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Cut-style Pollination Can Effectively Overcome Prefertilization Barriers of Distant Hybridization in Loquat

Qin Yang
College of Life and Health Science, Kaili University, Kaili, Guizhou 556000, China

Yan Fu
Qiandongnan Vocational and Technical College, Kaili, Guizhou 556000, China

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Abstract. **Loquat [*Eriobotrya japonica* (*E. japonica*)], a small genus of the subtribe Malinae that consists of ≈30 species, is an evergreen rosaceous fruit tree that is native to southeastern China, and some wild species that possess novel, favorable traits have excellent breeding potential. For example, *Eriobotrya bengalensis* blooms in late spring and ripens in early autumn in Guizhou Province, China, which prevents cold injury in winter by breeding spring-flowering cultivars using the special characters. Therefore, in the present study, the pollination treatments of cut-style pollination were evaluated that may promote successful distant hybridization in *Eriobotrya japonica* 'Dawuxing' × *Eriobotrya deflexa* and *E. japonica* 'Dawuxing' × *E. bengalensis*. The results indicated that the impairment of the pollen tube growth in the upper third of the style after pollen germination is an important factor leading to the failure of distant hybridization between the species tested in *E. japonica*, and that cut-style pollination can effectively overcome prefertilization barriers of the distant hybridization combination. Furthermore, the results of allele-specific polymerase chain reaction (AS-PCR) showed that *S*-genotypes, in accordance with the *S-RNase* heredity to separate the rule completely in offspring, should be both parents' *S-RNase*, and that the random 50 seedlings of *Eb*-2 and *Ed*-2 are true hybrids.**

Biodiversity is the material basis for human survival, sustainable development of agriculture and plant breeding, and the excellent germplasm resources provide a necessary foundation for breeding good varieties (Yang et al., 2004). Throughout the ages, a large number of innovative germplasms and new varieties of fruit trees have been created using conventional breeding methods, such as sexual hybridization and seedling selection, and this diversity plays an important role in the sustainable development of fruit industry (Wang et al., 2012). However, with the deepening of the breeding work, it is difficult to make a breakthrough in plant breeding by conventional methods due to the depletion of germplasm resources with good target traits in plant species, as well as genetic deficiencies in good or specific traits for some fruit trees (Yang et al., 2004). A large number of studies have shown that distant hybridization is a more effective method of enriching species and genetic diversity compared with close hybridization. This method combines the biological characteristics of distant species, which breaks species limits and amplifies genetic variations, resulting in creation of new variations or species (Chen et al., 2018; Li et al., 2016; Ma et al., 2015; Singh et al., 2011; Wang et al., 2012).

Distant hybridization refers to crosses between two different species, genera, or higher-ranking taxa (Chen et al., 2018; Li et al., 2016), and overcoming the prefertilization reproductive barrier is the key to its success. However, due to the reproductive isolation, distant hybridization tends to have a certain degree of incompatibility, which is an obstacle to the breeding process. Complications can include pollen not experiencing germination (Sui et al., 1999; Zhai et al., 2009), pollen tubes exhibiting lateral growth or twine and distortion (Luo et al., 1992; Rieseberg, 1997), pollen tubes bursting (Dutta, 2009), and pollen growth occurring slowly or briefly, preventing entrance to the ovary (Ma et al., 2005; Wu et al., 2006). Even if the pollen tube can enter the ovary, and reach the embryo sac, it may not be fertilized, or only the egg nucleus or polar nucleus produces single fertilization (Yang and Yu, 2004). Therefore, the distant hybridization incompatibility that has been a major concern for researchers may be overcome by several possible solutions, including pollination before anthesis (Chen et al., 2004), mixed pollen pollination (Zhao et al., 2008), repeated pollination (Xie et al., 2009), use of suitable parents (Yang et al., 2004), and obtaining the interspecific hybrids from *Prunus avium* × *Prunus pseudocerasus*, *Myrica rubra* × *Myrica nana*, *Dimocarpus longgana* × *Litchi chinensis*, and *Prunus salicina* × *Prunus armeniaca*. However, studies on how to overcome the distant hybridization incompatibility in the loquat have not been reported.

The loquat is an evergreen rosaceous fruit tree that is native to southeastern China, but is cultivated worldwide in more than 30 countries, where it grows predominantly in subtropical regions experiencing mild temperature (Yang et al., 2018). It flowers from late autumn to early winter, the fruit flesh is soft and succulent and rich in amino acids and microelements, and its taste makes it a popular choice among consumers (Yang et al., 2012). It also has ornamental significance and is grown for its use in the treatment of lung-related diseases, including cough, asthma, and chronic bronchitis, and as a result, it has been cultivated for more than 2000 years (Kikuchi et al., 2014; Yang et al., 2018; Zhang et al., 2015). Nevertheless, there are ≈30 species of loquats and only *E. japonica* was a cultivated species with narrow genetic diversity; when used as parents, several excellent varieties have been obtained by conventional cross breeding (Dong et al., 2008; Zheng, 2007). Through this process, some common flaws have been hard to overcome; for example, low edible percentage due to large seeds, easy lodging due to weak roots, low yield due to small, hydrophobic bud branches, and young fruits are susceptible to frost damage due to flowers from autumn to winter (Li et al., 2016).

In contrast, some wild species have many good traits, such as single seed, small seeds, high germination percentage, and strong branching ability, as well as spring flowers, but there are very few studies that have attempted to carry out distant hybridization with these wild species in loquats. For example, Fukuda et al. (2007) obtained three fruits and four seeds by distant hybridization of *E. japonica* 'Mogi' and *Rhaphiolepis indica*, among which three seedlings were true hybrid, but survived for only 4 months. It also was found that the opposite cross can also sit fruit but produce no seeds. Coombes and Robertson (2008) have obtained true hybrids from intergeneric hybridization of *E. deflexa* and *R. indica*. Li et al. (2016) tried breeding *E. prinoides*, *E. japonica*, and *E. prinoides* var. *dadunensis* as the male parent with *E. deflexa* and its variant that the pollen tube could not reach the ovule through the style after germination, which was determined by

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Q.Y. is the corresponding author. E-mail: yangqin1028518@126.com

fluorescence microscopy. However, although these three aforementioned articles reported success with obtaining the distant hybrid, the problem of low fruit setting percentage also exists, and there have been no reports discussing how to overcome the incompatibility of distant hybridization among species of loquat.

The present study uses *E. japonica* 'Dawuxing' as the female parent and *E. deflexa* (blooms in winter) and *E. bengalensis* (blooms in spring) as the male parents, to investigate different cut-styles of treatment during pollination. The analysis of difference in pollen germination, pollen tube dynamics, fruit set, and average number of seeds per fruit after different pollination treatments was conducted, and the *S-RNase* gene AS-PCR amplification was used to identify the true hybrid of the obtained hybrid seedlings. The effect of cut-style on distant hybridization affinity of loquats was studied systematically to provide a scientific basis for overcoming the incompatibility of distant hybridization between loquat species, and make full use of the fine characters of wild resources to improve the varieties, which is of great significance to germplasm resource innovations in the loquat.

Materials and Methods

Plant material. A total of 12 adult loquat trees were used in this study, including six *E. japonica* 'Dawuxing', three *E. deflexa*, and three *E. bengalensis*, which were planted in the experimental orchard of the Practice and Training Center for Horticulture, Kaili University, Kaili, Guizhou, China, in loamy clay soil. Pollen of the cultivars was respectively collected from flowers just before anthesis, dried at 25 °C in a constant temperature cabinet for 24 h, and kept at 4 °C until use. Before pollination, pollen viability was tested with the method described by Yang et al. (2012). Pollination was performed using a small brush.

Cut-style pollination. Taking the varieties of *E. japonica* 'Dawuxing' as the female parent, in the full bloom of 2016, 120 flowers at the balloon stage were emasculated and bagged to avoid self-pollination and natural pollination on the branches free from pests and diseases for each treatment, with three replicates. Pollination was carried out on the third day after emasculation by the pollen from *E. deflexa* (logogram "*Ed*-1"), and *E. bengalensis* (logogram "*Eb*-1"), respectively. Pollination was carried out on the third day after emasculation by pollen from *E. deflexa* (logogram "*Ed*-2") and *E. bengalensis* (logogram "*Eb*-2"). Before this step, the upper third of the style was cut 2 h after self-pollination, as preliminary experiments indicated that the upper third of the style is the key position for deposition of callose along the pollen tube wall, which stops pollen tube growth. In addition, pollination was carried out on the third day after emasculation by pollen from *E. deflexa* (logogram "*Ed*-3") and *E. bengalensis* (logogram "*Eb*-3"). Similarly, before this step, the upper third of the style was cut directly.

Five days after pollination, 100 pollinated pistils were collected from 20 flowers from each treatment, fixed in formaldehyde alcohol acetic acid solution [5:5:90 (v/v/v) 38% formaldehyde: acetic acid: 70% (v/v) ethanol] and prepared for fluorescence microscopy, as described by Yang et al. (2008). In addition, the fruit sets were determined for each treatment 80 d after pollination by the remaining 100 flowers, and the number of seeds was counted when the fruit was ripe.

Fluorescence microscopy of pollen tube dynamics. Pollen germination performance at the stigma, and pollen tube performance at the style and the ovary, were measured in squash preparations according to the methods described by Mesejo et al. (2007) and Yang et al. (2012). Pistils and ovaries were washed in distilled water (three times, 1 h each), softened until transparent by immersion in 1 mol·L^{-1} NaOH (25 °C), and stained in 0.1% aniline blue (0.1% K_3PO_4) for 48 h. An Olympus BX53 (Tokyo, Japan) fluorescence microscope equipped with a U-MWU filter (Olympus) was used for the measurements, with a DP70 camera taking pictures. We surveyed and calculated the percentage of styles with germinated pollen, as well as the percentage of styles at one-third, one-half, and at the base of the original style and ovary, traversed by pollen tubes for the treatments of *Ed*-1 and *Eb*-1. The percentage of styles with germinated pollen, as well as the percentage of styles at one-third, one-half, and at the base of the cut-style and ovary, traversed by pollen tubes were also surveyed and calculated for the treatments of *Ed*-2, *Eb*-2, *Ed*-3, and *Eb*-3.

Distant hybridization identification. The seeds were numbered for each treatment and sowed in nourishment bags with the substrates, a mixture of leaf mold, vermiculite, perlite, and sawdust in a 2:1:1:1 ratio and then disinfected with 900 × concentrated carbendazim for 7 d. Young expanding leaves from the 50 seedlings numbered *Ed*-2 and *Eb*-2, respectively, at random were collected during the following spring growing season, immediately frozen in liquid nitrogen, and stored at –80 °C. Total genomic DNA was subsequently isolated according to the method described by Yang et al. (2010). AS-PCR was conducted with the optimized reaction system as previously described for loquats (Yang et al., 2013). Hybrid offspring should theoretically have both parents' genotype according to the separation rule of the *S-RNase* gene in offspring, which is used to determine the presence of paternal *S-RNase* genetic information that has infiltrated the offspring, or the presence of new *S-RNase* genotype in offspring (hybrid offspring have the *S-RNase* gene, which is absent from both parents). Following these guidelines, it was determined if the seedlings were true hybrids by identifying the *S* gene of the progeny and determining the presence of a parent *S* gene or a new *S* gene. In this way, it was determined whether the seedlings were true hybrids (Deng et al., 2016; Wu et al., 2005; Zhao et al., 2008).

The obtained nucleotide and putative amino acid sequences were searched against National Center for Biotechnology Information (NCBI) (https://blast. ncbi.nlm.nih.gov/Blast.cgi) using BLASTn and BLASTx to identify homologous genes and putative intron sequences. The deduced protein sequences between the C1 and the C5 conserved regions were obtained, and the structures were analyzed using DNAMAN (Lynnon Biosoft, San Ramon, CA) and compared with available *E. japonica* sequences on GenBank (Yang et al., 2018).

Data analysis. All statistical analyses were performed using the SPSS 16.0 software (IBM Corp., Chicago, IL). Percentages were subjected to angular transformation to ensure normal distribution before analysis of variance ($P \leq 0.05$), followed by Student-Newman-Keuls multiple range test.

Results

Effects of cut-style pollination on pollen germination. Pollen germination in each treatment was estimated by counting the number of styles with germinated pollen grains using a fluorescence microscope. The percentage of styles with germinated pollen ranged from 48.7% (*Ed*-2) to 66.5% (*Eb*-1), with a mean percentage of 53.6% per treatment (Fig. 1A). There were no significant differences between *Ed*-1, *Ed*-2, *Ed*-3, *Eb*-2, and *Eb*-3 treatments, but *Ed*-1, *Ed*-2, *Ed*-3, *Eb*-2, and *Eb*-3 treatments were significantly lower than that of *Eb*-1 treatment. Further observation and comparison revealed that the cut-style can reduce the percentage of styles with germinated pollen, but the percentage can be maintained at ≈50% when pollen from *E. bengalensis* was used to pollinate the styles that had one-third of the upper part removed on the third day after emasculation of the *E. japonica* 'Dawuxing'.

Effects of cut-style pollination on pollen tube growth. The results of analysis on the effect of cut-style pollination on pollen tube growth are shown in Figs. 1B–D and 2A. The percentage of styles that had one-third of the style traversed by pollen tubes ranged from 59.4% (*Ed*-3) to 86.7% (*Eb*-2), with a mean percentage of 72.1% per treatment (Fig. 1B). In the treatment with the same source of pollen, there were no significant differences in the percentage of styles that had one-third of the style traversed by pollen tubes between *Eb*-1 and *Eb*-2, but both were significantly higher than *Eb*-3. Although there were no significant differences in the percentage of styles that had one-third of the style traversed by pollen tubes between *Ed*-1 and *Ed*-2 or *Ed*-2 and *Ed*-3, *Ed*-1 was significantly higher than *Ed*-3. The results showed that the cut-style pollination did not promote pollen tube growth in the upper third of styles, but rather it decreased significantly.

It was apparent that significant differences between the percentage of styles traversed by pollen tubes exist in one-half of the

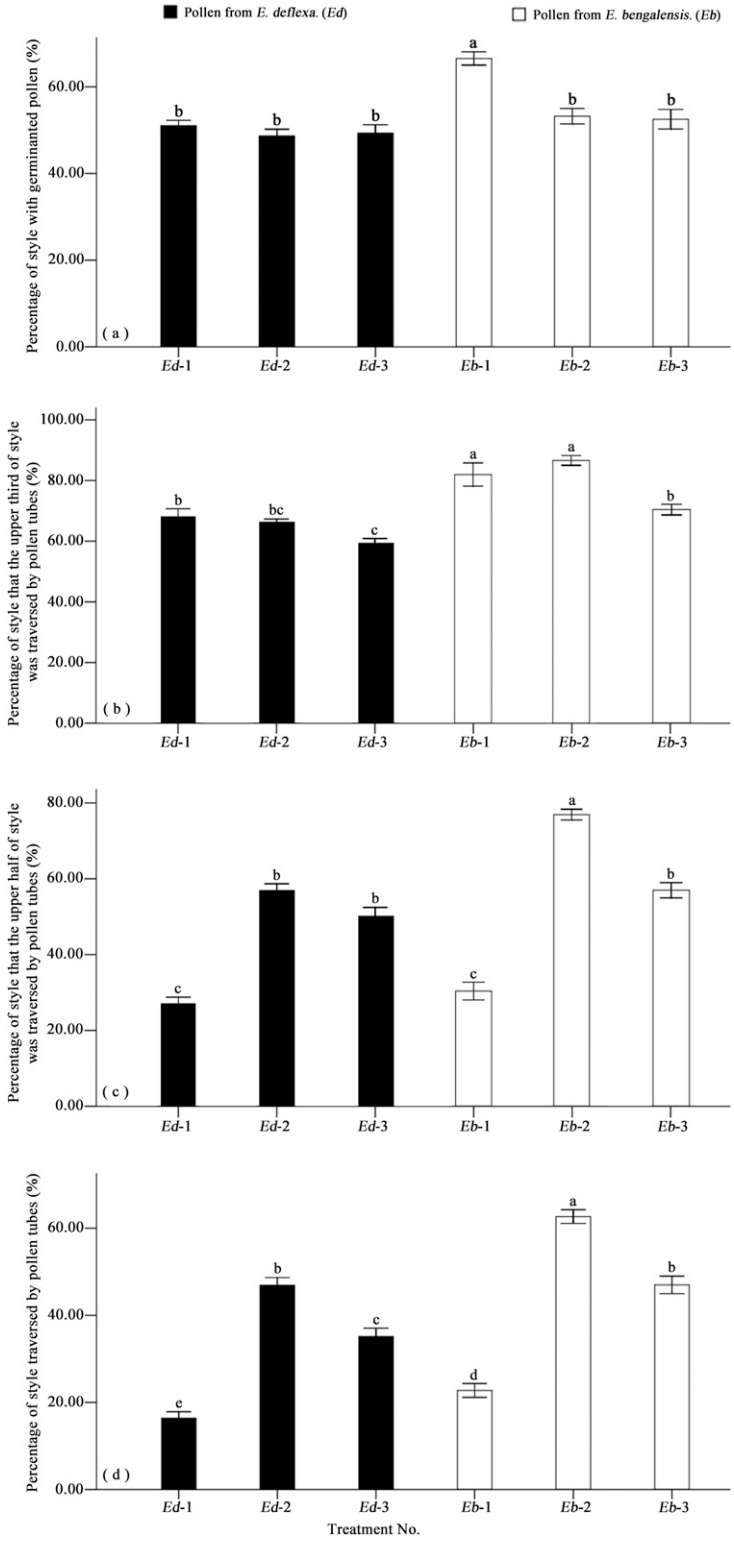

Fig. 1. Percentage of styles with germinated pollen (**A**), and that one-third (**B**), one-half (**C**), and base (**D**) of the style was traversed by pollen tubes among treatments of cut-style pollination. *Ed*-1 and *Eb*-1 indicate that the pollination was carried out on the third day after emasculation of *Eriobotrya deflexa* and *Eriobotrya bengalensis*, respectively. *Ed*-2 and *Eb*-2 indicate that the pollination was carried out on the third day after emasculation of *E. deflexa* and *E. bengalensis*, respectively, and before that, the upper third of the style was cut 2 h after self-pollination. *Ed*-3 and *Eb*-3 indicate that the pollination was carried out on the third day after emasculation of *E. deflexa* and *E. bengalensis*, respectively, and before that, it was cut at the upper third of the style. Data are means ± SE. Different letters above the bar graph indicate significant differences ($P \leq 0.05$), according to Student-Newman-Keuls test.

upper part of styles (Fig. 1C), the base of styles (Fig. 1D), and ovules (Fig. 2A) within treatment with the same source of pollen, as the pollen tube continues to grow in the style. The percentage of styles that had one-half of the style traversed by pollen tubes ranged from 27.1% (*Ed*-1) to 76.9% (*Eb*-2), with a mean percentage of 49.7% per treatment (Fig. 1C). Also, in the pollination treatment with the pollen from *E. deflexa*, the *Ed*-2 and *Ed*-3 were significantly higher than *Ed*-1, whereas in the pollination treatment with the pollen from *E. bengalensis*, the *Eb*-2 and *Eb*-3 were also significantly higher than *Eb*-1 in this study. However, there were no significant differences in the percentage of styles that had one-half of the style traversed by pollen tubes between *Ed*-2 and *Ed*-3, whereas the *Eb*-2 was significantly higher than *Eb*-3 by comparison.

Similar trends are found for the percentage of styles traversed by pollen tubes, with treatments of *Ed*-1, *Ed*-2, *Ed*-3, *Eb*-1, *Eb*-2, and *Eb*-3 having percentages of 16.4%, 46.9%, 35.2%, 22.7%, 62.7%, and 47.0%, respectively (Fig. 1D). In the pollination treatment with the pollen from *E. deflexa*, the *Ed*-2 and *Ed*-3 were significantly higher than *Ed*-1, whereas in the pollination treatment with the pollen from *E. bengalensis*, the *Eb*-2 and *Eb*-3 were also significantly higher than *Eb*-1, and the *Eb*-2 (62.7%) was significantly higher than *Ed*-2 (46.9%). However, the results are different from those with one-half of the style, as the *Ed*-2 was significantly higher than *Ed*-3, and the *Eb*-2 was also significantly higher than *Eb*-3 in this group.

The tracking observations of pollen tube growth also show that there were significant differences in the percentage of ovules traversed by pollen tubes on the premise of the same pollen source (Fig. 2A). The percentage of ovules traversed by pollen tubes of *Eb*-2 was as high as 52.7% and was significantly higher than *Eb*-3 (37.0%). Furthermore, the percentage of ovules traversed by pollen tubes of *Ed*-2 was as high as 26.9%, but there were no significant differences between *Ed*-2 and *Ed*-3 (25.2%). These results also indicate that prefertilization barriers were prevented by the methods of cut-style pollination.

Effects of cut-style pollination on the fruit set and average number of seeds per fruit. The results of fruit set analysis for each treatment revealed that the effect of cut-style pollination on fruit set and average number of seeds per fruit was consistent with the results of cut-style pollination effects on pollen tube growth (Fig. 2B). The fruit set of *Eb*-2 was as high as 50.3%, which was significantly higher than *Eb*-3 (37.0%) and *Eb*-1 (9.3%). In addition, the fruit set of *Ed*-2 was 26.3%, which was significantly higher than *Ed*-3 (20.0%) and *Ed*-1 (5.7%), whereas *Eb*-3 (37.0%) and *Ed*-3 (20.0%) were significantly higher than *Eb*-1 (9.3%) and *Ed*-1 (5.7%), correspondingly. Furthermore, there was a significant difference in fruit set between the same pollination methods of different pollen sources from *E. bengalensis* and *E. deflexa*. The results showed that the fruit set from pollination treatments with the pollen from *E. bengalensis* was higher compared with the pollination treatments with the pollen from *E. deflexa*. For example, the *Eb*-2 (50.3%) and *Eb*-3 (37.0%) were significantly

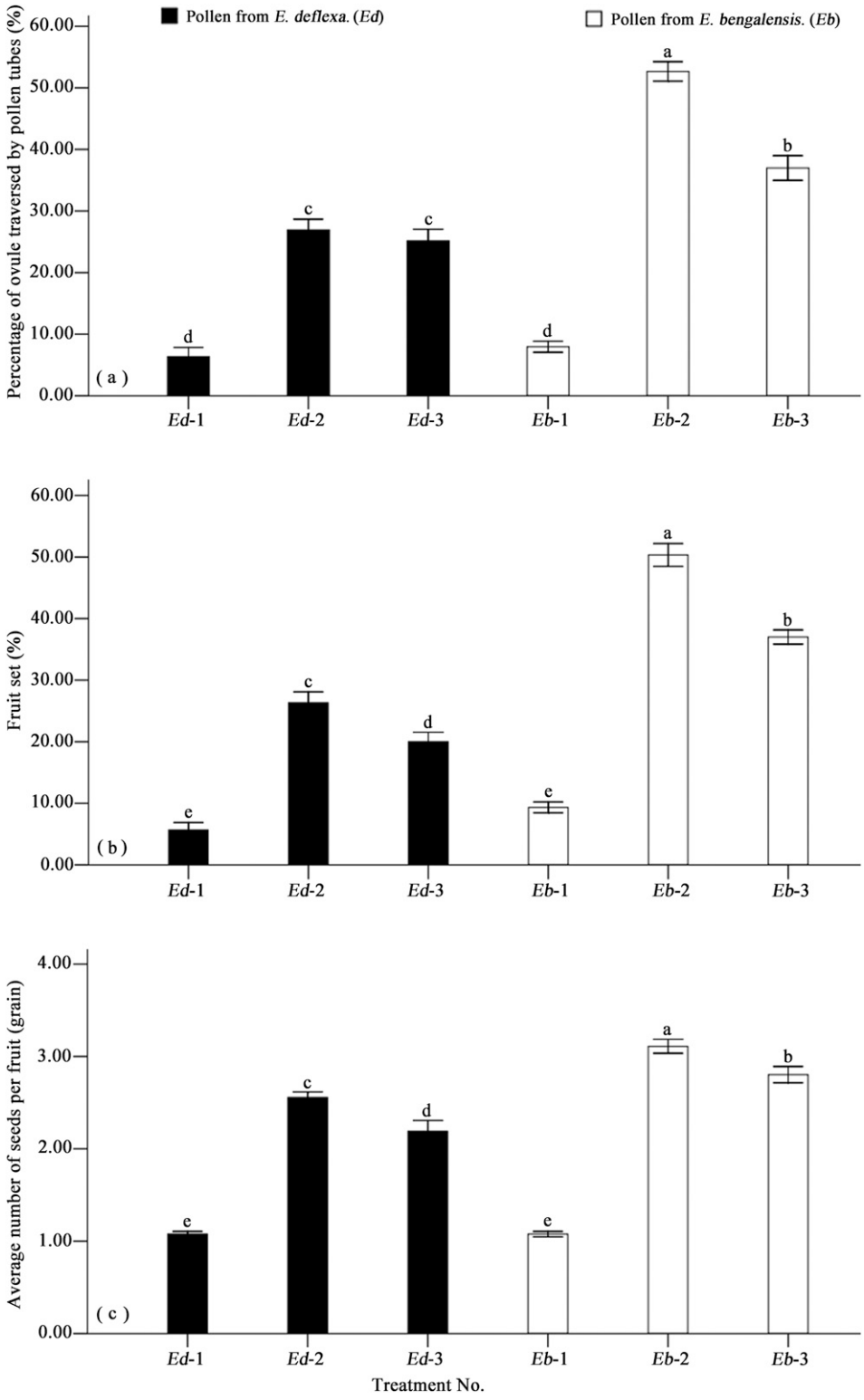

Fig. 2. Percentage of ovules traversed by pollen tubes (**A**), fruit set (**B**), and average number of seeds per fruit (**C**). Data are means ± SE. Different letters above the bar graph indicate significant differences ($P \leq 0.05$) according to Student-Newman-Keuls test.

higher than *Ed*-2 (26.3%) and *Ed*-3 (20.0%), respectively.

Figure 2C shows that the results of seeds per fruit were consistent with the results of cut-style pollination effects on fruit set. The *Eb*-2 treatment had an average of 3.1 seeds per fruit, which was significantly higher than *Eb*-3 (2.8) and *Eb*-1 (1.1), whereas the *Ed*-2 treatment had an average of 2.6 seeds per fruit, which was significantly higher than *Ed*-3 (2.2) and *Ed*-1 (1.1). The comprehensive analysis of the effect of cut-style pollination on pollen germination, pollen tube growth, fruit set, and seed number showed that cut-style pollination can effectively overcome the prefertilization barriers of distant hybridization combination of *E. japonica* 'Dawuxing' × *E. bengalensis*, *E. japonica* 'Dawuxing' × *E. deflexa*.

However, it is worth noting that the prefertilization barriers were better overcome by these treatments that the pollination was carried out on the third day after emasculation of *Eb*-2 and *Ed*-2. Before that, the upper

third of the style was cut 2 h after self-pollination. Within the same pollen source treatments, the percentage of styles or ovules traversed by pollen tubes, fruit set, and average number of seeds per fruit of *Eb*-2 was significantly higher than *Eb*-1 and *Eb*-3, respectively, whereas the percentage of styles or ovules traversed by pollen tubes, fruit set, and average number of seeds per fruit of *Ed*-2 was significantly higher than *Ed*-1 and *Ed*-3, respectively.

Identification of distant hybrids by AS-PCR. The *S*-genotypes have been identified in 'Dawuxing', *E. bengalensis*, *E. deflexa*, and their hybrids by AS-PCR with two combinations of primers [FTQQYQ- IIWPNV and FTQQYQ- FI (D/N) CP (H/R)]. All fragments were compared with the NCBI database using the BLASTN program to identify closer alleles in *E. japonica*. Before this analysis, the amplification fragments were recycled by gel extraction, using a DP210 DNA gel extraction kit (Tiangen Biotech, Beijing, China), cloned into the pGEM-T Easy Vector (Promega, Madison, WI), and sequenced by BGI Life Tech Co., Ltd. (Beijing, China). The *S*-genotypes of 'Dawuxing', *E. bengalensis*, and *E. deflexa* were determined to be S_2S_{41}, $S_{18}S_{21}$, and $S_{27}S_{30}$, respectively.

Fifty random seedlings from the F1 population of the *Eb*-2 cross were studied by means of S-allele-specific PCR, and five *S*-genotypes, 11 (S_2S_{18}), 12 (S_2S_{21}), 13 ($S_{18}S_{41}$), 12 ($S_{21}S_{41}$), and 2 ($S_{18}S_{30}$) were amplified so that the S_2: S_{18}: S_{21}: S_{41} were present in a 1:1:1:1 expected ratio by the χ^2 test, where $\chi^2 = 0.24 < \chi^2(0.05,3) = 7.81$ in this study. Meanwhile, 50 random seedlings from the F1 population of the *Ed*-2 cross were studied by means of S-allele–specific PCR, and five *S*-genotypes, 12 (S_2S_{27}), 13 (S_2S_{30}), 11 ($S_{27}S_{41}$), and 14 ($S_{30}S_{41}$) were amplified so that the S_2:S_{27}:S_{30}:S_{41} were present in a 1:1:1:1 expected ratio by the χ^2 test, where $\chi^2 = 0.32 < \chi^2(0.05,3) = 7.81$ in this study. The aforementioned results revealed that *S*-genotypes, in accordance with the *S-RNase* heredity to separate the rule completely in offspring, should represent both parents' *S-RNase*, and that the 50 random seedlings of *Eb*-2 and *Ed*-2, respectively, are true hybrids.

Discussion

Overcoming distant hybridization incompatibility. Plant pollination and fertilization is a complicated process. After a pollen grain lands on the stigmatic papillae, it usually undergoes immediate adhesion, hydration, and germination, which are prerequisites for subsequent pollen tube growth and fertilization (Yang et al., 2012). In this study, it was observed that the pollen tube lateral growth in some stigmatic papillae, the pollen tube growth in the one-third of the upper part of some styles of *Ed*-1 and *Eb*-1, and even a pollen tube with the tip expanded into the ball could stop growing for the deposition of callose along pollen tube wall (Fig. 3), which is similar to the results described by Luo et al. (1992), Ma et al. (2005), Rieseberg (1997), and Wu et al. (2006). The results confirmed that the greatest differences in the fruit set and average number of seeds per fruit were observed in different treatment.

Notably, within the same pollen source treatments, fruit set and average number of seeds per fruit of were the lowest for *Eb*-1 and *Ed*-1, as well as significantly lower than *Eb*-2 and *Eb*-3, *Ed*-2 and *Ed*-3, respectively. In addition, the upper third of the style was cut 2 h after self-pollination for the *Eb*-2 and *Ed*-2 treatments, whereas the upper third of

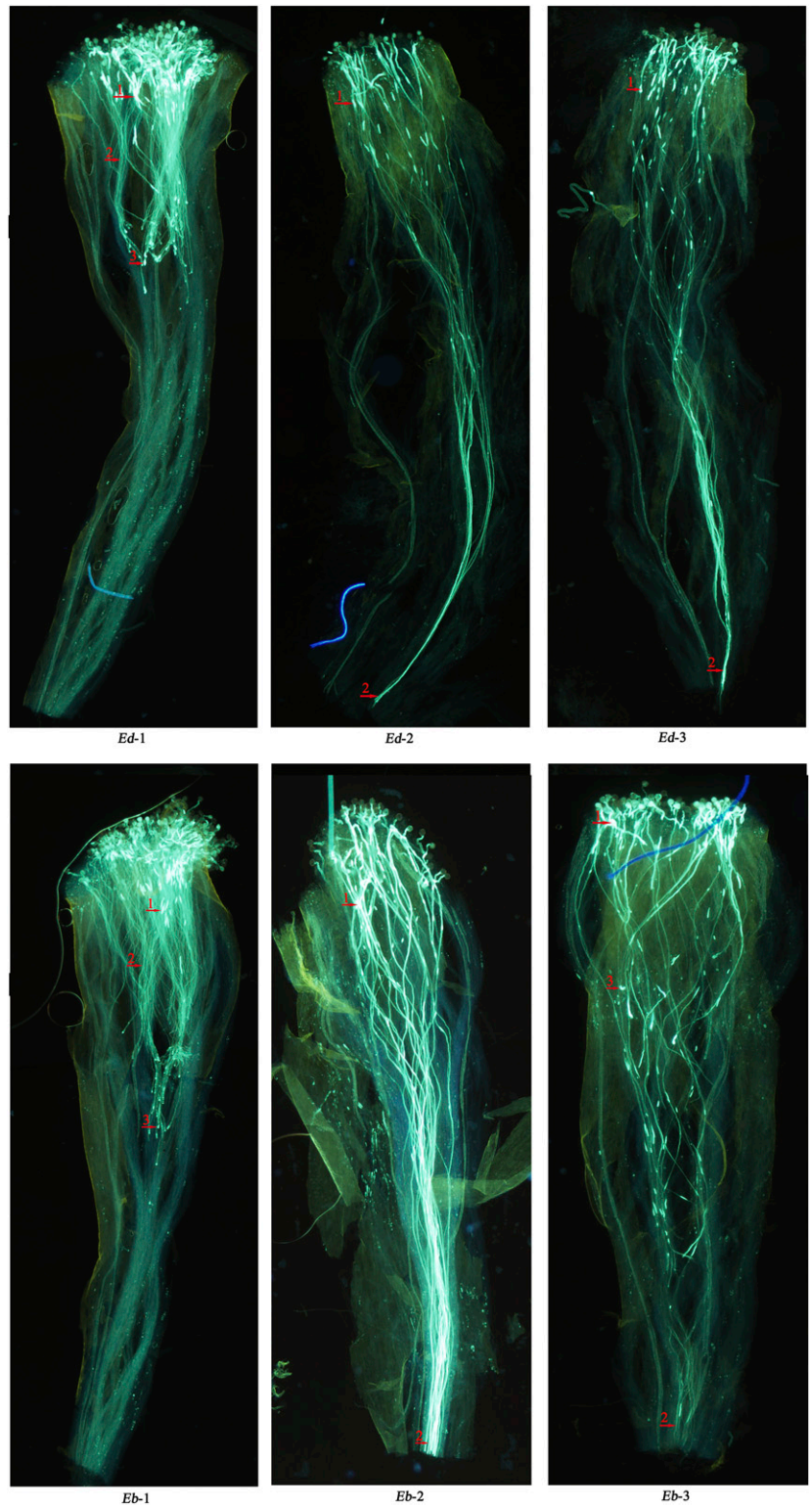

Fig. 3. Squash preparation using 0.1% (w/v) aniline blue for fluorescence microscopy of the pollen tube growth in the styles from treatments tested in the study. Callose deposits in emerging pollen tubes are shown by labeled arrows (1). Pollen tubes are indicated by labeled arrows (2). Pollen tube growth stoppage due to the deposition of callose along pollen tube wall are indicated by labeled arrows (3).

the style was cut directly for the *Eb*-3 and *Ed*-3 treatments. This might be because of the differences in the percentage of styles traversed by pollen tubes, fruit set, and average number of seeds per fruit among different tested treatments. Therefore, it can be presumed that distant hybridization incompatibility might be easily overcome, and more hybrids would be obtained by methods of cut-style pollination in loquats, which is contradictory to the results described by Zhang et al. (2012) in the study of distant hybridization in *Lilium*, except for the difference of post-zygotic reproductive isolation in *Lilium* (Zhang et al., 2012). This explains why the cut-style pollination is ineffective for overcoming distant cross-incompatibility, whereas the pre-zygotic reproductive isolation was observed in loquats.

Since the importance of distant hybridization breeding has been recognized, overcoming distant hybridization incompatibility has been a major concern for researchers, which could be resolved by several possible strategies, including pollination before anthesis (Chen et al., 2004), mixed pollen pollination (Zhao et al., 2008), repeated pollination (Xie et al., 2009), or use of suitable parents (Yang et al., 2004). These methods are effective because the pollination was carried out before the formation of substances that inhibit pollination, or after the substances of inhibition pollen germination or pollen tube growth were consumed. In this study, the pollination was carried out on the third day after emasculation in *Eb*-2 and *Ed*-2. Before that, the upper third of the style, which is the key part of self-incompatibility with callose deposition (Dumas and Knox, 1983; Yang et al., 2008), was cut 2 h after self-pollination. Therefore, cut-style pollination is an effective method to overcome the prefertilization barrier of distant hybridization in loquats.

Distant hybridization identification. Hybridization identification is an important and widely studied part of distant hybridization breeding, which has been carried out by many conventional methods, including morphological identification (Carlos de Oliveira et al., 2002), isozyme identification (Luo et al., 2006), and cytology identification (Dai et al., 2017; Zhang et al., 2012). However, these methods can be time-consuming because of complex identification procedures, and the results also may be influenced by environmental and cultivation conditions. In contrast, the application of molecular biology technologies can be used to identify distant hybrids, which is simple and enables molecular markers to be rapidly and accurately determined regardless of the environmental conditions (Chen et al., 2018).

A variety of molecular markers have accelerated hybridization identification and been used in canola (Marshall et al., 1994), by randomly amplified polymorphic DNA (RAPD), plum (Urbanovich et al., 2017), blueberry (Pathirana et al., 2016) and *Brassica napus* (Liu et al., 2018) by simple sequence repeat, tomato (Figueiredo et al., 2016) by intersimple sequence repeat, *Chrysanthemum* (Chen et al., 2013) by amplified fragment length polymorphism, and *Lilium* (Zhang et al., 2012) by sequence-related amplified polymorphism. In addition, some researchers have successfully identified distant hybrids of some plants using AS-PCR markers.

For example, the results of leaf shape investigation, S-allele–specific PCR and RAPDs revealed that distant hybrids of plum and apricot were true ones (Yang et al., 2004). Wang et al. (2006) carried out the identification of the interspecies hybrids in *Prunus*. The self-incompatibility gene of the parent was used as the molecular marker to validate the pedigree of hybrid G3, and the RAPD analysis further demonstrated that AS-PCR was an effective and credible method to identify the progeny of interspecies hybrids. In this study, *S-RNase* genes of 'Dawuxing', *E. bengalensis*, and *E. deflexa* were isolated, and 50 seedlings from the F1 population of the *Eb*-2 cross, composed of five *S*-genotypes: 11 (S_2S_{18}), 12 (S_2S_{21}), 13 ($S_{18}S_{41}$), 12 ($S_{21}S_{41}$), and 2 ($S_{18}S_{30}$), whereas 50 random seedlings from the F1 population of *Ed*-2 cross, with five *S*-genotypes: 12 (S_2S_{27}), 13 (S_2S_{30}), 11 ($S_{27}S_{41}$), and 14 ($S_{30}S_{41}$). The results showed that *S*-genotypes, in accordance with the *S-RNase* heredity to separate the rule completely in offspring, should represent both parents' *S-RNase*, and that the 50 random seedlings of *Eb*-2 and *Ed*-2, respectively, are true hybrids.

Conclusions

In this paper, the comprehensive analysis of the effect of cut-style pollination on pollen germination, pollen tube growth, fruit set, and seed number showed that cut-style pollination can effectively overcome the prefertilization barriers of distant hybridization combinations of *E. japonica* 'Dawuxing' × *E. bengalensis*, *E. japonica* 'Dawuxing' × *E. deflexa*. The results of AS-PCR amplification showed that *S*-genotypes, in accordance with the *S-RNase* heredity to separate the rule completely in offspring, should represent the *S-RNase* of both parents, or the presence of new *S-RNase* genotype in offspring, and that the 50 random seedlings of *Eb*-2 and *Ed*-2, respectively, are true hybrids. Therefore, cut-style pollination is an effective method to overcome the prefertilization barrier of distant hybridization in loquats. Therefore, breeding programs should focus on using this method by including breeding partners with some of the preferable traits to prevent potential future incompatibility issues.

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