.

CONCLUSION

There were great differences in pollen quantity and morphology between 'Longquan No. 5' loquat and its offspring lines obtained from the moderately degenerated seeds. While, 'Longquan No. 5' was male fertile, its offspring lines from the degraded-seeds had poor pollens, most anthers dehisced abnormally or delayed to dehisced after blossoming, there were many abnormal and poorly developed pollens and the pollen exine ornamentation was very different from their maternal plant. The pollen viabilities and germination rates in *in vitro* culture of the lines from the degraded seeds were obviously lower than their maternal plant (3.67-16.83 and 0.89-15.67%, respectively). The lines from the degraded seeds showed pollen abortion, belonging to sporogenesis male sterility. There were some differences in the male sterility degree among the materials.

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