Characterisation and evaluation of interspecific cross-incompatibility in two *Delphinium* species

Ying Cheng^{1,2}, Wenling Guan^{3,*}, Fan Li^{2,*}

¹ School of Agriculture, Yunnan University, Kunming 650504, China

² Floriculture Research Institute, Yunnan Academy of Agricultural Sciences, National Engineering Research Center for Ornamental Horticulture, Key Laboratory for Flower Breeding of Yunnan Province, Kunming 650200, China

³ College of Horticulture and Landscape, Yunnan Agricultural University, Kunming 650201, China

ABSTRACT

Delphinium yunnanense is an endemic species in the Yunnan Province of China, while *Delphinium elatum* ‘Guardian Blue’ is widespread as cut flowers in the world. They all bloom attractive blue flowers, which are a crucial ornamental trait for floriculture. In this experiment, the cross-compatibility between *D. yunnanense* and *D. elatum* was characterised and evaluated. The results showed that a strong cross-incompatibility was observed in *D. yunnanense* × *D. elatum*, resulting in hybrid sterility. The F₁ hybrid generation produced neither fruits nor seeds. The cross-incompatibility of the hybrids resulted from the disturbed structure of the pollen tubes penetrating pistil style and callose deposits on stigma and in style. Moreover, the germination rate of *D. elatum* × *D. yunnanense* F₁ seed was lower than their own allogamic crosses, although there was no difference in fruit set rate. The results confirmed the cross-incompatibility between *D. yunnanense* and *D. elatum*, suggesting that *D. elatum* is more suitable as a maternal parent for hybridisation breeding. Our study characterised and evaluated the interspecific cross-compatibility through the pollen–stigma interaction and the reproductive system in *Delphinium*, which could provide the basis for the development and utilisation of potential ornamental traits in *Delphinium*.

Keywords: allogamic cross, compatibility, *Delphinium*, pollen-stigma interaction, reciprocal cross, reproduction

INTRODUCTION

The genus *Delphinium*, a member of the Ranunculaceae family, is widely distributed in the temperate regions of the Northern Hemisphere (Malyutin, 2001). *Delphinium* comprises about 350 species of perennial flowering plants, of which 173 species have been described in China (Wang and Warnock, 2001). In particular, most of the species are concentrated in Southwest China, which is one of the most important distribution centres and covers most of the morphological primitive representatives (Yuan and Yang, 2008). Yunnan Province, known as the ‘Kingdom of Plant’, is a centre of species diversity and endemism of *Delphinium*, consisting of 49 species and 13 varieties (Yuan and Yang, 2019). According to

reports, there are 24 species with a high ornamental value that has potential for development and utilisation, such as *D. yunnanense* and *D. grandiflorum* (Guan and Li, 2002). Moreover, *Delphinium* is widely cultivated in the world for a long time, that is, *Delphinium elatum flore plena* was cultured by J. Parkinson in Western Europe as early as 1640 (Legro, 1961). In floriculture, the most important hybrid species is *D. elatum*, which developed as a series of 4,000 named cultivars from hundreds of different breeders and growers in the past few decades (Royal Horticultural Society, 1949). Besides, through hybridisation, mutagenesis, chemical and radiation treatment of different species within the

*Corresponding authors.

e-mail: 158066692@qq.com (Wenling Guan)/lifanla@foxmail.com (Fan Li).

genus *Delphinium*, a wide range of flower colours from white, yellow, red, violet to blue were introduced into the new cultivars as well as improved quality of cut flower, mildew resistance and toughness of the stems (Mehlquist et al., 1943; Wegulo and Vilchez, 2008; Chen et al., 2011; Lin et al., 2014). In addition to the ornamental purposes of showy flowers, the plants of *Delphinium* contain various alkaloids with spectacular medicinal values, such as diterpenoid alkaloids, trichodelphinines and flavonoids, which increase the utilisation and economic value of *Delphinium* (Chen et al., 2011; Agnihotri et al., 2014; Lin et al., 2014).

Delphinium yunnanense (*D. yunnanense*) is an endemic species in the Yunnan Province of China with blue flowers, which is considered a precious ornamental germplasm to be developed. However, *Delphinium elatum* (*D. elatum*) 'Guardian Blue' is one of the most popular flowers in the world as an attractive high-grade cut flower. Both species are the potential candidates for developing new *Delphinium* varieties with blue flowers through hybridisation. The chromosome number and the ploidy level of many species and varieties in the genus *Delphinium* have been investigated (Legro, 1961; Yuan and Yang, 2008). Most of the wild species, including *D. yunnanense*, are diploids with 16 chromosomes ($2n = 2x = 16$), whereas *D. elatum* was tetraploid ($2n = 4x = 32$). An intraspecific difference in chromosome number was detected neither in diploid nor in tetraploid species (Legro, 1961; Yuan and Yang, 2008). Nevertheless, the breeders have had success in interploidy crosses using different *Delphinium* species. For example, the frequently used tetraploid cultivars of *D. elatum* were widely crossed with diploid species to form sterile triploid hybrids, which, after the colchicine treatment, became fertile, produced seeds and viable seedlings (Lewis et al., 1951; Samuelson, 1957; Tjebbes, 2010). Moreover, the reproductive system with pollen–stigma interactions has been previously investigated in *D. grandiflorum*, *D. cardinale*, *D. nudicaule*, *D. barbeyi* and *D. nuttallianum* and their interspecific hybrids (Honda et al., 1999; Williams, 2007; Elliott and Irwin, 2009; Briggs et al., 2016). Some studies have shown that the breeding system of *Delphinium* is facultative xenogamy or obligate outcrossing, accompanying with partly self-compatibility requiring pollinators (Price and Waser, 1979; Williams et al., 2001; Zhang et al., 2014). Self-incompatibility (0–50%) and autogamy (1–90%) in different degrees among different species in *Delphinium* have been reported (Macior, 1975; Varney, 1979; Powell and Jones, 1983; Bosch et al., 2001). To our knowledge, both of the studied species are capable of selfing; however, no previous study concerned the cross-compatibility in the species of *D. yunnanense* and *D. elatum*.

In this study, we explored and investigated the interspecific cross-compatibility of *D. yunnanense* and *D. elatum* 'Guardian Blue' to establish the mating ability of these two *Delphinium* species, which could be helpful in developing new varieties of these ornamental plants.

MATERIALS AND METHODS

Plant material and growth conditions

D. yunnanense and a commercial cultivar of *D. elatum* 'Guardian Blue' were used in this study. The seeds of *D. yunnanense* used in this study were randomly collected in natural habitats (N25°7'33", E102°45'31") in Kunming, Yunnan Province at an altitude of 2,139 m. The seeds of *D. elatum* 'Guardian Blue' were obtained from the local flower market with 500 seeds in the same bag. A total of 100 seeds of each species were first germinated in the petri dish covering with a moist filter paper. Then, 50 seedlings of each species were separately transferred and planted in 100-well plant trays. After the root system filled the holes, the plants were separately planted in pots (diameter = 8 cm, height = 8 cm) filled with substrate (red soil:peat = 1:1) and grown in the solar greenhouse (without heating) at the experimental farm of Yunnan Agricultural University. The flowering dynamics were continuously observed when the plants begin to bloom.

Pollen viability and stigma receptivity

The examination of pollen viability was conducted with three plants of each species during anthesis based on *in vivo* germination method (He et al., 2017). In a full flowering stage, fresh pollen was collected every 2 h from three flowers per plant and was incubated for 4 h at 25°C in the liquid medium (5% sucrose, 150 mg · L⁻¹ boric acid and 20 mg · L⁻¹ CaCl₂) from 9:30 to 15:30. The total number of pollen grains and the number of germinating pollen grains were counted in three separate fields of view per slide under the light microscope. The percentage of germinating pollen was estimated by germinating pollen number/total pollen number × 100. The active pollen was identified by the length of the pollen tube longer than the diameter of pollen grain, otherwise recorded as inactive pollen (Rodriguez-Riano and Dafni, 2000).

Stigma receptivity was examined by the benzidine–hydrogen peroxide method (Li et al., 2018). During anthesis (from day 1 to day 8), three flowers at the full-open stage of each species were collected every day and were soaked in the benzidine–hydrogen peroxide solution (1% benzidine: 3% hydrogen peroxide: water = 4:11:22, v/v, Cato Research Chemicals, Inc.). The receptive stigma shows peroxidase activity and turns blue with a mass of bubbles surrounding it. The depth of the blue color and number of bubbles indicate the intensity of stigma receptivity.

Observation of pollen–stigma interaction

To observe the pollen–stigma interaction in interspecific hybridisation, the reciprocal crosses between *D. yunnanense* and *D. elatum* were conducted as well as intraspecific allogamic crosses. The *D. elatum* × *D. yunnanense* was treated as an orthogonal cross, and the *D. yunnanense* × *D. elatum* referred to reciprocal cross. To avoid self-pollination, the flowers were

emasculated and isolated by plastic bags until the anther dehiscence. This treatment was applied in each cross combination with a random selection of 10 plants (three flowers per plant). Before pollination, the stigma was examined by a magnifier for a secretion check. The pollen sac was then gently dipped onto the stigma with secretions using tweezers. Each stigma was pollinated several times to ensure a sufficient amount of pollen and was covered by bags after pollination.

The pistils of each cross were collected at 2, 4, 6, 24, 72 and 120 h after pollination and fixed in Carnoy's solution (ethanol: glacial acetic acid = 3:1, v/v, Shanghai Fusheng Industrial Co., Ltd.) for 24 h at 4°C and then stored in 70% of ethanol. The samples were then treated with 6 mol · L⁻¹ NaOH for 12 h. After repeated rinsing with distilled water, the pistils were immersed in a 0.1% water-soluble aniline blue solution (dissolved in 0.1 mol · L⁻¹ K₃PO₄, ScyTek Laboratories) for 24 h. The stained ovaries were then observed with Leica fluorescent microscope under ultraviolet light to investigate pollen adherence, pollen germination, callose formation and pollen tube growth. For each treatment, six pistils in three replications were used to calculate the average number of pollen grains.

Calculation of fruit setting and germination rate

Thirty flowers were used to evaluate the fruit setting for each cross. The number of mature fruits and seeds per fruit were counted, and the thousand kernel weight (TKW) was measured. The fruit-setting rate was calculated by the formula: the number of mature fruits/the number of pollinated flowers × 100.

The F₁ seeds of each cross were germinated in a pre-sterilised culture dish (Guangzhou Jet Bio-Filtration Co., Ltd., TCD000060) on wet filter papers. The seeds were then cultivated in environmentally controlled growth chambers (LRH-400-GSI, 400 L; Zhujiang, China) under the condition of 12 h photoperiod at 25°C. The experiment was repeated in triplicate (100 seeds per repeat). The germination rate was calculated as germinated seeds/total seeds × 100, in which germinated seeds refer to the sprout length equal to or longer than the seed length.

Statistical analysis

Data analysis and statistics were performed using Microsoft Excel 2016 and Data Processing System (Tang and Zhang, 2013). One-way and two-way ANOVA with Tukey's HSD post-hoc test was used to compare multiple samples at the 5% significance level.

RESULTS

Stages of flower development

The inflorescence of *D. yunnanense* and *D. elatum* is racemose, in which the development of individual flowers is from bottom to top. Eight flowering stages could be identified in the two species throughout the

entire flower developmental period, including flower bud stage, stem elongation stage, bud expansion stage, sepal open stage, petal open stage, flower semi-open stage, flower full-open stage, and anther cracked stage (Figure 1). The flower of *D. yunnanense* contains five large and bright bluish violet sepals, and the real petals are very small and hidden between the staminode and the upper sepal. Besides, there are two blue staminodes with yellow barbs on the ventral surface. For *D. elatum*, there are two layers of hyacinthine ovate sepals, and petals and staminodes are white. The flowering period of *D. yunnanense* was about 10 days, whereas the flowers of *D. elatum* can maintain a longer period of 15 days. This may be affected by the number of petals since *D. elatum* has double petal flowers.

Pollen viability and stigma receptivity

The pollen viability, estimated in pollen germination in vitro test, of *D. elatum* was significantly weaker than that of *D. yunnanense* during the day, and the diurnal variation displayed a trend of increase in the morning but decrease in the afternoon (Figure 2). Moreover, the pollen viability of *D. yunnanense* and *D. elatum* showed a peak at noon (11:30 AM) with germination ratio at 40.89% ± 15.65% and 18.78% ± 5.18%, respectively. This suggested that *Delphinium* is better to pollinated around noon, at least in these two tested species.

The observation of stigma receptivity revealed that the stigma of *D. yunnanense* was not receivable at the beginning of the flowering period, but displayed a positive reaction with pollen from 6th day to 8th day (Table 1). This positive reaction was strengthened at the 7th day of flowering and then declined until the end of the measurement. In contrast, the stigma receptivity of *D. elatum* presented one day earlier than that of *D. yunnanense* and had a strong receptivity the next day. However, the stigma receptivity of *D. elatum* had a shorter duration than that of *D. yunnanense*, since it became only partial receivable at the 7th day of flowering.

Pollen–stigma interaction

The number of pollen grains adhering to the surface of stigma and the germination rate was measured to reflect and evaluate the cross-compatibility of each cross combination.

The results showed that pollination time ($F = 73.3260$, $p = 0.0000$) and cross combination ($F = 110.5710$, $p = 0.0000$) had significant effects on the number of attached pollen grains in *Delphinium*, and their interaction was significant as well ($F = 18.9170$, $p = 0.0000$) (Table 2). The number of attached pollen grains significantly increased at 24 h after pollination in four crosses. The number of attached pollen grains of *D. yunnanense* was significantly higher than that of *D. elatum* after pollination on any type of stigma (Figure 3). This indicated that the pollen survival rate of *D. yunnanense* was higher than that of *D. elatum*, which was in line with the result of pollen viability. However, there was no statistically significant difference in the

Figure 1. Stages of flower development of *D. yunnanense* (A) and *D. elatum* 'Guardian Blue' (B). Bud stage (a), stem elongation stage (b), bud expansion stage (c), sepal open stage (d), petal open stage (e), flower semi-open stage (f), flower full-open stage (g) and anther cracked stage (h). Bar = 1 cm.

number of pollen grains attached to the stigma between interspecies crosses of *D. elatum* $\times$ *D. yunnanense* and intraspecific crosses of *D. yunnanense*, similarly as in the cross of *D. yunnanense* $\times$ *D. elatum* and intraspecific crosses of *D. elatum*.

From the overview of the dynamics of attached pollen grains in four crosses, some pollen grains had been attached to the stigma after 2 h of pollination (Figure 3). The number of attached pollens in the orthogonal cross (*D. elatum* $\times$ *D. yunnanense*) was higher than that of other three crosses, which were at the same low level in the early period after pollination. This

trend continued until 6 h after pollination, in which the attached pollen grains of *D. yunnanense* allogamic cross (*D. yunnanense* $\times$ *D. yunnanense*) dramatically increased over ten-fold (from 10.67 ± 1.53 to 118.67 ± 11.02 , $n = 3$). The same result as multiple comparisons, the attached pollens reached the peak after 24 h of pollination with a significant difference in each cross combination. Then, the attached pollens gradually decreased, except for that of *D. yunnanense* allogamic cross showing a sharp decrease.

The frequency of germinated pollen grains in different crosses after pollination displayed the same

Figure 2. The diurnal variation of pollen viability in *D. yunnanense* and *D. elatum*. One-way ANOVA was used to assess statistical significance, and *p* values were calculated with Tukey's HSD test ($\alpha = 0.05$). Multiple comparisons indicated by small letters were performed in different collection time within the same species. The data represent the mean value $\pm$ standard deviation from three replicates.

Table 1. Stigma receptivity of the full-open stage during the flower opening process in *D. yunnanense* and *D. elatum*. '–', stigma not receivable; '±', partial positive reaction; '+', positive reaction; '++' strong positive reaction.

Species	1 st day	2 nd day	3 rd day	4 th day	5 th day	6 th day	7 th day	8 th day
<i>D. yunnanense</i>	–	–	–	–	–	+	++	+
<i>D. elatum</i>	–	–	–	–	+	++	±	–

The data were obtained from 10 flowers.

Table 2. Results of statistical analysis about pollination time and cross combination on the number of attached pollen grains in *Delphinium*.

Source of variation	Sum of square	Degree of freedom	Mean square	<i>F</i> value	<i>p</i> value
Pollination time	27,746.7778	5	5549.3556	73.3260	0.0000
Cross combination	25,104.2778	3	8368.0926	110.5710	0.0000
Time $\times$ Combination	21,474.2222	15	1431.6148	18.9170	0.0000
error	3632.6667	48	75.6806		
Total variations	77,957.9444	71			

trend as the attached pollen grains in the corresponding cross combination (Figure 4). There was also a significant interaction of pollination time and cross combination on the germinated pollen number revealed by the multiple comparisons ($F = 6.0460$, $p = 0.0000$, Table 3). Moreover, the number of germinated pollen grains reached its peak 24 h after pollination. The frequency of germinated pollen of *D. yunnanense* was significantly higher than that of *D. elatum* in four

crosses. This was in line with the variation of attached pollens, indicating that the amount of germinated pollen grains was positively correlated with the amount of attached pollens in the species of *Delphinium*.

Dynamics of pollen tube growth in different cross combinations

In the orthogonal cross (*D. elatum* $\times$ *D. yunnanense*), most of the pollen grains germinated on stigma 2 h

Figure 3. The number of attached pollen grains of each cross combination after different pollination time. One-way ANOVA was used to assess statistical significance, and p values were calculated with Tukey's HSD test ($\alpha = 0.05$). Multiple comparisons indicated by small letters were compared in different cross combinations within the same time after pollination. The data represent the mean value $\pm$ standard deviation from three replicates.

Figure 4. The number of germinated pollen grains on each cross combination after different pollination time. One-way ANOVA was used to assess statistical significance, and p values were calculated with Tukey's HSD test ($\alpha = 0.05$). Multiple comparisons indicated by small letters were compared in different cross combinations within the same time after pollination. The data represent the mean value $\pm$ standard deviation from three replicates.

Table 3. Results of statistical analysis about pollination time and cross combination on the number of germinated pollen grains in *Delphinium*.

Source of variation	Sum of square	Degree of freedom	Mean square	F value	p-value
Pollination time	7034.9028	5	1406.9806	29.7860	0.0000
Cross combination	6178.2639	3	2059.4213	43.5980	0.0000
Time $\times$ Combination	4283.8194	15	285.5880	6.0460	0.0000
error	2267.3333	48	47.2361		
Total variations	19,764.3194	71			

after pollination, showing pollen tubes entered the style and few callose deposited in the style (Figure 5A). After 4 h, callose depositions appeared in large number in the style, some pollen tubes had entered the ovary at this stage. Six hours after pollination, pollen tubes arrived near the ovule and started to bend and twine. Finally, pollen tubes entered the ovule 24 h after pollination. In the reciprocal cross, pollen tube growth differs that in the orthogonal cross (Figure 5B). Only a small amount of pollen grains germinated on the stigma, whereas a large amount of callose deposition was visible in the style 2 h after pollination. Four hours after pollination, pollen tubes twined, knotted and spiraled, accompanied by callose formation. As a result, only a small number of pollen tubes reached the ovule 24 h after pollination, but none of them entered the ovule as observed.

In allogamic crosses of *D. yunnanense*, pollen tubes entered the ovule 24 h after pollination (Figure 5C). However, in the allogamic cross of *D. elatum*, a small amount of pollen germinated and callose deposition appeared 2 h after pollination. Six hours after pollination, the pollen tubes were visible in the style and reached the ovary 24 h after pollination (Figure 5D). Three days after pollination, the pollen tube entered the ovule. The pollen of *D. yunnanense* displayed a significantly strong interaction with stigmas of *D. yunnanense* and *D. elatum*, indicating that the pollen activity of *D. yunnanense* was stronger than that of *D. elatum*, which was in line with the results of pollen viability. It also suggested that *D. elatum* is more suitable as a maternal parent for hybridisation breeding.

Fruit and seed setting of *F₁* hybrids

After crossing, the fruit set rate was evaluated in each cross according to the between the fruit number and the number of previously pollinated flowers. The results showed differences in fruit set rate between the orthogonal and reciprocal cross of *D. yunnanense* and *D. elatum* (Table 4). In the orthogonal cross (*D. elatum* $\times$ *D. yunnanense*), the fruit set rate was the highest (90.0%) and generated 17.1 seeds per fruit. In contrast, there was no seed obtained in the reciprocal cross. This was consistent with the observation of microscopic dynamics that no pollen tube was found entering the ovule of *D. yunnanense* $\times$ *D. elatum*. On the other hand, there was no significant difference

in fruit set rate between the allogamic crosses of *D. yunnanense* and that of *D. elatum*, showing the rate of 86.7% and 83.3%, respectively. Moreover, the seed number per fruit of *D. elatum* in allogamic cross was the highest, suggesting that there were no disturbances in the sexual reproductive system of *D. elatum* since both male and female lines were fertile. Therefore, the cross-incompatibility probably caused the sterility of *D. yunnanense* $\times$ *D. elatum* *F₁* hybrids. Furthermore, the seed germination rate of *D. elatum* allogamic cross was the highest, while seeds derived from the cross of *D. elatum* $\times$ *D. yunnanense* displayed the lowest germination rate of 16.7% as well as the lowest thousand kernel weight. This may relate to the formation of abnormal seeds detected in the orthogonal cross of *D. elatum* $\times$ *D. yunnanense*.

DISCUSSION

The genus of *Delphinium* contains several species with a high ornamental value, which is crucial for the development of new floricultural varieties. In this study, we investigated the cross-compatibility of two *Delphinium* species, *D. yunnanense*, a wild endemic species of Yunnan province and a commercial variety *D. elatum* ‘Guardian Blue’. Strong cross-incompatibility was observed in the cross of *D. yunnanense* $\times$ *D. elatum*, whereas less cross-incompatibility was detected in the cross of *D. elatum* $\times$ *D. yunnanense*, which provided a better understanding of the hybridisation affinity in *Delphinium*.

The measurement of pollen viability has an important theoretical and practical significance in hybrid breeding. The level of pollen viability affects the success and efficiency of hybrid breeding. Therefore, it is essential to measure the pollen viability before hybridisation, which could provide a useful reference for the formulation of hybrid combinations. Our results showed that the diurnal variation of pollen viability displayed a trend of increased in the morning but decreased in the afternoon in both *D. yunnanense* and *D. elatum*. This was consistent with previous independent observations of *D. grandiflorum*, in which the pollen viability increased at the early stage and decrease later in daily variation (from 9:30 to 15:30) with a peak at 11:30 AM (Zhang et al., 2014). Moreover, in *Lilium davidii* var. unicolor,

Figure 5. Dynamics of pollen tube growth of the interspecific and allogamic crosses between *D. yunnanense* and *D. elatum*. **A:** *D. elatum* × *D. yunnanense*. A-2h: A bundle of pollen tubes (Pt) in the style of pistil, pollen grains (Pg) visible on stigma. A-4h: Large number of callose (Ca) depositions appeared in the style. A-6h: Pollen tubes arrived near the ovule (Ou) and started to bend and twine. A-24h: Pollen tubes entered the ovule. **B:** *D. yunnanense* × *D. elatum*. B-2h: Few pollen grains germinated on the stigma with a large amount of callose depositions visible in the style. B-4h: The twined, knotted and spiraled pollen tubes with callose. B-6h: Six hours after pollination, the state of pollen tube remains the same as 4 h after pollination. B-24h: Few pollen tubes were close to the ovule. **C:** *D. yunnanense* × *D. yunnanense*. C-2h: A number of pollen tubes in style of pistil, pollen grains also visible on stigma. C-4h: A bundle of pollen tubes grow along the style. C-6h: Pollen tubes arrived near the ovule. C-24h: Pollen tubes entered the ovule. **D:** *D. elatum* × *D. elatum*. D-2h: A small amount of pollen germinated and callose deposition appeared. D-6h: Pollen tubes were visible in the style. The pollen tubes were twined and knotted in the style. D-24h: Pollen tubes reached the ovary with visible callose deposition. D-72h: Pollen tube entered the ovule. ‘Pg’ refers to pollen grain, ‘Pt’ means pollen tube, ‘Ca’ is the abbreviation of callose and ‘Ou’ represents ovule. Bar = 200 μm.

the highest pollen vigor appeared at 08:00–14:00 with the highest germination rate appeared at 10:00 AM (Sun et al., 2019). The pollen vigor of *D. yunnanense* was significantly higher than that of *D. elatum*. This is probably because the pollen vigor of *D. elatum* has decreased after numerous artificial selections during the process of breeding.

Furthermore, we investigated the pollen–stigma interaction between *D. yunnanense* and *D. elatum*. The results showed that the number of pollen grains attached or germinated of *D. yunnanense* were relatively higher in the reciprocal or allogamic crosses. This was in line with the large pollen quantity and high pollen vitality of *D. yunnanense*. Moreover, it was detected that no pollen

Table 4. Characteristics of F_1 seeds in the interspecific and allogamic cross of *D. yunnanense* and *D. elatum*.

Cross combination	Pollinated flowers number	Fruit number	Fruit set rate	Seed number	Seed number per fruit	Thousand kernel weight (g)	F_1 seed germination rate (%)
<i>D. elatum</i> × <i>D. yunnanense</i>	30	27	90.0%	463	17.1	1.3	16.7 ± 2.08 a
<i>D. yunnanense</i> × <i>D. elatum</i>	30	0	0.0%	/	/	/	/
<i>D. yunnanense</i> × <i>D. yunnanense</i>	30	26	86.7%	421	16.2	1.7	24.7 ± 2.52 b
<i>D. elatum</i> × <i>D. elatum</i>	30	25	83.3%	527	21.1	2.0	80.0 ± 4.00 c

One-way ANOVA was used to assess statistical significance, and p values were calculated with Tukey's HSD test ($\alpha = 0.05$).

tube entered the ovule in the reciprocal cross, suggesting that there was a strong cross-incompatibility in the reciprocal cross of *D. yunnanense* × *D. elatum*. This was related to the cell recognition of the pollen–stigma interaction, which could prevent pollen with genetic variation from germinating on stigma and entering into ovule (Bedinger et al., 2017). It was reported that pre-fertilisation obstacles of interspecific hybridisation in plants were mainly caused by the abnormal growth of pollen tubes, the callose formed on stigma surface and excessive growth of pollen tubes (Heslop-Harrison, 1982; Dumas and Knox, 1983). In our study, the abnormal growth of pollen tubes was observed in the cross of *D. yunnanense* × *D. elatum*, such as winded, knotted and spiraled pollen tubes. The formation of callose in stigma mastoid cells was also observed after pollination, which prevented the pollen tube from entering the ovule and consequently resulted in the lack of fertilisation and seed production. Moreover, barriers to reproduction are the most important factor for cross-incompatibility, which can be effective before (prezygotic barriers) and after (postzygotic barriers) the formation of a zygote (Pease et al., 2016). Prezygotic barriers are arguably the most important and effective barriers that contribute to reproductive isolation acting before hybrids formation (Henrich and Kalbe, 2016). In the cross of *D. yunnanense* × *D. elatum*, although some pollen grains germinated, the pollen tubes were arrested in the pistils and were unable to reach the ovules. Thus, the pre-fertilisation reproductive barriers are the main factor affecting the cross of *D. yunnanense* × *D. elatum*. This phenomenon was also observed in the crosses of different petal phenotypes in *Jasminum sambac* Aiton (Deng et al., 2017).

Although the fruit set ratio of *D. elatum* × *D. yunnanense* was the highest and plenty of F_1 seeds were generated, yet the germination ratio of F_1 seeds was really low. In addition, we did not harvest any seed or fruit from the cross of *D. yunnanense* × *D. elatum*. These results demonstrated that the incompatibility indeed existed between *D. yunnanense* and *D. elatum*. One possible theory is that the development of embryo was disturbed and generated immature seed with

normal appearance but containing aborted embryos, which reduced the seed vitality (Zeng et al., 2010; Chai et al., 2011; Dai et al., 2014; Chong et al., 2018). This also indicated by the thousand kernel weight of F_1 seeds in *D. elatum* × *D. yunnanense*, which displayed the lowest thousand kernel weight. Moreover, the germination traits study of *D. chefoense* showed that the seed germination rate was about 38.0% and 7.3% under 20°C and 25°C, respectively (Qian, 2012). In our study, the seed germination rate of *D. yunnanense* was about 24.7% at 25°C. The similar low seed germination rate of the wild type in *Delphinium* species indicated that reproductive strategy of this genus was relatively conserved, which probably provides high recruitment rates for the existing populations with numerous, but the genetic variation and regeneration of natural population may not be able to maintain in the long term (Orellana et al., 2009). Thus, it is necessary to protect the species in *Delphinium*, since it was also reported that the species number of *Delphinium* decreased in China (Yuan, 2006). In conclusion, our study provided a fundamental information for the cross-incompatibility in *D. yunnanense* and *D. elatum* related to hybridisation. These findings may help ornamental breeders overcome reproductive barriers to develop new cultivars in the species.

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AUTHOR CONTRIBUTIONS

Y.C. and W.G. conceived and designed the experiments. Y.C. completed the experiments. Y.C. and F.L. conducted the data analysis and drafted the manuscript. F.L. critically revised the manuscript. W.G. and F.L. jointly provided supervision. All authors reviewed and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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