Ecological significance of deceptive pollination in Papilionanthe teres

tingting Yang[1] and xiang Zhou[1]

[1]Chinese Academy of Sciences

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Abstract

Though some hypotheses have obtained theoretical and empirical supports, it remains largely unknown in the aspect that how deception increases orchid fitness. This study used food-deceptive Papilionanthe teres as experimental material to explore the ecological significance of orchid deceptive pollination. Deception together with obvious pollinarium bending increases P. teres fitness by means of decreasing geitonogamy under the natural conditions. The proportions of full seeds, single fruit weight and seed weight per fruit after self-pollination and nectar addition were significantly lower than that after cross-pollination and natural conditions (all p ¡ 0.05). Seed viability (seed growth and development rate) after cross-pollination and natural condition were significantly higher than that after self-pollination and nectar addition (all p ¡ 0.05). However, there was no significant difference in all the above parameter values of fruits and seeds between cross-pollination and natural conditions (all p ¿ 0.05). These results confirmed that P. teres has high level of genetic load, and self-fertilization or geitonogamy will cause serious inbreeding depression. These conclusions support the outcrossing hypothesis that ecological significance of P. teres deception is to promote outcrossing and improve the ability of the offspring to adapt to the environment.

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Keywords: *Papilionanthe teres*, deception, geitonogamy, inbreeding depression, outcrossing

Introduction

Orchidaceae is a large family of plants, with about 763 genera and 28000 species (Crain Tremblay, 2014; Christenhusz Byng, 2016; Zhang et al., 2018). Of the 7500 angiosperm species that are pollinated through deception, approximately 6500 are orchids (Renner, 2006), suggesting that deception mainly occurs in orchids. Food deception is most prevalent in the orchid family, and several thousand species are found in 38 genera (Dafni, 1984; Ackerman, 1986; Nilsson 1992; Jersáková et al., 2006). The second is sexual deception,

and about 400 orchid species are found in 18 genera (Dafni Bernhardt, 1990; Cozzolino Widmer, 2006; Jersakova et al., 2006).

Visit frequency and natural fruit set of no rewarding species are lower than that of rewarding ones due to pollinator learning behavior. So the fitness of deceptive plants remains a focus for debate among biologists. Besides four orchid-specific hypotheses (Jersakova et al., 2006; Cozzolino Widmer, 2006; Scopece et al., 2010), there are two general hypotheses, resource-limitation hypothesis and outcrossing hypothesis, to explain how deception increases plant fitness (Jersakova et al., 2006).

Plant sexual reproduction, such as flower production and fruit set, is mainly limited by resources (Calvo, 1992; Mattila Kuitunen, 2000). The aim of deception is to invest more resources to maintain plant development and ensure a certain seed set (resource-limitation hypothesis) (Ackerman Montalvo, 1990; Barrett Harder, 1995). However, the sexual reproduction of deceptive orchids is often severely limited by pollens over a lifetime (Calvo, 1993; Tremblay et al., 2005). Hence, it is difficult to understand why resources in these orchids are not allocated to a component of pollinator attraction such as nectar. One possibility is that the attraction of investing more resources in floral display is more efficient than that in rewarding substances.

Deception results in lower visitation rates, fewer flowers probed per visit, lower level of geitonogamy, more pollen output and outcrossed progeny (outcrossing hypothesis) (Jersakova et al., 2006). Based on the above conclusions, it is widely believed that loss of rewarding substances contributes to decreasing geitonogamy and promoting outcrossing (Dressler, 1981; Nilsson, 1992; Johnson Nilsson, 1999). Outcrossing hypothesis emphasizes the importance of pollen resource and genetic quality in reproductive success, and postulates that self-fertilization or geitonogamy will cause inbreeding depression (Lammi Kuitunen, 1995). Therefore, the first problem of outcrossing hypothesis is to test whether self and cross-pollination result in different female reproductive success (Lammi Kuitunen, 1995). Though previous case studies confirm that deception promotes outcrossing, whether actual outcrossing rates are generally higher in deceptive orchids remains unknown (Jersakova et al., 2006).

Pollinarium bending occurres in many orchid species, it will result in a time delay before the pollinium assumes a position from which it can strike a stigma (Darwin, 1877; Johnson Edwards, 2000). The phenomenon will decrease geitonogamy when pollinators visit fewer flowers and stay for shorter time per visit. Some case studies confirm that pollinarium bending is an anti-selfing mechanism (Darwin, 1877; Johnson et al., 2004). These results suggest that pollinarium bending may increase orchid fitness under certain conditions. But it still requires further study that how pollinarium bending increases fitness in orchid deceptive pollination systems.

Papilionanthe teres is only found in a very limited region of southeast Xishuangbanna in China. It has adapted to high temperature, humidity and sunlight conditions. Its flowers exhibit color polymorphism of the corolla, such as white lateral sepal, purplish red petal, dorsal sepal and labellum (Zhou Gao, 2016). Its single inflorescence may bear 1-16 flowers. Floral longevity may reach more than 30 days under the conditions of no pollinarium removal, pollinia deposition and floral damage. *P. teres* is a typical food-deceptive species, with no reward in its flowers. The techniques of symbiotic and asymbiotic seed reproduction in *P. teres* were established in previous studies (Chen et al., 2007; Mazumder et al., 2010; Zhou Gao, 2016; Vishal, 2020). This study utilized these techniques and used *P. teres* as material to understand the ecological significance of orchid deceptive pollination. The following questions were addressed: (1) What are the mechanisms of promoting outcrossing in *P. teres* ? (2) What are the effects of self-pollination and nectar addition on fruit and seed development and seed viability of *P. teres* ? (3) Does self and cross pollination result in different female reproductive success? (4) Does *P. teres* have a high outcrossing rate (high levels of genetic load) under natural conditions?

Material and Methods

2.1 Study sites and species

Papilionanthe teres from six sites in the Xishuangbanna Tropical Botanical Garden (XTBG), Chinese

Academy of Science (CAS), was used as the experimental material for this study. The study species, XTBG geography and climate were previously described (Zhou Gao, 2016). The six study sites were located at the Fruit Garden (FG), Arboretum (AR), Tropical Seasonal Rainforest Area (TSRA), Vine Garden (VG), Energy Plant Garden (EPG), and Ethnic Medicinal Garden (EMG) in XTBG. About 97, 138, 87, 65, 58, 71 adult *P. teres* were found at the FG, AR, TSRA, VG, EPG and EMG, respectively. The study was conducted during the flowering and fruiting period of *P. teres* between 2019 and 2021.

The six study sites were selected for field observation and manual control experiments, such as observation of pollinator visit behavior, nectar addition experiments and hand pollination. All laboratory experiments, such as seed symbiotic and asymbiotic cultures, were conducted at the XTBG Institutional Center for Shared Technologies and Facilities, a research center for biodiversity conservation.

2.2 Manual control experiments

In order to obtain *P. teres* seeds from different sources, four types of manual control experiments including nectar addition, natural conditions, self and cross-pollination were conducted at the six study sites during the anthesis of *P. teres* in two consecutive years (2019-2020). All flowers at FG were selected to conduct nectar addition experiments. Due to partially overlapped antheses and the same pollinator of rewarding *Thunbergia grandiflora* and food-deceptive*P. teres* , we mimicked the open flowers of *T. grandiflora*to produce artificial nectar. Spur of each newly-open flower of *P. teres* was supplied with artificial nectar (20 μl volume and 50% sugar content) and the surplus nectar was removed. Fresh nectar was re-added using a microsyringe every morning at 08:00 am until the flower wilted. Nectar addition did not result in floral damage and had no effect on the floral longevity. So there was no necessity to set control of water addition. In order to obtain natural fruit and seed set, all flowers and inflorescences at AR, TSRA and VG were maintained in natural states. Two flowers of each inflorescence at EPG and EMG were used to conduct self (The pollinia were from the same or another flower in the same inflorescence) and cross (The pollinia were from the other flower at EPG) pollination, respectively. All flowers used in hand pollination were bagged before the flowers opened and they were bagged again after pollination. In next April, mature fruits of the four treatments were collected for subsequent experiments.

2.3 Observations of flower pollinator visit behavior

Five inflorescences and about 20 flowers at each study site (FG and AR) were selected to observe pollinator visit behavior annually for five days after nectar addition (FG) and natural conditions (AR) in May in two consecutive years (2020-2021). Observation date and time was from May 15 to 20, from 08:00 am to 18:00 pm every year, respectively. The time of flying between two flowers on a plant, total time of stay per visit, number of flowers visited per visit, and the length of time spent visiting each flower by pollinators were observed and recorded.

2.4 Collection of fruits and seeds, and measurement of their morphological characteristics

All mature fruits of the four types of manual control experiments at six study sites were collected in April 2020 and April 2021. For each treatment, 60 randomly selected fruits were used to measure their lengths, widths and weights. For each collected fruit, its seeds were collected to measure weight, number and proportion of shriveled seeds. We used vernier calipers to measure fruit length and width, and an electronic balance with high accuracy (one-millionth) to measure fruit and seed weights. Thereafter, 0.02 g seeds taken from each collected fruit were uniformly distributed in the solution of 20 ml 0.1% agarose. Average numbers of seed and shriveled seed in 5 μl of the above solution were counted using an optical microscope and 30 experimental replicates were selected. Seed total number per fruit (N) and proportion of shriveled seeds (P) was calculated using the following mathematical formula ($N = 2 * M * \tilde{n} * 10^5$, $P = (2 * M * n' * 10^5 / N) * 100\%$, M is seed weight per fruit, $\tilde{n}$ and n' are the average number of seeds and number of seeds with abnormal development in 5 μl of the above solution, respectively). All surplus seeds of each treatment were fully mixed, dried with anhydrous calcium chloride for 5 days at 22 and then stored at -20 for long-term preservation. Appropriate amount of mixed seeds from each treatment were taken to measure morphological characteristics using a scanning electron microscope.

2.5 Seed symbiotic and asymbiotic cultures

Seed symbiotic and asymbiotic cultures were conducted according to the methods described by Zhou and Gao (Zhou Gao, 2016). Mixed seeds of each treatment were removed from storage at -20 and kept at ambient temperature for 10 h. Appropriate amount of seeds of each treatment were surface-sterilized using sodium hypochlorite solution containing 0.1% available chlorine for 3 min followed by washing with ddH_2O for three times. Each sterilized circular nylon cloth with a diameter of 2.6 cm and spores of 45 μm was inoculated with 50-100 surface-sterilized seeds using a pipette. Then it was transferred individually to a cylindrical glass bottle (height 9 cm, diameter 6.5 cm) containing 35 mL of AGS (MS 0.9 L/L, natural mature coconut juice 0.1 L/L, carbon powder 1 g/L, sucrose 20 g/L, and agar 6 g/L) or $OMA^{+Epa\text{-}01}$ medium (OMA: oat 4 g/L, agar 8 g/L, pH = 5.8; Epa-01: a highly compatible fungus, which promotes seed germination and protocorm development of *P. teres*) such that its surface was completely covered with the Epa-01 strain colony. Each treatment had at least 60 replicates.

Thereafter, all glass bottles containing $OMA^{+Epa\text{-}01}$and AGS medium were incubated at 26.0 ± 0.5 and 14 / 10-h light/dark cycle for 60 and 70 days, respectively. Developmental stages of *P. teres* seeds were determined according to the methods described by Stewart and Kane (Stewart, 2008). *P. teres* seeds that reached developmental stage [?]2 were considered as germinated. *P. teres*seedlings with roots were considered as developmental stage 6. Seed states, including developmental stage, germination rate, and fresh weight of germinated seeds, were recorded after incubation. Electronic balance with high accuracy (one-millionth) was used to measure fresh weight of germinated seeds. During incubation, glass bottles contaminated with other fungi were discarded. The calculation of average developmental stage for each treatment was based on the methods described by Nontachaiyapoom et al (Nontachaiyapoom et al., 2011).

2.6 Statistical analysis

Statistical analysis was performed using the SPSS software 13.0. All comparisons of differences in seed germination, developmental stages, fresh weight of seed symbiotic and asymbiotic cultures, and fruit and seed morphological characteristics between different treatments were determined using one-way analysis of variance (ANOVA). The other comparisons of differences were conducted with nonparametric tests followed by a test for two independent samples.

Rusults

3.1 Observations of flower pollinator visit behavior

Time of pollinarium bending was significantly higher than the total time of pollinator flight between two flowers on a plant (U = 0.000, W = 105, p ¡ 0.01) and that of pollinator stay per visit (U = 3, W = 1833, p ¡ 0.01) (Figure 1A). However, it was significantly lower than the time of pollinator stay per visit under the condition of nectar addition (U = 17, W = 122, p¡ 0.01) (Figure 1A). The differences in visit behavior, such as total time of pollinator stay per visit (U = 0.000, W = 1953, p ¡ 0.01), rate of flowers visited to total number of flowers (U = 0.5, W = 21.5, p ¡ 0.01) and time of pollinator stay on a single flower (U = 6.5, W = 502.5, p¡0.01), showed significant differences between nectar addition and natural conditions (Figure 1A, B).

3.2 Fruit and seed characteristics

Self-pollination (Figure 2A) and nectar addition (Figure 2B) resulted in more shriveled seeds compared with cross-pollination (Figure 2C) and natural conditions (Figure 2D). The proportion of shriveled seeds after self-pollination and nectar addition was significantly higher than after cross-pollination and natural conditions (all p ¡ 0.01) (Table 1). There were significant differences in the proportion of shriveled seeds between self-pollination and artificial nectar addition (p ¡ 0.01) (Table 1), but not between cross-pollination and natural conditions (p = 0.703 ¿ 0.05).

There was no significant difference in fruit length and seed number per fruit among the four treatments (all p¿0.05) (Table 1). Fruit weight and seed weight per fruit of self-pollination and nectar addition was

significantly lower than that of cross-pollination and natural conditions (all p ¡ 0.01) (Table 1). There were no significant differences in fruit weight and seed weight per fruit between cross-pollination and natural conditions (p ¿ 0.05), and between self-pollination and artificial nectar addition (p ¿ 0.05) (Table 1). Though fruit width after self-pollination was significantly lower than natural conditions (p ¡0.05), there was no significant difference in fruit width after cross-pollination, artificial nectar addition and natural conditions (all p ¿ 0.05) (Table 1).

3.3 Seed symbiotic and asymbiotic cultures

More germinated seeds with low developmental stages (stage 2 and stage 3) were found after self-pollination (Figure 3A) and nectar addition (Figure 3B) compared with that after cross-pollination (Figure 3C) and natural conditions (Figure 3D) under the conditions of 30-day seed symbiotic culture. There were more seedlings with high developmental stages (stage 5 and stage 6) after cross-pollination (Figure 4C) and natural conditions (Figure 4D) compared with that after self-pollination (Figure 4A) and nectar addition (Figure 4B) under the conditions of 70-day seed asymbiotic culture.

There was no significant difference in seed germination among the four treatments under the conditions of 60-day symbiotic and 70-day asymbiotic cultures (all p ¿ 0.05) (Figure 5A). Average fresh weight of a single germinated seed after self-pollination and nectar addition was significantly lower than that after cross-pollination and natural conditions (all p ¡ 0.01). Moreover, there were significant differences in average fresh weight of a single germinated seed between self-pollination and nectar addition (p ¡0.05) but not between cross-pollination and natural conditions (p¿0.05) (Figure 5B). The results of average developmental stages were similar to that of average fresh weight of a single germinated seed (Figure 5C).

Under the condition of symbiotic culture, percentage of germinated seeds with high developmental stages (stage 5 and stage 6) after cross-pollination and natural conditions was significantly higher than that after self-pollination and nectar addition, while that with low developmental stages (stage 2 and stage 3) was significantly lower (all p ¡ 0.01) (Figure 5D; Table 2). Differences in percentage of germinated seeds with developmental stage 2-6 did not reach significant level between cross-pollination and natural conditions (all p ¿ 0.05) (Figure 5D; Table 2). There were significant differences in percentage of germinated seeds with developmental stage 3 and stage 6 between self-pollination and nectar addition (all p ¡ 0.01) (Figure 5D; Table 2).

Under the condition of asymbiotic culture, percentage of germinated seeds with high developmental stages (stage 6) after cross-pollination and natural conditions was significantly higher than that after self-pollination and nectar addition, while that with low developmental stages (stage 3 and stage 4) was significantly lower (all p ¡ 0.01) (Figure 5E; Table 2). In most cases (except stage 4), percentage of germinated seeds at different developmental stages showed no significant difference between cross-pollination and natural conditions (all p ¿ 0.05) (Figure 5D; Table 2). There were significant differences in percentage of germinated seeds with developmental stage 2, stage 4 and stage 6 between self-pollination and nectar addition (all p ¡ 0.01) (Figure 5D; Table 2).

Discussion

Like the other orchids (Johnson Edwards, 2000; Johnson et al., 2004), pollinarium bending obviously exists in *Papilionanthe teres* , and was reached in 62.43 +- 22.97 s (N = 30). Total time per visit under natural conditions and after nectar addition were significantly lower and higher than that of pollinarium bending, respectively (Figure 1A), suggested that the two conditions could delay and accelerate touching between pollinia and stigma, respectively. Nectar addition significantly increased the number of flowers visited in a single inflorescence and pollinator staying time in a single flower (Figure 1B). Besides, the proportions of full seeds and seed viability after nectar addition were significantly lower than that under natural conditions (Table 1; Figure 5). These results indicated that *P. teres* is similar to most deceptive orchids that avoid geitonogamy by the two mechanisms of deception and pollinarium bending (Darwin, 1877; Dafni Ivri, 1979; Johnson Edwards, 2000; Johnson et al., 2004), and deception contributes to pollinarium bending functioning in the aspect of anti-selfing.

Both self-compatibility and inbreeding depression can be checked by pollination experiments. There was no significant difference in fruit set between self and cross-pollination in the earlier stage of fruit development, while self-pollination led to serious fruit abscission in the late stages (data not shown). Compared with cross-pollination, there were more shriveled seeds, smaller weight of a single fruit and fewer seeds per fruit after self-pollination (Figure 2A, C; Table 1). These results indicated that self and cross-pollination result in different female reproductive success. Moreover, self-pollination seriously affects the development of fruits and seeds of *P. teres* .

Most orchids are self-compatible species, and the seed set and seed quality are usually greatly reduced after self-pollination (Tremblay et al., 2005). Compared with cross-pollination and natural conditions, self-pollination and nectar addition had more seeds with low developmental stages under the conditions of 30-day (Figure 3A, B), 60-day (Figure 5C, 5D; Table 2) symbiotic culture and 70-day asymbiotic culture (Figure 4A, B; Figure 5C, E; Table 2), suggesting that seed viability was greatly reduced after self-pollination and nectar addition. There were significant differences in average fresh weight of germinated seeds (Figure 5B), average developmental stage (Figure 5C), proportions of seeds with high developmental stage (stage 6) (Figure 5D, E) and shriveled seeds (Table 1) between self-pollination and nectar addition under the conditions of symbiotic and asymbiotic cultures, suggesting that nectar addition partially results in geitonogamy. It is important for orchids to reach higher developmental stage faster under natural conditions to enhance its survival and resistance to disadvantageous environmental and climate conditions (Smith et al., 2007; Stewart, 2008). Based on this conclusion, and combined with our study results, it is confirmed that germinated seeds after cross-pollination and natural conditions have higher ability to adapt to the environment than that after self-pollination and nectar addition.

Seed morphology (Figure 2C, D), and parameter values of fruits and seeds (Table 1) showed no significant differences between natural conditions and cross-pollination. Besides, there was no significant difference in average fresh weight of germinated seeds (Fig. 5B), average developmental stage (Figure 5C), proportions of seeds with different developmental stages (Figure 5D, E) between natural conditions and cross-pollination under the conditions of symbiotic and asymbiotic cultures. Taken together, these results suggested that *P. teres*has high outcrossing rate under natural conditions. Some case studies confirmed a negative correlation between inbreeding depression and selfing rate (Husband Schemske, 1996), indicating that the lower the selfing rate, the more obvious the inbreeding depression. Thus, if deceptive orchid species regularly experience high levels of outcrossing, a higher cost of selfing is expected in deceptive species than in rewarding ones (Jersakova et al., 2006). Compared with cross-pollination and natural conditions, there were more shriveled seeds (Figure 2), lighter weight of a single fruit and seeds per fruit (Table 1), and lower seed viability (Figure 5; Table 2) after self-pollination and nectar addition under the conditions of symbiotic and asymbiotic cultures, indicating that both self-fertilization and geitonogamy result in inbreeding depression. These results further confirmed that *P. teres* has high outcrossing rate under the natural conditions.

Nectar addition experiments along with field observation were used in this study to comparatively analyze the differences of pollinator visit behavior, and the results confirmed that*P. teres* decreased geitonogamy by the two mechanisms of deception and pollinarium bending. Deception contributes to pollinarium bending playing the function of anti-selfing in *P. teres* . Besides, the fruits and seeds from four sources (self-pollination, cross-pollination, nectar addition and natural conditions) were used to compare the differences in the characteristic parameters of seeds and fruits, and check the seed viability by symbiotic and asymbiotic culture techniques, which indirectly proved that *P. teres* had high level of genetic load. Both self-fertilization and geitonogamy resulted in inbreeding depression, such as high proportion of shriveled seeds and lower developmental speed of germinated seeds. These conclusions support the outcrossing hypothesis that ecological significance of *P. teres*deception is to promote outcrossing and improve the ability of the offspring to adapt to the environment.

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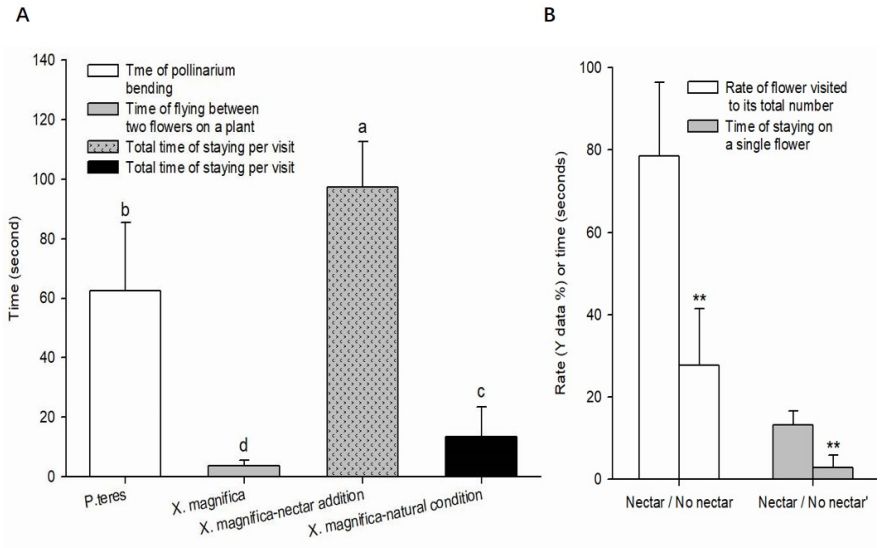

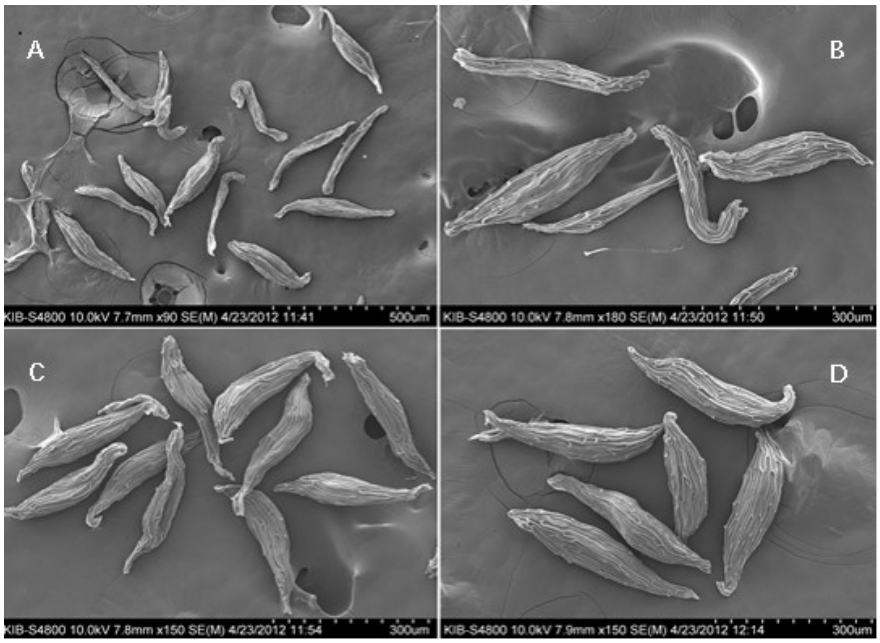

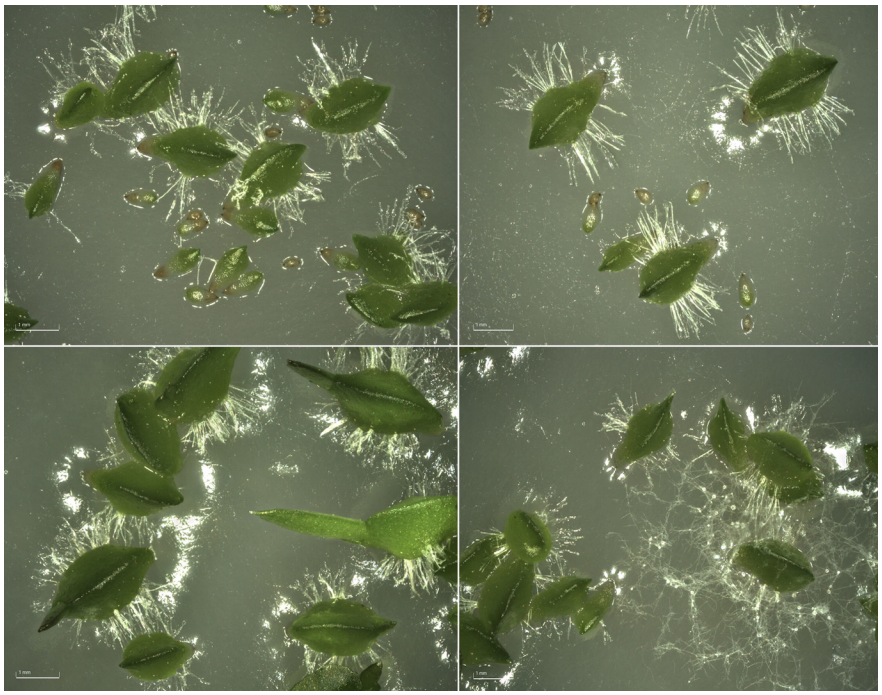

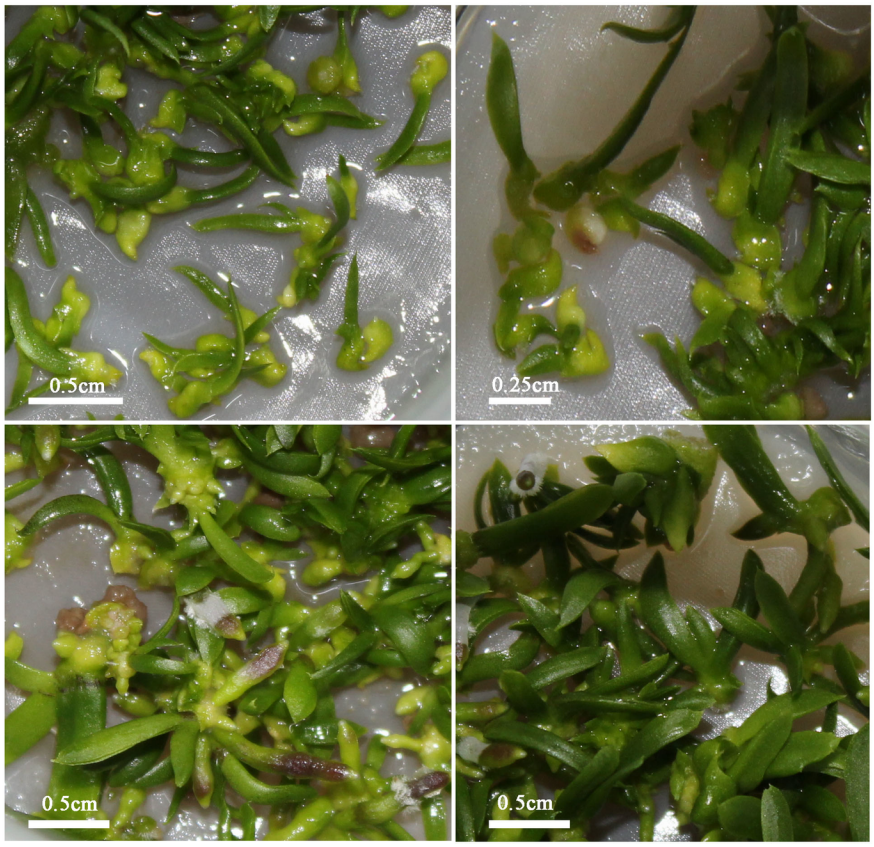

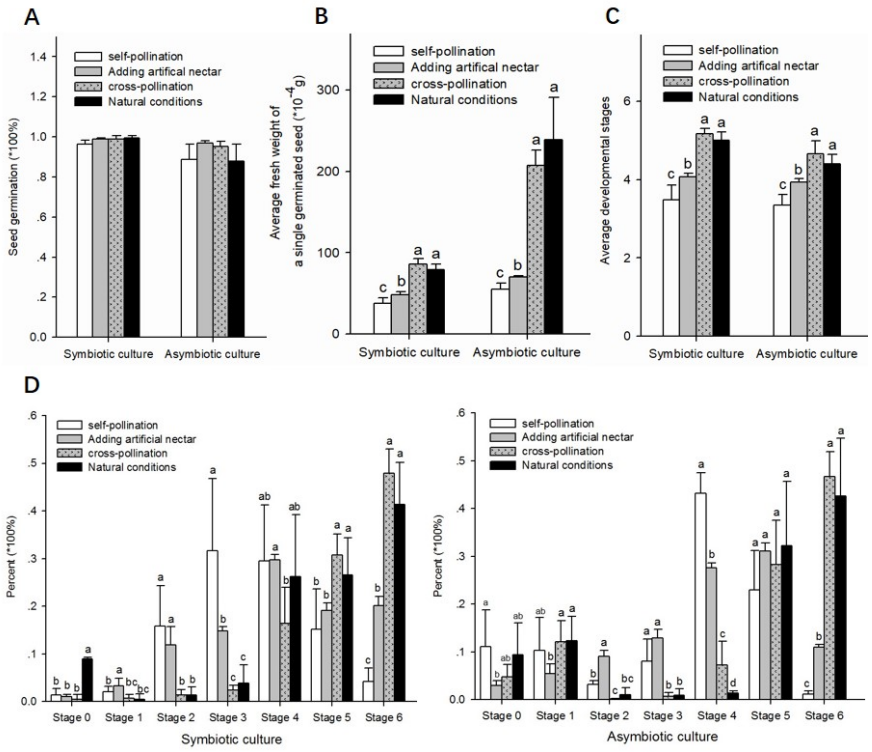

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