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Anatomical studies of ovule development in the post-bloom pistils of the 'Zuili' plum (*Prunus salicina* Lindl.)*

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Abstract: In order to investigate the cause of poor fruit set in 'Zuili' plums, anatomical examinations of post-bloom pistils were conducted and the dates of young fruit drop were recorded during the growing seasons of 2008 and 2009. Pistils of cv. 'Black Amber' were also examined as an abundant setting control. Two major dropping periods were detected in 'Zuili': one during the first 5 d after full bloom (DAF) and another between 10 and 17 DAF. Anatomical analyses of the pistils at the full bloom stage revealed that half of the ovules had not developed embryos, which may have caused their early drop. In most dropped pistils collected at 17 DAF, the micropyle had not been penetrated by a pollen tube, indicating that they were not fertilized. 'Zuili' ovules initiated embryo division at 10–12 DAF, although thereafter embryo development was retarded when compared to the rates observed in 'Black Amber'. Ovule fertilization failure and inactive embryo development after ovule fertilization may be the major causes of the later fruit drop observed in 'Zuili' plum trees.

Key words: Ovule, Development, Pistils, 'Zuili' plum
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1 Introduction

'Zuili' plums, mainly cultivated in the Jiaxing District of Zhejiang Province, China, produce very flavoursome fruit with juicy flesh and have high sugar content when they are fully ripe. However, they usually have poor fruit set, which is the main cause of the limited growing acreage of 'Zuili' production (Chen *et al*., 1982). In our previous report (Jia *et al*., 2008), we reported that high percentages (ca. 75%) of 'Zuili' pistils develop nonfunctional ovules with abnormal or undeveloped embryo sacs or degenerated nucelli, resulting in a poor fruit set. In addition to that, the common development of abnormal flowers with 'double pistils' is another reason for the poor set of 'Zuili' plums because the double pistils are more likely to produce nonfunctional ovules (ca. 90%) than normal pistils. However, we also found that the set percentage of 'Zuili' young fruit counted one month after pollination was significantly lower than that of ovaries with anatomically normal and fertilized ovules. This fact indicates that the young fruit drop after ovule fertilization is caused by some unidentified processes. It is generally accepted that the early drops of drupe fruits, such as apricot, sour cherry, and peach, are mainly caused by defective pistils and unfertilized ovules (Zucconi, 1986; Burgos and Egea, 1993; Cerović and Mićić, 1999; Alburquerque *et al*., 2004). In plums, Simons (1975) reported that flowers with defective pistils drop soon after blooming and that unfertilized fruitlets drop between 17 and 30 d after blooming. In grapes, Okamoto and Imai (1982) reported that the drop of post-bloom ovaries of cv.

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'Muscat of Alexandria' is mainly caused by the degeneration of fertilized ovules, which are significantly affected by shoot pinching and cluster trimming. However, information about the early drop of fertilized plum pistils is very limited.

This work was aimed at clarifying the cause and time of post-bloom young fruit drop in 'Zuili' plums by investigating the anatomical process of ovule development. Similar examinations were also carried out for 'Black Amber' plums as a control cultivar demonstrating abundant fruit setting. In addition, the effects of the type of fruiting branch on the post-bloom fruit drop and final fruit set were examined.

2 Materials and methods

2.1 Plant materials and pistil sampling

Six mature trees (seven years old) of both cv. 'Zuili' and 'Black Amber', growing in a commercial orchard located in the Jiaxing district in Zhejiang Province, Eastern China, were used for this investigation during 2008 and 2009. In the middle of March 2008, 500 flowers developing in the middle part of the tree canopy were marked at the pre-bloom stage with open pollination. Fifty pistils for each cultivar were sampled at 2-d intervals, starting from the time of the full bloom (Mar. 25 and 26) in 'Zuili' and 'Black Amber', respectively, until 20 d after full bloom (DAF). The sampled pistils were immediately soaked in FAA solution (50% EtOH:glacial acetic acid: formalin, 90:5:5 in volume) to fix the samples.

On Mar. 25, 2009, two types of fruiting branch (20 samples from 1-year shoots and spurs, respectively) were emasculated on each tree just prior to anthesis. Samples were immediately pollinated with a mix of compatible pollen from several *Prunus salicina*, labelled and wrapped with a plastic mesh bag. The numbers of dropped and retained pistils (young fruit) were counted at one- to two-week intervals thereafter until one month after full bloom. Pollen tube growth and ovule development, abscised pistils of branch types in 'Zuili' plum, and the final fruit set were investigated.

2.2 Anatomical investigation of sampled pistils

The maximum diameters of fixed pistil samples were measured using a micrometre inserted in a microscope (Olympus SZX 9), and then the pistils were dehydrated in an EtOH-BuOH series and embedded into paraffin blocks. The embedded pistils were cross-sectioned into 16 μm-thick slices using a microtome. The thin slices were pasted onto a glass slide, stained with Alcian blue and periodic acid solution (PAS), and sealed with a cover slip using albumen-glycerin mixture as an adhesive.

Sectioned and stained pistils were first measured for the maximum width of all ovules under a microscope (Olympus BH2). The sections were also examined to judge the developmental stages of each ovule, embryo sac, and zygote (embryo). Representative sections showing the average development were photographed using a digital camera (Nikon Coolpix 4500).

For the measurement of pollen tube growth in pistils, cross-sectioned samples were stained with aniline-blue solution (2 g/L in 0.1 mol/L K_2PO_3). The numbers of pollen tubes in the basal style, upper ovary, obturator, and micropyle were counted using a fluorescent microscope (Olympus FB-2). All data taken from the normal and double pistils were individually averaged.

3 Results

3.1 Growth of the ovary and ovule

As shown in Fig. 1, the ovary diameter of normal pistils of 'Zuili' increased at nearly uniform rates until 11 DAF, then the rate increased rapidly. The ovary diameters of double pistils were significantly smaller than those of normal ones. Double pistils stopped growing at 9 DAF, and most of them abscised thereafter. The ovary size of 'Black Amber' pistils was a little larger than that of normal 'Zuili' pistils and increased at a uniform rate until 14 DAF.

The average size of the 'primary ovule' (Gur, 1975), the developing ovule of the two generated in the nucellus, was a little larger in normal-type 'Zuili' pistils than in 'Black Amber' pistils 5 to 9 DAF. Ovules of the double pistils of 'Zuili' were smaller than those of normal pistils and stopped growing by 9 DAF, a trend also observed for whole ovary size. The sizes of degenerated ovules, designated as 'secondary ovules', were significantly smaller than those of normally developing ovules and became even smaller gradually until 9 DAF.

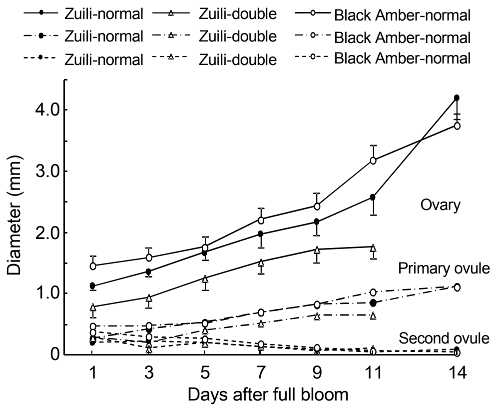

Fig. 1 Ovary and ovule development of 'Zuili' and 'Black Amber' plums after blooming

3.2 Development of ovule and embryo sac

At the full bloom stage, non-functional primary ovules lacking an embryo sac were found at higher percentages (50.0% in both types) in normal and double pistil samples from 'Zuili' trees than in 'Black Amber' pistils (30.4%) (Table 1). However, about 70% of the functional ovules with a viable embryo sac were found to be at the eight-nucleate stage in both cultivars. The normally developed mature egg apparatuses, composed of an egg cell and two synergids, as shown in Figs. 2a and 2b, were found in 50.0% of 'Zuili' pistils and 30.4% of 'Black Amber' pistils. The percents of fused polar nuclei (Fig. 2b), which indicate that the endosperm is ready for fertilization, were as low as 20.0% and 8.7% in 'Zuili' and 'Black Amber', respectively. Ten double pistils of 'Zuili' were examined, and the primary ovules in one half of the pistils had developed normally, but the ovules in the other pistils were undeveloped and degenerating.

At 5 and 6 DAF (the fertilizing stage according to Jia *et al*. (2008)), more than 70% of both normal and double pistil 'Zuili' ovules contained a normal mature egg apparatus (Table 2). The percentages of mature embryo sacs containing a mature egg apparatus and fused polar nuclei at this time were 60.0% in normal pistils and 50.0% in double pistils. In 'Black Amber' ovules, higher percentages of mature egg apparatuses (89.5%) and mature embryo sacs (84.2%) were found, as compared to those in both normal and double pistils.

At 7 to 9 DAF (the post-fertilized stage), shrinkage of the endosperm and/or nucellus in the primary ovules was found in 'Zuili' and 'Black Amber' pistils.

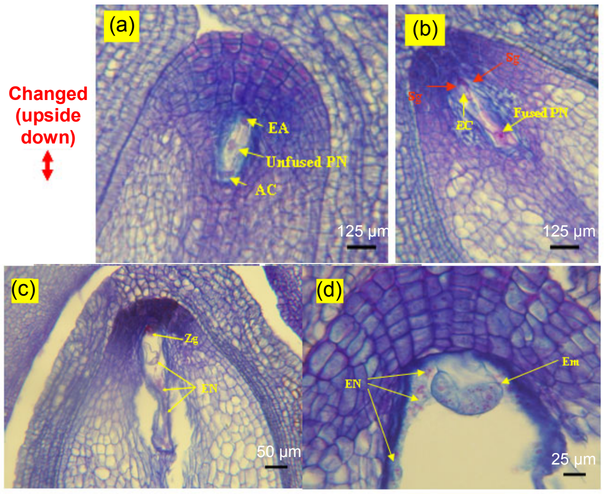

Fig. 2 Embryo sac development in a 'Zuili' pistil
Normally developed mature egg apparatus (EA) is composed of an egg cell (EC) with two synergids (Sg). (a) The pistil was sampled at 2 DAF: unfused polar nuclei (PN) and antipodal cells (AC). (b) The pistil was sampled at 4 DAF: fused PN. (c) The pistil was sampled at 8 DAF: dividing endosperm nuclei (EN) and quiescent zygote (Zg). (d) A developing embryo (Em) with active cell division to reach the globular Em: EN already divided to form numerous free nuclei along the margin of the endosperm

Table 1 Ovule development in 'Zuili' and 'Black Amber' pistils at the full bloom stage

Cultivar and pistil type	Number of ovules examined[z]	Number of ovules						
		Embryo sac undeveloped	Embryo sac with 0N	2N	4N	8N	Mature egg apparatus[y]	Fused polar nuclei
Zuili-normal	40	20 (50.0%)[x]	2	2	2	14	20 (50.0%)	8 (20.0%)
Zuili-double	10	5 (50.0%)	0	0	0	10	5 (50.0%)	0 (0.0%)
Black Amber-normal	46	14 (30.4%)	0	4	6	22	14 (30.4%)	4 (8.7%)

[z] Only the primary ovule in each ovule was examined; [y] Composed of an egg cell with one synergid on either side of it; [x] Figures in parenthesis indicate the percentage of ovules in each category

The percentages of such shrunken embryo sacs were lower in 'Zuili' (8.3% in normal pistils and 11.8% in double pistils) than in 'Black Amber' (23.1%) (Table 3). Seemingly normal egg apparatuses were found in 83.3% and 76.5% of the ovules in normal and double pistils of 'Zuili', respectively. A division of the endosperm nuclei (Fig. 2c), which indicates that the ovule has already been fertilized, was found in 41.7% and 47.1% of ovules in the normal and double pistils of 'Zuili', respectively. Lower percentages of ovules with normal egg apparatuses and divided endosperm nuclei (38.5% and 34.6%, respectively) were recorded in the ovules of 'Black Amber' pistils.

In the pistils taken at 10 to 12 DAF, cell division of the embryo was apparent in several ovules in both the normal and double 'Zuili' pistils (Fig. 2d). However, no distinct embryo division was observed in 'Black Amber' pistils, where all the zygotes maintained a quiescent stage (Table 4). However, the division of endosperm nuclei had taken place in most ovules of the 'Black Amber' pistils, although undivided endosperm nuclei were found in 'Zuili' ovules where the embryo had already begun cell division.

In 13 to 15 DAF pistils, embryo development was significantly enhanced in 'Black Amber'. In fact, 66.7% of the total numbers of ovules were actively dividing, and some of them had already reached the 2- to 8-cell or globular stage. The percentage of such highly developed embryos in ovules of normal 'Zuili' pistils was as low as 33.3% (Table 5). No sample of double pistils of 'Zuili' was available for microscopic observation.

3.3 Effect of fruiting branch type on fruit drop and pollen tube growth

As shown in Table 6, the major events of fruit abscission occurred from 0 to 5 DAF and from 10 to 17 DAF in 1-year shoots. The percentage of retained young fruit was as low as 3.2% in normal pistils and 0.0% in double pistils. However, in spur-born flowers, the early fruit drop was lower compared with the fruiting 1-year shoot flowers of normal pistils, although the percentage of retained young fruit from 10 to 30 DAF was 16.3% in the double and normal pistils. Significantly more retained young fruits were found on 'Black Amber' spurs than on 'Zuili' 1-year shoots. There was a significant difference in the percentage of 1-year shoots and spurs between 'Zuili' and 'Black Amber'. Fruit setting rate was as previously mentioned (Jia *et al.*, 2008).

As shown in Table 7, in both normal and twin pistils, only one-third of abscised pistils received a pollen tube in their basal style. Percentages of pistils reached by a pollen tube into the embryo sac through a micropyle were as low as about 7% to 9%. However, shrunken nucelli were observed at a lower percentage in normal-type pistils, especially in 1-year-shoots, than in double-type pistils.

Table 2 Developmental conditions of embryo sac and endosperm in 'Zuili' and 'Black Amber' pistils at the fertilizing stage^z

Cultivar and pistil type	Number of ovules examined	Number of ovules			
		Shrunken embryo sac	Mature egg apparatus^y	Fused polar nuclei	Mature embryo sac^x
Zuili-normal	30	2	22 (73.3%)	20 (66.7%)	18 (60.0%)
Zuili-double	8	0	6 (75.0%)	4 (50.0%)	4 (50.0%)
Black Amber-normal	30	4	34 (89.5%)	32 (84.2%)	32 (84.2%)

^{z} Samples were taken at 5 and 6 DAF. Values in parenthesis indicate the percentage of ovules in each category; ^{y} Composed of an egg cell with one synergid on either side of it; ^{x} The embryo sac containing a mature egg apparatus and fused polar nuclei

Table 3 Developmental conditions of embryo sac and endosperm in 'Zuili' and 'Black Amber' pistils at the post-fertilized stage^z

Cultivar and pistil type	Number of ovules examined	Number of ovules		
		Shrunken embryo sac	Normal egg apparatus	Dividing endosperm nuclei
Zuili-normal	24	2 (8.3%)	20 (83.3%)	10 (41.7%)
Zuili-double	17	2 (11.8%)	13 (76.5%)	8 (47.1%)
Black Amber-normal	26	6 (23.1%)	10 (38.5%)	9 (34.6%)

^{z} Samples were taken at 7–9 DAF. Values in parenthesis indicate the percentage of ovules in each category

Table 4 Developmental conditions of embryo sac and endosperm in 'Zuili' and 'Black Amber' pistils at the embryo formation stage[z]

Cultivar and pistil type	Number of ovules examined	Number of ovules					
		Shrunken embryo sac	Imperfect egg apparatus	Quiescent zygote	Dividing embryo	Undivided endosperm nuclei	Dividing endosperm nuclei
Zuili-normal	30	0	0	10 (33.3%)	12 (40.0%)	8 (26.7%)	12 (40.0%)
Zuili-double	10	0	5	1 (10.0%)	2 (20.0%)	5 (50.0%)	4 (40.0%)
Black Amber-normal	29	1	2	24 (82.8%)	0 (0.0%)	1 (3.4%)	25 (86.2%)

[z] Samples were taken at 10–12 DAF. Values in parenthesis indicate the percentage of ovules in each category

Table 5 Developmental conditions of embryo in 'Zuili' and 'Black Amber' pistils at the globular embryo stage[z]

Cultivar and pistil type	Number of ovules examined	Number of ovules				
		Shrunken embryo sac	Quiescent zygote	Developing embryo		
				2–8 cells	Globular	Total
Zuili-normal	36	12	12 (33.3%)	8 (22.2%)	4 (11.1%)	12 (33.3%)
Black Amber-normal	48	0	16 (33.3%)	16 (33.3%)	16 (33.3%)	32 (66.7%)

[z] Samples were taken at 13–15 DAF. Values in parenthesis indicate the percentage of ovules in each category

Table 6 Percentages of fruit drop and set in 'Zuili' and 'Black Amber' for flower position, pistil type and types of shoots[z]

Flower position	Pistil type	Fruit drop (%)				Total drop (%)		Final fruit set (%)	
		Mar. 30 (5 DAF)	Apr. 4 (10 DAF)	Apr. 11 (17 DAF)	Apr. 24 (30 DAF)	Zuili	Black Amber	Zuili	Black Amber
1-year shoot	Normal	37.0	11.4	29.0	16.4	93.8	89.2	0.5	8.9
	Double	31.4	14.3	31.0	23.3	100.0	0.0	0.1	0.0
Spur	Normal	18.4	8.5	33.9	22.8	86.6	70.1	2.6	15.0
	Double	5.1	4.6	37.6	24.5	97.1	0.0	0.0	0.0

[z] Two to three hundred flowers were examined for each position and pistil type

Table 7 Pollen tube growth in abscised pistils of 'Zuili' plum[z]

Flower position	Pistil type	Number of pistils examined	Pistils reached by pollen tube (%)				Shrunk nucellus (%)
			Basal style	Upper ovary	Obturator	Micropyle	
1-year shoot	Normal	15	33.3	26.7	20.0	6.7	33.3
	Double	20	35.0	35.0	30.0	15.0	75.0
Spur	Normal	20	35.0	20.0	15.0	10.0	75.0
	Double	24	33.3	25.0	20.8	0.0	83.3
Total	Normal	35	34.3	22.9	17.1	8.6	57.1
	Double	44	34.1	29.5	25.0	6.8	79.5

[z] Abscised pistils were collected on Apr. 11 (17 DAF)

4 Discussion

From the field investigation of the fruit drop process in 'Zuili' trees, two dropping periods were identified: one between 0 and 5 DAF and the other between 10 and 17 DAF (Table 6). The drop percentage and dropping pattern, however, were different to some extent depending on the flower position (in 1-year shoot vs. spur) and flower type (normal pistil or double pistil). The very poor fruit set of flowers born on 1-year shoots, regardless of the flower type, resulted from the high percentage of early drop 5 DAF. From our anatomical observation of 'Zuili' pistils at the full bloom stage (Table 1), half the ovaries had not developed an embryo sac in either the normal or double pistils. At 5 to 6 DAF, when pollen tubes begin to penetrate ovule micropyles (Jia *et al.*, 2008), 'Zuili' had a higher percentage of defective ovules than 'Black Amber' (Table 2). It is possible that many ovules matured later, and/or unfertilized ovules dropped during these days.

The second fruit drop may come from unfertilized ovules and/or inactive endosperm development. The active division of endosperm nuclei was first found in 'Zuili' pistils at the post-fertilized stage (7 to 9 DAF) just before the second fruit drop period (Table 3). However, more than 50% of the total number of ovules did not contain such actively developing endosperm nuclei in both normal and double pistils, although embryo sacs were not shrunken, and egg apparatuses were seemingly normal in most pistils. It can be surmised that pistils with such inactive endosperm nuclei after ovule fertilization might drop about one week later. Similar findings in 'Muscat of Alexandria' grapes have been reported by Okamoto and Imai (1982), where the pistil usually sets a berry if three or four ovules out of the four generated in an ovary are dividing endosperm nuclei, but if more than three ovules stopped their endosperm nuclei division, then the pistil usually drops. From the data on retained- and abscised-ovary diameter (Fig. 1 and Table 6, respectively), fruits that dropped at 17 DAF are estimated to have stopped growing approximately one week before the drop. The major reason for the halted ovary growth is likely an ovule fertilization failure. The normal and double pistils in 1-year shoots nearly abscised before 30 DAF (Table 6). The data of pollen tube growth in abscised pistils of 'Zuili' showed that no micropyle was penetrated by a pollen tube, indicating that ovule fertilization was not achieved (Table 7).

Our anatomical investigations have clarified that fertilized zygotes in 'Zuili' pistils begin to divide to form an embryo as early as 10 to 12 DAF, and globular embryos are found 13 to 15 DAF. Such rapid development of zygotes into globular embryos is similar to that observed in prune variety (Cheng *et al.*, 1999). Thompson and Liu (1973) reported that the first zygotic division occurs several days after ovule fertilization in Italian prune (*Prunus domestica* L.). In peaches, the fertilized zygotes usually remain quiescent for four to five weeks after fertilization and then begin to divide rapidly to form the embryo (Lilien-Kipnis and Lavee, 1971). No information has been reported about embryogenesis after fertilization in *Prunus salicina*, including 'Zuili'.

It is highly interesting that the zygotes in 'Zuili' pistils began to divide earlier than those of 'Black Amber' (Table 4), but thereafter, its development towards the globular stage was significantly slower than that observed in 'Black Amber' (Table 5). Such inactivity of embryo development in 'Zuili' pistils may be a factor in the high percentage of fruit drop from 10 to 30 DAF. The factors affecting the activity of early embryo development are unclear. However, it is well known in tree physiology that the early development of young stone fruits, such as plums, peaches, and cherries, is entirely supported by reserved nutrients accumulated in the tree body. Under the growing conditions we observed, 'Zuili' induced a lower proportion of 1-year shoots than 'Black Amber'. Although the lower early drop from spur buds indicates improvement of pistil development at the bloom stage, abundant spur shoots also reduce canopy growth, thereby affecting root growth (Li *et al.*, 2004). On the other hand, a high temperature event in summer causes leaf drop a month earlier than under normal conditions, which also plays an important role in floral initiation and differentiation. This phenomenon may be the result of a poor nutrient supply, due to the lack of carbohydrate accumulation (Kozlowski *et al.*, 1991) or to a deficient nutrient distribution within the plant because of disequilibrium between vegetative growth and flower differentiation (Goldhamer and Viveros, 2000). Beppu and Kataoka (1999) found that higher summer temperatures led to the development of double-pistils in sweet cherries. Although others have reported that a large tree size makes growers harder and more expensive to manage (Predieri *et al.*, 2003), in fruit production the strong long-shoots are the main organs for exchanging nutrients with the roots, and their export rate of carbon-assimilates was higher and more steady than that of weak short-shoots (Zhang, 1987). Therefore, a modification of the winter pruning system should be recommended to improve the fruit set in 'Zuili' plums. In addition to retaining many fruiting spurs in the canopy, there should be a 1-year shoot-to-adult shoot ratio of no less than 10% to increase underground carbon assimilation and nutrient exchange. Another recommendation would be to reduce the occurrence of double pistils through some means of cultivation techniques, such as canopy shading.

Compliance with ethics guidelines

Hui-juan JIA, Xia YANG, Feng-jie HE, and Bin LI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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