The long pollen tube journey and in vitro pollen germination of Phalaenopsis orchids

Jhun-Chen Chen$^{1,2}$ • Su-Chiung Fang$^{1,2}$

Key message  Pollen biology in P. aphrodite.

Abstract  Orchids have a distinct reproductive program. Pollination triggers ovule development and differentiation within flowers, and fertilization occurs days to months after pollination. It is unclear how pollen tubes travel through the developing ovaries during ovule development and when pollen tubes arrive at the mature embryo sac to achieve fertilization. Here, we report a robust staining protocol to image and record the timing of pollen germination, progressive growth of pollen tubes in ovaries, and arrival of pollen tubes at embryo sacs in Phalaenopsis aphrodite. The pollen germinated and pollen tubes entered the ovary 3 days after pollination. Pollen tubes continued to grow and filled the entire cavity of the ovary as the ovary elongated and ovules developed. Pollen tubes were found to enter the matured embryo sacs at approximately 60–65 days after pollination in an acropetal manner. Moreover, these temporal changes in developmental events such as growth of pollen tubes and fertilization were associated with expression of molecular markers. In addition, we developed an in vitro pollen germination protocol, which is valuable to enable studies on pollen tube guidance and tip growth regulation in Phalaenopsis orchids and possibly in other orchid species.

Keywords  Phalaenopsis aphrodite • Pollen tube • Reproductive development • Orchid • In vitro germination

Introduction

During the plant sexual cycle, pollen germinates on the stigma, and pollen tubes grow within the style and reach an embryo sac within a few hours to several days. Various modifications in reproductive systems have evolved in plants to coordinate pollination and fertilization and achieve successful reproduction. For example, pollen may travel as a single grain or as an aggregated entity. Pollen forms different types of aggregated entities called pollen dispersal units or PDUs (Pacini 1997). In angiosperms, Orchidaceae plants have the greatest number of PDU types and pollen is often packed in PDUs called pollinia (Pacini 2009; Pacini and Hesse 2002). The advantage of using PDU as a mode of fertilization is that it is highly effective with nearly all the ovules being fertilized and large amounts of seeds being produced by pollination.

In most angiosperms, the ovule structure and embryo sac have fully developed by the time pollen grains germinate. As a result, fertilization normally occurs hours after pollination (Christensen et al. 1997; Faure et al. 2002; Möl et al. 1994; Wu et al. 2011). In other plant species such as orchids and Fagales, female gametophyte development is either absent or incomplete before pollination (Liu et al. 2014; O’Neill 1997; Pimienta and Polito 1983; Sogo and Tobe 2006a, b). For these plants, pollination is important to trigger or regulate embryo sac development and ovule
maturation that subsequently allows fertilization. Orchid species provide notable examples of this kind of modified reproduction system (Arditti 1992; Yeung and Law 1997; Zhang and O’Neill 1993). For example, pollination triggers initiation and development of ovules that are absent in unpollinated ovaries of *Cattleya*, *Phalaenopsis*, and *Dendrobium* (Duncan and Curtis 1943; Israel and Sagawa 1964; Zhang and O’Neill 1993). In *Cyripedium* and *Paphiopedilum*, pollination triggers development of the ovule primordial that is present but arrested in the premeiotic stage before pollination (Duncan and Curtis 1942). It has been proposed that pollination-triggered female reproductive development ensures efficient investment in megagametophyte and ovary maturation for fertilization only after the low probability occurrence of pollination by highly specified pollinators (O’Neill 1997). In these cases, pollination not only provides paternal nuclei that contribute to the genetic makeup of the zygote, it also serves as a primary signal to coordinate developmental events required for successful fertilization (Zhang and O’Neill 1993).

Because of extended temporal separation between pollination and fertilization, germinated pollen and pollen tubes have to survive a significant period of time in the ovaries before entering the mature embryo sacs. It has been reported that this period of time can last as little as 4 days for *Gastrodia elata* to 10 months for *Vanda suavis* (Arditti 1992). However, the timely tracking of growth of the pollen tube between pollination and fertilization in orchid ovary remains limited. One reason for this is the lack of a reliable staining protocol to monitor pollen tubes in ovaries. Here, we report a robust pollen tube staining protocol that enables timely observation of the progression of pollen tube elongation in the developing ovaries of *Phalaenopsis aphrodite*. Using this protocol, we were able to pinpoint the timing when pollen tubes entered the matured ovaries. Pollen tubes are one of the best model systems for cellular process studies involved in polarity and tip growth (Kri-chevsky et al. 2007; Qin and Yang 2011; Yang 2008). To this end, we established an in vitro pollen germination and tube growth system, which will be valuable for studies of cell growth and morphogenesis of orchid pollen in the future.

**Materials and methods**

**Plant materials and growth conditions**

*Phalaenopsis aphrodite* subsp. *formosana* (m1663, tetraploid) seedlings in 2.5- or 3-inch pots were purchased from Chain Port Orchid Nursery (Ping Tung, Taiwan). Plants were grown in a growth chamber with alternating 12 h light (23 °C)/12 h dark (18 °C) cycles to induce flowering. The floral stalks (~0.5 to 1 cm long) became visible approximately 2 months after moving into the growth chamber. The first open flower appeared approximately three to 4 months after moving into the growth chamber. Flowers were hand pollinated 1 week after flower opening. Pollinia used for in vitro germination were taken from flowers from fully blooming flower spikes. For hand pollination, the pollinium was put in the stigmatic cavity. Following pollination, pollen grains adhered to the stigmatic cells. The stigma closed and then swelled to completely enclose the pollinium 1 day after pollination (DAP). The ovary started to develop and enlarge in size (Fig. 1a).

**Tissue fixation and pollen tube staining**

Capsules, the developing ovaries, were cut open longitudinally and dissected transversely into approximately 3-cm-long segments. The capsule segments were fixed in 8:1:1 of 80 % ethanol, glacial acetic acid, and formalin solution at 4 °C overnight. The fixed samples were subsequently rehydrated by passing through 70, 50, and 30 % ethanol for 10 min and left in H2O at 4 °C overnight. Samples were cleared in 8 N NaOH at room temperature overnight, washed three times with H2O, and left in H2O at room temperature overnight. Aniline blue staining of germinated pollen tubes was performed as previously described (Martin 1959) with slight modifications. The capsule segments were stained with 0.1 % aniline blue in 0.1 M K3PO4 buffer and 2 % glycerol (v/v) in the dark overnight. The stained segments could be kept in the aniline blue staining solution at 4 °C for at least a couple of weeks without tissue deterioration.

**Microscopy imaging**

LSM 710 confocal microscope (Zeiss) was used to score the arrival of pollen tubes at the micropyle end of ovaries and to image pollen germination on the stigma. Aniline blue fluorescence was excited using the 405 nm laser line from a diode laser and the emitted light was filtered through a 410–471 band-pass filter. The callose staining pollen tubes were photographed under a 40 × C-Apochromat lens or a 100× Plan-Apochromat lens.

Distribution of aniline blue-stained pollen tubes in the developing capsules was photographed on a Zeiss Axio Scope A1 fluorescence microscope under a 2.5 × Plan-Neofluar lens or 5 × N-Achroplan lens with an AxioCam HRc camera (Zeiss).
Fig. 1  Aniline blue staining of pollinia and pollen. a Structure of a *P. aphrodite* flower (complete and dissected); pollinia attached to the viscidium; and ovaries before and after pollination. White arrows point to the ovaries at 0 and 15 days after pollination (DAP). Notice the enlargement of ovary at 15 DAP. b Images showing aniline blue staining of pollinia 3 h after pollination (HAP), 1, 2, and 3 days after pollination (DAP). White arrowheads point to the pollinia. White arrow points to the germinated pollen tubes. c Image showing developing capsule and distribution of pollen tubes in ovary collected at 15 days after pollination (DAP). Aniline blue staining images were assembled from separate images of the same ovary. High magnification images of the indicated areas are shown at the bottom of the assembled ovary. d Image showing developing capsule and distribution of pollen tubes in an ovary collected at 30 days after pollination (DAP). Aniline blue staining pictures were assembled from separate images of the same ovary. High magnification images of the indicated areas are shown at the bottom of the assembled ovary. Black scale bar 500 μm. White scale bar 500 μm. Red scale bar 0.5 cm.
In vitro germination of pollen tubes

Stigma extract was prepared as previously described (Chen and Jiang 2014) with slight modification. Briefly, the stigma was removed and sterilized by 70 % EtOH for 20 s. Five removed stigma were vacuum drawn in 3 ml sterilized H2O to obtain soluble stigma extract. The stigma extract was filtered by 100-micron nylon mesh (Fisher Scientific, USA) and 100 l of filtered stigma extract was added into 1 ml germination media described below. Pollinia were briefly sterilized by 0.05 % NaClO and rinsed with sterilized H2O. Pollinia were then squashed into small pieces before adding into the germination medium. Pollen tubes were allowed to germinate in modified BK medium containing 100 mg/l H3BO3, 100 mg/l CaCl2·2H2O, 100 mg/l MgSO4·7H2O, and 100 mg/l KNO3 supplemented with 10, 20, or 30 % sucrose, pH 5.7 (Tsai and Chang 2010), or in 10, 20, or 30 % sucrose solution.

For acetocarmine staining, fresh pollen and germinated pollen tubes were directly fixed and stained in acetocarmine solution containing 10 % acetic acid and 2 % carmine (Tokyo Chemicals Industry, Japan) on a microscope slide before visualization. To determine the growth rate of the pollen tube, the fixed pollen tubes were photographed 10, 20, or 30 day after incubation. The length of pollen grains and pollen tubes was measured by ImageJ software (Abramoff et al. 2004). At least 50 pollen tubes were measured at each time point. Thirty-two pollen grains were measured to determine the average length of a pollen grain. The average length of a pollen grain is 17 µm.

Scanning electron microscopy

Samples were fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde in 67 mM PBS buffer (pH 7.0). A vacuum was applied to remove air bubbles from tissues. Samples were incubated in the fixative at 4 °C overnight. After washing the samples with PBS buffer, they were incubated in 1 % OsO4 at room temperature for 4 h followed by washing with PBS buffer. Samples were then dehydrated in an ethanol series, critical point-dried with a critical point dryer (Hitachi HCP-2, Japan), sputter-coated with gold and platinum with Hitachi E-1010 ion sputter (Japan), and observed under a scanning electron microscope (FEI Quanta 200, USA) with an accelerating voltage of 20 kV.

Sample collection and RNA extraction

Orchid flowers were hand pollinated, and developing ovaries were harvested on a specified day. For reproductive tissues, only the interior tissues of developing capsules were scooped and pooled for RNA extraction (Lin et al. 2014). Protocorm-like bodies (PLBs) and protocorms were collected. The samples were flash frozen in liquid nitrogen and stored in a freezer at −80 °C. For samples used in semi-quantitative RT-PCR, total RNA was isolated using OmicZol™ RNA Plus extraction reagent (Oomics Bio) according to the manufacturer’s instructions. For samples used in quantitative RT-PCR, total RNA was isolated using TRIzol reagent (Invitrogen) followed by purification using Direct-Zol RNA MiniPrep kit (Zymo Research) according to the manufacturer’s instructions. The isolated total RNA was treated with RNase-free DNase (Qiagen) followed by RNeasy mini-column purification according to the manufacturer’s instructions (Qiagen).

Quantitative and semi-quantitative RT-PCR

RNA was reverse transcribed in the presence of a mixture of oligo dT and random primers (9:1 ratio) using the GoScript Reverse Transcription System (Promega) as described previously (Lin et al. 2014). Ten microliters of quantitative RT-PCR reaction contained 2.5 µl of 1/20 diluted cDNA, 0.2 µM of primers, and 5 µl of 2 × KAPA SYBR FAST master mix (KAPA Biosystems). The following program was used for amplification: 95 °C for 1 min, 40 cycles of 95 °C for 5 s, and 58 or 60 °C for 20 s. PCR was performed in triplicate, and the experiments were repeated with RNA isolated from two independent samples. Primer pairs and the specified annealing temperature used for quantitative PCR are listed in Table 2. Ubiquitin (PaUB11) was used as an internal control (Lin et al. 2014).

For semi-quantitative RT-PCR, twenty microliters of PCR reaction contained 1 µl of 1/2 diluted cDNA, 0.5 µM of primers, 80 µM dNTP, 3 % DMSO, and 0.2 µl of Power Taq DNA polymerase (Genomics BioSci & Tech, Taiwan). The following program was used for amplification: 95 °C for 2 min, 36 cycles of 94 °C for 25 s, 60 °C for 25 s, and 72 °C for 40 s. Primer pairs and the specified annealing temperature used for semi-quantitative PCR are listed in Table 2. The Genbank accession numbers of PaC13L, PaLiM1, and PaEG1L1 are KU213915, KU213916, and KU213917, respectively (Table 1).

Results

Pollen germination and progressive growth of the pollen tube during pollination-induced ovary development in P. aphrodite

For most angiosperms, ovules and embryo sacs have reached maturity by the time of pollination. Ovule development of orchids on the other hand is pollination-dependent. The progressive development of ovule and embryo structures after pollination in Phalaenopsis orchids has
been reported (Chen et al. 2012; Tsai et al. 2008). How-
however, the coordinated pollen germination and develop-
mental processes are relatively limited. To monitor the
growth of the pollen tube, aniline blue staining followed by
confocal microscope imaging was used to visualize pro-
gressive growth of the pollen tube within the developing
ovaries. Callose and the callose plug, commonly found in
pollen grains and pollen tubes, can be stained selectively
by water soluble aniline blue (Currier 1957).

Phalaenopsis aphrodite was chosen as the system for our study because it
is an important parental plant for commercial breeding
programs in Taiwan and its pollination event has not been
documented in detail.

Phalaenopsis aphrodite has small white-colored flow-
ers. Each flower has two petals, one dorsal sepal, and two
lateral sepals. The median petal is enlarged and modified to
become a lip or labellum. The male and female reproduc-
tive parts are fused together and become the gynostemium
or column (Fig. 1a). Pollen grains of Phalaenopsis orchids
are packed together as a pollinium. Two pollinia were
connected together and attached to a sticky viscidium, a
disk-like structure that sticks to visiting insects. Pollinia sit
on the top of the gynostemium under the anther cap (Fig. 1a).

Following hand pollination, the pollen germinated and
pollen tubes started to enter the ovary 3 days after polli-
nation (Fig. 1b), which is earlier than previously reported
(Zhang and O’Neill 1993). Fifteen days after germination,
pollen tubes appeared to be evenly distributed in the cav-
ities of both sides of the placenta and continued to grow as
ovaries enlarged (Fig. 1c). The pollen tube continued to
grow and distributed over the entire ovary cavities as the
ovary grew at 30 DAP (Fig. 1d). To gain information about
when pollen tubes were attracted to and reached the
micropyle ends of the ovules to complete fertilization,
aniline blue was used to track pollen tubes at 50, 60, 65,
and 70 DAP. Only very few pollen tubes grew toward the
micropyle ends of the ovules at 50 DAP (Table 1). The tips
of pollen tubes started to enter the embryo sacs (30.6 %) at
approximately 60 DAP, and the frequency reached up to
48.7 % at 65 DAP (Table 1; Fig. 2a). Intriguingly, fertil-
ization events seemed to occur in an acropetal manner with
micropyle-guided pollen tubes starting at the basal half (60
DAP) and gradually expanded to the upper half (65 DAP)
of the developing ovaries (Table 1). Only a few pollen
tubes showed aniline blue staining at 70 DAP, the point
when fertilization took place. The presence of pollen tubes
at the micropyle end of embryo sacs, despite its lack of
aniline blue staining, at 73 DAP was confirmed by scan-
ning electron microscopy analysis (Fig. 2b). Taken toge-
ther, our data provide evidence that most fertilization
events of P. aphrodite occurred between 65 DAP and 70
DAP.

In vitro pollen germination system

The relatively long journey [from pollen germination to
fertilization (Arditti 1992)] that orchid pollen tubes have to
undergo within ovaries before reaching the embryo sacs to
complete fertilization suggests that orchid pollen grains
likely undergo unique development and differentiation
processes. Establishment of an in vitro pollen germination

---

**Table 1** Frequency of embryo sacs containing the pollen tubes at 50, 60, and 65 days after pollination

|                  | No. of embryo sacs with pollen tubes | No. of embryo sacs without pollen tubes | Total no. of examined embryo sacs | % of embryo sacs with pollen tubes |
|------------------|--------------------------------------|-----------------------------------------|-----------------------------------|-----------------------------------|
|                  | Up M Low                              | Up M Low                                |                                   |                                   |
| 50 DAP           | 0 0 3                                | 33 32 37                               | 105                               | 3.0                               |
| 60 DAP           | 8 14 31                              | 59 30 31                               | 173                               | 30.6                              |
| 65 DAP           | 31 12 13                             | 33 22 4                                | 115                               | 48.7                              |

*Up* upper parts of ovaries, *M* middle parts of ovaries, *Low* lower parts of ovaries. At least two capsules were used.

**Table 2** List of primer pairs used for RT-PCR and qRT-PCR

| Gene name       | Forward primer  | Reverse primer  | Amplicon size (bp) | Annealing temp (°C) |
|-----------------|-----------------|-----------------|--------------------|--------------------|
| PaC13L          | 5’-CGCTGAGTTCCGTTGAGAACA-3’ | 5’-GGCATACTGGAAGTGCAACA-3’ | 125                | 58                 |
| PaLIM1          | 5’-AACAGAGCAAGCAAGTAAGGT-3’   | 5’-CGTAGCTGTATGGTGAGGA-3’   | 172                | 60                 |
| PaEC1L1         | 5’-GCCATAACTGCTCTCCCTGCTCAT-3’ | 5’-GTAGTCGATCACGGCCTCC-3’ | 113                | 60                 |
| PaUB1           | 5’-AATCCATCGCTCTCCCCTTCTT-3’  | 5’-TGAAGCAGTAGCAATTTCTC-3’ | 101                | 58, 60              |
| PaC13L          | 5’-CAATTCCAGAGAGGCAAAGG-3’     | 5’-CCCCATGATTTGGTTCTTGA-3’  | 618                | 60                 |
| PaLIM1          | 5’-TTCTGCTCCCGTTTGGAAAT-3’     | 5’-ATATTATGATCTCTTGTTGTTG-3’ | 647                | 60                 |
and growth protocol provides a powerful tool to study pollen tube guidance and tip growth regulation (Boavida and McCormick 2007; Rodriguez-Enriquez et al. 2013) and is therefore an important tool to gain new insight into orchid pollen biology. To this end, the dissected pollinia of *P. aphrodite* flowers were allowed to germinate in a modified Brewbaker and Kwack (mBK) medium (Tsai and Chang 2010) or sucrose solution (Boavida and McCormick 2007). Germination is defined as the germinated tube growing to at least twice the length of a pollen grain. However, under the tested germination conditions, pollen grains failed to germinate. Because stigma extract has been shown to enhance pollen germination efficiency (Allen and Hiscock 2010; Roberts et al. 1983), stigma tissue extract was then added into the mBK medium or sucrose solution for pollen germination. In the presence of stigma tissue extract, pollen was able to germinate successfully in 10 and 20 % sucrose solution (Fig. 3a). In the presence of 10 % sucrose, pollen tubes grew steadily and continued to elongate 60 days after incubation. Pollen tubes germinated in 20 % sucrose on the other hand failed to elongate and appeared swollen 60 days after incubation. Some of the swollen pollen tubes eventually ruptured and broke into pieces. In the presence of stigma tissue extract, pollen grains were also able to germinate in mBK medium supplemented with either 10 or 20 % sucrose (Fig. 3b). With 10 % sucrose-supplemented mBK medium, pollen tubes elongated at a moderate rate but not as rigorously as with 10 % sucrose. With 20 % sucrose-supplemented mBK medium, some of the germinated pollen tubes became swollen and failed to elongate. Pollen tubes failed to germinate in the rest of the tested stigma extract supplemented conditions.

---

**Fig. 2** a Aniline blue staining images showing pollen tubes and developing ovules at 50, 65, and 70 days after pollination (DAP). The ovules under differential interference contrast (DIC) are outlined by fine red lines for better visualization. The white asterisk indicates the entry of the pollen tube into the embryo sac. The white arrowheads indicate the stained pollen tube. White scale bar 10 µm. Red scale bar 20 µm. b Scanning electron microscope image showing the entry of the pollen tube to the micropyle end of the matured female gametophyte at day 73 after pollination (73 DAP). White arrow indicates the micropyle end. White scale bar 100 µm.

**Fig. 3** Pollen germinated 60 days after treatment under various conditions. a Pollen germinated in 0, 10, 20, and 30 % sucrose. b Pollen germinated in modified BK medium supplemented with 0, 10, 20, or 30 % sucrose. Scale bar 20 µm. Stigma tissue extract was added under all of the described conditions.
of incubation. The pollen tubes elongated slowly initially (from day 10 to day 20) and grew at a relatively fast rate from day 20 to day 30 (Fig. 4). There was large variation in length of pollen tube after 30 days of incubation. It is likely caused by unsynchronized germination of pollen grains and/or uneven elongation rate of individual pollen tubes.

Expression of pollen-specific and egg-specific genes is associated with growing pollen tubes and the timing of fertilization in *P. aphrodite*

Two genes potentially involved in cellular processes related to pollen tube growth and development were isolated by reverse transcription polymerase chain reaction (RT-PCR). They are genes encoding homologs of the LIM domain-containing proteins (*Papuga et al. 2010; Wang et al. 2008*) and the C13-like pollen protein (*Hanson et al. 1989*). Consistent with their potential functions, *PaLIM1* and *PaC13L* mRNAs were preferentially expressed in in vitro germinated pollen tubes (Fig. 5a). In addition, accumulation of *PaLIM1* and *PaC13L* mRNAs was preferentially enriched in interior ovary tissues from 30 to 70 DAP during which pollen tubes were actively growing (Fig. 5b). Their accumulation levels reached a peak at 30 DAP and gradually declined as fertilization reached completion. These data confirmed pollen-specific expression of *PaLIM1* and *PaC13L* mRNAs and showed a positive correlation between their temporal expression patterns with pollen tube activities in developing ovaries.

In addition to the pollen-specific gene, a potential egg-specific gene named *EGG CELL 1 Like 1* (*EC1L1*) gene was isolated. Arabidopsis *EC1* is an egg-specific marker gene that plays an important role to activate female and male gametes by regulating exocytosis and sperm plasma membrane modifications (*Sprunck et al. 2012*). To assess the timing when fertilization occurred after pollination, the expression pattern of *PaEC1L1* mRNA was monitored in developing ovaries by qRT-PCR. As shown in Fig. 5b, the accumulation of *PaEC1L1* mRNA reached a peak at 70 DAP which coincided with arrival of pollen tubes within the embryo sacs (Fig. 2). The positive correlation between expression of *PaEC1L1* and arrival of the pollen tube at the embryo sac further supports that fertilization occurs at approximately 65–70 DAP in *P. aphrodite*.

Discussion

The long journey orchid pollen tubes embark upon after pollination and before fertilization have long fascinated biologists. Using the established aniline blue staining protocol, in this study we showed that pollen grains do not germinate until 3 days after pollination. It is possible that vacuolization and separation of tetrads are a prerequisite for pollen grains within the pollinia to germinate (Pacini et al. 2002; Pandolfi and Pacini 1995). After germination, pollen tubes continued to grow and quickly filled the entire ovary cavities as ovaries developed and elongated. Despite the continuous elongation of the pollen tubes, growth of the pollen tubes did not orient toward the developing ovules until 60–65 days after pollination. Coincidently, ovules reached maturity, marked by expression of the egg-specific marker *PaEC1L1*, at approximately the same time (Fig. 5b). It is likely that signals produced from the matured ovules are required to guide the tip-growing pollen tube in order to complete fertilization. Indeed, cysteine-rich proteins LUREs secreted from synergid cells in the matured ovules have been shown to be the key molecules that guide pollen tubes (*Okuda et al. 2009*).

During the course of monitoring pollen tube growth, we noticed that pollen tubes reached ovules in an acropetal manner. One of the possibilities is that ovules sequentially

### Table: Length of in vitro germinated pollen tubes over time

| Incubation time (days) | Average length (µm) | Standard error (µm) |
|------------------------|---------------------|---------------------|
| 10                     | 131                 | 6.4                 |
| 20                     | 321                 | 38.7                |
| 30                     | 875                 | 136.9               |

**Fig. 4** Length of in vitro germinated pollen tubes over time. At least 50 pollen tubes were measured and averaged after 10, 20, or 30 days of incubation.
mature starting from the base of the ovaries (close to the pedicle) and gradually move toward the top (close to the stigma). Acropetal succession of ovule development has been documented in some plants (Bittencourt and Mariath 2002; Endress 2011). It is also possible that pollen tubes respond to matured ovules in a sequential fashion. The detailed mechanism remains to be determined. Nevertheless, to our knowledge this is the first report on sequential fertilization in the multiovulated ovary of *Phalaenopsis* orchids.

While developing the in vitro germination protocol, we found that stigmatic tissue extract is required to initiate and promote pollen germination in vitro, indicating that stigmatic tissues provide cuing molecules to signal pollen to germinate. This is not too surprising because pistil-derived molecules such as sulfinylated azadecalins (Qin et al. 2011), pistil extracellular matrix (Cheung et al. 1993, 1995), and small cysteine-rich proteins (Chae et al. 2009; Dong et al. 2005; Kim et al. 2003) have been shown to be the important stimulants for pollen germination and growth. Our established in vitro germination protocol provides a screening platform to identify substances required for pollen germination/guidance in *Phalaenopsis* orchids and maybe in other orchid species.

**Author contribution statement** SCF conceived and designed the experiments. JCC performed the experiments and JCC and SCF analyzed the data. SCF wrote the paper. All authors read and approved the manuscript.

**Acknowledgments** We express our appreciation to Dr. Wann-Neng Jane for his assistance with scanning electron microscopy and Ms. Miranda Loney for English editing.

**Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

**References**

Abramoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. Biophot Intern 11:36–42
Allen AM, Hiscock SJ (2010) Molecular communication between plant pollen and pistils. Plant sciences reviews. CAB International, Wallingford, UK, pp 237–248

Arditti J (1992) Fundamentals of orchid biology. Wiley, New York

Bittencourt NS Jr, Mariath JEA (2002) Ovule ontogeny of Tabebuia pulcherrrima Sandwith (Bignoniaceae): megasporogenesis and integument development Brazilian. J Botany 25:103–115

Boavida LC, McCormick S (2007) Temperature as a determinant factor for increased and reproducible in vitro pollen germination in Arabidopsis thaliana. Plant J 52:570–582. doi:10.1111/j.1365-313X.2007.03248.x

Chae K, Kieslich CA, Morikis D, Kim SC, Lord EM (2009) A gain-of-function mutation of Arabidopsis lipid transfer protein 5 disturbs pollen tube tip growth and fertilization. Plant Cell 21:3902–3914. doi:10.1105/tpc.107.070854

Chen Y-C, Jiang S-W (2014) The effects of stigma extract solution and culture medium on pollen germination in vitro of sugar apple 'Taitung No. 2'. Res Bull Taitung District Agric Improv Stn 24:83–94

Chen YY et al (2012) C- and D-class MADS-box genes from Phalaenopsis aphrodite. Plant Mol Biol 84:203–226. doi:10.1007/s11103-013-0128-y

Liu J, Zhang H, Cheng Y, Kafkas S, Güney M (2014) Pistillate flower development and pollen tube growth mode during the delayed fertilization stage in Corylus heterophylla. Fisch Plant Reprod 27:145–152. doi:10.1007/s00497-014-0248-9

Martin FW (1959) Staining and observing pollen tubes in the style by means of fluorescence. Stain Technol 34:125–128

Möl R, Mathyssochon E, Dumas C (1994) The kinetics of cytological events during double fertilization in Zea-Mays L. Plant J 5:197–206. doi:10.1046/j.1365-313X.1994.05020197.x

Okuda S et al (2009) Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature 458:357–361. doi:10.1038/nature07882

O’Neill SD (1997) Pollination regulation of flower development. Ann Rev Plant Physiol Plant Mol Biol 48:547–574. doi:10.1146/annurev.arplant.48.1.547

Pacini E (1997) Tapetum character states: analytical keys for tapetum types and activities. Can J Bot 75:1448–1459

Pacini E (2009) Orchid pollen dispersal units and reproductive consequences. In: Kul T, Arditti J, Wong SM (eds) Orchid biology: reviews and perspectives, vol X. Springer, New York, pp 185–218

Pacini E, Hesse M (2002) Types of pollen dispersal units in orchids, and their consequences for germination and fertilization. Ann Bot Lond 89:653–664. doi:10.1039/aob/mcf138

Pandolli T, Pacini E (1995) The pollinium of Loroglossum-Hirconium (Orchidaceae) between pollination and pollen tube emission. Plant Syst Evol 196:141–151. doi:10.1007/BF00982955

Papuga J et al (2010) Arabidopsis LIM proteins: a family of actin bundlers with distinct expression patterns and modes of regulation. Plant Cell 22:3034–3052. doi:10.1105/tpc.110.075960

Pimenta E, Polito VS (1983) Embryo sac development in almond [Prunus dulcis (Mill) Webb, D.A.] as affected by cross-pollination, self-pollination and non-pollination. Ann Bot 51:469–479

Qin Y, Yang Z (2011) Rapid tip growth: insights from a new model. Semin Cell Dev Biol 22:816–824. doi:10.1016/j.semcdb.2011.06.004

Qin Y et al (2011) Sulfonlated azadecalins act as functional mimics of a pollen germination stimulant in Arabidopsis pistils. Plant J 68:800–815. doi:10.1111/j.1365-313X.2011.04729.x

Roberts IN, Gaude TC, Harrod G, Dickinson HG (1983) Pollen-stigma interactions in Brassica oleracea; a new pollen germination medium and its use in elucidating the mechanism of self-incompatibility. Theor Appl Genet 65:231–238. doi:10.1007/BF00308074

Rodriguez-Enriquez MJ, Mehdi S, Dickinson HG, Grant-Downton RT (2013) A novel method for efficient in vitro germination and tube growth of Arabidopsis thaliana pollen. New Phytol 197:668–679. doi:10.1111/nph.12037

Sogo A, Tobe H (2006a) Delayed fertilization and pollen-tube growth in pistils of Fagus japonica (Fagaceae). Am J Bot 93:1748–1756. doi:10.3732/ajb.93.12.1748

Sogo A, Tobe H (2006b) Mode of pollen-tube growth in Pistils of Myrica rubra (Myricaceae): a comparison with related families. Ann Bot 97:71–77. doi:10.1093/aob/mc015

Sprunk S, Rademacher S, Vogler F, Ghyselinck J, Grossniklaus U, Dresselhaus T (2012) Egg cell-secreted EC1 triggers sperm cell activation during double fertilization. Science 338:1093–1097. doi:10.1126/science.1229444

Tsai Y-C, Chang C (2010) The investigation of floriculture characteristics, chromosome numbers and in vitro pollen germination of Phaius tankervilleae and its allied genera. Hortic NCHU 35:99–112

Tsai WC, Hsiao YY, Pan ZJ, Kuoh CS, Chen WH, Chen HH (2008) The role of ethylene in orchid ovule development. Plant Sci 175:98–105
Wang HJ, Wan AR, Jauh GY (2008) An actin-binding protein, LILIM1, mediates calcium and hydrogen regulation of actin dynamics in pollen tubes. Plant Physiol 147:1619–1636. doi:10.1104/pp.108.118604

Wu CC, Diggle PK, Friedman WE (2011) Female gametophyte development and double fertilization in Balsas teosinte, Zea mays subsp. parviglumis (Poaceae). Sex Plant Reprod 24:219–229. doi:10.1007/s00497-011-0164-1

Yang Z (2008) Cell polarity signaling in Arabidopsis. Annu Rev Cell Dev Biol 24:551–575. doi:10.1146/annurev.cellbio.23.090506.123233

Yeung EC, Law SK (1997) Ovule and megagametophyte development in orchids. Orchid biology: reviews and perspectives, vol VII. Kluwer Academic Publishers, Dordrecht, pp 31–73

Zhang XS, O’Neill SD (1993) Ovary and gametophyte development are coordinately regulated by auxin and ethylene following pollination. Plant Cell 5:403–418. doi:10.1105/tpc.5.4.403