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EMBRYO DEVELOPMENT AND CORRESPONDING FACTORS AFFECTING *IN VITRO* GERMINATION OF *CYMBIDIUM FABERI* × *C. SINENSE* HYBRID SEEDS

Fengtong Li[1], Jianzhong Bao[1], Ye Sun[1], Chungui Liu[1], Hui Ma[1], Tian Zhang[1] and Xiulan Chen[1,*]

[1] *Jiangsu Lixiahe Region Research Institute of Agricultural Sciences*, Yangzhou 225007, Jiangsu Province, China

***Corresponding authors**: yzchxl@163.com

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Abstract: A better understanding of embryo development would provide insights into seed quality and subsequent germination events in the interspecific hybridization of *Cymbidium faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'. At the mature stage, 26.1% of the ovules were abnormal. Most of the hybrid embryos could develop normally. Abortions mainly occurred at the zygote (9.5%) and 2-4-celled embryo (15.1%) stages. No germination was observed at 90 and 105 days after pollination (DAP), when the embryo was at the early globular stage, with abundant organelles but no storage materials. During 110-130 DAP, the globular embryo was formed and the starch grains began to accumulate in plastids. The hybrid seeds collected at 120 DAP showed initiation of germination. Germination significantly increased at 135 DAP and was maximal at 150 DAP, during which period the hybrid embryos developed into the late globular stage. The storage materials, i.e. lipid and protein bodies, began to accumulate and the filamentary structures derived from suspensor cells still persisted. After the seeds matured (160 DAP), the germination percentage declined sharply. Safranin staining revealed that the outer seed coat was totally cuticularized and the inner seed coat appeared as a cuticle layer enclosing the embryo proper tightly, which may be the main factor inhibiting the subsequent germination of hybrid seeds. In conclusion, 150 DAP should be the opportune time for the *in vitro* germination of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' hybrid seeds.

Key words: *C. faberi* × *C. sinense*; embryo development; *in vitro*; interspecific hybridization; seed germination

INTRODUCTION

Chinese orchids, also known as Japanese orchids in Japan, are a family of terrestrial orchids; the species are perennial and herbaceous angiosperms, widely cultivated in eastern Asia, and the five main species are *Cymbidium faberi*, *C. goeringii*, *C. ensifolium*, *C. sinense* and *C. kanran* [1,2]. This family is characterized by its unique flower morphology, flower color, flower fragrance, as well as evergreen slender leaves. The different phenotypic traits of flower and leaf generally represent unique cultural connotations in China, Japan and South Korea. As a result, this family of orchids is charismatic to hobbyists and breeders in eastern Asia.

Chinese orchids have been cultivated for more than 2000 years in China [3]. The wild populations are distributed mainly in high altitude areas of southeast and southwest China [4]. Generally, the seeds of terrestrial orchids are very difficult to germinate in the natural conditions of temperate regions [5-7]. Thus, Chinese orchids are always propagated vegetatively by offshoots produced from the nodes of older shoots. The new varieties, with excellent ornamental traits, were historically selected from natural variants, thereby seriously reducing breeding efficiency. Moreover, due to the excessive collections of wild variants, many natural habitats have been destroyed [8]. With the changing appreciation standard and the successful application of germination technology *in vitro* in some orchid species, the demand for new Chinese orchid cultivars with novel characteristics has also increased. Breeders are making great efforts to carry out intraspecific and interspecific breeding within the five Chi-

nese orchid species, so as to develop new cultivars by using *in vitro* germination methods, and remarkable progress has been made in germinating intraspecific hybrid seeds [2,9-11]. However, to date, there are few reports on the successful germination of interspecific hybrid seeds. Although a number of researches have revealed that the harvesting time of seeds can significantly influence seed germination *in vitro* in many wild orchids [12-15], it is still difficult to determine the opportune harvesting time for distant hybrid seeds of Chinese orchids and no study has elucidated the reason for this. Orchids always produce large amounts of tiny seeds, with undifferentiated embryos containing some lipid droplets and proteins, and each capsule can contain up to four million seeds [16-18]. It is very difficult to identify the quality of developing hybrid seeds with the naked eye. Whether reproductive barriers exist in the interspecific hybridization of Chinese orchids or not is still unclear. Furthermore, a better understanding of zygotic embryogenesis would provide insights into subsequent germination events [12]. It is necessary to investigate the development of hybrid seeds anatomically, especially the process of embryo development.

C. faberi and *C. sinense* are two representative species of Chinese orchids. *C. faberi* is usually tolerant to cold and drought stress, with fragrant flowers and unique petal morphology, whereas *C. sinense* always comprises many elite cultivars differing from each other in flower color and leaf morphology. Interspecific hybridization was carried out using *C. faberi* as the maternal parent and *C. sinense* as pollen donor. Information regarding hybrid embryo development from fertilization to mature stage was detailed with the primary objective of investigating the compatibility of *C. faberi* × *C. sinense*, and finding the main corresponding factors affecting seed germination *in vitro* so as to maximize the germination percentage of hybrid seeds. The aim was to combine the flower color of *C. sinense* with the flower fragrance and extreme stress tolerance of *C. faberi* in the future.

MATERIALS AND METHODS

Plant materials

C. faberi 'Jiepeimei' ($2n = 2x = 40$) and *C. sinense* 'Qijianheimo' ($2n = 2x = 40$) were used in this study. Both cultivars were grown in pots in the greenhouse of the Jiangsu Lixiahe Region Research Institute of Agricultural Sciences in Yangzhou, Jiangsu province, China (32°24′ N, 119°26′ E), under a day/night temperature of 25/15°C, a 14/10-h (light/dark) photoperiod, light intensity of 50 μmol m^{-2} s^{-1} and 70% relative humidity. *C. faberi* 'Jiepeimei' (Fig. 1A), with 7-9 green flowers growing on the scape, was fragrant and tolerant to cold stress, whereas the flower color of *C. sinense* 'Qijianheimo' was purple (Fig. 1B). The anthesis of both cultivars occurs during March-April. It should be possible to obtain genotypes with novel traits by carrying out interspecific hybridization between the two cultivars. In order to collect a sufficient number of developing fruits for the present experiment, multiple plants of each cultivar were used. All the plants were propagated vegetatively by offshoots produced from the nodes of older shoots.

Interspecific hybridization

Artificial pollination was carried out by using *C. faberi* 'Jiepeimei' as the maternal parent and *C. sinense* 'Qijianheimo' as pollen donor. When the flowers of *C. faberi* 'Jiepeimei' opened fully, the stamens on the top of the gynandrium were removed before the anthers dehisced, and then the stigmas were pollinated

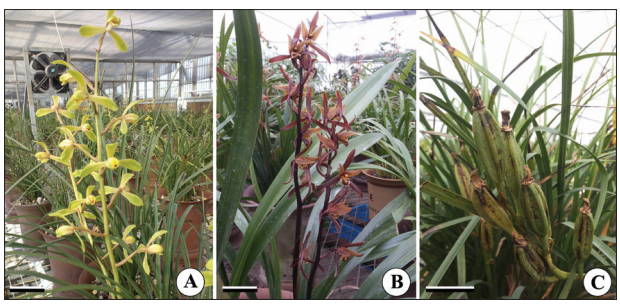

Fig. 1. Plants of *C. faberi* 'Jiepeimei' (**A**) and *C. sinense* 'Qijianheimo' (**B**) and the hybrid fruits of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' (**C**). Scale bar = 2 cm.

manually with fresh pollinia collected from *C. sinense* 'Qijianheimo' between 08:00-10:00 on sunny days. After pollination, bagging treatment was conducted on the pollinated flowers to avoid the visiting of insect pollinator, and the remaining unpollinated flowers on the same scape were eliminated.

Examination of fruit set and fruit quality

The fruit set was examined in 40 pollinated flowers from 5 plants for three repeats in three consecutive years of 2012, 2013 and 2014 to investigate whether it was stable in the cross behavior between *C. faberi* 'Jiepeimei' and *C. sinense* 'Qijianheimo' or not. At the beginning of November, the fruit set was calculated using the formula: fruit set = (the number of mature fruit/ the total number of pollinated flowers) × 100%. The fruit quality was evaluated through the measurements of fruit length, fruit diameter and seed weight in nine mature fruits selected randomly from three repeats.

Observation of embryo development

In 2014, developing ovaries and fruits were collected from the end of March to the beginning of November at regular intervals. Approximately 40 developing fruits were gathered after pollination, cut into 3-4-mm thickness, immediately immersed in FAA (1:1:18 formalin: acetic acid: 70% ethanol), and then stored at 4°C until use for the examination of embryo development. The developing ovaries were dehydrated through a graded series of alcohol solutions (70, 85, 95, and 100%, 2 h each), infiltrated with xylene (3 h) and embedded in paraffin wax [19,20]. Sections were cut into a thickness of 5-7 μm using a Leica RM2016 microtome (Shanghai Leica Instruments Co Ltd, China), stained with Heidenhain's hematoxylin and safranin combination. Digital images were observed and photographed using a CCD camera attached to an Olympus CX31 microscope. At each developmental stage, we collected about 100 ovules for determination of the ratio of abnormal ovules.

In addition, after the early globular embryo formed, the hybrid embryos at different developmental stages were also subjected to transmission electron microscopy (TEM), as carried out after Wang et al. [21] and Lee et al. [22] with some modifications. The fresh developing ovules were immediately immersed in 2.5% (v/v) glutaraldehyde (in 0.1 M phosphate buffer, pH 6.8) and stored at 4°C until use. Then the materials were post-fixed in 1.5% osmium tetroxide for 5 h after three 15-min rinses with the same phosphate buffer. Through a graded series of PHEM buffer (60 mM pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.8) and ethanol solutions, the materials were embedded in Epon 812. Ultrathin sections (70-80 nm) were cut using an LKB-V ultramicrotome (Bromma, Sweden) and stained with uranyl acetate and lead citrate. The sections were then examined and imaged under a transmission electron microscope (Hitachi H-7650) operated at 80 kV.

Seed harvesting and *in vitro* germination

In this experiment, after the early globular embryos were formed (90 DAP), the fruits were harvested at 15-day intervals in three consecutive years of 2012, 2013 and 2014 to confirm the validity of our findings. After decontaminating by dipping in 70% ethanol for 30 s, the fruit surface was sterilized with 1% sodium hypochlorite and then rinsed with sterile distilled water. Seeds were extracted from the capsules with forceps and directly sown onto the gelled KC medium [23] in 80×100-mm tissue culture bottles dispensed with 50 mL of medium. The medium was supplemented with 30 g L^{-1} sucrose and solidified with 7g L^{-1} agar. The pH value was adjusted to 5.8 and media were sterilized by autoclaving at 121°C (0.1 MPa) for 20 min. At each sampling date, 5 capsules were harvested. Approximately 100 seeds collected from each individual capsule were assigned to 5 replicates. After sowing, the culture bottles were incubated in complete darkness at 25°C for 180 days. Germination was defined as emergence of the embryo from the testa. The germination ratio was determined using the formula: germination ratio = (the number of germinated seeds/ the total number of sowed seeds) × 100%.

Statistical analysis

The data were subjected to one-way analysis of variance using SPSS 16.0 (SPSS Inc, Chicago, IL, USA) and presented as the mean value ± standard error. Duncan's multiple range test was employed to detect differences between means (with a level of significance of 0.05).

RESULTS

Fruit set and fruit quality

The cross behavior of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' was stable (Table 1, Fig. 1C). At 30 DAP, most of the ovaries began to enlarge and more than 90% of ovaries developed further into full fruits. The fruit set, fruit length, fruit diameter and seed weight showed no significant difference in the three consecutive years 2012-2014.

Embryo development

Major developmental events occurring within the hybrid ovaries of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' are shown in Table 2. At 60 DAP, most ovules were fertilized. The embryo sac contained an ovoid zygote and a complex that comprised of an endosperm nucleus and a chalazal nucleus (Fig. 2A). The zygote was characterized by a large nucleus, within which a prominent nucleolus was located, whereas the complex appeared as a distinct structure within the endosperm cavity. As a consequence of the metaphase plate being located toward the chalazal end (Fig. 2B), the zygote underwent mitosis asymmetrically and gave rise to a 2-celled embryo with a smaller apical cell and a larger basal cell (Fig. 2C). The apical cell had dense cytoplasm and a prominent nucleus (Fig. 2C). The division of the basal cell resulted in the formation of a 3-celled embryo (Fig. 2D) and was followed by division of the apical cell, resulting in a 4-celled embryo (Fig. 2E). The two basal cells developed into suspensor cells, while the two apical cells underwent further divisions. The apical cell near the micropylar pole gave rise to additional suspensor cells and that near the chalazal pole developed into embryo proper cells (Fig. 2F). The embryo proper cells had dense cytoplasm while the suspensor cells were vacuolated (Fig. 2F). The suspensor cells then expanded by the process of vacuolation and the three basal ones extended largely towards the micropylar end (Fig. 2G). Soon after the suspensor cells extended beyond the micropylar opening of the inner integument, two apical embryo proper cells continued to divide, which resulted in the formation of a uniseriate embryo (Fig. 2H). The endosperm complex began to degenerate (Fig. 2H). At the stage of proembryo formation by the oblique division of the terminal embryo proper cells (Fig. 2H, 2I), the suspensor cells were squeezed against the outer seed coat surrounding the proembryo by extending toward either the micropylar or the chalazal end, and the endosperm complex could no longer be observed (Fig. 2I).

According to anatomical observations, the development of the hybrid embryo proper could be divided

Table 1. Fruit set and fruit quality in the cross of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'.

Year	Fruit set (%)	Fruit length (cm)	Fruit diameter (cm)	Seed weight (g)
2012	91.67±1.44a	4.53±0.14a	1.68±0.07a	0.150±0.026a
2013	91.67±2.89a	4.49±0.11 a	1.68±0.08a	0.149±0.024a
2014	90.83±2.89a	4.48±0.16a	1.67±0.08a	0.145±0.024a

Values represent means±SE, and the same letters within the row indicate no significant differences at P±0.05 according to Duncan's test.

Table 2. Major developmental events occurring in the developing fruits of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' after fertilization.

DAP	Developmental stage of ovules	Percentage of abnormal ovules (%)
60	Zygote	9.5±1.4 a
70	2-4-celled embryo	15.1±2.1b
80	Proembryo	17.4±0.5 c
90	Early globular embryo	18.8±0.7 c
110	Globular embryo	21.4±1.0 d
130	Late globular embryo	23.7±1.2 e
160	Mature globular embryo	26.1±0.6 f

Values represent means ± SE, and different letters indicate significant differences at $P<0.05$ according to Duncan's test. DAP = days after pollination.

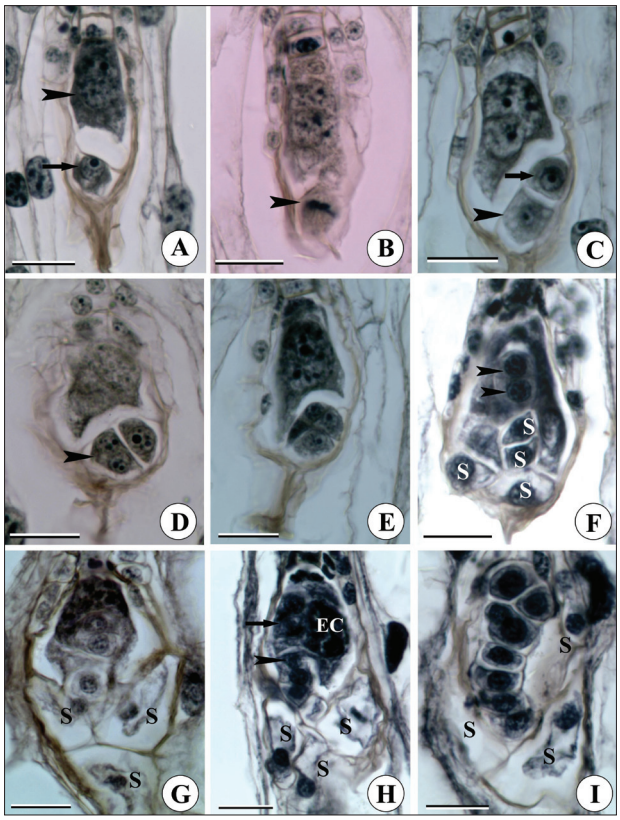

Fig. 2. Micrographs of proembryo development of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'. **A** – light micrograph of the embryo sac at 60 DAP, showing an ovoid zygote (arrow) and a complex (arrowhead) comprised of an endosperm nucleus and a chalazal nucleus. **B** – light micrograph of the zygote undergoing mitosis asymmetrically with the metaphase plate located toward the chalazal end (arrowhead). **C** – light micrograph of a 2-celled embryo with a smaller apical cell (arrow) and a larger basal cell (arrowhead). **D** – light micrograph of a 3-celled embryo with the larger basal cell undergoing division (arrowhead). **E** – light micrograph of a 4-celled embryo. **F** – light micrograph of the vacuolated suspensor cells (S) and the embryo proper cells (arrowhead) with dense cytoplasm; **G** – vacuolation and expansion of suspensor cells (S) with three basal ones extending largely toward the micropylar end. **H** – a uniseriate embryo (arrowhead) is formed by the divisions of the two apical embryo proper cells. One of the apical embryo proper cells divides obliquely (arrow). The endosperm complex (EC) begins to show degeneration. **I** – light micrograph of a proembryo and the suspensor cells (S) that surrounds the proembryo tightly by extending beyond the inner integument toward either the micropylar or the chalazal end. The endosperm complex is totally degenerated. Scale bar = 20 μm.

into 4 stages: early globular embryo, globular embryo, late globular embryo and mature stage. At 90 DAP, the early globular embryos, which were 8-10-cells long and 5-7-cells wide, were formed due to the cell division of proembryo cells. The suspensor cells began to degenerate and the degenerated products surrounded the embryo (Fig. 3A). Ridge-like structures were located on the epidermis of the outer seed coat, spaced at regular intervals. The top of the ridges began to appear cutinized (Fig. 3B). At 110 DAP, the globular embryos were formed. The internal cells of embryo proper became enlarged and showed a denser staining of nucleus and cytoplasm than that in the early globular embryo cells (Fig. 3C). The inner seed coat derived from the inner integument could be observed enveloping the embryo body (Fig. 3C). The degenerated products of suspensor cells appeared scarcely and only filamentary structures remained, connecting the outer seed coat with the embryo body (Fig. 3D). Safranin staining indicated that cuticular substance had accumulated in the outer seed coat (Fig. 3D). At 130 DAP, globular embryos developed further into the late stage. The high intensity of staining in embryo proper cells demonstrated that cell inclusion increased greatly (Fig. 3E). The inner seed coat toward the chalazal end appeared partially cuticularized, whereas no cuticular substance was detected at the micropylar end (Fig. 3E, 3F). At 160 DAP, the globular embryos had already matured. The inclusion of embryo proper cells increased further, resulting in a remarkable enlargement of the embryo body (Fig. 3G). The outer seed coat including ridges, as well as the inner seed coat, became largely cuticularized, appearing denser and thicker than in the former three stages (Fig. 3G, 3H). The inner seed coat appeared as a cuticle layer enclosing the embryo proper tightly (Fig. 3H). The filamentary structures could no longer be observed (Fig. 3H).

Most of the hybrid embryos could develop normally into the mature stage. Abortions mainly occurred at the zygote stage and the 2-4-celled embryo stage, which showed that the ovules could not be fertilized successfully (Fig. 4A) or the embryo cells failed to divide normally (Fig. 4B). Once the proembryos were formed, rare aborted embryos could be observed.

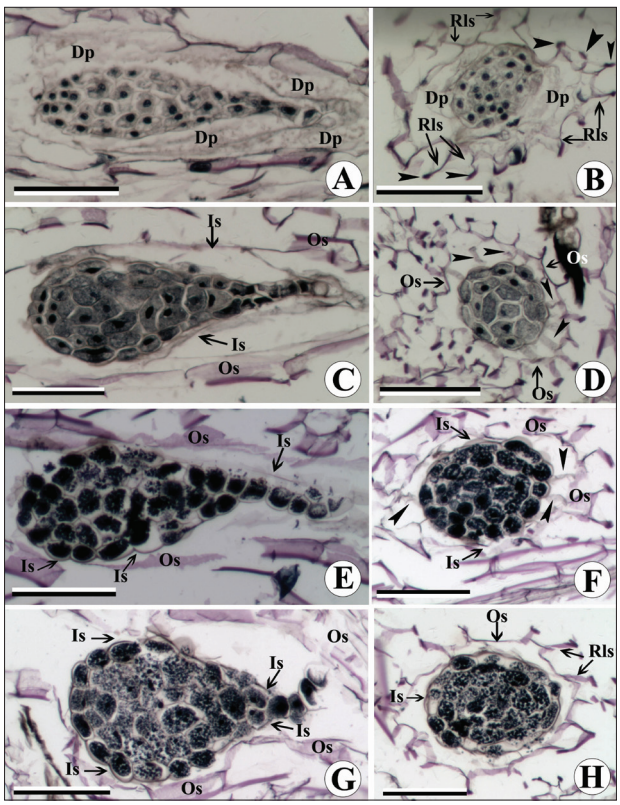

Fig. 3. Histological micrographs of embryo proper development of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'. **A** – light micrograph of the early globular embryo, which is 8-10-cells long and 5-7-cells wide, at 90 DAP. The degenerated products (Dp) derived from suspensor cells surrounds the embryo. **B** – ridge-like structures (Rls) with cutinized top (arrowhead) are located on the epidermis of the outer seed coat. Dp, degenerated products. **C** – light micrograph of the globular embryo at 110 DAP, showing the enlarged embryo proper cells with denser staining of nucleus and cytoplasm. The inner seed coat (Is) is visible enclosing the embryo body. Os, outer seed coat. **D** – light micrograph of the filamentary structures (arrowhead), connecting the seed coat with the embryo body. Arrows show the accumulation of cuticular substances in the outer seed coat (Os). **E** – light micrograph of the late globular embryo, showing a high intensity of staining in embryo proper cells (130 DAP). The inner seed coat (Is) toward the chalazal end begins to appear cuticularized. Os, outer seed coat. **F** – the inner seed coat (Is) surrounding the embryo body appears partially cuticularized and some filamentary structures are still visible (arrowhead). Os, outer seed coat. **G** – micrograph of the mature globular embryo (160 DAP), showing the large increase of inclusions in embryo proper cells and the remarkable enlargement of embryo body. Is, inner seed coat; Os, outer seed coat. **H** – micrograph of the outer seed coat (Os) and the inner seed coat (Is) at 160 DAP, showing the denser and thicker accumulation of cuticular substance. The filamentary structures are disappeared. Rls, ridge like structures. Scale bar = 100 μm.

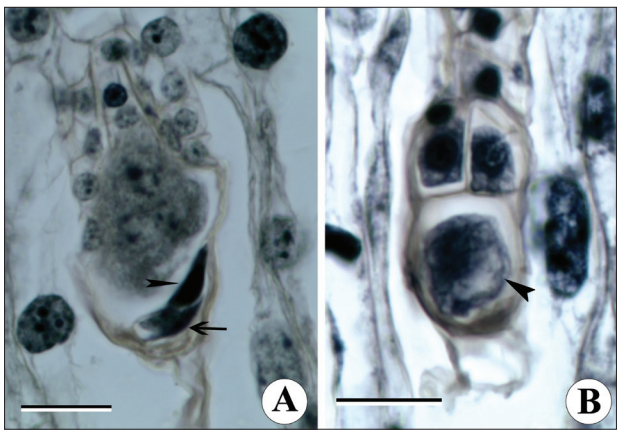

Fig. 4. Abnormalities occurring in embryo development of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'. **A** – micrograph of the embryo sac at 60 DAP, showing the degeneration of unfertilized egg cell (arrowhead) and a synergid (arrow). **B** – micrograph of the 3-celled embryo, showing vacuolated basal cell (arrowhead) that can't divide normally. The apical cell divides first and no endosperm complex can be observed in the sac. Scale bar = 20 μm.

The percentage of abnormal ovules at different developmental stages is shown in Table 2.

Ultrastructural changes during embryo proper development

Ultrastructural observations revealed changes in the distribution of organelles within the cells of the embryo proper at different stages of development. At the early globular embryo stage (90 DAP), different organelles were distributed evenly around the nucleus of the embryo proper cell (Fig. 5A), including plastids, rough endoplasmic reticulum (RER), ribosomes, dictyosomes, mitochondria, lipid bodies and small vacuoles. RER and ribosomes were abundant, while dictyosomes and lipid bodies were scarce (Fig. 5B, 5C). Wall ingrowths were present and the plasmodesmata were easily seen in the common walls of two adjacent embryo proper cells. No starch grains could be observed in plastids at this stage (Fig. 5B, 5C). At the globular embryo stage (110 DAP), large vacuoles were formed (Fig. 5D). Starch grains began to accumulate in plastids (Fig. 5D), and then the organelles and cytoplasm showed an uneven distribution (Fig. 5E). Most cytoplasm and organelles were located around the embryo proper cell nucleus (Fig. 5F). Only a few

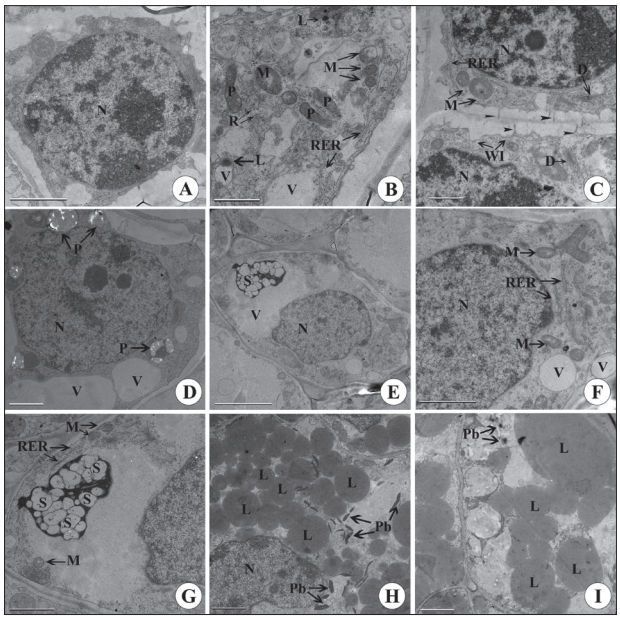

Fig. 5. Ultrastructural observations during embryo proper development of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'. **A** – electron micrograph of the embryo proper cell at 90 DAP, showing different organelles distributed evenly around the nucleus (N). Scale bar = 2 μm. **B** – higher magnification of **A**, showing several mitochondria (M), plastids (P) without starch grains, rough endoplasmic reticulum (RER), ribosomes (R), lipid bodies (L) and small vacuoles (V) in the cytoplasm. Scale bar = 1 μm. **C** – electron micrograph of two adjacent embryo proper cells showing dictyosomes (D) in the cytoplasm. Wall ingrowths (WI) are present and the two cells are interconnected by the plasmodesmata (arrowhead). M, mitochondria; N, nucleus; RER, rough endoplasmic reticulum. Scale bar = 1 μm. **D** – electron micrograph of the embryo proper cell at 110 DAP, showing the accumulation of starch grains in the plastids (P). N, nucleus; V, vacuole. Scale bar = 2 μm. **E** – electron micrograph showing that the organelles and cytoplasm are distributed unevenly. N, nucleus; S, starch granule; V, vacuole. Scale bar = 5 μm. **F** – higher magnification of **E**, showing that the cytoplasm and organelles are distributed mainly around the embryo proper cell nucleus (N). M, mitochondrion; RER, rough endoplasmic reticulum; V, vacuole. Scale bar = 2 μm. **G** – higher magnification of **E**, showing a few RER, mitochondria (M) and a plastid (P) full of starch grains (S) located by the side of the big vacuole (V). RER, rough endoplasmic reticulum. Scale bar = 2 μm. **H** – electron micrograph of the embryo proper cell at 130 DAP, showing the abundant lipid bodies (L) of various sizes and the protein bodies (Pb) distributed sporadically. Most of the organelles can't be observed any more. N, nucleus. Scale bar = 2 μm. **I** – electron micrograph of the mature embryo proper cell at 160 DAP. Lipid bodies (L) develop into larger ones while few small protein bodies (Pb) are readily found. Scale bar = 2 μm.

cisterns of RER and mitochondria, as well as plastids full of starch granules, were observed at the side of the big vacuole (Fig. 5G). The amounts of organelles, especially the RER and ribosomes decreased. At the late globular embryo stage (130 DAP), starch-containing plastids gradually disappeared, whereas the storage materials, i.e. lipid and protein bodies, began to accumulate. Lipid bodies were abundant and of various sizes (Fig. 5H), while protein bodies were distributed sporadically in the cytoplasm. Most organelles could not be observed any more (Fig. 5H). At 160 DAP, the embryo proper became fully matured. Few protein bodies were readily found. The lipid bodies developed into larger ones and became the main storage materials (Fig. 5I).

Effect of harvesting time on germination *in vitro*

The *in vitro* germination ratio of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' hybrid seeds was significantly affected by harvesting time, which showed a great degree of similarity in three consecutive years of 2012, 2013 and 2014 (Table 3). No germination could be observed at 90 and 105 DAP. Seeds germinated poorly at 120 DAP, while the germination percentage showed a significant increase in the 135- and 150-DAP seeds (from 3% to 17%). The highest seed germination percentage was observed in 150 DAP seeds (≈17%), significantly higher than any other harvesting time. After 150 DAP, the seed germination percentage decreased significantly. Rare germination could be observed at 165 and 180 DAP. After 195 DAP, the germination percentage decreased further to almost 0%.

DISCUSSION

In orchids, distant hybridizations are always evaluated by fruit sets because of the large number and tiny seeds. It is difficult to distinguish the quality of developing hybrid seeds visually. Interspecific hybridizations among species of Chinese orchids are considered to be easy because of high fruit sets [24]. However, little information is available on the development of hybrid seeds. It is still unclear whether the

Table 3. Effect of harvesting time on *in vitro* germination of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' hybrid seeds.

Year	Germination ratio (%)								
	90 DAP	105 DAP	120 DAP	135 DAP	150 DAP	165 DAP	180 DAP	195 DAP	210 DAP
2012	0 e	0 e	3.15±1.82 d	10.72±1.06 b	16.38±2.10 a	7.84±1.16 c	6.82±2.80 c	3.27±1.08 d	0.78±0.82 e
2013	0 e	0 e	2.56±1.26 d	13.99±2.36 b	16.91±3.71 a	7.14±0.65 c	6.92±2.22 c	3.18±1.10 d	0.36±0.49 e
2014	0 e	0 e	2.68±0.82 d	10.46±3.28 b	17.12±5.71a	6.16±1.14 c	6.30±2.06 c	2.71±1.26 d	0.31±0.68 e

Values represent means±SE, and different letters within the same row indicate significant differences at P<0.05 according to Duncan's test. DAP = days after pollination.

hybrid seeds in the pods develop normally or not. In the present study, there was no significant difference in the cross behavior of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' in three consecutive years, which showed that the fruits were full and the seeds were of large amounts. Ovule development is always triggered by pollination in orchids [25]. As a result, most of the ovules can accomplish the process of macrogametogenesis, which was also the case in our study. However, a cross barrier can be caused by many factors, such as pollen viability, pollen-pistil interaction, fertilization and embryo development [26-29]. The post-fertilization processes are always associated with seed quality. Therefore, we focused on the hybrid embryo development from fertilization to mature stage with the aim to investigate whether the hybrid seeds develop normally or not in the cross of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'. The anatomical results indicated that most of the abnormalities occurred at the zygote and 2-4-celled embryo stages. Although the percentage gradually increased, only 26.1% of abnormal ovules could be observed and most of the hybrid embryos could develop normally into the mature stage. Therefore, it is feasible to obtain plenty of fertile hybrid seeds by carrying out the hybridization of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'. Generally, cross compatibility requires a certain chromosomal or genomic balance, so that crosses are more likely to be successful when the ploidies of the parents are similar [26,30]. The parents in our research were both diploid species of *Cymbidium*, which may be mainly responsible for the high cross compatibility.

In Chinese orchids, although more and more researches have revealed that harvesting time is crucial for seed germination *in vitro* [2, 11], little information is available about embryo development characteristics when the seeds are excised for culture. In the present study, seed germination was initiated at 120 DAP, at which stage the embryo was globular and starch grains began to accumulate in plastids. It was reported that germination in *Cypripedium formosanum* could be achieved by cultivating immature seeds at the proembryo stage [12]. However, in our study no germination was observed at the early stages of 90 DAP and 105 DAP. Although abundant organelles are readily found at 90 DAP, the accumulation of storage materials at 110 DAP seems to be more essential for the germination of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo' hybrid seeds when placed on culture media. Germination significantly increased at 135 DAP and maximized at 150 DAP. During this period, the hybrid embryos developed into the late globular stage. The storage materials, i.e. lipid and protein bodies, began to accumulate. In orchids, an endosperm fails to develop during seed development [18,31]. Consequently, the internal storage materials in an embryo proper may play an important role in seed germination *in vitro.* Besides, filamentary structures derived from suspensor cells were still persistent, connecting the outer seed coat and embryo proper body, which may serve as a potential conduit for nutrient flow from the maternal tissues and external environment and may provide unique metabolites for the growth of the embryo proper in the process of embryo germination *in vitro* [22,32]. A significant decrease in germination ratio occurred in the hybrid seeds collected at 165 DAP. Fully mature seeds always encounter more difficulties, such as the low permeability of the seed coat [33] and the presence of inhibitors in mature seeds [34]. In our study, according to the safranin staining, the inner seed coat toward the chalazal end began to show partial cuticularization, whereas no cuticular substance was detected at the micropylar end at 130

DAP. However, at the mature stage (160 DAP), the outer seed coat was totally cuticularized and the inner seed coat appeared as a cuticle layer enclosing the embryo proper tightly, which may be the main factor that causes the low permeability of the seed coat and inhibits the subsequent germination of hybrid seeds. Moreover, the lack of suspensor filamentary structures at late stages may be another important barrier to the uptake of water and nutrients from culture medium by the hybrid embryos.

CONCLUSIONS

We report on the embryonic development and corresponding factors affecting *in vitro* germination of the hybrid seeds of *C. faberi* 'Jiepeimei' × *C. sinense* 'Qijianheimo'. It is feasible to obtain sufficient amounts of fertile interspecific hybrid seeds because of high compatibility. The storage materials and the persistence of filamentary structures derived from suspensor cells are essential for the germination of hybrid seeds. The most favorable time for seed harvesting should be at 150 DAP, at which stage the outer and inner seed coats are not totally cuticularized and the seed germination percentage is maximized. The defined time frame is expected to support germplasm innovation of Chinese orchids.

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Authors' contributions: FL and XC designed the experiments. FL, YS, HM and TZ performed the experiments and analyzed the data. FL, XC, JB and CL wrote and revised the manuscript. All authors read and approved the final manuscript.

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