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Full Length Article

Comparison of Self- and Cross-Pollination in Pollen Tube Growth, Early Ovule Development and Fruit Set of *Camellia grijsii*

Huan Xiong[1], Feng Zou[1*], Deyi Yuan[1*], Xiaofeng Tan[1], Jun Yuan[1], Ting Liao[1] and Genhua Niu[2]
[1]*Key Laboratory of Cultivation and Protection for Non-Wood Forest Trees, Ministry of Education Central South University of Forestry and Technology, Changsha 410004, Hunan, China*
[2]*Texas AgriLife Research at El Paso, Texas A&M University System, 1380 A&M Circle, El Paso, TX 79927, USA*
[*]For correspondence: zoufeng06@126.com; csuftyuanyi@126.com

Abstract

Camellia grijsii Hance is one of the most important woody edible oil tree species in Southern China; however, it often has a low fruit set rate. To elucidate the causes of poor fruit set in *C. grijsii*, self-pollination (SP) with *C. grijsii* and cross-pollination (CP) of *C. grijsii* × *C. villosa* tests were conducted. Pollen germination and pollen tube growth into pistils, and early ovule development after SP and CP, were examined using a paraffin section and fluorescence microscopy. The fruit set percentage in SP and CP was also investigated. The results showed that pollen germinated normally on the stigma, and the pollen tubes both reached the style base after SP and CP, but the growth rates of pollen differed significantly between SP and CP, being faster for CP. The pollen tubes arrived at the style base 48 h after SP, but only 24 h after CP. No barriers to SP acted at the stigmatic or stylar level in *C. grijsii*; however, SP pollen tubes stopped at the upside of the ovary at 72 h due to the presence of ovarian self-incompatibility (OSI). There was also no callose deposition in the ovules at 84 h after SP. The inability of SP pollen tubes to penetrate the ovule and the absence of a mature embryo sac in the ovule were the critical factors that led to ovule abortion. Fruit set following SP (2.1%) was consistently and significantly lower than that obtained from CP (72.9%). Thus, we conclude that the presence of strong OSI gives rise to ovule abortion and is the main cause of the poor fruit set in *C. grijsii*.

Keywords: *Camellia grijsii*; Ovule abortion; Fruit set; Ovarian self-incompatibility

Introduction

Camellia grijsii Hance is one of the most important non-wood forest trees in the Theaceae family. It is native to China and is widely distributed in the provinces of Hunan, Zhejiang, Fujian, Jiangxi, Guangdong, Guangxi, Hubei, Shanxi, Guizhou and Yunnan (Zhuang *et al*., 2012; Zou *et al*., 2013a). *C. grijsii* can be used not only as an evergreen ornamental shrub but also as an oilseed species. The oil extracted from the seeds of *C. grijsii* can be used for medicinal purposes and a range of other uses such as in cosmetics. The edible oil (called tea oil) is also known as "oriental olive oil" and has high oleic acid content, typically exceeding 80% and low saturated fat content (Gao *et al*., 2018). *C. grijsii* is a valuable germplasm species due to its excellent characteristics of oil quality, high oil rate and thin shell (Zhuang *et al*., 2012). In addition, its fruit capsules are highly resistant to anthracnose disease (*Colletotrichum camelliae* Massee), which causes severe premature drop of the fruit capsules of *C. oleifera* (Weng, 1997).

China is the largest producer of tea oil worldwide, which is a main source of income for many rural communities in the hilly areas (Zhuang *et al*., 2012; Zou *et al*., 2014). However, in many cases, the productivity is not satisfactory, and this is particularly true for *C. grijsii*, which blooms profusely but has a relatively low fruit set (Zou *et al*., 2013b). The low productivity has discouraged growers from cultivating this species, impeding the development of the tea oil industry in China.

The reproduction process of any plant has several important stages, namely, flowering, fruit development and seed maturation. Each stage is strongly related to fruit set. Abnormal floral organs in plants, which are generally formed after pollen abortion, play a key role in the fruit set (Ye *et al*., 2009). Although some histological studies that have investigated sexual reproduction have considered different *Camellia* species (Cao, 1965; Chen *et al*., 2011; Liao *et al*., 2014a; Gao *et al*., 2015a), only one study (Chen *et al*., 2011) observed that pollen of irregular shape and shrinkage was aborted in *C. oleifera*. Zou *et al*. (2013a) reported that male gametes are fertile in *C. grijsii*, but Ye *et al*. (2009) reported embryo sac sterility, with few or no ovules able to develop into fully mature seeds, resulting in a poor fruit set. Although aborted ovules were responsible for the low seed set of *C. grijsii* (Zou *et al*., 2013b), whether this is caused by poor pollination and fertilization or other

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factors is unknown. The processes of pollen germination, pollen tube growth and ovule fertilization are regulated by pollen–pistil interactions (Radunić *et al*., 2017). Pollen-pistil compatibility interactions are essentially controlled by the genotype. Fluorescence microscopy has been used to study the compatibility of pollen tube growth into the pistil in many *Camellia* species including *C. oleifera* (Liao *et al*., 2012; Gao *et al*., 2015b) and *C. sinensis* (Chen *et al*., 2012; Zheng *et al*., 2014). Thus, information on pollen tube growth into the pistil can be useful for interpreting the variation in fruit set (Feijó *et al*., 1999; Jahed and Hirst, 2017). However, current information about pollen germination and pollen tube growth rates following self-pollination (SP) and cross-pollination (CP) and their roles in *C. grijsii* fruit set are limited.

In this study, we investigated pollen germination on the stigma, pollen tube growth into the pistil and early ovule development in *C. grijsii* following SP and CP. We also investigated the effects of SP and CP on *C. grijsii* fruit set. Our aim was to determine the reason for poor fruit set in *C. grijsii.*

Materials and Methods

Plant Materials

The plant of *Camellia grijsii* were grown at the *Camellia* Garden at the Central South University of Forestry and Technology in Zhuzhou city, Hunan province (27°55′30″N, 113°09′50″E; approximately 70 m above sea level) were used in this study. This site is in a typical subtropical moist climate with a mean annual precipitation of 1392 mm and a mean annual temperature of 17.5°C. Another *Camellia* species, *C. villosa* Chang was planted in the *Camellia* Garden and used as the pollen source for CP. *C. villosa* is an important species with large fruit and good oil quality, and is distributed across the Hunan, Zhejiang, Guangxi, Guizhou provinces (Wang *et al*., 1990). *C. grijsii* and *C. villosa* usually bloom from February to early April and fruiting occurs in late October. To record the climate conditions in 2009 and 2010 during the full flowering phase, daily mean temperatures were collected by a weather station located less than one mile from experimental plots. When more than 50% of flowers were open, this was considered the full flowering phase (Zhuang *et al*., 2012). The flowering period lasted for about 40 days for *C. grijsii.* Temperature differences among the years were mostly small during flowering (Fig. 1) and thus the effects of temperature on the fruit set were assumed to be negligible. Experimental trees in the orchard had no diseases or pests and were planted with a spacing of 2 m × 3 m. All materials were obtained from three 10-year-old trees selected for their good yielding potential and were representative of the *C. grijsii* population from the *Camellia* orchard. All trees were grown under the same environmental conditions and performed the same irrigation and fertilization treatments (Zou *et al*., 2013a).

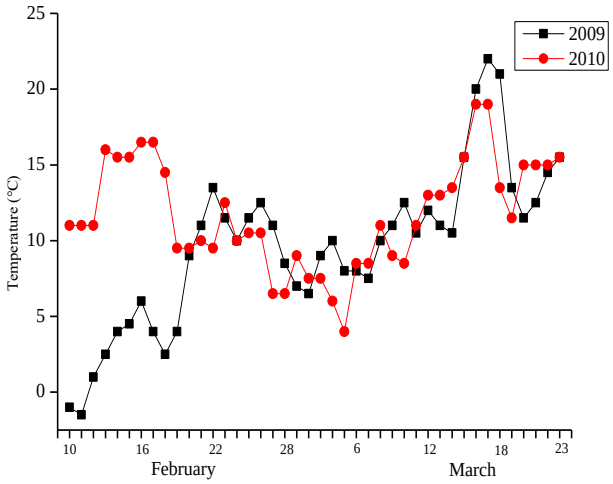

Fig. 1: Mean daily air temperatures during two flowering seasons of *C. grijsii* at Zhuzhou. Data presented are from 10 February to 23 March

Pollination Treatments

Flower buds were emasculated during the white bud stage and covered in parchment paper bags to prevent biotic pollination. To determine whether pollen grains were viable after *in vitro* germination before hand-pollination experiments, anthers were collected from the *Camellia* orchard at the balloon stage. The germination medium was prepared by dissolving 2% agar, 10% sucrose and 0.01% boric acid in boiling water at pH 5.6 (Zou *et al*., 2009). The percentage of germinated pollens was determined using a BX-51 microscope (Olympus, Tokyo, Japan) according to Xiong *et al.* (2016). SP (*C. grijsii* × *C. grijsii*) and CP (*C. grijsii* × *C. villosa*) were performed the next morning from 8:00 to 11:00 am, and the buds were re-bagged quickly after pollination. The bags were removed in early April when all of the flowers were withered. A total of 300 flowers were used in each treatment for histological observation. Other 200 pollinated flowers with chosen randomly from three trees were performed to investigate fruit set after SP and CP. SP and CP of *C. grijsii* were conducted in the spring of both 2009 and 2010.

Pollen Germination and Pollen Tube Growth in the Pistil following SP and CP

Ten pistils for each treatment were sampled at different intervals (1, 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 120, 144, and 168 h) after pollination to observe pollen tube growth into pistils or ovules. Ten pistils were removed by dissecting female flowers in a solution of 50% ethanol. The samples were fixed in a formalin: glacial acetic acid: 70% ethyl alcohol (FAA, 1:1:18, v/v) solution, and then stored at 4°C (Zou *et al*., 2014). For each treatment, the pistils or ovules were hydrated and softened in 2 M NaOH for 3–4 h. The samples were stained with 0.05% aniline

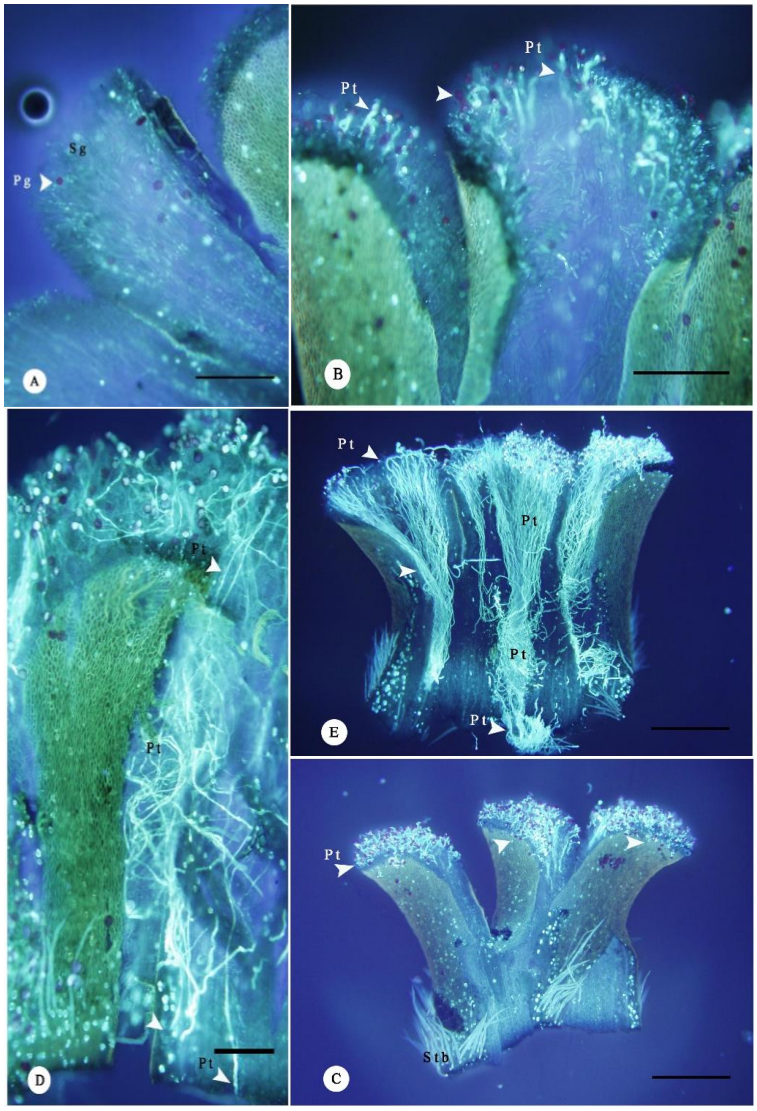

Fig. 2: Pollen germination and pollen tube growth in the style after self-pollination in *C. grijsii*. (A) Pollen grains adhered to stigmata at 1 h after SP (100×). (B) Pollen tube germination on stigmata at 4 h after SP (100×). (C) Pollen tube growth on stigmata at 8 h after SP (40×). (D) Pollen tube growth in the base of the style at 48 h after SP (100×). (E) Many pollen tubes passed through the base of the style by 60 h after SP (40×). SP, self-pollination; Sg, stigmata; Pt, pollen tube; Pg, pollen grain

blue in 0.15 M K_2HPO_4 for 4 h and covered with a cover glass (Liao *et al*., 2014b; Gao *et al*., 2015b). Pollen tube growth in the pistil and ovules was observed under a BX-51 fluorescence microscope (Olympus, Japan). Each microscopic field was photographed and the mean lengths of pollen tubes (10 tubes per field) were measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

Early Ovule Development Following SP and CP

Self- and cross-pollination pistils were harvested at 1, 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 120, 144 and 168 h and every 7 days from 7–105 days after pollination. Ten pistils were collected at each time point and fixed in the solution. Early ovule development was detected by the paraffin sectioning method (Zou *et al*., 2016). Most sections were stained with hematoxylin and eosin, but some were stained with 0.5% decolorized aniline blue in 0.1 M K_3PO_4 for 3–12 h, after which the pollen tubes were examined to ascertain whether they had penetrated into the ovules (Zou *et al*., 2013c). The sections were observed using a BX-51 microscope (Olympus, Japan).

Fruit Set

Fruit set percentages were calculated in each pollination treatment 3 months after pollination, from 2009 to 2010.

Statistical Analyses

The SPSS 19.0 statistical software (IBM, USA) was used for most statistical analyses. We performed a chi-square test (χ^2) to investigate significant differences in the average pollen tube length and fruit set among the different treatments (Streher *et al*., 2018). Figures were generated by Origin 8.5 software (Origin Laboratory, USA).

Results

Pollen Viability

Pollen collected from dehiscing anthers on culture medium showed a high percentage of germination in *C. grijsii* and *C. villosa*. The average percentage of germination was 66.5% for *C. grijsii* and 94.4% for *C. villosa*.

Pollen Germination and Pollen Tube Growth in the Pistil following SP and CP

No morphological or structural differences were observed in pollen germination or pollen tube growth in the style after SP (Fig. 2) and CP (Fig. 3). However, the mean lengths of the pollen tubes in the style at various time points after SP and CP differed significantly ($\chi^2 = 0.05 = 3606.88$, $p<0.001$; Fig. 4). At 1 h after SP, the pollen grains had not germinated on the stigma (Fig. 2A); however, at 1 h after CP, pollen grains begun to germinate on the stigma and some pollen tubes had passed through the mastoid cell (Fig. 3A). Subsequently, at 4 h after SP some pollen grains begun to germinate on the stigma and some pollen tubes had passed through the mastoid cell (Fig. 2B). At 8 h after CP, a large number of pollen grains had germinated on the stigma (Fig. 3B) and pollen tubes had grown to approximately one-quarter of the style length after SP (Fig. 2C). However, at 12 h after CP, many pollen tubes had grown to approximately one-half of the style length (Fig. 3C). The SP tubes required 48 h to grow and reach the base of the style (Fig. 2D), but the CP tubes required only 24 h. At 60 h after SP, many pollen tubes were observed to have passed through the base of the style (Fig. 2E), whereas the time required after CP was 36 h (Fig. 3E). At 72 h after SP, the pollen tubes had stopped growing and become twisted at the upper side of the ovary (Fig. 5A and B), whereas after CP the pollen tubes had entered the ovule through the micropyle by 60 h (Fig. 5G). SP tubes remained at the upper side of the ovary (Fig. 5A), without penetrating the ovule (Fig. 5C) and no callose invaded the ovules 84 h after SP (Fig. 5C).

Early Ovule Development following SP and CP

Most CP ovules were fertilized and developed into mature embryo sacs. When the pollen tube penetrated the micropyle (Fig. 5G), two male nuclei appeared at the micropyle end by 72 h after CP (Fig. 5H). An egg cell and a synergid cell located at the micropyle end and another degenerated synergid cell were observed (Fig. 5H). Then one male nucleus moved towards the egg cell and fused 84 h after CP (Fig. 5I). The double fertilization was the premitotic type of syngamy. Subsequently, a zygote was observed in the embryo sac by 96 h after CP. The zygote remained there for 2 months and a globular embryo was observed at 105 days after CP (Fig. 5K). The other male nucleus moved towards the polar nucleus and began to fuse by 120 h after CP. The primary endosperm nuclei split in metaphase by 144 h after CP (Fig. 5M). The primary endosperm nuclei continued to develop into free endosperm nuclei by 168 h and formed a free nuclear layer encircling the embryo sac wall by 63 days after CP. The development of endosperm conformed to the nuclear type. However, SP ovules mostly aborted during seed formation. Ovules with abnormally shaped embryo sacs were observed at 28 days after SP (Fig. 5D). Subsequently, abortive ovules and dead tissues (Fig. 5E and F) began to wither at 56 and 70 days after SP, respectively. Thus, it was impossible for the ovule in an abortive ovary to be fertilized.

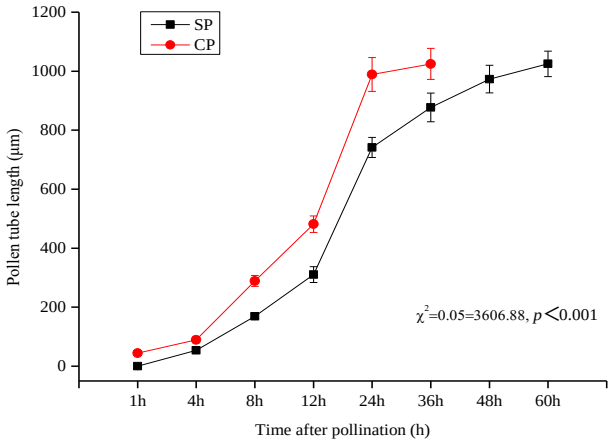

Fig. 4: Mean lengths of pollen tubes in the style at various times after self- and cross-pollination. SP, self-pollination; CP, cross-pollination. Significant difference in the mean pollen tube lengths between SP and CP treatments, as determined by the chi-square test (χ^2)

Fruit Set Following SP and CP

There was a significant difference in fruit set between the SP and CP treatments ($\chi^2 = 0.05 = 134.43$, $p<0.001$) (Table 1). SP of *C. grijsii* resulted in a lower rate of mean fruit set (2.1%) than that obtained by CP (72.9%).

Discussion

There were significant differences between the pollination types as measured based on pollen tube growth and ovule development. Pollen adheres to the stigma, after which pollen tubes begin to grow into the pistil (Radunić *et al*., 2017). Pollen tubes that grow into the pistil are affected by

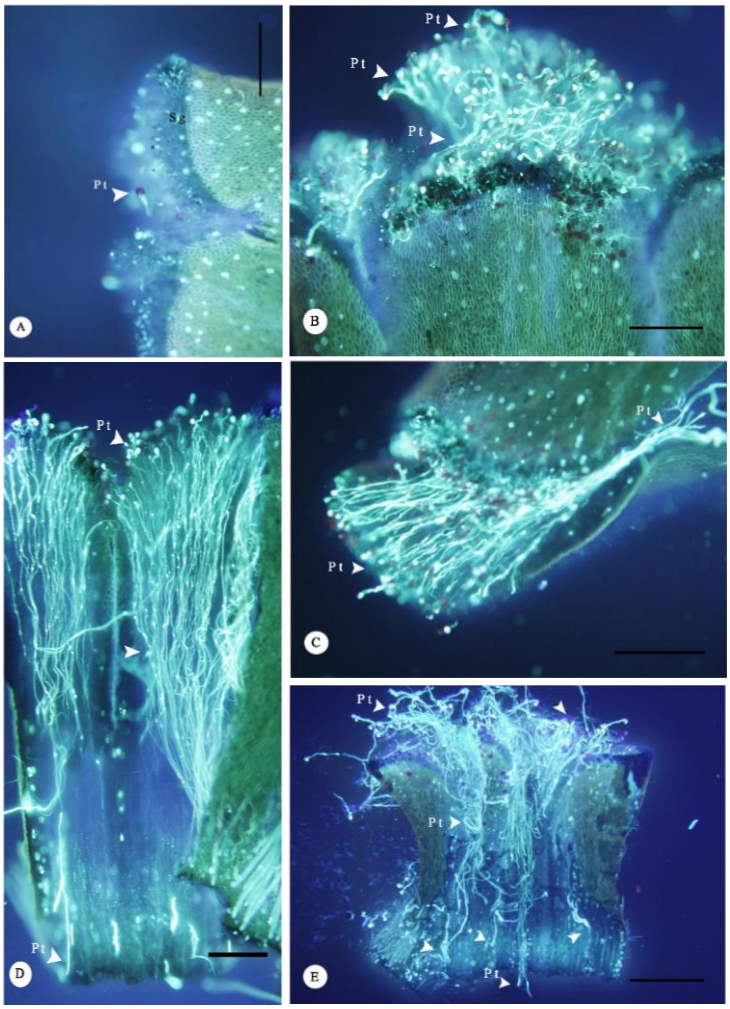

Fig. 3: Pollen germination and pollen tube growth in the style after cross-pollination in *C. grijsii*. (A) Pollen grain germination on stigmata at 1 h after CP (100×). (B) Growth of pollen tubes on stigmata at 8 h after CP (40×). (C) Pollen tube growth to the middle of the style at 12 h after CP (100×). (D) Pollen tube growth to the base of the style at 24 h after CP (100×). (E) A large number of pollen tubes passing through the base of the style at 36 h after CP (40×). CP, cross-pollination; Sg, stigmata; Pt, pollen tube

many biotic and abiotic factors. The main biotic factors are pollen viability and pistil receptivity (Nepi and Pacini, 2001). Pistil receptivity includes stigma, style, and ovule receptivity; pollen must be able to germinate, penetrate the stigma and style, and reach the ovule, which partially determines the reproductive success of a species. We found that *C. grijsii* and *C. villosa* pollen grains were capable of germinating in the *C. grijsii* stigma and able to elongate through the style (Fig. 2 and 3), yet the timings of these events differed. Chen *et al*. (2012) reported that pollen tubes successfully passed through the style base at 24–48 h after SP and CP in *C. sinensis*. Liao *et al*. (2014b) observed that pollen tubes crossed the style by 60 h after SP and by 48 h after CP.

In the present study, SP tubes had reached the style base by 48 h (Fig. 2D), while CP tubes had reached the style after only 24 h (Fig. 3D). The growth rate of CP pollen tubes was greater than that of SP pollen tubes and similar observations have been obtained in *C. oleifera* 'Huashuo' (Liao *et al*., 2012). However, SP pollen tubes with swelling tube tips failed to fertilize and seemed to experience difficulty in entering the ovule after 72 h (Fig. 5B), whereas CP pollen tubes successfully fertilized and penetrated the ovule by 60 h (Fig. 5G). Furthermore, we did not detect callose in the ovules after SP (Fig. 5C). Similar observations have been reported in *C. sinensis* (Chen *et al*., 2012). The fate of these selfed ovules was likely due to ovule abortion, non-fertilization, or death (Fig. 5D and F). However, in our study, crossed ovules were fertilized, formed a zygote, and developed into a globular embryo (Fig. 5G and K), similar to earlier reports on *C. oleifera* (Liao *et al*., 2014a, b).

Production of fruit set under CP treatment indicated that failure of fertilization in SP treatments resulted in poor fruit set. The fertilization and development of normal seeds in crops is determined by several processes, such as self-

Table 1: Effects of self- and cross-pollination on fruit set of *C. grijsii*

Combination	No. of flowers tested	Percentage fruit set (%)[a]			Average fruit rate (%)
		2009	2010		
Cross-pollination (CP)	100	72.7	73.1	$\chi^2 = 0.05 = 134.43$, $p < 0.001$	72.9
Self-pollination (SP)	100	2.0	2.1		2.1

[a] Significant difference in the fruit set between SP and CP treatments, as determined by the chi-square test (χ^2)

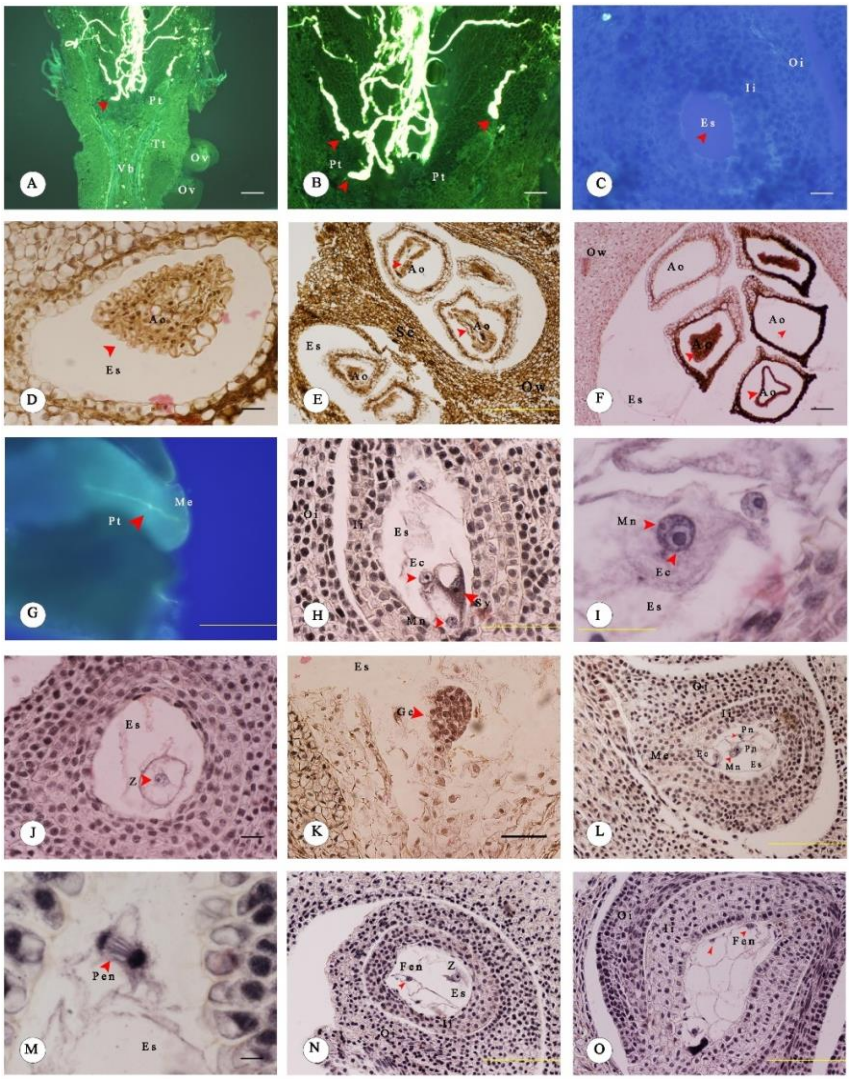

Fig. 5: Pollen tube growth into the pistil and early ovule development after self- and cross-pollination. (A) Distorted pollen tubes with a reserving tube and swelling tip at the upper side of the ovary at 72 h after SP (40×). (B) High magnification of A (100×). (C–F) Degenerate and abortive ovules in self-pollinated ovaries at various stages. (C) Abortive ovules with an abnormally shaped embryo sac at 84 h after SP (200×). (D) Abortive ovules with abnormally shaped embryo sacs at 28 days after SP (400×). (E) Abortive ovules containing dead tissue at 56 days after SP (100×). (F) Abortive ovules containing dead tissue at 70 days after SP (100×). (G) Pollen tubes penetrating into the ovule at 60 h after CP (200×). (H) Longitudinal section showing inner and outer integuments, embryo sac with an egg cell, a synergid cell and two male nuclei located at the micropylar end at 72 h after CP (400×). (I) A male nucleus beginning to fuse with an egg cell at 84 h after CP (1000×). (J) A zygote resting in the embryo sac at 96 h after CP (400×). (K) The globular embryo at 105 days after CP (200×). (L) The other male nucleus close to the polar nucleus, embryo sac with an egg cell, and one polar nucleus at 120 h after CP (200×). (M) The primary endosperm nuclei split at the metaphase at 144 h after CP (1000×). (N) Free endosperm nuclei and dormant zygote in the embryo sac at 168 h after CP (200×). (O) The free endosperm nuclei continued to divide and formed a free nuclear layer circle around the embryo sac wall by 63 days after CP (200×). Ao, abortive ovule; CP, cross-pollination; Ec, egg cell; Es, embryo sac; Fen, free endosperm nuclei; Ge, globular embryo; Ii, inner integument; Me, micropylar end; Mn, male nucleus; Oi, outer integument; Ov, ovule; Ow, ovary wall; Pt, pollen tube; Pn, polar nucleus; Pen, primary endosperm nuclei; SP, self-pollination; Sy, synergid cell; Tt, transmitting tissue; Vb, vascular bundle; Z, zygote

incompatibility (SI) (Kakade *et al*., 2017), ovule or embryonic abortion (Jia *et al*., 2008; Guerra *et al*., 2011; Dai *et al*., 2014; Liu *et al*., 2014), adverse climatic conditions (Ebadi *et al*., 1995; Dorđević *et al*., 2017) and other factors. Jia *et al*. (2008) reported that the main cause of poor fruit set in Zuili plums (*Prunus salicina*) was a lack of embryo sac. Low temperatures during the blooming period lead to weak pollen tube growth, which contributes to poor fruit set in some plum cultivars (Dorđević *et al*., 2017). Fruit set is greatly reduced by SI interactions in *C. sinensis* (Chen *et al*., 2012; Zhang *et al*., 2016), which is a genetically controlled mechanism. SI is an inability of a flowering plant with functional male and female gametes to set seed when self-pollinated (Pounders *et al*., 2006). SI may occur in the stigma, style, or ovary. In sporophytic SI, the germination of incompatible pollen is inhibited at the stigmatic surface (Tangitcharoen and Owens, 1997), but in gametophytic SI, the site of pollen tube inhibition is typically in the upper third of the style (Peralta *et al*., 2014; Zhang *et al*., 2014). Apart from conventional SI, a lack of fruit set following selfing, despite the apparently successful growth of self-pollen tubes in the ovary, which was called ovarian SI has been reported (Thimmaiah *et al*., 2018). In our study, pollen germinated freely in *C. grijsii* in all pollination treatments, suggesting the absence of a strong barrier to self-fertilization acting at the stigmatic or stylar level; however, the growth of SP pollen tubes in the upper aspect of the ovary was inhibited. The restricted ability of self-pollen tubes to reach the ovary was indicative of SI in *C. grijsii*. Hence, SP resulted in a significantly lower fruit set than did CP between 2009 and 2010 (Table 1), implying that SI in *C. grijsii* is most likely ovarian SI and occurs at a later stage (ovary level). Thus, ovarian SI may be the cause of the low fruit set in *C. grijsii*. The inhibition of pollen tubes has been reported in other members of the Theaceae family. Liao *et al*. (2014b) and Gao *et al*. (2015b) found that most SP tubes grew slowly and they failed to enter the locule, suggesting that *C. oleifera* was a ovarian SI plant. In contrast, *C. sinensis* exhibits GSI, because inhibition of self-pollen tube growth occurs at the style base (Wang *et al*., 2008). The different positions at which self-pollen tube inhibition occurs may differ among species. Corroboration of these results and a more detailed exploration of the mechanisms of ovarian SI in *C. grijsii* will require further genetic and molecular studies.

Conclusion

There was a reduction in fruit set following SP in *C. grijsii*, and differentiated the processes leading to such reduction. The results revealed that a main cause of poor fruit set in *C. grijsii* was explained by the existence of OSI mechanism.

Acknowledgements

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