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Pollen biology of ornamental ginger (*Hedychium* spp. J. Koenig)

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ABSTRACT

An improved *in vitro* pollen germination assay was developed to assess the viability of stored *Hedychium* pollen. The effect of polyethylene glycol (PEG) (10, 15, and 20%, w/v) on pollen germination and tube growth was evaluated for *Hedychium longicornutum* and two commercial *Hedychium* cultivars, 'Orange Brush' and 'Filigree'. Overall, the inclusion of PEG 4000 in the medium improved both pollen germination and tube growth for the three different genotypes tested and the results varied depending on genotype. *In vitro* germination was used to assess the viability of *Hedychium* pollen stored up to two months. Pollen nucleus status was determined for four *Hedychium* cultivars, 'Orange Brush', 'Anne Bishop', 'Filigree', and 'Daniel Weeks'. Pollens of 'Orange Brush', 'Anne Bishop', and 'Daniel Weeks' were found to be binucleate but 'Filigree' was shown to possess both binucleate and trinucleate pollens. High pollen:ovule ratio values were obtained in several *Hedychium* taxa. The results obtained on the nuclear pollen status and pollen:ovule ratios will further our understanding of the pollination biology and help clarify the taxonomy and phylogeny of *Hedychium* species.

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1. Introduction

The genus *Hedychium* consists of about 80 species characterized by beautiful foliage as well as diverse, showy, and fragrant flowers. Some species are grown for their edible flowers and others for their medicinal or industrial properties (He, 2000; Gopanraj et al., 2005; Gao et al., 2008). Most *Hedychium* species are native to central and southeastern Asia with high concentrations in southern China and the Himalayan regions (Hayes et al., 2009). They are increasingly becoming popular as ornamental plants worldwide because of their showy and scented flowers. For example, in Great Britain where no commercial sources for *Hedychium* existed in 1982 (Schilling, 1982), the 2009 Royal Horticultural Society (RHS) Plant Finder lists 116 taxa that are available to British gardeners. Many more taxa are found in Japan and the United States (Branney, 2005). Despite the potential ornamental, medicinal, and industrial values of the *Hedychium* genus, very little work has been done to understand the pollen biology such as germinability, storage, viability, nuclear status, and pollen/ovule ratio.

In the United States, *Hedychium* plants are mostly grown in the southern part of the country where they generally flower in the summer and fall, but some species bloom in winter and spring times. This asynchronous flowering could constitute an impediment for breeders of that region to fully take advantage of the tremendous diversity that exists within *Hedychium* genus. Therefore, optimal storage conditions for *Hedychium* pollen to be used later on for pollination need to be investigated, but to assess the viability of the stored pollen, a convenient and reliable method of testing the pollen quality is required. Currently, there are no published reports available on *Hedychium* pollen storage and viability. Assessing pollen viability on the basis of its functions of delivering sperm cells to the embryo sac is not only tedious and time-consuming but also not always feasible (Heslop-Harrison et al., 1984), so other techniques including staining pollen with vital dyes and *in vitro* pollen germination and tube growth are often used to evaluate pollen viability. Furthermore, successful fertilization depends greatly not only on the ability of pollen to germinate but also on its elongation rate, assuring the successful delivery of the sperm cells to the ovules (Daher et al., 2009). *In vitro* pollen germination and pollen tube growth are greatly influenced by different factors including the composition of the germination medium, genotype of the accession, temperature and humidity. For example, addition of polyethylene glycol (PEG) to the germination medium has been reported to improve *in vitro* pollen germination frequency and tube growth by preventing tube bursting (Read et al., 1993; Shivanna and Sawhney, 1995).

Pollen of flowering plants is either binucleate or trinucleate. Compared to binucleate pollens, trinucleate pollens are known to have a rapid germination rate but short viability (Brewbaker, 1967).

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The generative nucleus of binucleate pollen divides in the pollen tube after germination to form two sperm nuclei while that of trinucleate pollen divides in the anther before pollen is shed (Sugiura et al., 1998). Among the 2000 species surveyed by Brewbaker (1967), (70)% were found to be binucleate and the rest trinucleate, and among the 265 families surveyed by this author, 179 had binucleate genera, 54 had trinucleate genera, and 32 had both types of genera. Furthermore, the ratios of pollen grains to number of ovules (P:O ratios) have been shown to correlate with the mating system of a plant. Cruden (1977) was the first to demonstrate that in general outcrossing species had higher P:O ratios than predominantly selfing species and conclude that outcrossed species had higher P:O ratios while selfed species had lower P:O ratios. This pollination efficiency hypothesis is based on the argument that maximum seed set in outcrossing plants would require more pollens grains as a result of inefficient pollen transfer. Pollen development and morphology are often used by taxonomists and paleobotanists to clarify the classification and identity of plant species (Fogle, 1977; Lanza et al., 1996; Mert, 2009). Ultrastructural observations under scanning electron microscopy (SEM) or transmission electron microscopy (TEM) help define pollen characteristics such as shape, size, and presence or absence of exines.

Knowledge about *in vitro* germination, tube growth, storage, nucleus status, ultrastructure of *Hedychium* pollen as well as pollen:ovule ratio can be of the utmost importance for understanding not only the basic characteristics of *Hedychium* pollen but also its pollination biology, which could ultimately lead to better breeding strategies for the *Hedychium* genus. Furthermore, it is worth mentioning that despite the attractiveness of *Hedychium* plants for industrial, medicinal, and ornamental potential, some *Hedychium* species spread so quickly that they are considered invasive or pest plants in certain regions of the world, including Australia, New Zealand, Hawaii, and Brazil (Funk, 2001; Williams et al., 2003; Soares and Barreto, 2008). Data and knowledge gained on *Hedychium* pollen through this study could contribute to the development of better control strategies for *Hedychium* species that are or could be invasive. However, information on any of the pollen characteristics listed above is either scarce or totally lacking. Therefore, the objectives of the current study are to (1) evaluate pollen germination and tube growth in different genotypes of *Hedychium*; (2) assess viability of stored *Hedychium* pollen; (3) study pollen development and biology including nucleation and ultrastructure; and (4) determine pollen:ovule ratios for selected *Hedychium* taxa.

2. Materials and methods

2.1. In vitro pollen germination and tube growth

For pollen germination test, the hanging drop technique was employed following published procedures (Loupassaki et al., 1997; Deng and Harbaugh, 2004) with some modifications as described below. A "basic" liquid medium containing 1.2 M sucrose, 0.42 g L^{-1} calcium nitrate [$Ca(NO_3)_2$], 0.20 g L^{-1} boric acid (H_3BO_3), 0.1 g L^{-1} potassium nitrate (KNO_3), and 0.22 g L^{-1} magnesium sulfate ($MgSO_4 \cdot 7H_2O$) was used. The effect of polyethylene glycol (PEG) concentrations (10, 15, and 20%, w/v) on pollen germination and tube growth was evaluated in *Hedychium longicornutum* and two other *Hedychium* commercial cultivars, 'Orange Brush' and 'Filigree'. For the germination test with the PEG-based media, both pollen germination and pollen tube growth were recorded after a 3 h incubation period. Pollen germination was determined by direct microscopic observation (at 10×) in three fields of view per slide. Four replicates (slides) were used for each plant. A pollen grain was considered to have germinated when pollen tube length equaled or exceeded the grain diameter. Germination percentage was determined by dividing the number of germinated pollen grains by the total number of pollen grains per field of view and multiplying by 100. To measure pollen tube growth, samples were prepared as described above. The slides were observed using a compound microscope (ACCU-SCOPE, Inc., Sea Cliff, NY) at 1000× oil immersion magnification. The germinated tubes were photographed with an M Eye Digital camera (Ken-A-Vision, Kansas City, MO), and tube growth was measured using the Vision Explore software (Ken-A-Vision, Kansas City, MO).

2.2. Pollen viability test

For the germination test, both pollen germination and pollen tube growth were recorded after a 3 h incubation period. For the rest of the germination test, pollen viability was evaluated after 24 h incubation. The effect of storage temperature (4 °C and −20 °C and duration (0, 4, and 8 weeks) on pollen germination was evaluated for the commercial cultivars 'Orange Brush', 'Filigree', 'Tai Golden Goddess', 'Pink V', and *H. forrestii*. Pollen germination was determined by direct microscopic observation (at 10× magnification) in three fields of view per slide. Four replicates (slides) were used for each plant. A pollen grain was considered to have germinated when pollen tube length equaled or exceeded the grain diameter. Germination percentage was determined by dividing the number of germinated pollen grains by the total number of pollen grains per field of view and multiplying by 100.

2.3. Pollen nucleus status and ultrastructure

To stain pollen nucleus, a 1% acetocarmine was prepared by diluting 1 g carmine and 0.2 g in boiling 100 ml of 45% (v/v) glacial acetic acid, which was rapidly cooled and then filtered into a dark amber glass. Pollens were collected from greenhouse grown *Hedychium* plants, transferred in 1.5 ml centrifuge tubes containing 1 (acetic acid):3 (ethanol), vortexed for about 10 s, and fixed for 24 h. Afterwards, they were centrifuged for 3 min at 1300 rpm and the liquid was poured off. Pollens were hydrolyzed in 1N HCl at 60 °C for 15 min, centrifuged for 3 min, and the acid poured off. Three to four drops of deionized water were added to the pollens, which were vortexed for 3 min before the water was removed. Thereafter, 3–4 drops of 1% acetocarmine staining solution was added to entirely cover the pollens and let set for 1–24 h. Two drops of the staining liquid containing pollens were transferred on a slide and covered with a cover slip. A bibulous filter paper was placed on top of the slide and the pollens were given a gentle squeeze to extrude pollen cytoplasm. Pollens were then examined under a light microscope (10× and 40×) to determine the nuclear number. Pollen nuclear number determination was achieved for pollens from the *Hedychium* cultivars 'Filigree', 'Orange Brush', 'Anne Bishop', and 'Daniel Weeks'.

2.4. Scanning electron microscopy

The pollen samples were collected from fresh flowers and processed for scanning microscopy without the usual fixation and dehydration procedures. The samples were directly sputter-coated with gold/palladium (200 nm) before imaging at 10–13 kV with an XL30 ESEM (FEI Instruments, Hillsboro, OR).

2.5. Pollen:ovule ratios

To count pollen grains and ovules, flowers were collected at dehiscence from seven *Hedychium* taxa, *Hedychium* cv. 'Anne Bishop', *Hedychium coronarium*, *Hedychium* cv. 'Disney', *Hedychium* cv. 'Kwense', *Hedychium* cv. 'Daniel Weeks', *Hedychium coccineum*, and *Hedychium* cv. 'Double Eagle'. Five drops of 1% acetocarmine